Membrane targeting of p21-activated kinase 1 (PAK1) induces neurite outgrowth from PC12 cells

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Rho-family GTPases regulate cytoskeletal dynamics in various cell types. p21-activated kinase 1 (PAK1) is one of the downstream effectors of Rac and Cdc42 which has been implicated as a mediator of polarized cytoskeletal changes in fibroblasts. We show here that the extension of neurites induced by nerve growth factor (NGF) in the neuronal cell line PC12 is inhibited by dominant-negative Rac2 and Cdc42, indicating that these GTPases are required components of the NGF signaling pathway. While cytoplasmically expressed PAK1 constructs do not cause efficient neurite outgrowth from PC12 cells, targeting of these constructs to the plasma membrane via a C-terminal isoprenylation sequence induced PC12 cells to extend neurites similar to those stimulated by NGF. This effect was independent of PAK1 ser/thr kinase activity but was dependent on structural domains within both the N- and C-terminal portions of the molecule. Using these regions of PAK1 as dominant-negative inhibitors, we were able to effectively inhibit normal neurite outgrowth stimulated by NGF. Taken together with the requirement for Rac and Cdc42 in neurite outgrowth, these data suggest that PAK(s) may be acting downstream of these GTPases in a signaling system which drives polarized outgrowth of the actin cytoskeleton in the developing neurite.

Keywords: cytoskeleton/neurite outgrowth/p21 activated kinases/PC12 cells/Rho-GTPases

Introduction

Nervous system function is dependent upon the highly specific connections which form between neurons during development. The patterning and specificity of these connections requires neurite extension toward the proper targets guided by the growth cone in response to environmental signals. Neurite extension is thus an extremely important and highly complex, yet poorly understood process that involves signal-induced morphological changes ultimately resulting in coordinated cytoskeletal remodeling in the specialized growth cone at the tip of an extending neurite (Gordon-Weeks, 1991; Bentley and O'Connor, 1994). Studies on neuronal cell lines, such as the rat adrenal pheochromocytoma cell line PC12, have demonstrated that a variety of extracellular signals can

lead to changes in neurite outgrowth and cellular morphology. In PC12 cells, the most well characterized pathway involves signaling by nerve growth factor (NGF) via its tyrosine kinase receptor (Trk). Ligand binding to receptor in this system is believed to activate a Ras-dependent MAP kinase cascade which leads to cellular differentiation and neurite outgrowth (Thomas *et al.*, 1992; Wood *et al.*, 1992; Cowley *et al.*, 1994; Kamata *et al.*, 1996). Indeed there is substantial evidence linking Ras superfamily GTPases to the signaling pathways that lead to neuronal differentiation (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985; Ayala *et al.*, 1990). However, it is less clear which signaling elements regulate the actinomyosin-based cytoskeleton in order to generate the cytoskeletal extensions required for neurite growth, and to regulate growth cone dynamics.

Recent studies on PC12 and other neuronal cell lines have suggested that the Rho family of GTPases are critical regulators of the cytoskeletal changes required for neurite extension and retraction. A central finding has been that activation of Rho itself results in neurite retraction, while inactivation of Rho can induce neurite outgrowth (Tigyi *et al.*, 1996a,b; Gebbink *et al.*, 1997; Kozma *et al.*, 1997). Thus, current evidence suggests a role for Rho in determining the overall balance between neurite formation and retraction (Tigyi *et al.*, 1996a; Kozma *et al.*, 1997). However, studies on the dynamics of neurite outgrowth and growth cone remodeling suggest that this process is analogous to the events at the leading edge of a motile cell and may well involve the other Rho family members Rac and Cdc42 (Kozma *et al.*, 1997). Evidence in support of this comes from genetic studies showing a role for Rac in the formation of dendritic spines in developing mouse axons (Luo *et al.*, 1996), and that defects in Rac and Cdc42 signaling affect the development of *Drosophila* neural pathways (Luo *et al.*, 1994). Dominant-negative forms of Rac and Cdc42 have been shown to inhibit both acetylcholine-induced actin extensions and botulinum C3 exoenzyme-mediated neurite formation in N1E-115 neuroblastoma cells (Kozma *et al.*, 1997). Despite the evidence linking Rho family GTPases to these processes, little is yet known about the effector mechanisms involved in GTPase-mediated cytoskeletal remodeling in neuronal cells.

The p21-activated kinase (PAK) family of ser/thr kinases (PAK1, 2 and 3) were first identified as targets for active Rac and Cdc42 (Manser *et al.*, 1994). Binding of Rac-GTP or Cdc42-GTP to a specific p21-binding domain in the N-terminus of PAK (Burbelo *et al.*, 1995) causes a change in the conformation of the kinase, inducing autophosphorylation and an increase in its kinase activity (Manser *et al.*, 1994). There is increasing evidence that PAK family members may play a critical role in cytoskeletal changes associated with both enhanced motility and

apoptosis. PAK phosphorylates both the heavy and light chains of myosin, modulating actin-stimulated ATP hydrolysis (Tauzon and Traugh, 1984; Wu *et al.*, 1996; Brzeska *et al.*, 1997). Studies from this laboratory have shown that PAK1 translocates to areas of active cytoskeletal rearrangement, and certain activated PAK1 mutants induce lamellipodia and ruffles when microinjected into fibroblasts (Dharmawardane *et al.*, 1997; Sells *et al.*, 1997). The action of PAK in regulating the fibroblast cytoskeleton appears to be partially independent of its kinase activity and more dependent on structural changes in the N-terminus which occur following the binding of GTPases. Recent evidence has also demonstrated that PAK can be activated in a GTPase-independent manner via interactions with specific lipids (G.M.Bokoch, A.M.Reilly, R.H.Daniels, C.C.King, A.Olivera, S.Spiegel and U.G.Knaus, submitted), and that targeting of PAK to membranes leads to its activation and the stimulation of downstream signaling cascades (Lu *et al.*, 1997; Manser *et al.*, 1997; G.M.Bokoch, A.M.Reilly, R.H.Daniels, C.C.King, A.Olivera, S.Spiegel and U.G.Knaus, submitted).

Studies on the yeast homolog of PAK, Ste20, have identified this molecule as being important in coupling the mating response to MAP kinase cascades, in the polarized outgrowth of emerging buds and in pheromone-induced polarized morphogenesis of yeast (Chant, 1994; Cvrckova *et al.*, 1995; Leberer *et al.*, 1997b). These latter responses involve the polarized outgrowth and dynamic regulation of the actin cytoskeleton, and Ste20 has been shown to link pheromone signaling and Cdc42 activation to elements of the actin cytoskeleton in a signaling complex which regulates polarized morphogenesis (Leeuw *et al.*, 1995). It is intriguing to speculate, therefore, that in mammalian cells PAK family members may also have a dual signaling role in which PAK functions as both a component of kinase cascade signaling and as a downstream mediator of signaling events associated with polarized morphological changes. In this respect, PAK is therefore a likely candidate for mediating effects of Rac and Cdc42 in the extending growth cone of neurites.

