

# Regulation of anchorage-dependent signal transduction by protein kinase A and p21-activated kinase

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**Activation of the canonical mitogen-activated protein kinase (MAPK) cascade by soluble mitogens is blocked in non-adherent cells. It is also blocked in cells in which the cAMP-dependent protein kinase (PKA) is activated. Here we show that inhibition of PKA allows anchorage-independent stimulation of the MAPK cascade by growth factors. This effect is transient, and its duration correlates with sustained tyrosine phosphorylation of paxillin and focal-adhesion kinase (FAK) in non-adherent cells. The effect is sensitive to cytochalasin D, implicating the actin cytoskeleton as an important factor in mediating this anchorage-independent signalling. Interestingly, constitutively active p21-activated kinase (PAK) also allows anchorage-independent MAPK signalling. Furthermore, PKA negatively regulates PAK *in vivo*, and whereas the induction of anchorage-independent signaling resulting from PKA suppression is blocked by dominant negative PAK, it is markedly prolonged by constitutively active PAK. These observations indicate that PKA and PAK are important regulators of anchorage-dependent signal transduction.**

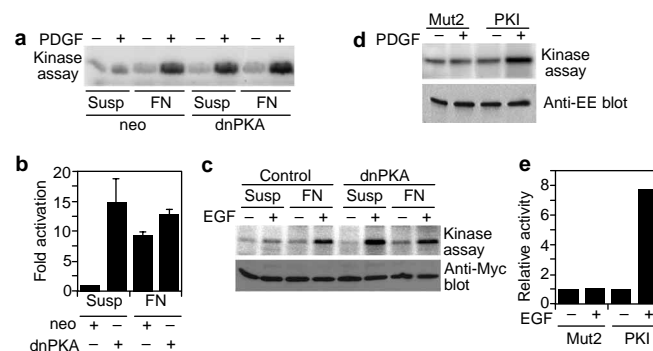
The adhesion of a cell to the extracellular matrix (ECM) conveys positional and morphological information that profoundly influences the cellular response to soluble factors<sup>1–3</sup>. In this way, cell adhesion can regulate many aspects of the behaviour of a cell. The ability of cell adhesion to influence the activation of the MAPK cascade is likely to be an important facet of adhesion-mediated cell regulation. The MAPK cascade is activated by a wide variety of extracellular signals and is an essential component of pathways that regulate cell division, motility and differentiation<sup>4</sup>. It has been shown that activation of the MAPK cascade by soluble factors is inhibited in non-adherent cells<sup>5–8</sup>. We have found that in non-adherent cells stimulated with peptide growth factors, receptor tyrosine phosphorylation and loading of Ras with GTP occur efficiently whereas activation of the downstream kinases Raf, MEK and MAPK is inhibited<sup>7</sup>.

This inhibition is reminiscent of another instance in which growth-factor signalling to MAPK is blocked, namely when adherent cells are treated with agents that increase levels of cAMP (such as forskolin (Fsk), isoproterenol and cholera toxin) and thereby activate PKA<sup>9</sup>. This effect has been mapped to inhibition of Raf activation, although the mechanism through which this occurs has not been clearly defined<sup>9</sup>. PKA is a well-established inhibitor of proliferation in many cell types, and its inactivation or downregulation may predispose cells to transformation or may itself contribute to the transformation process<sup>10,11</sup>. As well as its effects on signal transduction and cell growth, activation of PKA is known to disrupt the actin cytoskeleton<sup>11,12</sup>. This is particularly significant given the recent demonstration that an intact actin cytoskeleton, specifically cortical or peripheral actin structures, is required for anchorage-dependent activation of MAPK by growth factors<sup>13</sup>.

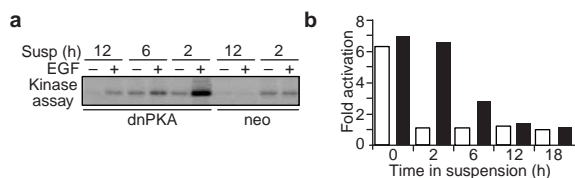
Further evidence of a function of the actin cytoskeleton in regulating growth-factor signaling is provided by the observation that activation of MAPK by growth factors in non-adherent cells can be partially restored by constitutively active Cdc42 (ref. 13), a Rho-family GTPase that regulates cortical actin structures<sup>14</sup>. Potential effectors for Cdc42 in this regard are the PAKs, which are activated

by both Cdc42 and Rac and can regulate cell morphology through changes in cortical actin<sup>15</sup>. Interestingly, PAKs can also directly regulate components of the MAPK pathway itself, specifically Raf<sup>16,17</sup> and MEK<sup>17</sup> — phosphorylation of these kinases by PAK is required for their efficient activation by upstream regulators<sup>16,17</sup>.

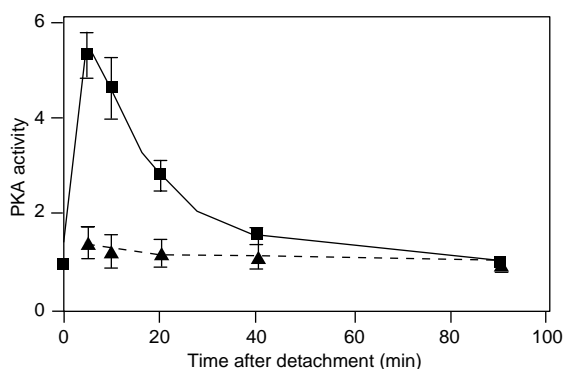
Here we examine the function of PKA and PAK1 in regulating the anchorage-dependence of the MAPK cascade. Our results



**Figure 1 Inhibition of PKA induces anchorage-independent activation of the MAPK cascade.** **a**, *In vitro* kinase assay of Raf immunoprecipitated from control (neo) or dnPKA cell lines incubated in suspension (Susp) or re-plated onto fibronectin-coated dishes (FN) for 2 h, then stimulated with 10 ng ml<sup>-1</sup> PDGF for 5 min as described<sup>7,15</sup>. **b**, Mean fold activation  $\pm$  s.d. from seven experiments carried out as in **a**. **c**, *In vitro* kinase assay from cells transiently co-transfected with Myc-Raf and either control vector or dnPKA-encoding plasmid and cultured as in **a**. **d**, *In vitro* kinase assay of non-adherent cells co-transfected with plasmids encoding wild-type PKI or an inactive point mutant (Mut2) and stimulated with PDGF or EGF (10 or 20 ng ml<sup>-1</sup>, respectively). Equivalent expression of the reporter kinases was confirmed by western blotting with anti-Myc (**c**) or anti-EE (**d**) antibody.



**Figure 2 Induction of anchorage-independence by PKA inhibition is transient.** **a**, *In vitro* kinase assay of MAPK immunoprecipitated from control neo or dnPKA-expressing cell lines incubated in suspension (susp) for the indicated times before stimulation with growth factor. **b**, Results from a separate experiment carried out as in **a**, showing fold activation of MAPK in neo (white bars) and dnPKA-expressing (black bars) cells as a function of time in suspension (time = 0 represents adherent cells before detachment).

