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PAK promotes morphological changes by acting upstream of Rac

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The serine/threonine kinase p21-activated kinase (PAK) has been implicated as a downstream effector of the small GTPases Rac and Cdc42. While these GTPases evidently induce a variety of morphological changes, the role(s) of PAK remains elusive. Here we report that overexpression of βPAK in PC12 cells induces a Rac phenotype, including cell spreading/membrane ruffling, and increased lamellipodia formation at growth cones and shafts of nerve growth factor-induced neurites. These effects are still observed in cells expressing kinase-negative or Rac/Cdc42 binding-deficient PAK mutants, indicating that kinase- and p21-binding domains are not involved. Furthermore, lamellipodia formation in all cell lines, including those expressing Rac binding-deficient PAK, is inhibited significantly by dominant-negative RacN17. Equal inhibition is achieved by blocking PAK interaction with the guanine nucleotide exchange factor PIX using a specific N-terminal PAK fragment. We conclude that PAK, via its N-terminal non-catalytic domain, acts upstream of Rac mediating lamellipodia formation through interaction with PIX.

Keywords: guanine nucleotide exchange factor/lamellipodia formation/neurite outgrowth/PAK/Rac

Introduction

Important aspects of the mechanisms by which nerve growth factor (NGF) and its receptor, Trk, transmit their signals to initiate neuronal differentiation are understood (Obermeier *et al*., 1994, 1995; reviewed in Kaplan and Stephens, 1994). However, little is known about the complex processes which follow these immediate signal transduction events and which provide a lasting basis for neurite formation and other features of a differentiated phenotype.

As neuronal differentiation is associated with one of the most dramatic cell morphological changes known, we considered the Rho family of small GTPases, which are implicated in cytoskeletal rearrangements and subsequent morphological changes (reviewed in Lim *et al*., 1996a; Tapon and Hall, 1997; Van Aelst and D'Souza-Schorey, 1997), as potentially involved candidates. Indeed, recent evidence indicates that two members of this family, namely Rac and Cdc42, play important roles in neurite outgrowth and neuronal development (Luo *et al*., 1997). More specifically, Rac and Cdc42 regulate the formation of lamellipodia and filopodia at neurite growth cones (Kozma *et al*., 1997). Such structures sample, collect and process important clues about the environment and, together with other structures, provide adhesive and tractive forces to determine and enforce an appropriate and directed movement of the growth cone. It is not known, however, how these GTPases mediate these cytoskeletal rearrangements that are crucial for a proper neurite outgrowth.

Previous findings describing the serine/threonine p21-activated kinase (PAK) as a target of Cdc42 and Rac (Manser *et al*., 1994) prompted us to investigate possible roles of the predominantly brain-expressed β-isoform of PAK (Manser *et al*., 1995) in morphological changes associated with neuronal differentiation. Here we report that PAK induces cytoskeletal rearrangements characteristic of Rac, including formation of lamellipodia. Interestingly, this does not involve PAK as a downstream effector, but rather as an upstream activator of Rac.

Results

Expression of PAK, PAK-KA and PAK-HL in PC12 cells results in distinct neurite outgrowth properties and neurite morphology

We generated two mutants of βPAK, a kinase-negative PAK (PAK-KA), in which Lys297 in the ATP-binding consensus sequence has been changed to alanine, and a p21(Rac/Cdc42) binding-deficient PAK (PAK-HL), in which His78 and His81 in the p21-binding domain (Manser *et al*., 1994; Burbello *et al*., 1995) have been changed to leucine residues (Sells *et al*., 1997), and expressed wild-type and mutant PAKs in PC12 cells by means of retrovirus-mediated gene transfer. For each construct, three independent cell lines were generated by recombinant virus infection and pooling of G418-selected clones. G418-resistant pools of PC12 cells infected with a virus carrying the empty retroviral expression vector (pLXSN) served as a control. To determine the expression levels of the PAK proteins in the different cell lines, equal amounts of protein from corresponding cell lysates were analysed by Western blotting using anti-βPAK antibodies. Figure 1A shows strong anti-βPAK signals from all transfectants, with the exception of control cells expressing no or very little endogenous βPAK.

Treatment of PC12 cells with NGF for several days revealed characteristic differences in neurite outgrowth and neurite morphology between the different cell lines (Figure 1B). Cells expressing wild-type PAK, PC12/PAK (WT), display a strong neurite outgrowth, developing long, robust and straight neurites; process formation in these cells is even more pronounced and faster than in

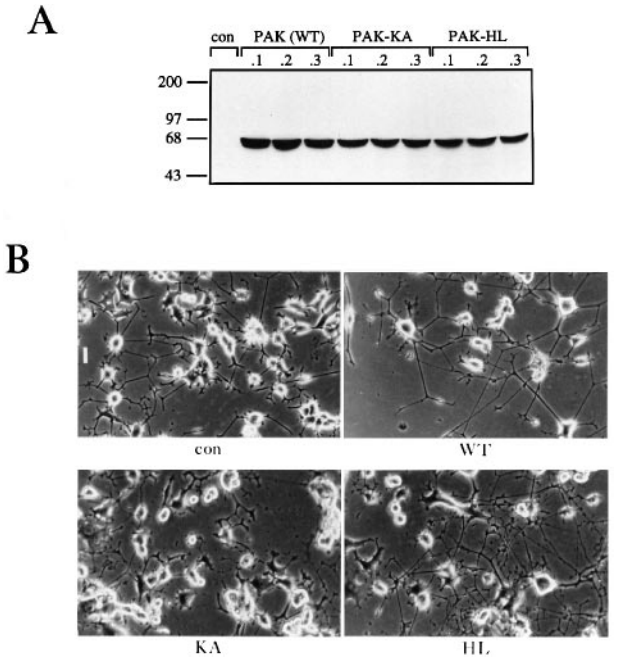

Fig. 1. Expression of PAK, PAK-KA and PAK-HL in PC12 cells results in distinct neurite outgrowth properties and neurite morphology. (**A**) Expression levels of PAK, PAK-KA and PAK-HL. PC12 transfectants stably expressing wild-type βPAK, the kinase-inactive mutant, PAK-KA or the p21 binding-deficient mutant, PAK-HL (three different cell lines each), or control transfectants (con) were lysed, and equal amounts of protein were separated by SDS–PAGE. After transfer to nitrocellulose membrane, proteins were detected with anti-βPAK antibodies. (**B**) Neurite outgrowth properties and neurite morphology of PC12 transfectants. Representative photographs of PC12 cells treated with NGF (100 ng/ml) for 4 days are shown. con, control transfectants; WT, PC12/PAK.2 cells; KA, PC12/PAK-KA.2 cells; HL, PC12/PAK-HL.2 cells. In each case, phenotypes of clones .1, .2 and .3 are essentially identical. Bar, 10 μm.

control cells. As compared with control cells, PC12/PAK-HL cells generate rather irregularly shaped, branched and varicose neurites. Furthermore, at early stages of process formation (i.e. ~2 days), the number of processes per cell is comparatively high and their length is generally reduced. Neurite outgrowth in PC12/PAK-KA cells is inhibited compared with control or any of the other cell lines. However, this does not reflect a general inability of these cells to generate proper neurites, but rather a slower and delayed response; after longer (>1 week) exposure to NGF, long, complex and fully developed neurites are formed. Having a moderate tendency to form neurites of irregular shape and varicose appearance, their overall neurite morphology is intermediate between that of control and PC12/PAK-HL cells.