In this study, we demonstrate that NGF-induced neurite outgrowth from PC12 cells is dependent upon Rac and Cdc42. The activation of these GTPases is not, however, sufficient to induce the outgrowth of neurites from these cells. PAK1 alone is also not sufficient to induce neurite outgrowth when cytoplasmically expressed; however, targeting of PAK1 to the plasma membrane of PC12 cells induces the outgrowth of neurite-like structures. We demonstrate that this process is largely independent of the kinase activity of PAK1, but requires structural elements present in both the PAK1 N- and C-terminus. Truncated N- or C-terminal PAK1 fragments are able to act as dominant-negatives, preventing NGF-induced neurite outgrowth in PC12 cells. These results suggest that Rac and Cdc42 and their downstream effector PAK may comprise part of a signaling pathway which is involved in growth factor-induced neurite outgrowth.

Results

Activation of Rac and Cdc42 is required but not sufficient to induce PC12 neurite outgrowth

PC12 cells grown on collagen-coated dishes in the presence of 50 ng/ml NGF for 72 h develop a network of neurite

Fig. 1. Dominant-negative Rac2 and Cdc42 inhibit NGF-induced neurite outgrowth. Dominant-negative (T17N) and wild-type Rac2 and Cdc42 were transiently expressed in PC12 cells which were stimulated with 50 ng/ml NGF for 72 h. Cells were then fixed and stained for expression of myc-tagged protein and TRITC-phalloidin. Untransfected control cells, either unstimulated or stimulated with NGF over the same time course, were stained with TRITC-phalloidin to visualize the cells and neurite extensions. Cells with neurite extensions of greater than two body lengths were scored as neurite bearing, and at least 100 cells from each treatment were counted. Results are expressed as the mean percentage of cells ($\pm$ SD) from three separate experiments.

outgrowths, with ~70% of cells elaborating neurites at least two cell bodies in length. Transient overexpression of dominant-negative forms of either Rac2^{T17N} or Cdc42^{T17N} effectively prevented NGF-induced neurite formation (Figure 1). Expression of the wild-type constructs had no effect on basal or NGF-induced neurite outgrowth. This inhibitory effect of the dominant-negative Rac2 and Cdc42 contrasts with the stimulatory effect of blocking Rho activity (Kozma *et al.*, 1997).

PC12 cells transiently transfected with activated constructs of these GTPases, Rac2^{Q61L} and Cdc42^{Q61L}, did not develop significant numbers of neurites over 72 h. As Figure 2 shows, these constructs did induce substantial morphological changes in the cells, causing the cells to flatten, the soma to enlarge, the generation of ruffles at the cell periphery and, in the case of Cdc42, the extension of membrane processes. These changes are similar to those observed in fibroblasts which overexpress these constructs (Ridley and Hall, 1992; Ridley *et al.*, 1992; Nobes and Hall, 1995).

Membrane targeting of PAK1 induces neurite outgrowth in PC12 cells

Recent work has implicated PAK1 as a downstream mediator of Rac and Cdc42 effects on polarized cytoskeletal assemblies (Sells *et al.*, 1997). We postulated that PAK1 might, therefore, mediate the effects of these small GTPases during the process of polarized neurite outgrowth. To test this hypothesis, PC12 cells were transfected with wild-type PAK1 and with the GTPase binding-deficient but cytoskeletally active construct PAK1^{H83,86L} (Sells *et al.*, 1997). Neither of these constructs was able to induce substantial neurite outgrowth. However, the PAK1^{H83,86L}

Fig. 2. Activated Rac2 and Cdc42 induce morphological changes in PC12 cells. Fluorescence micrographs (400 $\times$) magnification of PC12 cells expressing either pCDNA3 Rac2^{Q61L} or pCDNA3 Cdc42^{Q61L} for 72 h. The left-hand panels show PC12 cells expressing the myc-tagged Rac2^{Q61L} (upper) or Cdc42^{Q61L} (lower) detected after fixation and staining with the appropriate antibodies. The right-hand panel shows the same cells stained with TRITC-phalloidin to visualize polymerized actin. These fluorescent images were typical of cells expressing these constructs. Similar results were recorded in four separate experiments.

mutation caused a clear morphological change, inducing a 'spiky' morphology with short actin-rich protrusions extending from the cell surface (Figure 3A).

One likely effect of the association of PAK with an activated Rac or Cdc42 would be to localize the kinase at the cell membrane and/or membrane-associated cytoskeleton. Recent studies have demonstrated that membrane localization of PAK stimulates its activity, possibly by GTPase-dependent and -independent mechanisms (Lu *et al.*, 1997; G.M.Bokoch, A.M.Reilly, R.H.Daniels, C.C.King, A.Olivera, S.Spiegel and U.G.Knaus, submitted). Since neurite outgrowth may require spatially restricted reorganization of the actin cytoskeleton at or near the membrane level, we tested whether artificially targeting PAK constructs to PC12 membranes would affect their morphology. Transfection of PC12 cells with either wild-type PAK1 or PAK1^{H83,86L} to which we have added the 17 amino acid C-terminal membrane-targeting sequence from Ras leads to the induction of neurite outgrowth from transfected cells (Figure 3A). The wild-type PAK1-CAAX induced neurite formation in $46 \pm 7\%$ of the cells, while the PAK1^{H83,86L}-CAAX mutation was even more effective, causing neurite outgrowth from $65 \pm 8\%$ of the cells, a figure strikingly similar to the NGF-treated controls ($67 \pm 7\%$). These extensions were microscopically very similar to NGF-stimulated neurites (Figure 3B); most had actin-rich growth cones at the tips of extending neurites and stained positively for tubulin and the neurite-specific antigen GAP-43, the latter of which redistributed from the cell body into the extending neurite and growth cone (data not shown). PAK1-CAAX-induced neurite outgrowth was inhibited effectively by cytochalasin B (8 μ g/ml) and colchicine (10 μ M) in a similar manner to NGF-induced outgrowth. This suggests a requirement for both microfilament- and microtubule-based cytoskeletal elements in neurites extended by PC12 cells overexpressing membrane-targeted PAK.

The induction of neurite outgrowth was entirely dependent on the presence of a functional membrane-targeting

CAAX motif, as control constructs in which the terminal cysteine was mutated to an alanine (AlaAAX) did not induce this phenotype (Figure 3A). Moreover, PAK1-CAAX was localized to the extending neurites and growth cones, along with F-actin (Figure 3B).

In order to verify that the CAAX motif actually led to membrane localization of the PAK1 constructs, we overexpressed them in the COS 7 cell line (which expressed the constructs at greater efficiency than PC12 cells) and prepared a crude high-speed membrane pellet and supernatant fraction from cellular homogenates. As Figure 3C shows, the CAAX-tagged constructs were localized mainly in the high-speed pellet, whereas wild-type PAK resided exclusively in the cytosolic fraction.

PAK-induced neurite outgrowth is independent of Rho GTPases and PAK kinase activity

The fact that the PAK1^{H83,86L}-CAAX construct was effective at inducing neurite outgrowth suggested that membrane targeting was able to bypass the normal requirement for GTPases and that PAK was acting downstream of GTPases in this pathway. Support for this hypothesis was obtained when PAK1-CAAX constructs were co-expressed with Rac2^{T17N} or Cdc42^{T17N} dominant-negative mutants. As Figure 4 shows, there was a only slight inhibition of both PAK1-CAAX- and PAK1^{H83,86L}-CAAX-stimulated neurite outgrowth by the dominant-negative GTPases, while NGF was effectively blocked.