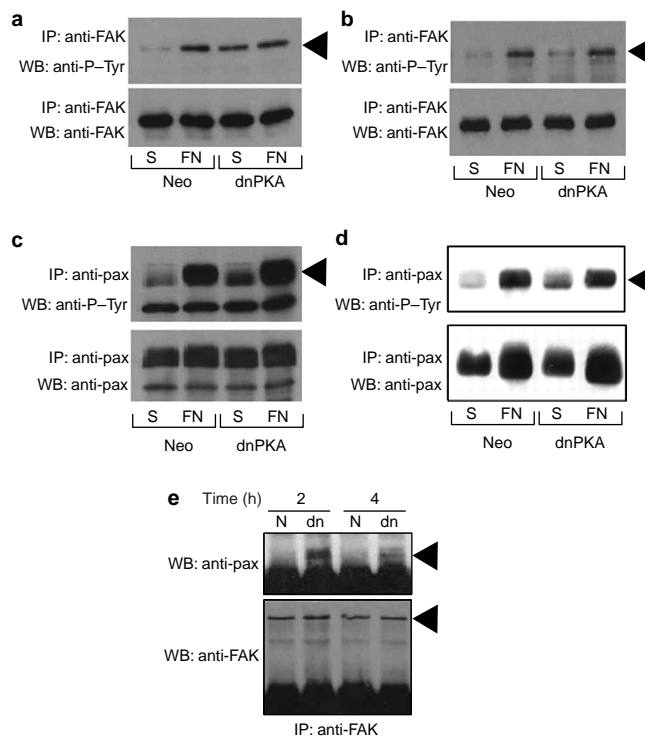


**Figure 3 PKA is activated upon detachment of cells.** PKA activity was measured in NIH3T3 cells (solid line) and in cells stably expressing dnPKA (dashed line) at the indicated times after detachment (time = 0 represents adherent cells before detachment). Data are expressed relative to PKA activity in adherent cells; values are means  $\pm$  s.d. ( $n = 4$ ).

establish these two kinases as important regulatory elements of anchorage-dependent signal transduction and also implicate PKA as a regulator of PAK function *in vivo*.

## Results

**Inhibition of PKA allows anchorage-independent activation of MAPK.** To determine whether PKA is involved in restricting the growth-factor response in non-adherent cells, we used a stable cell line<sup>18</sup> expressing a dominant negative regulatory subunit of PKA (dnPKA)<sup>19</sup>. Expression of this mutant subunit reduced Fsk-stimulated PKA activity by >95% compared with that in control (neo) cells (data not shown). Platelet-derived growth factor (PDGF)-stimulated activation of Raf was robust in control cells that were re-plated onto the ECM protein fibronectin, but was minimal in non-adherent control cells, whereas in dnPKA-expressing cells, Raf activation occurred efficiently regardless of adhesion state (Fig. 1a, b). Experiments involving transient co-transfection of dnPKA or neo control plasmids with an epitope-tagged 'reporter kinase' (in this case, Myc-Raf) gave results that were similar to those obtained with stable cell lines, indicating that the induction of anchorage-independence was not due to a clonal anomaly (Fig. 1c). Furthermore, transient expression of the naturally occurring, heat-stable PKA-inhibitor peptide (PKI)<sup>20</sup> allowed efficient activation of co-transfected, epitope-tagged MEK by growth factors in

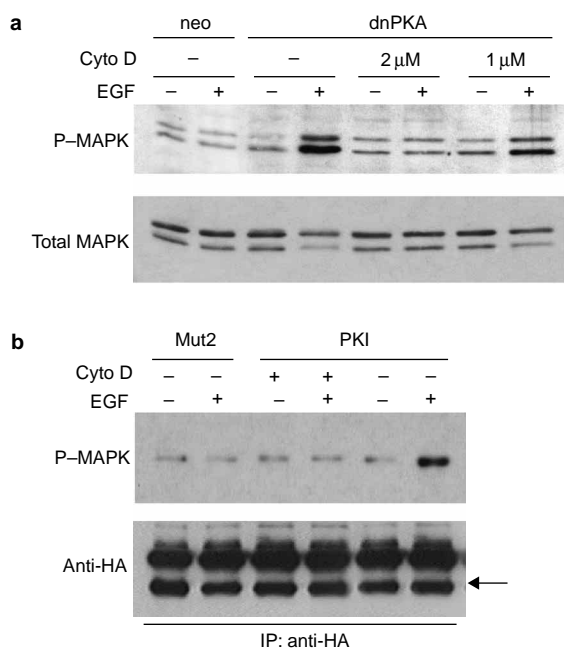


**Figure 4 PKA inhibition delays dephosphorylation and dissociation of FAK and paxillin after detachment.** Stable control neo or dnPKA-expressing cells were incubated in suspension (S) or replated onto fibronectin-coated dishes (FN) for 2 h (**a**, **c**) or 4 h (**b**, **d**). Anti-FAK (**a**, **b**) and anti-paxillin (**c**, **d**) immunoprecipitates (IP) were analysed by western blotting (WB) with anti-phosphotyrosine (P-Tyr) antibodies and with anti-FAK or anti-paxillin (Pax) antibodies as indicated. **e**, Control neo (N) or dnPKA-expressing (dn) cells were incubated in suspension for the indicated time periods before collection and immunoprecipitation of FAK. Immunoprecipitates were analysed by western blotting with anti-paxillin and anti-FAK antibodies as indicated. The relevant band in each case is marked with an arrowhead.

non-adherent cells, whereas an inactive double point mutant of PKI (Mut2) had no such effect (Fig. 1d, e). This indicates that inhibition of PKA activity, rather than sequestration of cAMP by the regulatory subunit, is responsible for promoting the anchorage-independent growth-factor response.

**Anchorage-independent signalling induced by PKA inhibition is transient.** We determined the duration of dnPKA-induced, anchorage-independent signalling by assaying growth-factor activation of MAPK in cells cultured in suspension for prolonged periods of time. The induction of anchorage-independent signalling by PKA inhibition was transient, as the robust growth-factor response seen after 2 h in suspension gradually fell to control levels after 6–12 h in suspension (Fig. 2). Together, these data indicate that the environment that restricts growth-factor signalling in non-adherent cells may ultimately prevail, and that inhibition of PKA activity may significantly delay, rather than irreversibly inhibit, this process.