PAK, JNK and ERK1/2 activities, and phosphorylation/co-precipitation by PAK of specific substrates

To examine the activity and specificity of βPAK and the two mutants, PAK-KA and -HL, proteins were precipitated from corresponding lysates with anti-βPAK antibodies and subjected to immune complex kinase assays with or without myelin basic protein (MBP) as exogenous substrate. Cellular proteins serving as substrates for phosphorylation by PAK (or possibly co-precipitated kinases) were visualized by autoradiography. As indicated in Figure 2A, specific phosphorylation products include proteins migrating as a triplet of bands of ~90/87/82 kDa and as doublets of 67/62 kDa and 36/33 kDa. The strongest phosphorylation occurs in PC12/PAK (WT) cells. PAK-HL, although having constitutive kinase activity, exhibits only ~50% of wild-type PAK's activity perhaps because it is unable to bind to and be activated by p21s. PAK activities do not change significantly upon addition of NGF (the slightly increased signals in the 2d NGF lanes of Figure 2A were not reproducible). The above-mentioned proteins are hardly phosphorylated in control immune complexes and even less so in PAK-KA precipitates, demonstrating that PAK-KA is kinase-dead and may even exert a dominant-negative effect on residual activity from endogenous PAK. Pp67 and possibly also pp62 probably correspond to autophosphorylated PAK. The identity of pp36/33 should be of particular interest as the protein(s) are specifically phosphorylated and/or associated with PAK after prolonged (2 days) NGF treatment; NGF treatment for 5–8 h or epidermal growth factor treatment for 2 days was much less effective (data not shown). This protein(s) may thus be functionally associated with neuronal differentiation. The possible nature of pp82 is discussed below (see last subsection of Results).

Quantitative determination of PAK activity towards MBP (not shown) accords with results from the above-described kinase assays without MBP. Addition of glutathione-*S*-transferase (GST)–Cdc42·GTPγS *in vitro* increased the activity of PAK isolated from PC12/PAK cells ~15-fold, but not, as expected, the activities of the kinase-dead PAK-KA and the Cdc42 binding-incompetent PAK-HL (Figure 2A, graph).

As c-Jun N-terminal kinase1 (JNK1) has been suggested to be activated downstream of Rac, Cdc42 (Coso *et al*., 1995; Minden *et al*., 1995) and PAK (Bagrodia *et al*., 1995; Brown *et al*., 1996), we examined JNK1 activity in the different cell lines by means of anti-JNK1 immune complex kinase assays using GST–Jun$^{1–79}$ as a substrate (Figure 2B). We could not find any correlation between PAK activity and JNK activity in our PC12 cell lines. NGF stimulation slightly increased JNK activity. Anisomycin, known as a potent JNK activator, was used as a positive control. Correspondingly, anisomycin had no effect on PAK activity (not shown).

ERK1 and ERK2 MAP kinase activities, which are known to be strongly induced by NGF treatment of PC12 cells (Boulton *et al*., 1991), were likewise independent of PAK (Figure 2C and data not shown).

Lamellipodia formation rate and cell spreading/membrane ruffling

Since activated Rac induces formation of lamellipodia (Ridley *et al*., 1992) and PAK has been implicated as a Rac effector, we examined the lamellipodia formation rates of the different cell lines. Video recording-assisted high resolution phase-contrast microscopy of differentiated cells under incubator conditions allowed us to examine hundreds of growth cones and neurites in several independent experiments and to deduce lamellipodia formation rates. These rates were determined separately for growth cones and neurite shafts (Figure 3B, left graph) as well as without such discrimination (combined values) (Figure 3B, right

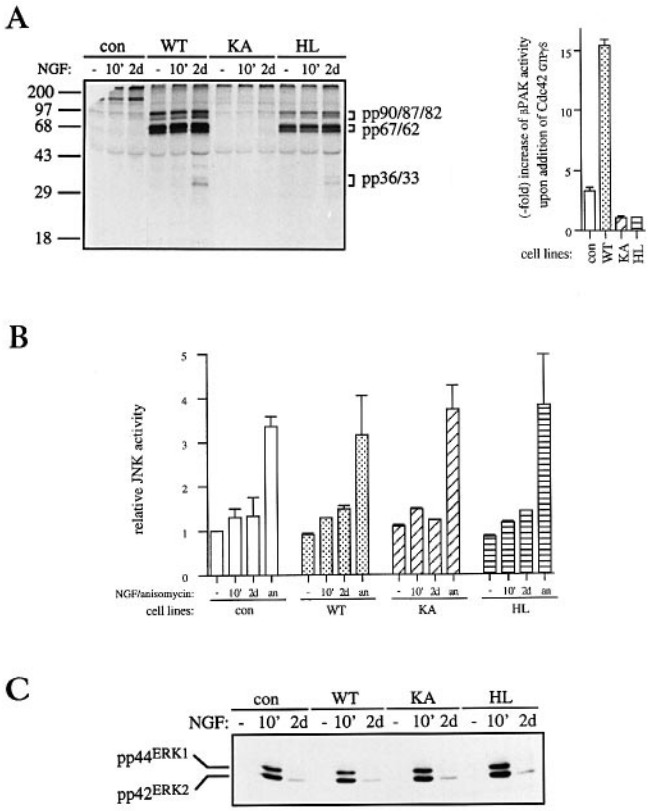

Fig. 2. Kinase activities of PAK/PAK mutants, JNK and ERK1/2 in PC12 transfectants. (**A**) PAK kinase activities and phosphorylation/co-precipitation of specific substrates. Left: PC12 control transfectants (con), PC12/PAK.2 cells (WT), PC12/PAK-KA.3 cells (KA) and PC12/PAK-HL.1 cells (HL) were grown to 20–40% confluency, starved in serum-free medium for 18 h, and then lysed either immediately (–) or after treatment with NGF (100 ng/ml) for 10 min (10') or 2 days. Then 350 μg of protein from each lysate was immunoprecipitated with anti-βPAK antibodies, and precipitates were subjected to an *in vitro* kinase assay using [γ-^{33}P]ATP, followed by SDS–PAGE. Gels were dried and phosphorylation products revealed by autoradiography. Specifically phosphorylated proteins are indicated at the right (see text for details). Identical results were obtained with the other clones (PC12/PAK.2 and .3, -KA.1 and .3, -HL.2 and .3) (not shown). Right: to determine the stimulation of PAK (WT, KA, HL) kinase activities by Cdc42, their activities towards MBP were examined as described above, adding 1 μg of MBP to the kinase reactions and including (or not) excess GTPγS-loaded GST–Cdc42, and quantified using a phosphoimager. Results displayed are from 3–4 independent experiments combining data from all cell lines (error bars: SEM). Similar values were obtained from cell lines expressing the same constructs. (**B**) JNK kinase activity. Lysates of all 10 cell lines were prepared as described in (A); additionally, as a positive control, some cells were stimulated with anisomycin (100 ng/ml; 30 min) prior to lysis (an). Proteins were immunoprecipitated using anti-JNK1 antibodies, and precipitates were subjected to an *in vitro* kinase assay with GST–Jun$^{1–79}$ as a substrate. Data shown are from 2–3 independent experiments combining data from all cell lines (error bars: SEM). (**C**) MAP kinase (ERK1/2) activity. Protein (110 μg) from each cell lysate, as described in (A), was subjected to SDS–PAGE and immunoblotted with phospho-specific p44/42MAPK antibodies. As confirmed by band shift and in-gel kinase assays (not shown), detected phosphorylation levels correspond to kinase activity. Equivalent results were obtained from all cell lines expressing the same constructs.

graph). Details on determination and quantification procedures are given in the legend to Figure 3B. PC12/PAK cells had an ~2-fold higher combined lamellipodia formation rate than control cells. Surprisingly, PC12/PAK-KA and /PAK-HL cell lines displayed even higher lamellipodia formation rates (combined rates are ~4-fold above control level), indicating that neither PAK activity nor PAK binding to Rac is required for PAK to mediate formation of lamellipodia. The increased rates involved lamellipodia at both growth cones and neurite shafts.

Another striking feature that distinguishes cell lines expressing PAK or PAK mutants from control cells was the frequent appearance of huge, flattened cells (Figure 4A). This morphology is a consequence of extensive cell spreading. Smoothly spread cells (without peripheral ruffling) were observed 2- to 3-fold more frequently in PAK-, PAK-KA- and PAK-HL-expressing cells as compared with control cells (Figure 4B). Spread cells with extensive membrane ruffling were essentially absent from control cells (frequency 0.05%), rarely observed in PC12/PAK cells (~0.3%) and comparatively frequent in PC12/PAK-KA (~4.8%) and -HL (~3.2%) cells (Figure 4). Besides lamellipodia formation, cell spreading and membrane ruffling can be another morphological consequence of Rac activation (Ridley *et al*., 1992; Altun-Gulekin and Wagner, 1996; Gebbink *et al*., 1997). As with lamellipodia formation, cell spreading/ruffling increases in the order PC12/con<PC12/PAK<PC12/PAK-KA, PC12/PAK-HL, corroborating the finding that PAK and, in particular, PAK-KA and PAK-HL induce a Rac phenotype in PC12 cells. However, the kinase activity of PAK is obviously not of importance in this context. Furthermore, the results with the Rac binding-incompetent PAK-HL mutant demonstrate that PAK does not induce Rac-type effects as a downstream effector of Rac. Thus, PAK functions either independently of Rac or, more likely, as an (upstream) activator of Rac, via its non-catalytic N-terminal domains.