Surprisingly, the ability of membrane-targeted PAK to induce neurite outgrowth seems to be largely independent of its ser/thr kinase activity. Introduction of a kinase-inactivating mutation PAK1^{K299R} (Hanks *et al.*, 1988; Sells *et al.*, 1997; Tang *et al.*, 1997) into either wild-type PAK1-CAAX or PAK1^{H83,86L}-CAAX had no effect on the ability of these proteins to induce neurite outgrowth (Figure 5A). Moreover, the expression of a constitutively active PAK mutation PAK1^{T423E} or membrane-targeted PAK1^{T423E}-CAAX resulted in phenotypes similar to the wild-type constructs (Figure 5A). The expression of kinase-dead

Fig. 3. Membrane targeting of PAK constructs induces neurite outgrowth from PC12 cells. (A) Typical fields at 400 $\times$ magnification of PC12 cells expressing wild-type PAK1 variants (a–c) or PAK1^{H83,86L} variants (d–f) fixed and stained for the myc epitope. (a) and (d) show the effects of cytoplasmically expressed constructs, (b) and (e) show typical fields of PC12 cells overexpressing myc-tagged wild-type-PAK1-CAAX (b) and PAK1^{H83,86L}-CAAX (e) fixed and stained with the appropriate antibodies. (c) and (f) show cells expressing the same variants of PAK with the terminal cysteine mutated to an alanine to prevent isoprenylation. Similar results were obtained in at least five independent experiments. (B) PC12 cells transfected with membrane-targeted PAK1 extend neurites with actin-rich and PAK1-rich growth cones at the tip. A 1000 $\times$ magnification of growth cone-bearing neurites from PC12 cells treated with 50 ng/ml NGF for 72 h (upper panel) or transiently overexpressing wild-type PAK1-CAAX for 72 h (lower panel) and stained for actin with TRITC–phalloidin (left side) or for expression of the myc epitope (lower right panel). (C) Western blots of myc-tagged PAK constructs present in the supernatant (S) or pellet (P) of COS 7 homogenates subjected to high-speed centrifugation (see Materials and methods). Blots were probed with the anti-myc epitope antibody, 9E10, and detection was performed using the alkaline phosphatase detection kit (Bio-Rad). Similar results were obtained in two independent experiments.

mutations of PAK has been shown to inhibit certain PAK-dependent pathways in other cells, presumably due to their ability to form non-productive complexes with downstream kinase targets (Rudel and Bokoch, 1997; Tang *et al.*, 1997). We therefore tested whether NGF-induced neurite outgrowth could be inhibited by overexpression of kinase-dead PAK1^{WT}K299R or PAK1^{H83,86L}K299R in PC12 cells. As Figure 4B shows, neither of these kinase-dead constructs had any effect on NGF-induced neurite outgrowth.

Endogenous PAK localizes to the membrane-enriched fraction in NGF-stimulated PC12 cells

In lysates of PC12 cells, the predominant isoform of PAK had an apparent mol. wt of 62 kDa and was recognized by antibodies raised against the N-terminus of PAK2 (whole lysate, Figure 6) (Knaus *et al.*, 1995) and an antibody raised against the highly conserved PAK p21-binding domain (PBD) of PAK1 (data not shown). It is important to note that, while these antibodies are raised

against fusion proteins that represent these particular regions of PAK1 or PAK2, they do exhibit a degree of cross-reactivity between isoforms and hence cannot be used to define the isoform. However, the apparent molecular weight of the band, coupled with its lack of binding to an anti-peptide antibody raised against a specific sequence in PAK1 (not shown), does suggest that the predominant isoform in PC12 cells is more closely related to PAK2. In this respect, it is also important to note that the kinase domain, the PBD domain and the putative SH3-binding domains of PAKs are highly conserved between PAK1 and PAK2.

To investigate whether this endogenous protein was recruited to a membranous location upon NGF stimulation of PC12 cells, we prepared membrane-enriched fractions of cells stimulated for various times with 50 ng/ml NGF. As Figure 6 shows, there is a clear increase of the 62 kDa PAK2-immunoreactive band after 2 h of NGF stimulation. This elevated level of PAK in the membrane-enriched fraction is maintained over the time course of the experi-

Fig. 4. Effect of dominant-negative GTPases on PAK1-CAAX-induced neurite outgrowth from PC12 cells. PC12 cells were either exposed to NGF or transfected with PAK1-CAAX constructs for 72 h in the presence or absence of Rac2^{T17N} or Cdc42^{T17N}. Cells were then assessed for neurite outgrowth as described in Materials and methods. The results show the mean percentage of cells bearing neurites ($\pm$ SD). At least 60 cells were counted in each experiment and these results show the average from three independent experiments.

ment (24 h), and in separate experiments with longer time courses the amount of membrane-associated PAK remained above background for at least 48 h.

PAK-induced neurite outgrowth is dependent on both the N- and C-terminal domains of the protein

Studies of the effects of PAK1 on cytoskeletal changes in fibroblasts had demonstrated that many of these effects could occur independently of functional PAK1 protein kinase activity, and suggested that structures within the N-terminus of PAK were required for protein interactions which directed cytoskeletal reorganization (Sells *et al.*, 1997). To analyze further the regions of PAK which were important for the induction of neurites, we constructed a number of expression plasmids of PAK encoding different regions of the molecule (Figure 7).

We generated two truncated constructs which comprised the N-terminus (amino acids 1–205) and the C-terminus (amino 206–end) of PAK1, as well as a third construct which incorporated the PAK1^{H83,86L} GTPase-binding domain mutation within the N-terminal fragment (Figure 7); all three constructs were prepared both as non-targeted and CAAX-tagged membrane-targeted forms. Neither membrane-targeted or untagged versions of the C- or N-terminal PAK1 fragments resulted in neurite outgrowth, suggesting that neither fragment alone contained sufficient structural information to induce the differentiated phenotype (Figure 8A). The untagged N-terminal PAK1^{H83,86L} construct induced morphological changes

Fig. 5. Quantification of neurite outgrowth from unstimulated PC12 cells (A) or NGF-stimulated PC12 cells (B) expressing various PAK1 constructs. (A) Neurite outgrowth from PC12 cells overexpressing wild-type PAK1-CAAX and PAK1^{H83,86L}-CAAX constructs was compared with kinase-dead (PAK1^{K299R}) and constitutively active (PAK1^{T423E}) variants. The y-axis indicates the number of cells with neurite-like protrusions of greater than two soma, as described in Materials and methods. (B) PC12 cells transiently transfected with pCMV6M wild-type PAK1, the kinase-dead mutations and a pCMV6-LacZ construct (as an irrelevant overexpressed control) were stimulated with 50 ng/ml NGF for 72 h. The percentage of cells bearing neurites in each case is shown. Results are expressed as the mean $\pm$ SD of at least 100 cells counted in three independent experiments.

similar to those obtained with full-length PAK1^{H83,86L}. In contrast, the N-terminal PAK1^{H83,86L}-CAAX membrane-targeted construct had more dramatic effects on cell morphology, and >50% of the cells resembled those transfected with activated Rac or Cdc42. Moreover, a few cells appeared to generate neurites after 72 h (Figure 8A).