**Detachment activates PKA.** The observations described above indicate that PKA may be involved in creating or maintaining a non-permissive environment for growth-factor signalling in non-adherent cells. This, in turn, implies that cell adhesion may regulate PKA — specifically, that PKA activity may be upregulated in non-adherent cells. Adhesion and cytoskeletal organization are known to be negatively regulated by increased activity of cAMP and PKA (reviewed in ref. 12), but reports of control of cAMP and PKA by cell adhesion are



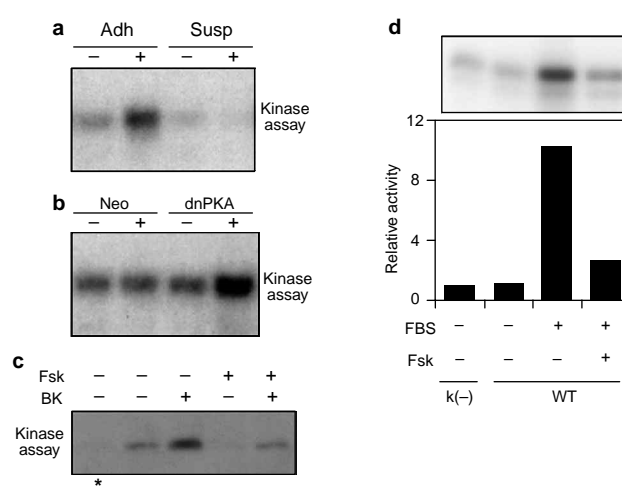
**Figure 5 Anchorage-independent MAPK activation is blocked by cytochalasin D.** **a**, Stable control neo or dnPKA-expressing cells were placed in suspension and incubated for 2 h in the presence (+) or absence (-) of the indicated concentrations of cytochalasin D (cyto D) before stimulation with 20 ng ml<sup>-1</sup> EGF for 5 min as indicated. Cell lysates were subjected to SDS-PAGE and analysed by western blotting with antibodies against total MAPK or active, phosphorylated MAPK (P-MAPK). **b**, NIH3T3 cells were transiently co-transfected with plasmids encoding HA-tagged MAPK and either the wild-type or Mut2 allele of PKI, and then incubated in suspension for 2 h in the presence (+) or absence (-) of 2 μM cytochalasin D before stimulation with 20 ng ml<sup>-1</sup> EGF for 5 min as indicated. The tagged kinase was immunoprecipitated (IP) from lysates, subjected to SDS-PAGE and analysed by western blotting with anti-HA or anti-P-MAPK antibodies.

scarce<sup>21-23</sup>. To investigate the latter process, we assayed intracellular PKA activity in cells while adherent and at various times after detachment. In control NIH3T3 cells, PKA activity increased rapidly after detachment, peaking at ~5-fold after 10–15 min (Fig. 3, solid line). As expected, there was no significant increase in PKA activity in the dnPKA cell line (Fig. 3, dashed line).

Interestingly, PKA activity in detached NIH3T3 cells returned to basal levels 60–90 min after detachment, well before the time point at which activation of the MAPK cascade was shown to be inhibited in normal non-adherent cells (2 h after detachment; see Figs 1 and 2). Thus, non-adherent NIH3T3 cells remain refractile to growth-factor stimulation well after PKA activity has returned to baseline levels. This indicates that PKA may not directly inhibit signalling itself, but rather may initiate an event that persists past the decline of peak PKA activity to restrict the growth-factor response.

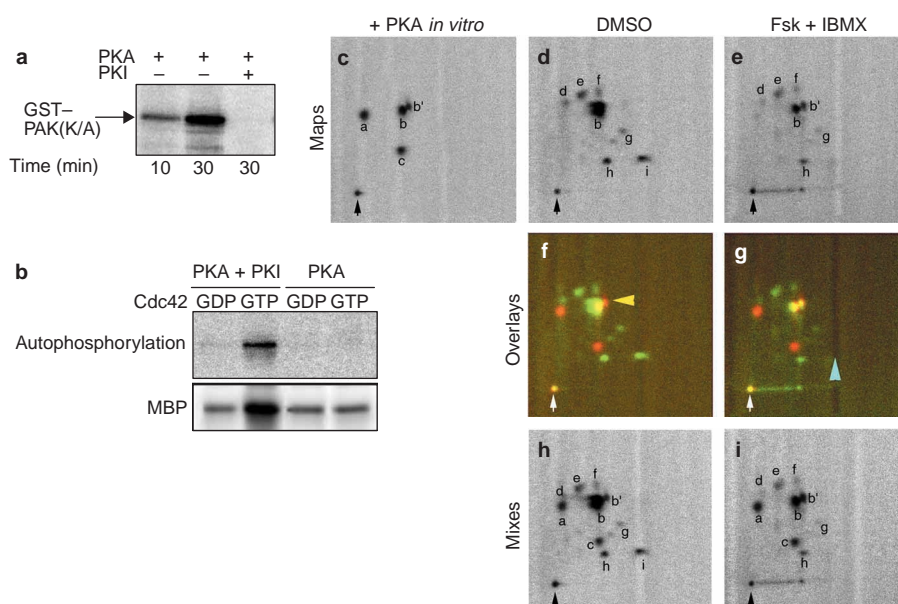
One hypothesis that is compatible with our findings is that PKA activity may be required for inactivation of adhesion-related proteins or structures that may be involved in regulating the efficiency of growth-factor signalling<sup>24</sup>. This idea is supported by the ability of PKA to disrupt the actin cytoskeleton in adherent cells<sup>14</sup> and induce tyrosine dephosphorylation of the focal-adhesion proteins paxillin<sup>25-28</sup> and FAK<sup>29,30</sup>. These proteins, which are important constituents of focal contacts, are highly tyrosine-phosphorylated in adherent cells and are normally rapidly dephosphorylated after detachment of cells from the ECM.

**Regulation of tyrosine phosphorylation of FAK and paxillin in dnPKA cells.** To determine whether inhibition of PKA affects



**Figure 6 Negative regulation of PAK by PKA in vivo.** **a**, Anchorage-dependence of PAK activation. Serum-starved NIH3T3 cells were maintained on their tissue-culture plates (Adh) or were placed in suspension for 2 h (Susp), then either left unstimulated (-) or stimulated with 10% FBS for 5 min (+). PAK was immunoprecipitated from cell lysates and assayed by *in vitro* kinase assay using myelin basic protein as a substrate. **b**, Anchorage-independent activation of PAK in dnPKA cells. Serum-starved control neo or dnPKA-expressing cells were placed in suspension for 2 h and either left untreated (-) or stimulated with serum (+) as in **a**. PAK activity was assessed by immunoprecipitation and *in vitro* kinase assay. **c**, **d**, Forskolin inhibits activation of PAK. Confluent, serum-starved NIH3T3 cells (**c**) or NIH3T3 cells transiently transfected with Myc-tagged, kinase-inactive (k(-)) or wild-type (WT) PAK (**d**) were incubated with dimethylsulphoxide (0.1% v/v) or 25 μM forskolin (Fsk) for 20 min before stimulation with 500 nM bradykinin (BK) (**c**) or 10% FBS (**d**) for 5 min. PAK activity was assessed by immunoprecipitation with anti-PAK (**c**) or anti-Myc (**d**) antibodies, followed by *in vitro* kinase assay. Asterisk in **c** denotes the activity in the reaction mixture alone. In **d**, data for activity of transfected PAK are expressed relative to the activity of wild-type PAK in unstimulated cells; the relative activity of kinase-inactive PAK was typically 0.8–0.9. Autoradiograph (**d**, upper panel) shows the results of a typical experiment; values in the histogram (**d**, lower panel) are means from three experiments.