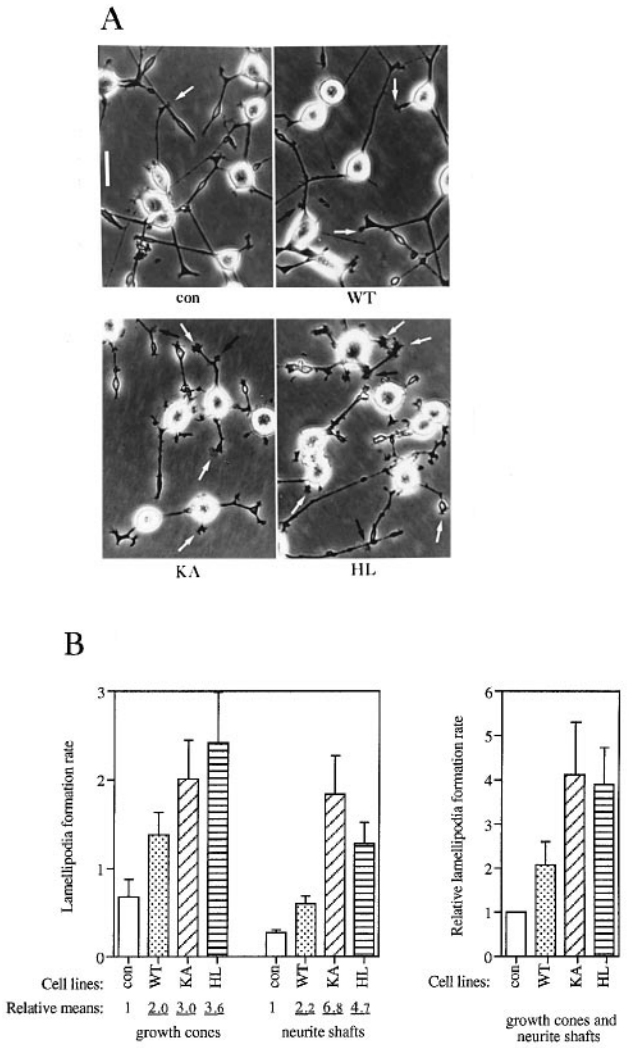

Fig. 3. Lamellipodia formation. (**A**) Cells were treated with NGF (100 ng/ml) for 2 days in the absence of serum. Photographs displayed are from control transfectants (con) and from PC12/PAK.2 (WT), /PAK-KA.2 (KA) and /PAK-HL.2 (HL) lines, and are in each case representative of all three clones. A few selected lamellipodia are marked by white (growth cones) and black arrows (neurite shafts). Bar, 10 μm. (**B**) For quantification purposes substantial lamellipodial structures formed during 3 min of video recording-assisted observation were counted. Lamellipodia formation rate, as depicted in the left graph, is defined as the ratio between the number of lamellipodia at growth cones or along neurite shafts, and the number or length (in multiples of 20 μm), respectively, of neurites subjected to counting. Values (means ± SEM) are derived from five independent experiments with four different determinations each; NGF treatment was for 2–4 days. Means (± SEM) of the combined, relative values (con = 1) are depicted in the right graph and reflect the average of the relative values for lamellipodia formation rate at both growth cones and neurite shafts, weighted according to the number of lamellipodia counted, respectively.

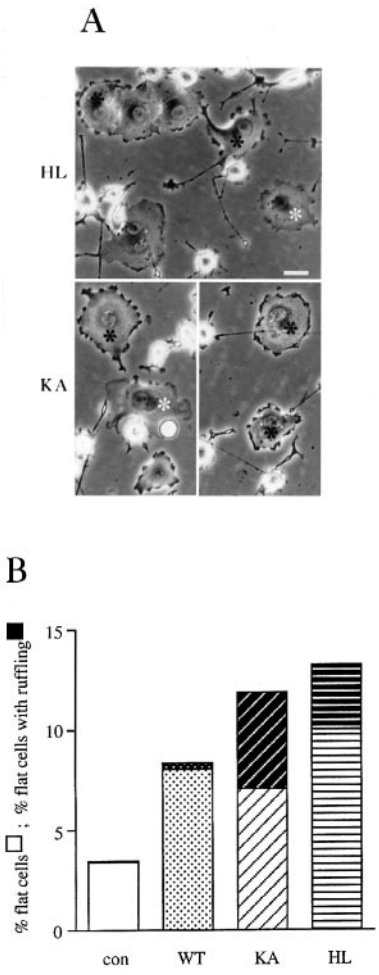

Fig. 4. Cell spreading and membrane ruffling. (**A**) Typical examples of huge flattened cells with and without peripheral membrane ruffling, which frequently are found in PC12/PAK-KA (bottom photographs are from the -KA.2 line) and PC12/PAK-HL cells (top photograph is from the -HL.2 line). Cells were treated with NGF (100 ng/ml) for 3 days in serum-free medium. Smoothly spread cells (i.e. without ruffling) are marked by white asterisks; some of the spread cells with ruffling, giving them a characteristic 'fried egg-like' appearance, are marked by black asterisks. (**B**) The frequencies of extensively spread cells are expressed as percentages of all living cells counted; the percentages of spread cells without ruffling and with ruffling ('fried eggs') are depicted by the lower (white) and by the upper (black) portions of the bars, respectively. Values are based on a total number of 1977 (PC12/con), 1605 (PC12/PAK.2), 1515 (PC12/PAK-KA.2) and 1788 (PC12/PAK-HL.2) counted cells from three independent cell cultures; 20 different, randomly chosen areas were counted per cell line per experiment. Similar results were obtained examining the other clones (PC12/PAK.1, .3, -KA.1, .3, -HL.1, .3) (not shown).

Subcellular localization of PAK, PAK-KA and PAK-HL and formation of focal complexes

Immunocytochemistry with NGF-differentiated cells was performed using affinity-purified βPAK antibodies. While there was only a faint nuclear background staining with control cells (not shown), cells expressing PAK, PAK-KA or PAK-HL displayed a strong *in situ* immunoreactivity. PAK appears to be evenly distributed over the whole cell body and out into the neurite(s) (Figure 5A). Growth cones often show a more intense PAK staining [and vinculin co-staining as well (not shown)]. Nuclei are more or less exempted from PAK staining. The subcellular distribution of PAK-HL differs from that of PAK only in the neurites. PAK-HL staining of neurites is not even, but rather interrupted, giving most neurites a 'beads-on-a-string'-like appearance (Figure 5A). In some of the huge spread cells with extensive peripheral membrane ruffling [as exemplified under phase contrast in Figure 4A (black asterisks)], PAK-HL localizes to areas of ruffling (Figure 5A). PAK-KA distribution is strikingly spotty and granular. Spots of intense PAK-KA localization are frequently found all over the cell body and especially along neurites, giving them a strikingly irregular and granular appearance (Figure