Fig. 6. Translocation of endogenous PC12 PAK to the membrane fraction upon NGF stimulation. Membrane-enriched fractions of PC12 cells prepared as described in Materials and methods were separated by SDS-PAGE and blotted onto nitrocellulose. The blots were probed with a polyclonal anti-PAK antibody and [^{125} I]protein A-beads prior to exposure of the blot to a Molecular Dynamics Phosphor-screen. The screen was then scanned by a PhosphorImager (Molecular Dynamics) and the bands quantitated to give their average density relative to background. The graph shows the average phosphorimage units on the y-axis and the inset shows the appearance of the 62 kDa PAK band over time in 20 μ g of membrane-enriched fractions. The final lane shows the immunoreactive bands in a total lysate of PC12 cells (equivalent to 10^6 cells). Similar results were obtained in three independent experiments.

Fig. 7. Schematic diagram of PAK1 constructs inserted into the pCMV6M expression vector. The map shows proline-rich regions, the PBD domain and the kinase domain and their location in the constructs used to map PAK activity in unstimulated and NGF-stimulated PC12 cells.

Portions of PAK1 serve as dominant-negative inhibitors and block NGF-induced neurite outgrowth

The redistribution of an endogenous PAK isoform to PC12 membranes following NGF stimulation suggests that PAK is involved in the NGF signaling pathway which leads to neurite outgrowth. We expressed the N- and C-terminal portions of PAK1, each of which was inactive in itself, to see if we could block NGF signaling. Both the N- and C-terminal fragments acted as dominant-negative inhibitors of the NGF-induced neurite outgrowth (Figure 8B). This further supported the notion that structural elements present in each domain were needed to provide the appropriate

functional interactions. Full-length wild-type PAK1 and PAK1^{H83,86L} had no inhibitory effect on NGF signaling.

The PAK1^{H83,86L} N-terminal construct caused less inhibition of neurite outgrowth when overexpressed in NGF-stimulated PC12 cells than the unmutated N-terminus (Figure 8B). This suggested that the intact PBD domain of the N-terminal fragment could be contributing to the inhibition of NGF signaling by titrating out activated GTPases in these cells. To investigate the relative contribution of the PBD or other N-terminal domains to this effect, we generated three PAK1 fragments: amino acids 1–74, the PBD domain (amino acids 67–150) and amino acids 169–206, which covered virtually the entire sequence of the N-terminal fragment (Figure 7). The 1–74 fragment had no inhibitory effect on NGF signaling when overexpressed in stimulated cells, but both the PBD domain construct and, surprisingly, the 169–205 domain caused a substantial inhibition of NGF-induced neurite outgrowth (Figure 9). These results suggest that, in addition to the GTPase-binding property of the PBD contained within amino acids 67–150 of the PAK1 N-terminus, an additional structural domain C-terminal to the PBD is critical for interactions of PAK1 with other signaling components in this system.

Discussion

Rho GTPases in neurite outgrowth

The signal transduction pathways responsible for neuronal cell differentiation and neurite outgrowth have been the subject of intense investigation over recent years. In PC12 cells, a well-characterized model of neuronal differentiation, a Ras-dependent MAP kinase cascade has been shown to regulate differentiation and neurite outgrowth

Fig. 8. Expression of N- and C-terminal PAK constructs in PC12 cells. Cells expressing constructs were detected by staining with the anti-myc antibody as previously described and actin-rich neurite-like extensions were visualized by co-staining with TRITC-phalloidin. (A) Quantitation of neurite outgrowth in PC12 cells overexpressing N- and C-terminal PAK1-CAAX-tagged constructs compared with wild-type PAK1-CAAX. (B) Effects of cytoplasmically expressed truncated N- and C-terminal constructs on NGF-induced neurite outgrowth in PC12 cells. Fifty to 150 cells were counted each time and the results are expressed as the mean $\pm$ SD from three independent experiments.

following growth factor stimulation (Thomas *et al.*, 1992; Wood *et al.*, 1992; Cowley *et al.*, 1994; Kamata *et al.*, 1996). More recently, a growth factor-independent c-Jun N-terminal kinase (JNK) pathway which does not require Ras has also been demonstrated to induce neurite outgrowth in PC12 cells (Kobayashi *et al.*, 1997; Yao *et al.*, 1997). These studies have concentrated on early signaling events which lead to cellular differentiation and subsequent neurite outgrowth. It is apparent that as a result of these events, other signaling systems in the cell must be activated

Fig. 9. Quantitation of neurite outgrowth from NGF-stimulated PC12 cells expressing various regions of PAK1. Cells expressing each construct were detected by staining for the myc epitope, and actin-rich neurite extensions were visualized more easily by co-staining with TRITC-phalloidin. At least 100 cells were counted in each experiment, and the results show the mean $\pm$ SD from three independent experiments.

to initiate the coordinated turnover of the actin cytoskeleton necessary for neurite outgrowth. In fibroblasts and other cell types, it is now widely accepted that members of the Rho family of GTPases are key mediators of the cytoskeletal changes that occur following cell stimulation by growth factors (Ridley and Hall, 1992; Ridley *et al.*, 1992; Nobes and Hall, 1995). It is becoming more apparent that these signaling molecules are also critical components of the signaling systems which lead to cytoskeletally driven neurite outgrowth in neuronal cell lines (Kozma *et al.*, 1997).

In this study, we establish a key role for both Rac and Cdc42 in mediating NGF-induced neurite outgrowth from PC12 cells. Dominant-negative constructs of these GTPases, which have been used to confirm roles for Rac and Cdc42 in regulating other systems, completely block the response of PC12 cells to NGF. However, Rac and Cdc42 activity was necessary, but not sufficient, for neurite formation, as expression of activated variants of these GTPases in unstimulated cells was unable to induce polarized neurite outgrowth. The active constructs did, however, have dramatic effects on the morphology of the PC12 cells, causing a spreading out of the cell, peripheral membrane ruffling and, in some cases, filopodia production. These effects are similar to those reported for Rac and Cdc42 in fibroblasts (Nobes and Hall, 1995).

PAK regulates the PC12 cytoskeleton and neurite outgrowth

The search for effectors of Rac and Cdc42 led to the discovery of the PAK family of p21-activated kinases. These ser/thr kinases have been implicated in the morphological changes associated with Rac and Cdc42 in other cell types (Dharmawardane *et al.*, 1997; Manser *et al.*, 1997; Sells *et al.*, 1997). In particular, overexpression of the PAK1^{H83,86L} variant, which is mutated in the GTPase-

binding domain, caused changes in the fibroblast cytoskeleton similar to those induced by activated Rac (Sells *et al.*, 1997). This construct, although unable to bind Rac or Cdc42, is believed to mimic the structural changes which occur upon GTPase binding, resulting in both constitutive kinase activity and enhanced binding of SH3-containing regulatory proteins to proline-rich motifs in the N-terminus (Sells *et al.*, 1997). We find in PC12 cells that overexpression of wild-type PAK1 had little effect on cell morphology, the cells remaining rounded and appearing completely undifferentiated. However, expression of the PAK1^{H83,86L} variant did induce the appearance of short actin-rich 'spikes' from the surface of the cells, which suggested that PAK1 was capable of regulating membrane and cytoskeletal outgrowth in these cells.