adhesion-related signalling components, we monitored the tyrosine phosphorylation of paxillin and FAK in neo and dnPKA cells in suspension or re-plated onto fibronectin. At 2 h after detachment, the level of tyrosine phosphorylation of both FAK and paxillin was significantly higher in non-adherent dnPKA cells than in neo control cells (Fig. 4a, c). The level of tyrosine phosphorylation of FAK in non-adherent dnPKA cells was comparable to that in cells plated on fibronectin. However, the level of tyrosine phosphorylation of paxillin in non-adherent dnPKA cells, although greater than in non-adherent control cells, was considerably less than in cells on fibronectin. The tyrosine phosphorylation of both FAK and paxillin in non-adherent dnPKA cells was significantly reduced by 4 h after detachment (Fig. 4b, d), and by 6 h was no greater than the level observed in non-adherent control cells (data not shown). This time course is consistent with that observed for the effect of dnPKA expression on MAPK activation. In addition to the effects on tyrosine phosphorylation, co-immunoprecipitation experiments showed that the association between FAK and paxillin was also transiently preserved in non-adherent dnPKA cells (Fig. 4e). Although not mediated by tyrosine phosphorylation, the interaction between FAK and paxillin is usually dependent upon cell anchorage<sup>31</sup>. These data show that inhibition of PKA impedes dephosphorylation of tyrosine and dissociation of FAK and paxillin after detachment of cells. This indicates that inhibition of PKA activity might delay detachment-induced inactivation



**Figure 7 PKA phosphorylates PAK *in vitro* and *in vivo*.** **a**, Purified, recombinant, kinase-inactive PAK fused to GST (GST-PAK(K/A)) was incubated for the indicated times with the PKA catalytic subunit and  $^{32}\text{P}$ - $\gamma$ -ATP, in the presence or absence of PKA-inhibitor peptide (PKI) as indicated. Reaction products were separated by SDS-PAGE and visualized by autoradiography. **b**, PAK, immunoprecipitated from adherent, serum-starved NIH3T3 cells, was incubated with PKA in the absence or presence of PKI, then incubated with purified Cdc42 (preloaded with GDP or GTP- $\gamma$ -S as indicated) and assayed for autophosphorylation and phosphorylation of MBP (see Methods). **c–e**, Two-dimensional tryptic phosphopeptide maps of recombinant PAK1 phosphorylated by PKA *in vitro* (**c**), or isolated from  $^{32}\text{P}$ -labelled cells treated with dimethylsulphoxide (DMSO; **d**) or with forskolin and isobutylmethylxanthine (Fsk

+ IBMX; **e**). The pattern of phosphopeptides for each sample was almost identical in at least two separate experiments. The small arrow in the lower-left corner of each panel indicates the origin. **f**, **g**, To help illustrate distinct and co-migrating phosphopeptides, *in vitro* and *in vivo* maps were pseudo-coloured red and green, respectively, and overlaid. **f** represents the pseudo-colour overlay of **c** and **d**; **g** represents **c** and **e**. As in dual-colour immunofluorescence, co-localization or, in this case, co-migration, appears yellow. The arrowhead in **f** indicates phosphopeptide b'; the arrowhead in **g** indicates the position at which phosphopeptide i should run (see text). **h**, **i**, Equal c.p.m. of phosphopeptides from *in vitro*-phosphorylated PAK and PAK isolated from control (**h**) or Fsk/IBMX-treated (**i**) cells were mixed, spotted onto thin-layer cellulose plates and separated (see Methods).

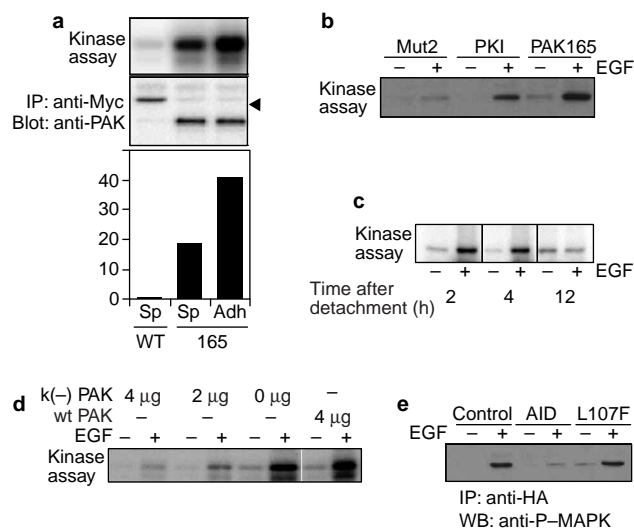
or disassembly of adhesion-related protein complexes that may regulate the efficiency of growth-factor signalling.

**Cytochalasin D blocks signalling in non-adherent dnPKA cells.**

As discussed earlier, activation of PKA can disrupt the actin cytoskeleton in many cell types<sup>11,12</sup>, and dissolution of microfilament integrity can inhibit growth-factor signalling to MAPK<sup>13,24</sup>. Thus, inhibition of PKA activity might delay the dissolution of actin structures that are required for efficient growth-factor signalling. It is also possible that, rather than preserving cytoskeletal integrity, inhibition of PKA may promote anchorage-independent signalling by causing the MAPK cascade to bypass the requirement for cytoskeletal integrity altogether. If this were true, then one might expect the anchorage-independent signalling behaviour of dnPKA cells to be insensitive to pharmacological agents that disrupt the cytoskeleton. To test this hypothesis, we measured growth-factor activation of MAPK in non-adherent neo and dnPKA cells treated with cytochalasin D, a potent actin-depolymerizing agent. The efficient activation of MAPK observed in control-treated, non-adherent dnPKA cells was significantly reduced by 1  $\mu\text{M}$  cytochalasin D (Fig. 5a). Treatment of non-adherent dnPKA cells with 2  $\mu\text{M}$  cytochalasin D completely inhibited the response to growth factors, and the level of activation of MAPK in these cells was no greater than that seen in non-adherent neo cells. Similar results were obtained when cells transiently transfected with the PKI-expressing plasmid were treated with cytochalasin D (Fig. 5b). The observed sensitivity of anchorage-independent signalling to cytochalasin D indicates that some filamentous actin is preserved in, and is required for, the signalling-permissive cellular environment created by suppression of PKA activity.

**PKA inhibits PAK activity *in vivo*.** The findings that PKA can affect phosphorylation of FAK and paxillin and that anchorage-independent signalling induced by PKA inhibition is blocked by cytochalasin D support the hypothesis that adhesive and/or cytoskeletal elements have a crucial function in regulating the anchorage-dependence of MAPK activation. Indeed, the importance of FAK and cortical actin in regulation of anchorage-dependent signalling to MAPK has been directly demonstrated<sup>13,32</sup>. The ability of PAK to regulate the formation of cortical actin structures, as well as to regulate the activation of Raf and MEK, indicates that PAK may also be involved in anchorage-dependent signalling events. Previous studies have shown that inhibition of PAK function can block signalling through the MAPK cascade in adherent cells<sup>16,33,34</sup>. Furthermore, it has recently been demonstrated that activation of PAK by soluble factors (serum and PDGF) is highly dependent upon cell adhesion<sup>35</sup>. It follows that inhibition or restriction of PAK activity in non-adherent cells may be involved in the inhibition of signalling to MAPK.