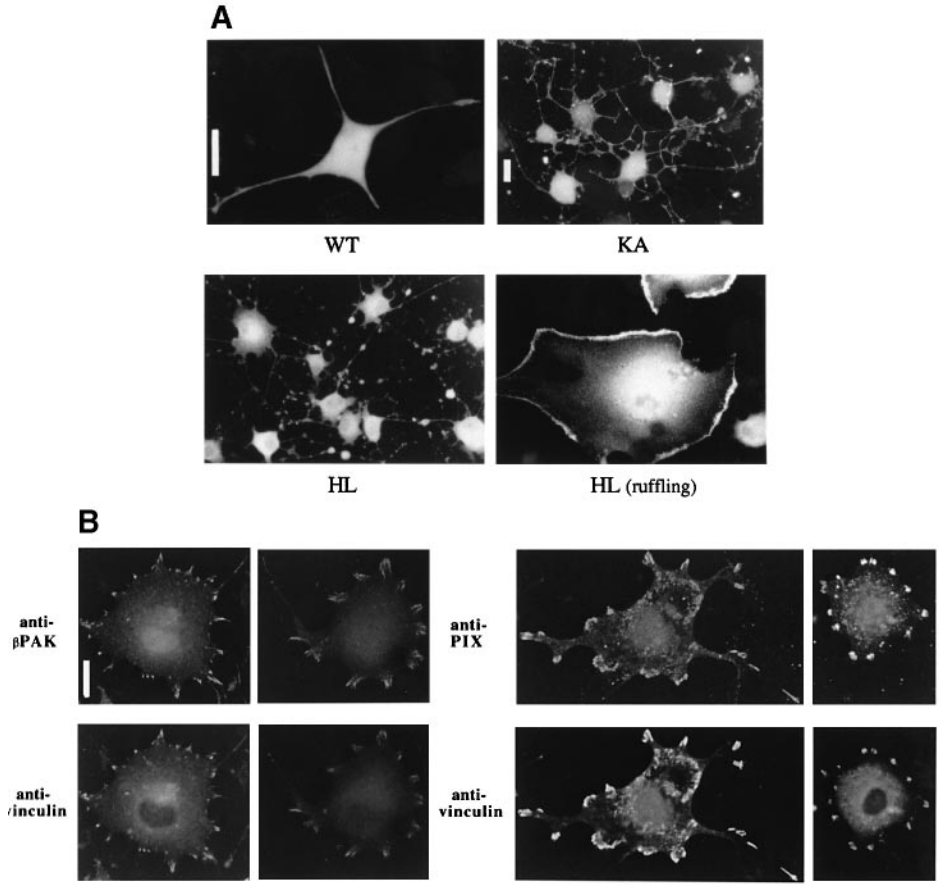

Fig. 5. Subcellular localization of PAK, PAK-KA and PAK-HL, and formation of vinculin-containing focal complexes. (**A**) Fluorescence micrographs showing typical anti-βPAK staining of differentiated PC12/PAK.2, /PAK-KA.2 and /PAK-HL.2 cells. Cells were grown on Matrigel-coated glass coverslips and treated with NGF (100 ng/ml) for 5 days prior to fixation and staining. Some of the huge, flattened PAK-HL-expressing cells show PAK-HL localization to regions of membrane ruffling. Bars, 10 μm; the bar in the KA picture also applies to the lower two pictures taken from -HL cells. (**B**) PAK-KA/vinculin and PIX/vinculin co-staining of FCs in PC12/PAK-KA cells. Cells were treated and processed as in (A). Two spread, FC-bearing cells are shown in each case as representative examples (four micrographs on the left: anti-PAK/anti-vinculin; four micrographs on right: anti-PIX/anti-vinculin). Bar, 10 μm.

5A). Particularly interesting is a strong and specific localization of PAK to focal complex (FC)-like structures, usually present in spread cells (Figure 5B). There is a very precise co-localization of PAK-KA and vinculin, as well as other FC-associated markers, such as paxillin or phosphotyrosine (not shown), to these sites. Moreover, endogenous PIX, the recently identified PAK-interacting guanine nucleotide exchange factor for Rac and Cdc42 (Manser *et al*., 1998) was also localized to such structures (Figure 5B). A co-localization of both PAK and PIX to FCs has also been demonstrated in transfected HeLa cells (Manser *et al*., 1998). This is consistent with the observation that PAK and PIX are strongly associated *in vitro* as revealed by co-immunoprecipitation experiments (see Figure 7B). There are only few cases of PAK (and PIX) co-staining with FCs in PC12/PAK-HL cells, and even fewer such cases in PC12/PAK or control cells (not shown). Stronger staining of FCs was detected for PAK-KA than for PAK or PAK-HL. In addition, vinculin staining revealed that FC-bearing cells are much more frequent amongst PAK-KA cells as compared with the other cell lines. Besides lamellipodia formation and membrane ruffling, FC formation is yet another Rac phenotype (Nobes and Hall, 1995). Why kinase-inactive PAK-KA but not PAK-HL or PAK expression results in a significant proportion of FC-bearing cells may be explained by our observations that kinase-active PAK can dissolve FCs (Manser *et al*., 1997). Thus, while PAK and PAK-HL may indeed also induce the formation of FCs, they would concurrently promote FC dissolution as a part of a dynamic process.

Microinjection of dominant-negative Rac reduces lamellipodia formation

To investigate whether PAK is indeed upstream of and signalling through Rac, as can be inferred from the data presented so far, we microinjected dominant-negative RacN17 (GST–RacN17) into differentiated PC12 cell lines and compared lamellipodia formation rates after and just before injection. Evaluating 898 injected cells, we found that microinjection of RacN17 significantly inhibited lamellipodia formation in all cell lines. The inhibition ranged from 32 to 46% and was typically ~40% (Figure 6B). Lamellipodia at growth cones and neurite shafts were counted separately and also determined without such discrimination (combined values are closer to the growth cone than to neurite shaft values, because the majority of neurites were usually formed at growth cones). The inhibition data obtained from PC12/PAK-HL cells (43.5%, growth cones; 34.4%, neurite shafts; 41.1%, combined)

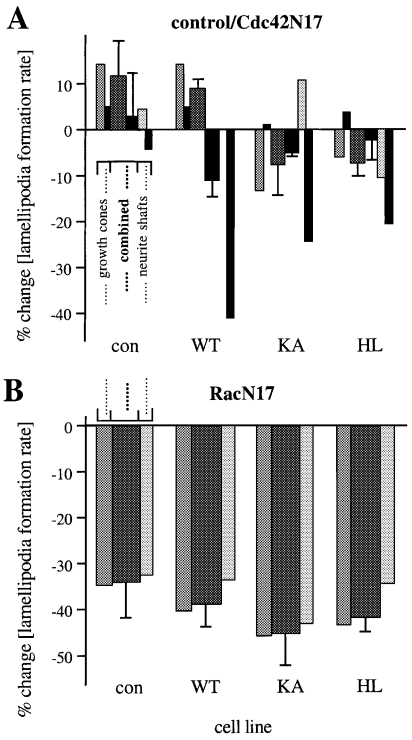

Fig. 6. Microinjection of dominant-negative Rac reduces lamellipodia formation. After treatment with NGF (100 ng/ml) for 2–4 days, cells were microinjected with GST–RacN17 (~1 mg/ml) (**B**) or, as a control, with buffer alone or GST–Cdc42N17 (~1 mg/ml) (**A**). Substantial lamellipodial structures formed over a period of 2.5 min directly prior to injection as well as during a 2.5 min period starting 3–5 min after injection were counted. The ratio between the first count and the difference between the first and the second count reflects the change in the lamellipodia formation rate, which in each case has been determined separately for growth cones and neurite shafts as well as without such discrimination (combined). Means of combined values are depicted by the broad central bars (dark grey) (error bars: SEM), which are each flanked by two thin bars, separately showing values for growth cones (left, medium grey) and shafts (right, light grey). In (A), these three bars are each split up into two bars: grey bars depict control, black bars Cdc42N17 values. Data shown are means derived from 11 (RacN17) or four (control/Cdc42N17) independent experiments. Altogether, the numbers of cells successfully injected and evaluated amount to 139 (RacN17)/38 (control)/42 (Cdc42N17) for PC12/control (con) cells, 142/25/42 for PC12/PAK.2 (WT) cells, 132/29/36 for PC12/PAK-KA.2 (KA) cells, and to 241/48/53 for PC12/PAK-HL.2 (HL) cells. Equivalent data have been obtained from analogous experiments using the other clones (PC12/PAK.1, .3, -KA.1, .3, -HL.1, .3) (not shown).

are most informative. This mutant neither binds to, nor is activated by Rac, yet it induces a strong Rac phenotype, which can be blocked by dominant-negative RacN17, providing compelling evidence that PAK can function upstream of Rac. Since we have shown that the PAK-KA mutant lacking kinase activity can also induce Rac-type morphological changes (see Figure 3), the non-catalytic N-terminal domain of PAK is likely to be responsible for Rac activation.