The process of neurite formation requires assembly of cytoskeletal elements at specific membrane sites where appropriate connections with partner neurons can be established. This polarized process is akin to the polarized bud assembly process in yeast, as well as development of a leading edge in motile fibroblasts, both of which appear to involve PAK-related kinases, perhaps in a specific membrane-associated localization (Cvrckova *et al.*, 1995; Peter *et al.*, 1996; Dharmawardane *et al.*, 1997; Leberer *et al.*, 1997a). Binding of activating GTPases to PAKs not only stimulates kinase activity but would also be expected to alter the subcellular localization of PAKs, since the GTPases themselves are activated at the membrane level and bind to membranes via their isoprenylated C-terminus. We and others have demonstrated that localization of PAK to membranes results in an increase in its kinase activity (Lu *et al.*, 1997; G.M.Bokoch, A.M.Reilly, R.H.Daniels, C.C.King, A.Olivera, S.Spiegel and U.G.Knaus, submitted). Hypothesizing that PAK localization may be an important component of its effect on PC12 cells, we targeted PAK to membranes by the addition of a CAAX motif. Membrane targeting of PAK1 was sufficient to induce the polarized outgrowth of neurites from the cell surface (Figure 3A). As expected, the cytoskeletally active PAK1^{H83,86L} mutant was the most effective at inducing this phenotype, causing a similar percentage of cells to extend neurites as with NGF treatment. The membrane-targeted PAK appeared to be acting downstream of Rac or Cdc42, since the induced neurite formation was largely insensitive to dominant-negative Rac and Cdc42. The slight effect of the dominant-negative GTPases on PAK-induced neurite formation may reflect the requirement for Rac and Cdc42 in recruiting additional signaling components necessary for the process of neurite extension.

We identified the requirement for structural elements in both the N- and C-terminal regions of PAK1 for neurite formation, although PAK kinase activity was not necessary (Figures 5 and 8). In particular, both the PBD (amino acids 67–150) and an adjacent region (169–206) were required for PAK1 activity. The latter contains both a putative SH3-binding motif and an acidic glutamic acid/aspartic acid-rich region (ED domain) conserved in all PAK isoforms. We speculate that these regions may act to localize PAKs properly and/or to recruit signaling proteins necessary to initiate neurite extension.

PAK(s) are required components of NGF-induced neurite formation

Reasoning that important functional domains on PAK when expressed in isolation might serve to block the

function of the endogenous protein, we used these constructs as dominant-negative antagonists to establish that NGF-induced neurite extension is dependent upon PAK (Figures 8B and 9). This was supported by our data which show that an endogenous PAK in PC12 cells, which appears to be PAK2 based upon size and antibody reactivity, is recruited to the membrane fraction in response to stimulation by NGF (Figure 6). Although the experiments described in this study utilized PAK1 constructs, the high level of sequence conservation within the PAK family suggests that the isoforms are likely to be functionally conserved as well, and this is borne out by the fact that the truncated PAK1 constructs acted as dominant-negative inhibitors of NGF signaling. Moreover, PAK1 constructs have been used to block endogenous PAK2 cytoskeletal activity in other cell models where PAK2 was the predominant isoform (Rudel and Bokoch, 1997). In addition to the fact that the PAK1-CAAX-induced neurites appear to be morphologically similar to those induced by NGF in terms of neurite thickness and the presence of growth cones (Figure 3B), the extensions also stain positively for tubulin and GAP-43, two markers which indicate a level of complexity above simple actin microfilament-based outgrowths. Moreover, pharmacological modulators of the cytoskeleton have similar inhibitory effects on PAK-CAAX- and NGF-induced neurites. Both structures were inhibited by long-term treatment with the actin-capping drug cytochalasin B, and the microtubule-disrupting agent colchicine caused complete retraction of both structures (data not shown). The similarities between the neurites induced by membrane-targeted PAK and those induced by NGF, coupled with the evidence for a dominant-negative effect of PAK constructs toward NGF, strongly support a role for PAK(s) in normal NGF signaling.

A model for PAK action in NGF signaling

The model shown schematically in Figure 10 suggests that localization and activation of PAK by Rho family GTPases result in the formation of a protein complex which regulates polarized cytoskeletal outgrowth. Although the identification of other interacting proteins remains to be accomplished, the analogies to polarized cytoskeletal rearrangements mediated by PAK homologs in lower eukaryotic systems are striking. Ste20 (the yeast homolog of PAK) appears to exist in a complex of proteins which mediate polarized morphogenesis and cytoskeletal organization during cytokinesis (Cvrckova *et al.*, 1995; Leeuw *et al.*, 1995). In *Caenorhabditis elegans*, a nematode homolog of PAK is involved in the cytoskeletal reorganization during embryonic body elongation (Chen *et al.*, 1996), and a *Drosophila* homolog (DPAK) has been described which localizes to adhesive complexes and is involved in maintenance of cytoskeletal structures during dorsal closure in embryonic development (Harden *et al.*, 1996). These studies, along with evidence from fibroblast models, and the data presented here suggest that PAK family members are important molecular elements in dynamic cytoskeletal processes.

It is of interest that inactivation of PAK1 kinase activity (Sells *et al.*, 1997; Tang *et al.*, 1997) has no effect on the ability of membrane-targeted wild-type PAK1 or PAK1^{H83,86L} to stimulate neurite formation. This finding is consistent, however, with our previous observation that

Fig. 10. A model for the role of PAK in NGF-induced signaling of neurite outgrowth in PC12 cells. The schematic shows a recruitment of PAK to the membrane by isoprenylated, GTP-bound Rac or Cdc42, interaction with a putative protein/multiprotein complex (X) and subsequent polarized outgrowth driven by polymerizing cortical actin filaments. (1) NGF binding to its receptor (Trk) leads to a signal transduction cascade (Ras-dependent MAP kinase cascade, etc.; see text) resulting in activation of Rac/Cdc42, probably by release from a cytoplasmic GDI and subsequent nucleotide exchange at the membrane level by a guanine nucleotide exchange factor. (2) Isoprenylated Rac/Cdc42-GTP localized to the membrane recruits PAK by binding to the N-terminal p21-binding domain. Specific membrane lipids may contribute to PAK recruitment and activation as well (G.M.Bokoch, A.M.Reilly, R.H.Daniels, C.C.King, A.Olivera, S.Spiegel and U.G.Knaus, submitted). (3) This localization and unfolding of Pak allows its interaction with an unknown protein or proteins, generating a complex which mediates (4) polarized actin polymerization and subsequent neurite extension. This model suggests a possible scaffolding role for PAK, independent of its kinase activity, which provides a localized cue for the polarized extension of the neurite. It is likely that other GTPase-dependent and/or -independent proteins will also be recruited to the site of cytoskeletal reorganization and contribute to the dynamics of actin assembly and neurite elongation. It is possible that the kinase activity of PAK may also exert effects on this signaling pathway *in vivo*, e.g. in the dissolution of adhesive interactions important for allowing growth cone extension through a complex extracellular matrix.