To investigate the function of PAK in regulation of anchorage-dependent MAPK signalling, we first determined the anchorage-dependence of PAK1 activation in our system. We measured PAK1 activity in non-adherent cells and in stably attached cells, before and after stimulation with serum (Fig. 6). As was found for MAPK, activation of PAK by serum was seen to be strongly anchorage-dependent in control cells (Fig. 6a), but independent of cell anchorage in dnPKA cells (Fig. 6b). This indicates that, like activation of the MAPK cascade, activation of PAK may be regulated, at least in part, by PKA activity. To assess this possibility more directly, we stimulated control and Fsk-treated NIH3T3 cells with bradykinin

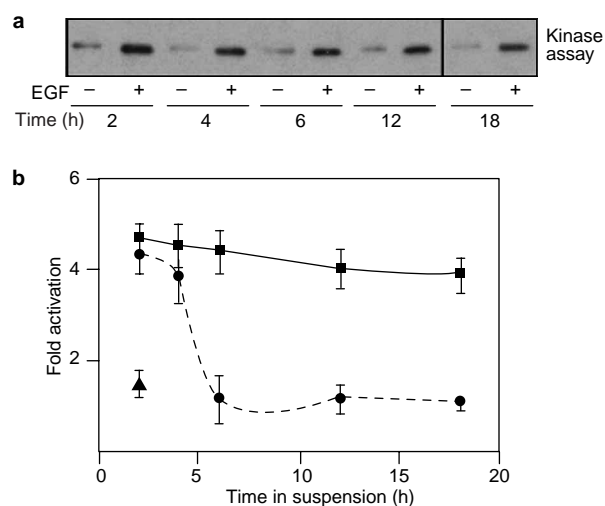


**Figure 8 PAK and PKA regulate anchorage-dependent signalling to MAPK.**

**a**, A truncated mutant of PAK1 is highly active in non-adherent cells. NIH3T3 cells were transiently transfected with plasmids encoding Myc-tagged wild-type PAK (WT) or a tagged mutant of PAK lacking the first 165 amino acids (165). Cells were serum-starved overnight and maintained on tissue-culture dishes (Adh) or placed in suspension for 2 h (Sp) before collection. Anti-Myc immunoprecipitates (IP) were subjected to an *in vitro* kinase assay or were analysed by western blotting with anti-PAK antibody as indicated. The arrowhead indicates the position of a marker of relative molecular mass 62,000. Values in the histogram represent activity relative to that of wild-type PAK and are means from two separate experiments. **b**, Constitutively active PAK induces anchorage-independent activation of MAPK by growth factors. Cells were co-transfected with HA-MAPK and the indicated plasmids, incubated in suspension and stimulated with growth factor as indicated. The kinase activity of HA-MAPK is shown. Similar results were obtained from three separate experiments. **c**, The anchorage-independent phenotype induced by constitutively active PAK is transient. NIH3T3 cells were transfected with PAK165, and cultured as in **a**; activation of HA-MAPK by EGF was measured at the indicated times after detachment. **d**, Catalytically inactive PAK inhibits activation of MAPK in non-adherent dnPKA cells. The indicated amounts of wild-type (wt) or catalytically-inactive (k(-)) PAK plasmids were co-transfected with HA-MAPK into the dnPKA cell line, and activation of the tagged kinase was measured after culturing the cells in suspension for 2 h and stimulation with EGF as indicated. **e**, Expression of the PAK autoinhibitory domain (AID) blocks activation of MAPK in non-adherent dnPKA cells. Empty vector (control), or a plasmid expressing the wild-type PAK1 AID or an inactive AID point mutant (L107F) was co-transfected with HA-MAPK into the dnPKA cell line, and activation of the tagged kinase was measured after culturing the cells in suspension for 2 h and stimulation with EGF as indicated. WB, western blot; P-MAPK, phosphorylated MAPK.

(an activator of Cdc42; ref. 14) or serum, and measured activation of endogenous (Fig. 6c) or transfected (Fig. 6d) wild-type PAK1. Stimulation of PKA by Fsk inhibited activation of PAK by both bradykinin (Fig. 6c) and serum (Fig. 6d, lanes 2–4). These data indicate that activation of PKA results in inhibition of PAK1 *in vivo*.

**PKA phosphorylates PAK *in vitro* and *in vivo*.** Previous data support a growing body of evidence that PKA functions in regulating PAK-family kinases. Functional connections between PKA and PAK have been demonstrated in the regulation of oocyte maturation in *Xenopus*<sup>36</sup> and chemotaxis in *Dictyostelium*<sup>37</sup>. A more direct connection has recently been reported for MST3b (ref. 38), a kinase of the STE20 family, to which PAK also belongs; in this example, MST3b is directly phosphorylated and inhibited by PKA. Interestingly, mammalian PAKs contain several potential sites for phosphorylation by PKA, indicating that PKA may also regulate



**Figure 9 PAK and dnPKA synergize to prolong the anchorage-independent phenotype.** **a**, dnPKA cells were transfected with PAK165 and cultured in suspension for the indicated times before assaying for HA-MAPK activity. **b**, Fold activation of HA-MAPK in PAK165-transfected dnPKA cells (squares; *n* = 4) and PAK165-transfected NIH3T3 cells (circles; *n* = 3) is shown for reference. **c**, Proposed scheme connecting PKA, PAK and activation of the MAPK pathway. Arrows denote positive/activating relationships; bars denote negative/inhibitory relationships. See Discussion for details.

mammalian PAKs, at least in part, by direct phosphorylation. To explore this possibility, we determined whether purified PKA could phosphorylate recombinant PAK1 *in vitro*. We used a kinase-inactive point mutant of PAK1 fused to glutathione-S-transferase (GST-PAK(K/A)), as wild-type GST-PAK proteins are highly active and are readily autophosphorylated (refs 17, 18 and references therein). Incubation of GST-PAK(K/A) with purified PKA and radiolabelled ATP resulted in a time-dependent increase in incorporation of radioactive phosphate into the band corresponding to the full-length fusion protein (Fig. 7a). The observed phosphorylation was solely due to the activity of PKA, as it was completely blocked by addition of purified PKI to the reaction. Phosphorylation was not due to modification of the GST moiety, as a similar degree of phosphorylation was observed using purified PAK(K/A) from which GST had been removed by thrombin cleavage (data not shown). We next determined the effect of PKA phosphorylation of PAK on PAK kinase activity. Immunoprecipitated PAK phosphorylated by PKA *in vitro* was refractory to Cdc42-mediated activation, whereas unmodified PAK (that is, PAK incubated with PKA in the presence of PKI) was readily activated by GTP-bound Cdc42 (Fig. 7b). These data show that PKA can phosphorylate and inhibit PAK *in vitro*.