Control injections with buffer alone did not result in any significant reduction in lamellipodia formation rates; the changes observed were usually in the range of 0–10% (involving a decrease as well as an increase of lamellipodial activity) (Figure 6A). Injections using GST–Cdc42N17 also did not result in any significant changes in lamellipodia formation rates at growth cones, which ranged between 1 and 5% only. However, interestingly, lamellipodia formed at neurite shafts were clearly affected, resulting in a reduction of 20, 24 and 41% for formation rates of PAK-HL-, PAK-KA- and PAK-expressing cells, respectively; this suggests a role for Cdc42 in PAK-mediated lamellipodia formation along neurite shafts.

Interfering with PAK–PIX interaction reduces lamellipodia formation

PIX, which can act as a Rac guanine nucleotide exchange factor (GEF), binds via its Src homology (SH)3 domain with high affinity and in a highly specific manner to a non-conventional proline-rich sequence in the non-catalytic N-terminal domain of PAK (Manser *et al*., 1998). This sequence comprising PAK residues E175–E206 is depicted in Figure 7A as PID100 (PIX-interacting domain). Figure 7B shows that an endogenous PIX of 82 kDa is co-precipitated strongly with PAK and PAK mutants from the different PC12 cell lines used in this study (PIX probably corresponds to pp82, the PAK substrate co-precipitating with PAK as depicted in Figure 2A). When lysates from PIX-overexpressing PC12 cells (see below) were added to the co-precipitation assays in order to increase PIX concentrations, it was revealed that PAK mutants bound ~2-fold more PIX than wild-type PAK (Figure 7B, graphic display), correlating with their 2-fold stronger potentials to induce Rac-type morphological effects. As expected, a PID100 peptide competitively inhibited the PAK–PIX interaction *in vitro* (Figure 7C). In contrast, a mutated version of this peptide (PID100-MUT), in which residues P191,R192 have been changed to G,A, lost its ability to bind to PIX-SH3 and, thus, to interfere with PAK–PIX interaction (Figure 7C).

To examine whether the functional link between PAK and activation of Rac may be provided by PIX, we microinjected GST–PID100 and GST–PID100-MUT into neurite-bearing cells. Experiments were performed and evaluated analogously to the GST–RacN17 injections described in Figure 6B. After injection of GST–PID100, lamellipodia formation rates dropped in all cells, with the inhibition ranging from 34 to 47% and with typical values of ~40% (Figure 7D). Both growth cones and neurite shafts were affected equally. The extent and characteristics of the inhibition of lamellipodia formation by PID100 are thus practically equivalent to the inhibition caused by RacN17. Injection of GST–PID100-MUT did not result in any significant change of lamellipodia formation rates (Figure 7D), strongly suggesting that the inhibitory effects of PID100 are due to its interference with the PAK–PIX interaction. To confirm further that PAK–PIX interaction is important for PAK-induced lamellipodia formation, we have also performed the reciprocal experiment: microinjection of the PIX-SH3 domain inhibits lamellipodia formation to a similar extent as PID100 (data not shown). From these findings, we conclude that PAK acts upstream of Rac and mediates lamellipodia formation through interaction with PIX.

We have also generated several PC12 cell lines expressing FLAG-tagged versions of either PIX (PC12/PIX.1–.4), PIX-SH3^{m} (Manser *et al*., 1998), a PIX mutant, which is unable to bind to PAK (PC12/PIX-SH3^{m}.1, .2) or PIX-DH^{m} (Manser *et al*., 1998) which, due to a mutation in the Dbl homology (DH) domain, is catalytically inactive