the effect of PAK1 on the fibroblast cytoskeleton was similarly not dependent on PAK1 kinase activity (Sells *et al.*, 1997), and suggests that it is the ability of PAK1 to interact with other proteins through its N-terminus, perhaps via the four putative SH3-binding domains present, which is critical for polarized cytoskeletal reorganization. One role of PAK(s) may thus be to serve as an adaptor or scaffolding protein, linking cytoskeletal regulatory molecules together at appropriate sites (Figure 10). Localization of PAK to appropriate sites may normally be dependent upon targeting via Rac and/or Cdc42, which in turn may require specific regulation by guanine nucleotide dissociation inhibitors and membrane-localized guanine nucleotide exchange factors (Bokoch *et al.*, 1994; Michiels *et al.*, 1997). That localization cues are necessary for neurite formation is supported by the ineffectiveness of constitutively active Rac and Cdc42 at inducing neurites (Figure 2), even though Rac and Cdc42 are normally required for this process to occur (Figure 1). The overexpression of the unregulated active GTPases simply appears to cause gross, unpolarized, morphological changes in the PC12 cells. The targeting of PAK to the membrane, acting downstream of the GTPases (Figure 4), may act as a polarization cue, triggering sustained outgrowth from a distinct focus.

The importance of localizing signaling complexes during neural development has also been shown in genetic studies by Zipursky and colleagues (Garritty *et al.*, 1996). This study demonstrated that axon guidance and targeting in the *Drosophila* photoreceptor system require an SH2/SH3-containing adaptor protein homologous to the mammalian Nck protein. It is intriguing to note that Nck binds to the first proline-rich SH3-binding motif on PAK1 and has been suggested to be a means of specific membrane and/or receptor targeting of PAK(s) (Bokoch *et al.*, 1996; Galisteo *et al.*, 1996). A role for mammalian Nck in PC12 neurite outgrowth mediated by PAK1 appears doubtful, however, as we find no requirement for the first SH3 domain-binding/Nck-binding site of PAK1 in neurite induction (Figure 9). Furthermore, preliminary experiments in which we attempted to induce neurite formation by membrane targeting of either Nck itself or the Nck second SH3 domain were negative (R.H.Daniels and G.M.Bokoch, unpublished data). Although we cannot rule out an interaction of Nck with one of the other PAK1 SH3-binding domains in the PC12 cell, our data suggest that a protein interacting with the amino acid region 169–206 in PAK(s) may be critical. It is of interest that a protein target for PAK recently has been described which binds to the proline-rich area within this domain (R.Cerione, personal communication). The possible role of this and other N-terminal-interacting proteins in the process of PAK-mediated neurite outgrowth will be investigated in future studies.

Materials and methods

Plasmids

cDNA expression plasmids containing full-length PAK1 and its various mutants using the pCMV6M (CMV promoter, N-terminal myc tag) have been described elsewhere (Sells *et al.*, 1997). Wild-type PAK1 with the C-terminal 17 amino acid farnesylation signal sequence of K-Ras (KDGKKKKKKSKTKCVIM) also in the pCMV6M vector was a gift of J.Chernoff (Fox Chase Cancer Center). All other full-length CAAX-tagged PAK1 constructs were generated by subcloning into the *Bam*HI and *Nhe*I sites of pCMV6M. Mutation of the terminal cysteine to an alanine in the CAAX motif was achieved by PCR amplification using a 3' oligonucleotide containing the mutated base pairs (5' CCGGAATTCT-CACATAATTACAGCCTTTGTCTT 3'). Truncated versions of non-CAAX-tagged and CAAX-tagged PAK1 were generated by using internally derived oligonucleotides containing the appropriate restriction sites and amplifying the desired region by PCR. PCR fragments were then digested and subcloned into either pCMV6M (*Bam*HI and *Eco*RI) or pCMV6M-CAAX (*Bam*HI and *Nhe*I).

Full-length cDNAs encoding wild-type and mutations of Rac2 (WT, T17N and Q61L) and Cdc42 (WT, T17N and Q61L) were subcloned into the pcDNA3 expression plasmid containing an N-terminal myc tag via *Bam*HI and *Eco*RI sites generated by PCR using oligonucleotides flanking the cDNA coding sequence.

Transfection of COS cells and detection of membrane localization

COS 7 cells grown to 75% confluence on 10 cm tissue culture dishes were transiently transfected with 10 µg of pCMV6M vector containing either wild-type PAK1, wild-type PAK1-CAAX or other constructs with or without the CAAX tag using the lipofectamine transfection protocol (Gibco BRL). The cells were allowed to express the protein for 48 h post-transfection and were then washed in phosphate-buffered saline (PBS) and scraped into 150 µl of detergent-free lysis buffer [25 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 5% glycerol]. The cell suspension was then lysed by homogenizing with 50 strokes of a tight-fitting Dounce homogenizer and the homogenate was spun at 10 000 r.p.m. in a benchtop Eppendorf microfuge to remove unbroken cells and large

debris. A protein assay was carried out on the clarified homogenate using a BCA reagent protein assay (Pierce). Equivalent amounts of this clarified homogenate was then transferred to fresh tubes and spun at 100 000 g for 1 h in a Beckman TL100 rotor to pellet the cell membranes. The membrane pellet was resuspended in 50 µl of SDS-PAGE sample buffer, and both the crude membrane fraction and the cytosol-enriched supernatant were run on 10% polyacrylamide gels to resolve the proteins. Following electrophoresis, the proteins were transferred to nitrocellulose and probed with 9E10 anti-myc antibody to detect the expression and localization of the transfected proteins (Evan *et al.*, 1985; Robertson *et al.*, 1995).

Culture and differentiation of PC12 cells

PC12 cells were grown in culture flasks (Falcon) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum (HS) and 5% fetal calf serum (FCS). For transfection and differentiation, cells were transferred into 35 mm Petri dishes (Falcon), which had been pre-coated with 10 µg/ml Type I collagen (Upstate Biotechnology) to facilitate neurite outgrowth, at a density of 2×10^5 cells/well and grown overnight in standard medium. Differentiation was stimulated with 50 ng/ml NGF (Grade II, 2.5S, Boehringer Mannheim) and allowed to proceed for 72 h. Cells were exposed to NGF in both complete medium and medium containing 2% HS and 1% FCS, with little difference in the percentage of cells generating neurites observed in either condition. To test the effects of cytoskeletal inhibitors, PC12 cells were cultured and exposed to NGF or transfected as described. Cytochalasin B (8 µg/ml) or colchicine (10 µM) were added after either 24 or 48 h exposure to NGF or post-transfection, and neurite extension was assessed as described below.