Figure 6 shows that activation of PKA can inhibit PAK *in vivo*. The data in Fig. 7a, b indicate that this may be achieved, at least in part, through direct phosphorylation. We therefore examined the effect of PKA activation on phosphorylation of PAK *in vivo* by two-dimensional phosphopeptide mapping (Fig. 7c–i). PAK isolated from control-treated cells routinely generated seven distinct phosphopeptides (Fig. 7d). Stimulation with Fsk and IBMX resulted in the appearance of a unique phosphopeptide that was not present in PAK from control-treated cells. (peptide b', Fig. 7e). Significantly, this peptide co-migrated precisely with one of the phosphopeptides generated by phosphorylation of purified, recombinant, kinase-inactive PAK by PKA *in vitro* (Fig. 7c, e, g, i). Interestingly, the intensity of the remaining phosphopeptides was reduced (peptides b, d–h) or completely ablated (peptide i) by activation of PKA. Together, the data in Figs 6 and 7 show that PKA can directly phosphorylate and inhibit PAK both *in vitro* and *in vivo*.

**Constitutively active PAK promotes anchorage-independent MAPK signalling.** If the anchorage-dependence of PAK activation is involved in restricting MAPK signalling in non-adherent cells, then rescuing PAK activity in non-adherent cells should restore signalling to MAPK. To test this hypothesis, we sought to obtain a PAK mutant that, unlike wild-type PAK, exhibits high levels of activity in non-adherent cells. We transfected NIH3T3 cells with PAK165, a truncated and constitutively active form of PAK1 (ref. 17). After recovery from the transfection, we left cells attached to the tissue-culture plate or placed them in suspension and collected them to assay PAK165 activity. As expected, PAK165 was highly active in attached cells, but this level of activity was reduced by ~50% when cells were placed in suspension (Fig. 8a). Despite this reduction, the level of activity of PAK165 in non-adherent cells was still roughly twice that of serum-stimulated wild-type PAK (Figs 6d, 8a and data not shown). Although the mechanism that underlies the relative decrease in PAK165 activity in non-adherent cells is currently unknown, the high level of residual activity (relative to wild-type PAK) justifies the use of PAK165 as a source of constitutive PAK activity in non-adherent cells.

We next determined the effect of expression of PAK165 on the anchorage-dependence of growth-factor signalling to MAPK. We transiently co-transfected NIH3T3 cells with a plasmid encoding haemagglutinin (HA)-tagged MAPK together with plasmids encoding wild-type (Fig. 8b, lanes 3 and 4) or mutant (lanes 1 and 2) PKI, or PAK165 (lanes 5 and 6). Like inhibition of PKA, constitutive PAK activity induced anchorage-independent activation of MAPK by growth factors (Fig. 8b, lanes 5 and 6). As with dnPKA, the anchorage-independent growth-factor response induced by PAK165 was transient (Fig. 8c).

**PKA and PAK coordinately regulate anchorage-dependent MAPK activation.** We determined whether PAK activity is required for the anchorage-independent MAPK signalling elicited by inhibition of PKA. Expression of full-length, kinase-inactive PAK inhibited MAPK activation in non-adherent dnPKA cells in a dose-dependent manner, whereas expression of wild-type PAK had no effect (Fig. 8d). Similar inhibition was elicited by expression of the PAK auto-inhibitory domain (AID; Fig. 8e), comprising amino acids 83–149 of PAK1, which inhibits PAK by direct binding to the kinase domain<sup>39</sup>. No inhibition was observed after expression of the AID containing a L107F point mutation (Fig. 8e); this construct cannot bind to the PAK kinase domain and therefore does not inhibit PAK activity. These data indicate that PAK may be important for anchorage-independent signalling to MAPK in dnPKA cells. This is not surprising, as it has previously been shown that inhibition of PAK function can block signalling through the MAPK cascade in adherent cells<sup>16,33,34</sup>.

Finally, we sought to determine the effect of concurrent inhibition of PKA and constitutive activation of PAK on anchorage-dependent signalling. Expression of PAK165 in dnPKA cells had no apparent effect on the level of MAPK activation after stimulation of non-adherent cells (Fig. 9a). However, coincident expression of

dnPKA and PAK165 induced a state of anchorage-independent growth-factor signalling that persisted for far longer than that observed with dnPKA or PAK165 alone (Fig. 9a, b). Thus, PAK and PKA cooperate or synergize in regulating anchorage-dependent growth-factor signalling to MAPK. These observations indicate that PAK has an important function in supporting the state of anchorage-independent signalling elicited by inhibition of PKA. Furthermore, the synergy observed between PKA inhibition and PAK activation indicates that, rather than being connected in a purely linear fashion, PKA and PAK may cooperate to affect several pathways that regulate anchorage-dependent signal transduction.

## Discussion

We have shown that inhibition of PKA induces a transient state of anchorage-independence with respect to growth-factor signalling to MAPK. The duration of this effect correlates with the persistence of tyrosine-phosphorylated FAK and paxillin after detachment of cells from adhesive substrates. A function of PAK in this effect is indicated by three sets of observations. First, PKA phosphorylates and inhibits PAK both *in vitro* and *in vivo*. Moreover, an environment that restricts activation of PAK (that is, non-adherence) will permit PAK activation when PKA is inhibited. Second, inhibition of PAK activity can block the anchorage-independent MAPK signalling elicited by inhibition of PKA. Third, constitutively active PAK can synergize with PKA inhibition to prolong the duration of anchorage-independent signalling. To our knowledge, this is the first work to implicate either PKA or PAK in regulation of anchorage-dependent signal transduction through the MAPK pathway.

The data presented here prompt three important questions. First, how does PKA regulate PAK activity? There is growing evidence to suggest that PKA may directly or indirectly regulate several PAK-family kinases. Previous reports have shown functional connections between PKA and PAK in the regulation of oocyte maturation in *Xenopus*<sup>36</sup> and chemotaxis in *Dictyostelium*<sup>37</sup>. A more direct connection has recently been reported for MST3b (ref. 38), a kinase of the STE20 family, to which PAK also belongs. Here we have shown that PKA can directly phosphorylate PAK1 at at least one site *in vivo*, which seems to lead to net dephosphorylation at other sites. Although the mechanism of this latter effect is currently unknown, possibilities include inhibition of PAK autophosphorylation and regulation of the availability of PAK to various phosphatases, such as PP2A (ref. 40). Control of PAK activity *in vivo* is achieved by a complex milieu of signalling and structural factors, many of which can be affected by PKA and may therefore also contribute to the regulation of PAK by PKA. Most prominent among these are paxillin<sup>41</sup> and the adaptor protein Nck<sup>42</sup>. Detailed investigation of this regulatory pathway, including identification of the phosphorylated residues that are affected by PKA and elucidation of how their modification controls PAK function *in vitro* and *in vivo*, is a subject for future study.

A second question raised by our results is how inhibition of PKA allows anchorage-independent signalling to MAPK. One hypothesis that is compatible with our findings is that PKA may regulate cytoskeletal structures that govern the efficiency with which growth factor signals are transduced. This model is depicted in Fig. 9c. Such signalling 'scaffolds' certainly exist in yeast, and there is growing evidence that they are also present in higher eukaryotes<sup>43,44</sup>. As mentioned above, activation of PKA in adherent cells is known to disrupt the actin cytoskeleton. Therefore, the increase in PKA activity upon cellular detachment may contribute to the disassembly of such structures, thereby impeding growth-factor signalling. Conversely, inhibition of PKA may slow disassembly, allowing certain structures to remain intact after detachment and therefore allowing a transient period of anchorage-independent growth-factor response. The idea that a certain degree of cytoskeletal integrity is preserved under these conditions is strongly supported by our data that indicate that anchorage-independent

signalling induced by PKA inhibition is blocked by cytochalasin D.