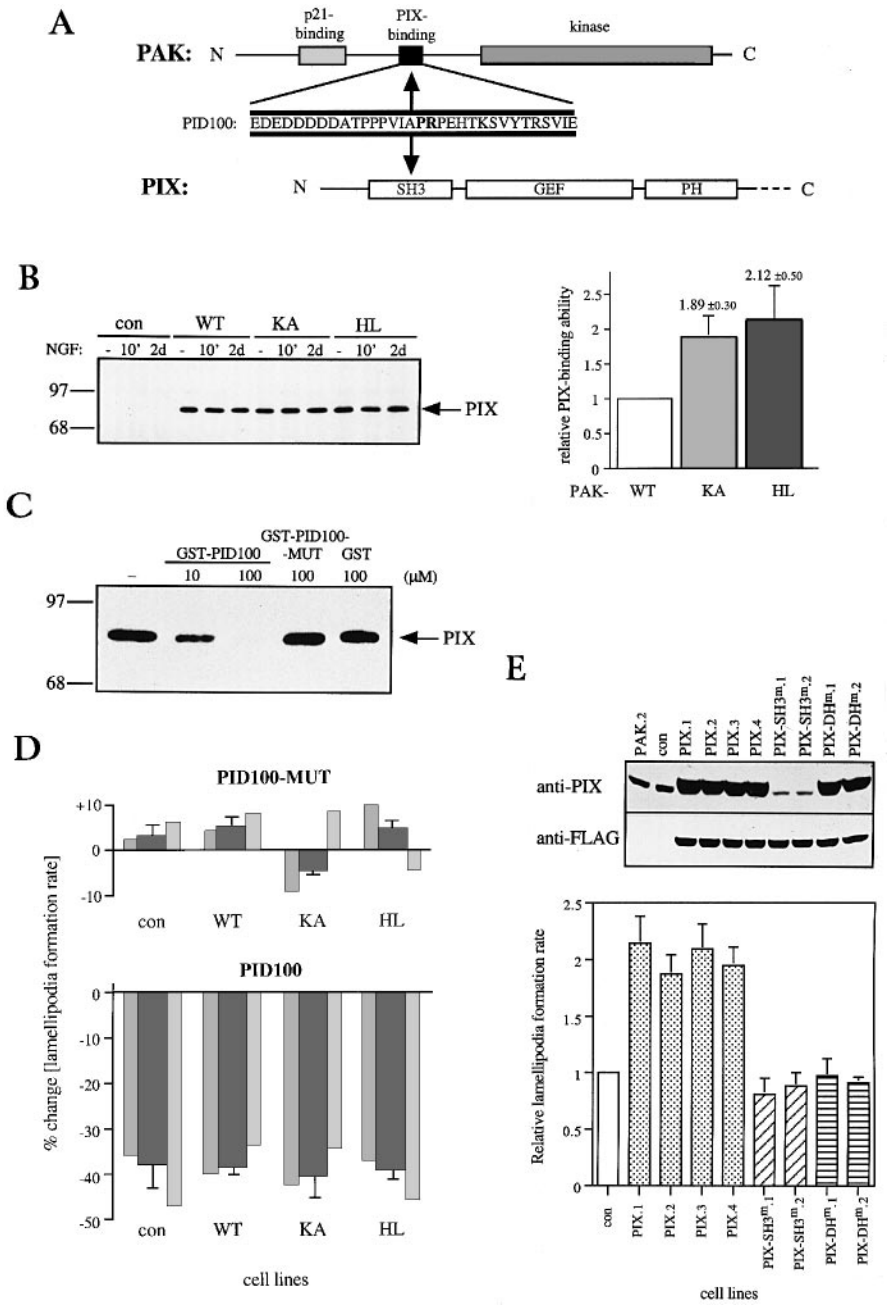

Fig. 7. Interfering with PAK–PIX interaction reduces lamellipodia formation. (**A**) Interaction between PAK and PIX is mediated by a small portion of PAK's N-terminal non-catalytic domain, containing a proline-rich core sequence, and the SH3 domain of PIX. The sequence of the PIX-binding domain of PAK is indicated (single letter amino acid code). A peptide of this sequence (PID100) competitively interferes with PAK–PIX interaction. In contrast, a mutated version of this peptide (called PID100-MUT), in which amino acids P191,R192 (typed in bold face) have been changed to G,A, does not. (**B**) Co-precipitation of PIX with PAK. Left panel: equal amounts of protein from untreated or NGF-treated (10 min or 2 days) PC12/con, /PAK.2, /PAK-KA.2 and /PAK-HL.2 cells were precipitated with anti-βPAK antibodies, and co-precipitated PIX was detected by anti-PIX immunoblotting. Amounts of PAKs were equivalent (not shown). Right panel: equal amounts of lysates from PIX-overexpressing PC12 cells [see below: (E)] were added during anti-βPAK immunoprecipitations. Amounts of co-precipitated PIX were determined by densitometric scanning. Data shown are means from five experiments (± SE). (**C**) Co-precipitation of PIX with PAK is inhibited by PID100 but not by PID100-MUT. Equal aliquots from pooled lysates (PC12/PAK, PC12/PAK-KA, PC12/PAK-HL) were immunoprecipitated with anti-βPAK antibodies (N-19; Santa Cruz Biotech.) in the absence (–) or presence of 10 or 100 μM GST–PID100 or 100 μM GST–PID100-MUT or, as a control, 100 μM GST, followed by immunoblotting with anti-PIX antibodies. Equivalent results were obtained in several independent experiments [irrespective of (duration of) NGF treatment or which cell line was used]. (**D**) Microinjection of GST–PID100 reduces lamellipodia formation. After treatment with NGF (100 ng/ml) for 2–3 days, cells were microinjected with GST–PID100 or GST–PID100-MUT (~1 mg/ml), and resulting changes in lamellipodia formation rates were determined as described for Figure 6 and displayed analogously. Data are based on 87(PID100)/174(PID100-MUT) (con), 89/166 (WT), 84/80 (KA) and 123/153 (HL) successfully injected cells from five independent experiments. (**E**) Lamellipodia formation rates of PC12/PIX, PC12/PIX-SH3^m and PC12/PIX-DH^m lines. Upper panels: expression levels of FLAG-tagged PIX (^{F}PIX) and ^{F}PIX mutants. PC12 transfectants stably expressing ^{F}PIX, the PAK binding-deficient mutant ^{F}PIX-SH3^m or the GDP/GTP exchange-inactive mutant ^{F}PIX-DH^m (two to four different cell lines each) were lysed, and equal amounts of protein were separated by SDS–PAGE; as controls, equal amounts of protein from PC12/con and PC12/PAK.2 lysates were included. After transfer to nitrocellulose membrane, proteins were detected with anti-PIX and anti-FLAG antibodies. Graph: lamellipodia formation rates were determined as described for Figure 3.

(PC12/PIX-DH^m.1, .2). The expression levels of these various PIX constructs are shown in Figure 7E. As the PIX antibody is directed against the PIX-SH3 domain, PIX-SH3^m is only recognized by the FLAG antibody. We found that expression of FLAG-PIX (^{F}PIX) induced an ~2-fold increase in lamellipodia formation rates compared with control cells (Figure 7E), which is similar to the results obtained with PC12/PAK cells (Figure 3). In contrast, cells expressing either ^{F}PIX-SH3^m or ^{F}PIX-DH^m did not display any increased lamellipodial activity. These findings corroborate a role for PIX in PAK-induced lamellipodia formation and show that both GEF activity and PAK binding are required. A significant dominant-negative effect on lamellipodia formation by ^{F}PIX-SH3^m or ^{F}PIX-DH^m was not observed, probably because the levels of these PIX mutants were not high enough in relation to endogenous PIX; furthermore, about one half of the ^{F}PIX protein was localized in the cytosol, whereas endogenous PIX is found almost exclusively at the membrane (data not shown).

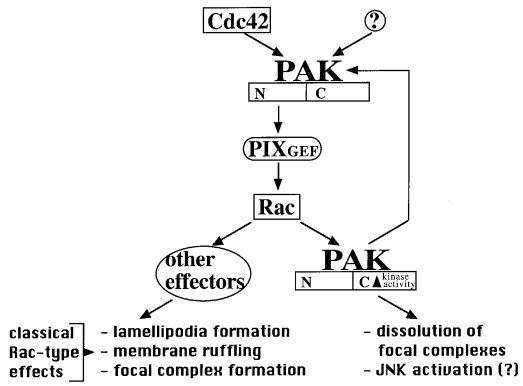

Fig. 8. A model showing PAK to be upstream of Rac mediating Rac-type morphological effects through interaction with the putative Rac-GEF PIX. The N-terminal, non-catalytic domain of PAK is responsible for binding to PIX and thereby for activation of Rac and induction of all Rac-type effects, placing PAK upstream of Rac. At the same time, this does not rule out a role for PAK as a downstream effector of Rac, possibly mediating down-regulation of focal complexes and stress fibres and, in some cell types, activation of JNK and other MAP kinase cascades, by means of its kinase activity.

Discussion

PAK produces a Rac phenotype in PC12 cells

By expressing βPAK in PC12 cells, we have found a phenotype for PAK that closely resembles a Rac phenotype. As with Rac, PAK mediates actin cytoskeletal rearrangements leading to a variety of morphological changes, including lamellipodia formation (Figure 3), cell spreading and membrane ruffling (Figure 4). As PAK has generally been regarded as a downstream effector of Rac (and Cdc42), being catalytically activated upon binding to the active, GTP-bound forms of these p21s (Manser *et al*., 1994), it initially was unexpected that, instead of being attenuated or abolished, these morphological Rac-type effects were even more pronounced in cells expressing two mutants of PAK, namely PAK-KA, which is catalytically inactive, and PAK-HL, which cannot bind p21s. These unexpected findings reveal that neither kinase activity nor binding to Rac is essential for PAK to promote these Rac-type effects, and that in this action PAK does not behave as a downstream effector/target of Rac. That RacN17 significantly inhibited lamellipodia formation in all cell lines, including those expressing Rac binding-deficient PAK-HL (Figure 6B), indicates that PAK can function upstream of Rac. In addition, specifically blocking the interaction of PAK with PIX, a recently cloned putative exchange factor for Rac (Manser *et al*., 1998), resulted in an inhibition of lamellipodia formation that was essentially equivalent to the inhibition by RacN17 (Figure 7D). Finally, overexpression of PIX resulted in increased lamellipodial activity, for which both GEF activity and an intact PAK interaction (SH3) domain were required (Figure 7E). Thus it is possible, at least in PC12 cells, that PIX may turn out to be the only significant functional link between PAK and Rac activation. From these results, a model can be derived, in which PAK binding to PIX is required to activate Rac, most likely mediated by the GEF activity of PIX (Figure 8).

As the inhibition of lamellipodia formation by injection of RacN17 averages in the range of 40%, we cannot exclude a role for PAK in promoting lamellipodia formation independently of Rac. However, it seems likely that higher inhibition rates are unattainable because of the relatively complex morphology of process-bearing PC12 cells and consequent practical difficulties in efficient delivery of injected proteins into neurites; even in fibroblasts, injection of dominant-negative p21s does not completely abolish, but rather usually reduces, relevant morphological changes by 60–80%. Sells *et al*. (1997) claimed that RacN17 was ineffective in blocking mutant PAK-induced morphological changes in fibroblasts (only as unpublished data). Whether this reflects cell type specificity (as their morphological changes represented a mixture of Cdc42-, Rac- and Rho-type effects) remains to be determined, in the absence of documented evidence.

PAK, neurite outgrowth and neurite morphology

Whereas lamellipodia formation and cell spreading/ruffling was promoted similarly by both PAK mutants and to a lower extent by wild-type PAK, accentuated FC formation, which is another Rac phenotype (Nobes and Hall, 1995), was only observed in PC12/PAK-KA, but not in PC12/PAK-HL or PC12/PAK cells (Figure 5B). These observations suggest that PAK kinase activity is counteracting the formation of such structures. Indeed, we have demonstrated recently that PAK activity functions to dissolve stress fibres and FCs (Manser *et al*., 1997). Thus, on the one hand, PAK-HL and PAK may very well induce the formation of FCs via their N-terminal domains, but on the other hand (and in contrast to PAK-KA), through their kinase activity promote FC dissolution, with the nett result that no significant accumulation of FCs occurs. A strong tendency to form stable FCs in PC12/PAK-KA cells may, at least in part, be responsible for the delayed neurite outgrowth response exhibited by these cells (Figure 1B) as proper neurite extension depends on a highly dynamic regulation of growth cone (and shaft) attachment to substratum, for which a high turnover of the adhesion-providing FCs is a prerequisite. In this regard, a kinase-inactive PAK which has lost its negative regulatory device to counteract its FC-promoting activity may lack sufficient flexibility to enable rapid neurite outgrowth.