Transient transfection of PC12 cells

PC12 cells grown overnight on collagen-coated 35 mm dishes as described were washed and then exposed to Superfect transfection agent (Qiagen) and 2 µg of DNA for 6 h at 37°C. Cells were then allowed to express the constructs for 72 h post-transfection prior to immunostaining either in the presence or absence of 50 ng/ml NGF. For co-transfection experiments using the PAK1-CAAX constructs and the Rac2^{T17N} and Cdc42^{T17N}, the GTPase plasmids were added in 6-fold excess of the PAK constructs (2.4:0.4 µg). PAK transfectants were specifically detected using the PAK1 antibody (Knaus *et al.*, 1995), which did not stain the untransfected PC12 cells or those expressing only the GTPase. These cells were then scored for neurite outgrowth, as described below.

Immunofluorescent staining and F-actin staining of PC12 cells

PC12 cells grown on the 35 mm dishes were fixed in 3% paraformaldehyde in PBS for 20 min at room temperature. The fixed cells were washed twice in PBS and then permeabilized for 5 min in PBS containing 0.2% Triton X-100. Cells were washed once more and then incubated with 2 µg of monoclonal 9E10 anti-myc antibody or 1:300 dilution of in-house polyclonal anti-PAK1 (Knaus *et al.*, 1995) to detect transfected cells or 2 µg of monoclonal anti-GAP43 (Calbiochem) as a neurite marker, in antibody diluent (PBS/0.5% BSA/1% goat serum) for 1 h at room temperature. The cells were then washed three times for 5 min in immunowash buffer (PBS/0.1% BSA/0.02% saponin) prior to the addition of 4 µg/ml of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (or rabbit) IgG and 0.1 µg/ml tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma) for a further 1 h. The stained cells were washed 3 × 10 min in immunowash buffer and 2 × 10 min in PBS prior to addition of 10 µl of fluoromount (Southern Biotechnology Associates, Inc.), and covered with a 22 mm² glass coverslip.

Microscopic evaluation of neurite outgrowth

Fluorescently stained PC12 cells were visualized on a Nikon Labphot-2 microscope equipped with a Nikon EF-D episcopic fluorescence attachment. The total number of cells expressing myc-tagged proteins was counted and cells with one or more neurite extensions of more than two body lengths were regarded as neurite positive. The number of cells with such outgrowths was then expressed as a percentage of the total number of cells. All cells on any individual dish were counted up to a total of 250 cells. Representative photographs of expressing cells were taken using a Nikon FX-35 DX camera attachment.

Localization of endogenous PAK to membrane-enriched fractions

PC12 cells (4×10^6 cells/ml) were seeded out onto collagen-coated (10 µg/ml, UBI) 10 cm tissue culture dishes (Corning) and allowed to

adhere for 6 h. The cells were then placed into low serum (2% HS, 1% FCS) overnight at 37°C to quiesce. Quiescent cells were then stimulated for various time points (0–48 h) with 50 ng/ml NGF. Membrane-enriched fractions were prepared by nitrogen cavitation in 2 ml of bomb buffer (10 mM PIPES, pH 7.3, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂), removal of unbroken cells and large debris by centrifuging at 1500 r.p.m., and pelleting of the membrane-enriched fraction by high-speed centrifugation at 45 000 r.p.m. in a Beckman Ti-70.1 rotor using a Beckman LP-55 ultracentrifuge. The membrane-enriched pellet was then solubilized in 100 µl of lysis buffer (lysis buffer + 1% NP-40) by passing through a 21-gauge syringe needle and by sonication for 20 s in a bath sonicator. Protein determinations were made using a BCA reagent protein assay (Pierce) and the membrane fractions were adjusted to 0.5 µg/µl using SDS sample buffer and then boiled for 5 min. Twenty µg of membrane-enriched fraction was separated by SDS-PAGE (9% gels) and the separated proteins were transferred to nitrocellulose. The nitrocellulose membrane was then probed with in-house polyclonal anti-PAK peptide antibodies (Knaus *et al.*, 1995) and either alkaline phosphatase-conjugated secondary antibody or ¹²⁵I-conjugated protein A.

Acknowledgements

Thanks to Dr Roberta Gottlieb for initially supplying the PC12 cells. Thanks also to Drs Ulla Knaus and Shelley Halpain for helpful comments during the preparation of this manuscript. Antonette Lestelle provided expert secretarial assistance. Technical assistance was provided by Brooke Emerling, and preparation of PAK-PBD antibody by Alaina M. Reilly is gratefully acknowledged. This work was supported by USPHS grants numbers GM44428 and GM39434. This is publication No. 11091-IMM of The Scripps Research Institute.

References

- Ayala, J., Touchot, N., Zahraoui, A., Tavitian, A. and Prochianyz, A. (1990) The product of rab2, a small GTP binding protein, increases neuronal adhesion and neurite outgrowth *in vitro*. *Neuron*, **4**, 797–805.
- Bar-Sagi, D. and Feramisco, J.R. (1985) Microinjection of the ras oncogenic protein into PC12 cells induces morphological differentiation. *Cell*, **42**, 841–848.
- Bentley, D. and O'Connor, T.P. (1994) Cytoskeletal events in growth cone steering. *Curr. Opin. Neurobiol.*, **4**, 43–48.
- Bokoch, G.M., Bohl, B.P. and Chuang, T.-H. (1994) Guanine nucleotide exchange regulates membrane translocation of rac/rho GTP-binding proteins. *J. Biol. Chem.*, **269**, 31674–31679.
- 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.
- Brzeska, H., Knaus, U.G., Wang, Z., Bokoch, G.M. and Korn, E.D. (1997) p21-activated kinase (PAK) has substrate specificity similar to *Acanthamoeba* myosin I heavy chain kinase and activates *Acanthamoeba* myosin I. *Proc. Natl Acad. Sci. USA*, **94**, 1092–1095.
- Burbelo, P.D., Dreschel, D. and Hall, A. (1995) A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J. Biol. Chem.*, **270**, 29071–29074.
- Chant, J. (1994) Cell polarity in yeast. *Trends Genet.*, **10**, 328–333.
- Chen, W., Chen, S., Yap, S.F. and Lim, L. (1996) The *Caenorhabditis elegans* p21-activated kinase (CePAK) colocalizes with CeRac1 and CDC42Ce at hypodermal cell boundaries during embryo elongation. *J. Biol. Chem.*, **271**, 26362–26368.
- Cowley, S., Paterson, H., Kemp, P. and Marshall, C.J. (1994) Activation of MAP kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell*, **77**, 841–852.
- Cvrckova, F., DeVirgilio, C., Manser, E., Pringle, J.R. and Nasmyth, K. (1995) Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. *Genes Dev.*, **9**, 1817–1830.
- Dharmawardane, 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.
- Evan, G.I., Lewis, G.K., Ramsay, G. and Bishop, J.M. (1985) Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol. Cell. Biol.*, **5**, 744–756.
- 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.