PKA can negatively regulate several factors that are important for establishing or maintaining adhesive and cytoskeletal structures. A partial list includes FAK, paxillin, the vasodilator-stimulated phosphoprotein (VASP)<sup>45</sup>, Rho<sup>46</sup>, actin itself<sup>47</sup> and, as shown here, PAK. The delayed dephosphorylation of FAK in detached dnPKA cells is particularly interesting in light of a recent report showing that FAK has a crucial function in mediating the anchorage requirement for signalling through the MAPK cascade<sup>32</sup>. This indicates that the ability of PKA inhibition to elicit anchorage-independent growth-factor signalling may be directly linked to the concomitant preservation of tyrosine phosphorylation of FAK (Fig. 4). Regulation of PAK function may also contribute to the ability of PKA to modulate anchorage-dependent signalling. Indeed, the importance of PAK in this regard is underlined by the finding that constitutive PAK activity alone can transiently rescue signalling in non-adherent cells. PAK may function by promoting assembly of cortical actin structures, which may then provide purchase for signalling molecules (Fig. 9c). Thus, inhibition of PAK through activation of PKA would facilitate disassembly of the cytoskeleton and inhibition of growth-factor signalling. Conversely, suppression of PKA may allow PAK activity to persist, thereby promoting maintenance of cytoskeletal integrity. However, given the complexity of cytoskeletal regulation and the large number of PKA substrates, PAK, although certainly important, is unlikely to be the sole target for PKA in regulation of anchorage-dependent signalling.

In addition to cytoskeletal effects, PAK may also contribute to controlling the anchorage-dependence of MAPK signalling through direct phosphorylation of Raf and MEK<sup>16,15</sup> (Fig. 9c). This is particularly interesting in light of the fact that both Raf and MEK have been shown to represent points of convergence between cell adhesion and growth-factor signalling<sup>7,8</sup>. Thus, activation of PKA would inhibit PAK, preventing phosphorylation of Raf and MEK and lowering the efficiency of their activation. Conversely, increased PAK activity would increase phosphorylation of Raf and MEK and thereby facilitate their activation. Indeed, the synergy observed between inhibition of PKA and activation of PAK indicates that, rather than being connected in a purely linear fashion, these proteins may cooperate to affect several pathways, structural and otherwise, that regulate anchorage-dependent signalling. This idea is also supported by the large number of potential effectors for both PKA and PAK<sup>11,15-17</sup>.

A third question raised by this study concerns the downstream biological event(s) that is/are affected by this regulatory mechanism. The MAPK pathway is highly promiscuous, being involved in the regulation of many cellular events. It is possible that the prolonged anchorage-independent response to growth factors induced by simultaneous PKA inhibition and PAK activation may promote anchorage-independent growth. However, it has become increasingly clear that pathways other than the MAPK cascade also regulate anchorage-dependent cell growth<sup>24</sup>, and so persistent MAPK signalling may not be sufficient to permit anchorage-independent growth. It is also possible that prolonged responsiveness to growth factors gives rise to cells that are resistant to apoptosis or anoikis<sup>48</sup>. Finally, it is conceivable that PKA and PAK may cooperate to regulate crosstalk between cell adhesion and growth-factor signalling at a more localized level, in order to influence cytoskeletal organization during directed cell motility. Investigation of these possibilities is currently underway. □

## Methods

### Stable cell lines and transfections.

The dnPKA and neo control cell lines were generated by infection of NIH3T3 cells with bicistronic retroviral vectors<sup>9</sup> containing the dnPKA coding sequence or an empty multiple cloning site upstream of the IRES/neo sequence, and were maintained in DMEM containing 10% FBS and 1 mg ml<sup>-1</sup> active G418. Transient transfections were carried out using SuperFect (Qiagen) according to the manufacturer's protocol. Constructs used for transient transfections include derivatives of the neo and dnPKA plasmids used to generate the retroviral vectors<sup>9</sup>, as well as CMV-based plasmids expressing wild-type or mutant PKI (ref. 20 and D. Howe, UNC, Chapel Hill, North Carolina). Plasmids encoding Myc-tagged wild-type PAK-1, kinase-inactive PAK-1(K298A) and PAK165 (ref. 17) were gifts from M. Cobb

(Southwestern Med. Center). Plasmids encoding the wild-type PAK1 AID (amino acids 83–149) and the inactive L107F point mutant were provided by G. Bokoch (Scripps Research Institute). Plasmids encoding Myc-Raf, EE-MEK and HA-MAPK were as described<sup>11,49</sup>. For experiments involving non-adherent and re-plated cells, cells were cultured as described<sup>7,15</sup>. Briefly, confluent cells were serum-starved overnight, then detached by trypsinization and collected in DMEM containing 1 mg ml<sup>-1</sup> soybean trypsin inhibitor. Cells were washed once with DMEM plus 2% BSA, resuspended in the same, and then incubated in suspension with gentle rocking for 45 min. Cells were then maintained in suspension or re-plated onto fibronectin-coated plates for 2 h (unless otherwise indicated) before stimulation and lysis. Where indicated, cytochalasin D was added to cells 2 h before collection. Forskolin, IBMX and bradykinin were used at 25 mM, 50 μM and 500 nM, respectively.

### Immunoprecipitation, western blotting, and immunoprecipitation kinase assays.

Cells were washed twice with ice-cold PBS before extraction in NP40 lysis buffer<sup>49</sup>. Lysates were clarified by centrifugation in a microcentrifuge at 4 °C at 16,500g for 15 min. Protein concentrations were measured by bicinchoninic acid reaction (Sigma), and equal amounts of soluble protein were used in subsequent steps. FAK and paxillin were immunoprecipitated and western blotted using commercially available monoclonal antibodies (Upstate Biotechnology and Transduction Laboratories for FAK and paxillin, respectively). Phosphotyrosine was measured in western blots using monoclonal antibody 4G10 (Upstate Biotechnology). Endogenous and epitope-tagged kinases of the MAPK cascade were assayed as described<sup>7,15,49</sup>. Phospho-specific anti-MAPK antibody was from Promega. PAK kinase assays, using myelin basic protein (MBP) as a substrate, were carried out as described<sup>7</sup> after immunoprecipitation either with anti-α-PAK antibody (C-20; Santa Cruz) or with anti-Myc antibody (9E10; Boehringer). Kinase reaction products were routinely separated by SDS-PAGE and visualized from stained, dried gels by exposure to a phosphorimager plate (Molecular Dynamics). Incorporation of radioactive phosphate into reaction products was quantified using a phosphorimager, or in some cases, by spotting 10% of the material from stopped reactions onto phosphocellulose spin columns (Pierce), which were then washed with 75 mM phosphoric acid and analyzed in a scintillation counter.