Lamellipodia are instrumental for neurites to sense the

environment, to expand and grow directionally, and are linked intimately to new process formation (Tanaka and Sabry, 1995). Perhaps the extraordinarily high lamellipodia-forming activity in PC12/PAK-HL (and -KA) cells may underlie their irregular, varicose neurite morphology (Figure 1B; see Results for further details), in as much as too many scouts and guides can prevent concerted unidirectional movement. In line with our results, observations from transgenic flies and mice expressing dominant-negative or constitutively active Rac indicate that perturbation of Rac activity in either way results in abnormal neurite outgrowth and neurite morphology (Luo *et al*., 1997), consistent with a role for PAK in regulating Rac activity.

While this manuscript was under review, Daniels *et al*. (1998) reported that neurite outgrowth is induced by expression of a membrane-targeted version of PAK1 (αPAK), engineered by attaching to its C-terminus the 17 C-terminal amino acid residues of Ras, including the so-called CAAX-box, responsible for membrane-anchoring. In line with our data, the authors did not observe any neurite outgrowth by expression of non-membrane-targeted forms of PAK. Thus, this study and ours suggest that PAK is involved in neurite outgrowth, although PAK is not sufficient to induce neurite outgrowth under physiological conditions, i.e. when PAK can cycle between membrane and cytoplasm. Our data on PAK inducing actin cytoskeletal rearrangements implicated in growth cone steering and neurite outgrowth provide a mechanistic explanation for these observations. Interestingly, Daniels *et al*. (1998) find that expression of a PAK fragment comprising amino acids 169–205 inhibited NGF-induced neurite outgrowth. As this fragment is similar to PID100 (residues 175–206) containing the PIX-binding site of PAK, these results are very supportive of our findings about the importance of the PAK–PIX interaction for PAK-induced morphological changes.

PAK mutants induce a stronger Rac phenotype than wild-type PAK

Given that the Rac activation signal is conveyed by the non-catalytic N-terminal domain(s) of PAK, how can it be explained that PAK mutants induce an even stronger Rac phenotype than wild-type PAK? The N-terminal domain(s) is probably not easily accessible for other factors to bind, unless PAK has undergone conformational changes, which can be accomplished either by p21-mediated kinase activation or by mutations in the kinase or the p21-binding domain, such as KA or HL (Manser *et al*., 1997; Sells *et al*., 1997). Therefore, KA and HL mutants would be in a constitutively open conformation facilitating a robust signalling via N-terminal sequences, whereas wild-type PAK activity/autophosphorylation status and consequently its open conformation and N-terminal signalling capacity may be subject to down-regulation by phosphatases (Manser *et al*., 1997). That wild-type PAK activity is still 15-fold below the maximal achievable level (Figure 2A) suggests that its N-terminal signalling potential is clearly below that of the PAK mutants with a constitutively exposed N-terminus.

Equal amounts of endogenous PIX co-precipitate with PAK, PAK-KA and PAK-HL from corresponding cell lysates; however, and in support of the above, increasing amounts of PIX during the anti-PAK immunoprecipitation (by including some lysate from PIX-overexpressing cell lines) reveals an ~2-fold increased PIX-binding capacity of PAK-KA and -HL as compared with wild-type PAK (Figure 7B). This corresponds well to the 2-fold stronger Rac phenotype induced by the mutants as compared with wild-type PAK. High local concentrations of PIX at the membrane and FCs of living cells, where PAK and Rac signalling is thought to occur, suggest that the different affinities for PIX of wild-type and mutant PAKs may be effective *in vivo*, whereas PIX concentrations are obviously limiting under *in vitro* conditions. It is also possible that other PAK N-terminus-interacting proteins which are possibly involved in PIX activation, such as Nck, may be associated more efficiently with PAK mutants than with wild-type PAK.

PAK–PIX interaction and Rac activation

It will be interesting to determine how PAK–PIX interaction leads to PIX activation of Rac. Although PIX is phosphorylated by PAK (Manser *et al*., 1998), this is very unlikely to lead to PIX activation because PAK-KA incapable of phosphorylating PIX still induces a strong Rac phenotype, and *in vitro* PIX GEF activity is apparently unaffected by phosphorylation (E.Manser and C.-G.Koh, unpublished). It is possible that conformational changes in PIX may unmask/activate PIX GEF activity. Such a conformational activation could be induced directly by PIX binding to PAK and/or by any other protein present in the PAK–PIX signalling complex, such as p90 (Figure 2A; Manser *et al.*, 1998). Nck has been reported to bind to PAK (Bokoch *et al*., 1996; Galisteo *et al*., 1996). Interfering with this interaction inhibits the ability of PAK to induce membrane ruffling and focal complexes (Sells *et al*., 1997), and a stronger binding of Nck to PAK-HL as compared with wild-type PAK (Sells *et al*., 1997) correlates with a stronger Rac phenotype induced by the mutant. Thus, Nck could be involved in PIX activation. Nck and PIX via their respective SH3 domains bind separately to two of the several proline-rich sequences in the N-terminal, non-catalytic half of PAK, which also contains other potential sites for protein–protein interaction. PAK could function as a scaffolding protein, assembling different proteins which may work together to achieve Rac activation and/or may also be involved in additional signal transduction pathways. Such a signal-integrating function of PAK is also suggested by its modulation of Ras signalling to ERK1 MAP kinase in certain cell lines (Tang *et al*., 1997), although this does not seem to be the case in PC12 cells (Figure 2C). PAK–PIX interactions could also accomplish Rac activation by bringing PIX within the vicinity of its target Rac, reminiscent of the case of the Ras exchange factor Sos, which is brought to the membrane by binding to Grb2, thereby enabling Ras activation (McCormick, 1993; Aronheim *et al*., 1994).

Rac effector mutants and PAK function

PAK binding-deficient mutants of activated Rac can still mediate morphological changes, including membrane ruffling and lamellipodia formation, while other Rac effector site mutants which retain PAK binding are defective in inducing morphological changes (Joneson

et al., 1996; Lamarche *et al*., 1996; Westwick *et al*., 1997). These observations have led to the conclusion that PAK is not involved in actin (re)organization by Rac. On the other hand, PAK has been functionally associated with at least some aspects of Rac-type cytoskeletal rearrangements (Sells *et al*., 1997; Tang *et al*., 1997) and this is confirmed and extended by our present study. Resolution of this apparent contradiction is provided by our findings that PAK function is not confined to a Rac effector role, but rather that PAK can function upstream of Rac and effect Rac activation (Figure 8). This upstream role of PAK is mediated by its N-terminal non-catalytic domain(s) via protein–protein interaction(s). As discussed above, PAK may need to adopt an open conformation to allow its N-terminus to interact stably with PIX and other factors, and to activate Rac; in a wild-type setting, this could be accomplished by p21-mediated PAK activation. Thus activated Cdc42 or some other as yet unknown upstream factor(s) may activate PAK and at the same time enable PAK to associate with PIX and mediate Rac activation. This model also provides a possible mechanistic explanation for the frequently observed functional Cdc42–Rac hierarchy (reviewed in Lim *et al*., 1996a,b). As PAK can also be activated by Rac, heretofore activated by the PAK–PIX pathway or by any other means, both PAK and Rac may constitute a positive feedback loop. It is likely that the loop is under the tight control of corresponding phosphatases.