- Garrity,P.A., Rao,Y., Salecker,I., McGlade,J., Pawson,T. and Zipursky,S.L. (1996) *Drosophila* photoreceptor axon guidance and targeting requires the dreddlocks SH2/SH3 adapter protein. *Cell*, **85**, 639–650.
- Gebbink,M.F.B.G., Krannenburg,O., Poland,M., van Horck,F.P.G., Houssa,B. and Moolenaar,W.H. (1997) Identification of a novel, putative rho-specific GDP/GTP exchange factor and a rhoA-binding protein: control of neuronal morphology. *J. Cell Biol.*, **137**, 1603–1613.
- Gordon-Weeks,P.R. (1991) Growth cones: the mechanism of neurite advance. *BioEssays*, **13**, 235–239.
- Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**, 42–52.
- Harden,N., Lee,J., Loh,H.-Y., Ong,Y.-M., Tan,I., Leung,T., Manser,E. and Lim,L. (1996) A *Drosophila* homolog of the Rac- and Cdc42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures. *Mol. Cell Biol.*, **16**, 1896–1908.
- Kamata,H., Tanaka,C., Yagisawa,H., Matsuda,S., Gotoh,Y., Nishida,E. and Hirata,H. (1996) Suppression of nerve growth factor-induced neuronal differentiation of PC12 cells. *J. Biol. Chem.*, **271**, 33018–33025.
- 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.
- Kobayashi,M. *et al.* (1997) Expression of a constitutively active phosphatidylinositol 3-kinase induces process formation in rat PC12 cells. *J. Biol. Chem.*, **272**, 16089–16092.
- Kozma,R., Sarner,S., Ahmed,S. and Lim,L. (1997) Rho family GTPases and neuronal growth cone remodeling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Mol. Cell Biol.*, **17**, 1201–1211.
- Leberer,E., Wu,C., Leeuw,T., Fourest-Lieuvin,A., Segall,J.E. and Thomas,D.Y. (1997a) Functional characterization of the Cdc42p binding domain of yeast Ste20p protein kinase. *EMBO J.*, **16**, 83–97.
- Leberer,E., Thomas,D.Y. and Whiteway,M. (1997b) Pheromone signaling and polarized morphogenesis in yeast. *Curr. Opin. Genet. Dev.*, **7**, 59–66.
- Leeuw,T., Fourest,A., Wu,C., Chenevert,J., Clark,K., Whiteway,M., Thomas,D.Y. and Leberer,E. (1995) Pheromone response in yeast: association of Bem1p with proteins of the MAP kinase cascade and actin. *Science*, **270**, 1210–1213.
- 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.
- Luo,L., Liao,Y.J., Jan,L.Y. and Jan,Y.N. (1994) Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev.*, **8**, 1787–1802.
- Luo,L., Hensch,T.K., Ackerman,L., Barbel,S., Jan,L.Y. and Jan,Y.N. (1996) Differential effects of the Rac GTPase on purkinje cell axons and dendritic trunks and spines. *Nature*, **379**, 837–840.
- Manser,E., Leung,T., Salihuddin,H., Tan,L. and Lim,L. (1994) A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature*, **367**, 40–46.
- Manser,E., Huang,H.-Y., Loo,T.-H., Chen,X.-Q., Dong,J.-M., Leung,T. and Lim,L. (1997) Expression of constitutively active α -PAK reveals effects of the kinase on actin and focal complexes. *Mol. Cell Biol.*, **17**, 1129–1143.
- Michiels,F., Stam,J.C., Hordijk,P.L., van der Kammen,R.A., Ruuls-Van Stalle,L., Feltkamp,C.A. and Collard,J.G. (1997) Regulated membrane localization of tiam 1, mediated by the NH₂-terminal pleckstrin homology domain, is required for rac-dependent membrane ruffling and c-jun NH₂-terminal kinase activation. *J. Cell Biol.*, **137**, 387–398.
- Nobes,C.D. and Hall,A. (1995) Rho, Rac and Cdc42 GTPases regulate the assembly of multi-molecular focal complexes associated with actin stress fibers, lamellipodia and filopodia. *Cell*, **81**, 53–62.
- Noda,M., Ko,M., Ogura,A., Lim,D.G., Amano,T., Takano,T. and Ikawa,Y. (1985) Sarcoma viruses carrying *ras* oncogenes induce differentiation-associated properties in a neuronal cell line. *Nature*, **318**, 73–75.
- Peter,M., Neiman,A.M., Park,H.-O., van Lohuizen,M. and Herskowitz,I. (1996) Functional analysis of the interaction between the small GTP binding protein Cdc42 and the Ste20 protein kinase in yeast. *EMBO J.*, **15**, 7046–7059.
- Ridley,A.J. and Hall,A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibres in response to growth factors. *Cell*, **70**, 389–399.
- Ridley,A.J., Paterson,H.F., Johnson,C., Diekmann,D. and Hall,A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*, **70**, 401–410.
- Robertson,D., Paterson,H.F., Adamson,P., Hall,A. and Monaghan,P. (1995) Ultrastructural localization of ras-related proteins using epitope-tagged plasmids. *J. Histochem. Cytochem.*, **43**, 471–480.
- Rudel,T. and Bokoch,G.M. (1997) Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. *Science*, **276**, 1571–1574.
- 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.
- Tang,Y., Chen,Z., Ambrose,D., Liu,J., Gibbs,J.B., Chernoff,J. and Field,J. (1997) Kinase deficient Pak1 mutants inhibit Ras transformation of rat-1 fibroblasts. *Mol. Cell Biol.*, **17**, 4454–4464.
- Tauzon,P.T. and Traugh,J.A. (1984) Activation of actin-activated ATPase in smooth muscle by phosphorylation of myosin light chain with protease-activated kinase I. *J. Biol. Chem.*, **259**, 541–546.
- Thomas,S.M., DeMarco,M., D’Arcangelo,G., Halegona,S. and Brugge,J.S. (1992) Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell*, **68**, 1031–1040.
- Tigyi,G., Fischer,D.J., Sebök,A., Yang,C., Dyer,D.L. and Miledi,R. (1996a) Lysophosphatidic acid-induced neurite retraction in PC12 cells: control by phosphoinositide signaling and rho. *J. Neurochem.*, **66**, 537–548.
- Tigyi,G., Fischer,D.J., Sebök,A., Marshall,F., Dyer,D.L. and Miledi,R. (1996b) Lysophosphatidic acid-induced neurite retraction in PC12 cells: neurite-protective effects of cyclic AMP signaling. *J. Neurochem.*, **66**, 549–558.
- Wood,K.W., Sarnecki,C., Roberts,T.M. and Blenis,J. (1992) Ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1 and RSK. *Cell*, **68**, 1041–1050.
- Wu,C., Lee,S., Furmaniak-Kazmierczak,E., Cote,G.P., Thomas,D.Y. and Leberer,E. (1996) Activation of myosin-I by members of the Ste20 protein kinase family. *J. Biol. Chem.*, **271**, 31787–31790.
- Yao,R., Yoshihara,M. and Osada,H. (1997) Specific activation of a c-Jun NH₂-terminal kinase isoform and induction of neurite outgrowth in PC12 cells by staurosporine. *J. Biol. Chem.*, **272**, 18261–18266.

Received September 18, 1997; revised October 23, 1997;
accepted November 17, 1997