### PKA assays.

Adherent cells or cells in suspension were washed with ice-cold PBS, covered or resuspended in PKA assay buffer<sup>49</sup>, then snap-frozen in liquid nitrogen. Lysates were thawed on ice, scraped into tubes (for adherent cells) and then sonicated and clarified by centrifugation at 10,000g for 10 min at 4 °C. Supernatants were assayed for PKA activity by measuring transfer of radioactive phosphate from ATP into Kemptide (Sigma) in the presence or absence of purified PKI, as described<sup>49</sup>.

### In vitro biochemical methods.

For purification of GST-PAK, log-phase bacteria transformed with a plasmid expressing kinase-inactive PAK1(K299A) fused to GST (G. Bokoch) were induced with 0.1 mM isopropyl-β-thiogalactopyranoside for 4 h at 30 °C, collected by centrifugation and sonicated on ice. The lysate was clarified by centrifugation at 10,000g and the supernatant was mixed with glutathione agarose (final concentration 1% v/v) for 15 min at room temperature. The resin was washed twice with 50 ml PBS containing 1% Triton X-100 and then 4 times with PBS, and then either resuspended in 25 mM Tris pH 7.6, 10 mM NaCl and 50% glycerol and stored at -80 °C or incubated for 1 h at room temperature with reduced glutathione. Eluted proteins were dialysed against 25 mM Tris pH 7.6, 10 mM NaCl and 50% glycerol overnight at 4 °C and stored at -80 °C. To remove the GST tag, some of the resin-bound fusion protein was washed twice in 50 mM Tris pH 7.5 and 150 mM NaCl, and once in thrombin-cleavage buffer (50 mM Tris pH 7.5, 150 mM NaCl and 2.5 mM CaCl<sub>2</sub>), and then incubated with 0.5 μg thrombin for 1 h at 25 °C. Thrombin was removed from the released protein by incubation with *p*-diaminobenzamide-agarose (Sigma). PKA phosphorylation reaction mixtures contained 1–2 μg recombinant PAK, 2.5–5.0 U of purified PKA catalytic subunit (New England Biolabs), 10 μCi <sup>32</sup>P-γ-ATP (3,000 Ci mM<sup>-1</sup>) and 20 μM unlabelled ATP in 50 μl PKA buffer (50 mM Tris pH 7.5 and 10 mM MgCl<sub>2</sub>). Reactions were incubated at 30 °C for 30 min (unless otherwise indicated) and stopped by addition of an equal volume of 2× Laemmli sample buffer and boiling for 5 min. In some instances, purified PKI peptide was added (final concentration 400 μg ml<sup>-1</sup>) to block PKA activity. To determine the effect of PKA phosphorylation on PAK activation by Cdc42 *in vitro*, PAK immunoprecipitates from 3–4 mg of soluble protein from NIH3T3 detergent lysates were washed 4 times with modified RIPA lysis buffer (1% NP40, 0.5% sodium deoxycholate, 100 mM NaCl, 2 mM each of EDTA and EGTA and 25 mM Tris, pH 7.6), once with 100 mM Tris pH 8.6 and 500 mM LiCl, and once with PKA buffer (50 mM Tris pH 7.5 and 10 mM MgCl<sub>2</sub>), and then either used immediately or resuspended in PKA buffer containing 10% glycerol and frozen in aliquots at -80 °C. PAK beads were washed once with PBS and then resuspended in 50 μl PKA buffer containing 2 μl purified PKA catalytic subunit (New England Biolabs), 20 μM ATP, and in some instances, 10 μCi <sup>32</sup>P-γ-ATP (3,000 Ci mM<sup>-1</sup>). PAK1 isolated in this way was also specifically phosphorylated by PKA with kinetics similar to those seen with recombinant PAK (data not shown). PAK was quenched with an equal volume of 20 mM EDTA and 0.8 g l<sup>-1</sup> PKI in PKA buffer and removed over several washes; PAK beads were then incubated at room temperature for 10 min with 0.5 μg purified recombinant Cdc42 (a gift from J. Sondek, UNC) and preloaded with GDP or GTP-γ-S as described<sup>51</sup>. PKA buffer (40 μl) containing 20 μM ATP, 10 μCi <sup>32</sup>P-γ-ATP and 100 μg ml<sup>-1</sup> MBP were added to the complexes and reactions were incubated at 30 °C for 15 min. Reactions were stopped by addition of an equal volume of 2× sample buffer and boiling for 5 min.

### Phosphopeptide mapping

NIH3T3 cells at ~90% confluence were placed in serum-free DMEM for 8 h, then re-fed with phosphate-free DMEM containing 1 mCi ml<sup>-1</sup> <sup>32</sup>P-orthophosphate (NEN) and incubated at 37 °C for 6 h. Where indicated, Fsk and IBMX were added during the last 20 min of labelling before collection. Labelled cells were washed extensively with ice-cold PBS and lysed in 2× modified RIPA buffer (0.5 ml per 100-mm plate). Lysates were scraped into microfuge tubes, brought to a final volume of 1 ml per 100-mm plate with ice-cold PBS, vortexed for 15 s and placed on ice for 15 min. Lysates were pre-cleared by centrifugation (16,500g at 4 °C for 10 min) and further clarified by incubation with protein A/G-agarose for 1 h at 4 °C. Cleared lysates were incubated at 4 °C with an antibody against the amino terminus of PAK1 (N-20, Santa Cruz) for 2 h, then with protein A/G-agarose for 45 min. Immunoprecipitates were washed extensively (>5 times) with modified RIPA buffer and then boiled in

1x Laemmli sample buffer. Radiolabelled proteins were separated on 7.5% SDS-PAGE gels, which were soaked for 1 h in 20% aqueous methanol, then dried. A duplicate gel containing ~10% of each sample was run, transferred to nitrocellulose and analysed by immunoblotting with anti-PAK antibody to ensure equal loading. Gels were exposed to phosphorimager plates and bands corresponding to PAK1 were localized, excised, rehydrated in fresh 50 mM NH<sub>4</sub>CO<sub>3</sub> and fine-ground with disposable pestles (Kontes, Vineland, New Jersey). Phosphopeptides were released from ground gel slices by incubation with 2.5 µg sequencing-grade trypsin in 50 mM NH<sub>4</sub>CO<sub>3</sub> (total volume 100 µl) for 4 h at 37 °C; a further 2.5 µg trypsin was then added and the mixture was incubated overnight at 37 °C. Phosphopeptides were recovered by washing ground gel slices twice with 50 µM NH<sub>4</sub>CO<sub>3</sub> (500 µl for each wash); they were then lyophilized, resuspended in deionized H<sub>2</sub>O and re-lyophilized, and then resuspended in 5 ml deionized H<sub>2</sub>O. Phosphopeptides (equal volumes for *in vivo* samples, equal c.p.m. for PAK phosphorylated *in vitro*) were applied to thin-layer cellulose plates and separated in one dimension by electrophoresis at 1 kV for 35 min in buffer at pH 1.9 (ref. 52), and in a perpendicular dimension by chromatography for 10 h in isobutyric acid buffer<sup>22</sup>. Plates were air-dried, wrapped in plastic and exposed to phosphorimager plates, typically for 12–24 h.

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