Activation of JNK by Rac and PAK

Rac can mediate activation of JNK in some cell types (Coso *et al*., 1995; Minden *et al*., 1995) although this mediation is not seen in other cell lines (Teramoto *et al*., 1996). It was also suggested that PAK can mediate JNK activation and may thus provide a link between Rac and the JNK kinase cascade (Bagrodia *et al*., 1995; Brown *et al*., 1996). In PC12 cells, we could not find any correlation between PAK and JNK activities, the latter appearing equal in all cell lines (Figure 2B). Since our findings indicate a role for PAK in Rac activation, by implication Rac does not activate JNK in PC12 cells. The ability of PAK and/or Rac to activate JNK possibly depends on cell type-specific expression of other kinases, e.g. MEKKs or mixed lineage kinases (Sells and Chernoff, 1997; Van Aelst and D'Souza-Schorey, 1997, and references therein), which potentially can link PAK and Rac to JNK. Differential expression of phosphatases that down-regulate the JNK kinase cascade may also be a contributing factor. JNK activity correlates with apoptosis in PC12 cells (Xia *et al*., 1995). As PAK appears to be necessary for a rapid and continuous neurite outgrowth, possibly by regulating FC turnover and lamellipodia formation (see above), differentiating neuronal-type cells may well need to possess mechanisms that prevent JNK (and consequently apoptosis) from being activated by PAK.

Notably, PAK activity is not affected by NGF stimulation (Figure 2A). Thus, PAK as a kinase may not be involved directly in NGF/Trk-induced signalling pathways responsible for differentiation, but rather plays a permissive role in regulating cytoskeletal rearrangements and morphological changes associated with and necessary for neurite outgrowth, as discussed above.

Conclusions

PAK's role in Rac signalling is 2-fold. Firstly, PAK is an effector of Rac. As such, acting through its kinase domain, it can dissolve FCs and may also induce transcriptional up-regulation through activation of JNK and other MAP kinase cascades in appropriate cellular settings. Secondly, it can promote Rac activity and thereby has the potential to regulate many of the effects ascribed to Rac, including membrane ruffling, FC assembly and lamellipodia formation. The actual Rac effectors responsible for these effects are not known, although the recently cloned POR1 may be a good candidate as an effector for membrane ruffling (Van Aelst *et al*., 1996).

Activated Rac can induce a tumorigenic phenotype and is also essential for Ras transformation (Khosravi-Far *et al*., 1995; Qiu *et al*., 1995). As cellular transformation and tumorigenesis is a multistep process that involves activation of MAP kinase cascades and subsequent changes in gene expression and cell growth, as well as actin cytoskeletal rearrangements and consequent changes in cell morphology, motility and adhesion, PAK is likely to play an important role in this process, both upstream and downstream of Rac.

Materials and methods

Generation of expression constructs and PC12 cell lines

The βPAK-cDNA (Manser *et al*., 1995) was cloned into the *Eco*RV site of pBluescript-SK+ and subjected to oligonucleotide-directed (K297A: 5′-GTTGAAGGTTCATTTGTGCTATAGCCACCTC-3′; H78/81L: 5′-CAAAACCCACAAGAATCGTAAGCTCAAAGTC-3′) M13K07 helper phage-assisted Kunkel mutagenesis as described (Obermeier *et al*., 1994). For expression, wild-type and mutant sequences were retrieved from the SK vector as *Sma*I–*Xho*I fragments, cloned into *Hpa*I–*Xho*I-opened retroviral vector pLXSN, and verified by DNA sequencing.

The βPIX-cDNA [wild-type, PIX-SH3^{m} (W43P,W44G) and -DH^{m} (L238R,L239S) versions] including the N-terminal FLAG-tag were retrieved from the corresponding pXJ vector constructs (Manser *et al*., 1998) as *Eco*RI–*Bgl*II fragments and cloned into *Eco*RI–*Bam*HI-opened pLXSN.

Generation of retroviruses using expression vector-transfected BOSC 23 cells and infection and selection of PC12 cells were as described (Pear *et al*., 1993; Obermeier *et al*., 1994).

GST fusion proteins and antibodies

For GST–PID100, a PCR fragment corresponding to αPAK amino acids E175–E206 (for sequence, see Figure 7A) was generated, containing a *Bam*HI site (5′) and an *Eco*RI site (and a stop codon) (3′), and cloned into pGEX-2T-K; PID-100-MUT was derived analogously by PCR from an αPAK cDNA template mutated to replace P191,R192 by G,A. Purification of GST–PID100(-MUT) as well as cloning and purification of GST–RacN17 and GST–Cdc42N17 was as described previously (Ahmed *et al*., 1995; Kozma *et al*., 1995).

Rabbit polyclonal anti-βPAK antibodies were raised against GST–βPAK$^{1–221}$, produced from pGEX-4T-2 carrying a *Bam*HI–*Sca*I(*Sma*I) insert coding for the corresponding βPAK sequence. Rabbit polyclonal anti-PIX antibodies were raised against GST–PIX(SH3) (Manser *et al*., 1998). Both antibodies were purified by affinity chromatography. Anti-JNK1 and anti-βPAK (N19) antibodies were obtained from Santa Cruz Biotechnology Inc., anti-phospho-p44/42MAPK was from NEB, and anti-vinculin monoclonal antibody (hVIN-1) from Sigma.

Cell culture and microinjection

PC12 cells were grown as described (Obermeier *et al*., 1994) on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum (Sigma) and 5% fetal bovine serum (Gibco). For differentiation, 100 ng/ml NGF (human recombinant; Sigma) was added to the medium. NGF-containing medium was changed every 60 h.

After 2–4 days of NGF treatment, cells exhibiting a typical phenotype were microinjected with proteins (diluted to ~1 mg/ml) in phosphate-buffered saline (PBS) using an Eppendorf injector and a Zeiss axiovert

microscope. Cells were maintained on a 37°C heated platform with 5% CO_2 (incubator conditions) and monitored under phase contrast, assisted by video recording. Legends to Figures 3 and 6 contain details on determination and quantification of lamellipodia formation.

Cell lysis, immunoprecipitation, kinase assays and immunoblotting analysis

Agonist- or growth factor-treated cells were starved in serum-free medium for 18 h before treatment. After aspiration of culture medium, cells were lysed on ice with 750 µl per 10 cm dish of ice-cold lysis buffer [50 mM HEPES pH 7.3, 150 mM NaCl, 15 mM NaF, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 20 mM β-glycerophosphate, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml aprotinin, 1 µg/ml leupeptin].

After 10–20 min, lysates were pre-cleared by centrifugation at 15 000 *g* for 7.5 min at 4°C. Pre-cleared lysates were either directly mixed with SDS sample buffer, boiled and subjected to SDS–PAGE, or used for immunoprecipitation: 100–200 µl of lysates were topped up with lysis buffer to 300 µl and incubated with 1 µl of serum or ~0.5 µg of purified antibodies on a mixer for 2 h at 4°C; after addition of 20 µl of protein A–Sepharose slurry (0.2 mg/µl; pre-washed in 20 mM HEPES pH 7.3), incubation was continued for 1 h. Precipitates were washed three times in 50 mM HEPES pH 7.3, 150 mM NaCl, 15 mM NaF, 2 mM EDTA, 10% glycerol, 0.2% Triton X-100 and either taken up in SDS sample buffer or further subjected to *in vitro* kinase assays.

For kinase reactions, precipitates were washed with 50 mM HEPES pH 7.3, 10 mM $MgCl_2$, 2 mM $MnCl_2$ and then incubated in 15 µl of the same buffer containing 20 µM ATP, 5 µCi [γ-^{33}P]ATP (Amersham). Where appropriate, 1 µg of MBP (and 4 µg of GTP-γS-pre-loaded GST–Cdc42) or 2 µg of GST–Jun$^{1–79}$ were added. After 15 min at 21°C and 10 min at 37°C, reactions were stopped by addition of SDS sample buffer. After SDS–PAGE, proteins were fixed, stained with Coomassie and subjected to autoradiography.

For immunoblotting, proteins separated by SDS–PAGE were electrophoretically transferred onto nitrocellulose filters. Filters were blocked with 5% milk powder solution in PBS for 45 min, and antibody solutions (in PBS) were added overnight at 4°C (first antibody) and for 1 h (second horseradish peroxidase-coupled antibody). Signals were developed using the ECL system (Amersham).

Immunocytochemistry

Cells were seeded at low density on 4-well chamber slides (Nunc), which had been coated with Matrigel (Collaborative Research, Inc.), pre-diluted 1:50 in DMEM. After 5 days of NGF treatment, cells were fixed and stained as previously described (Manser *et al*., 1997) using affinity-purified anti-βPAK (1:250) or anti-PIX (1:30), and anti-vinculin (1:300) antibodies.

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