PAK4 is activated via PI3K in HGF-stimulated epithelial cells

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Accepted 1 August 2002
Journal of Cell Science 115, 3947-3956 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00080

Summary

The p21-activated kinases (PAKs) are divided into two subgroups based on sequence homology. Group 1 PAKs (PAK1-3) are involved in cell migration, and are activated by pro-migratory stimuli and by Cdc42/Rac GTPases. In contrast, little is known about the regulation of the recently identified group II PAKs (PAK4-6). Here we report that PAK4 is activated by HGF, a migratory stimulus for epithelial cells. In unstimulated MDCK cells, activated PAK4 induces a decrease in stress fibres, and when cells are stimulated with HGF, it induces a loss of focal complexes and cell rounding. This response is dependent on PAK4 kinase activity but does not require Cdc42 interaction. Activated PAK4 localises to the cell periphery but not specifically in lamellipodia, and HGF induces localisation of wild-type PAK4 to the cell periphery. LY294002, a phosphoinositide 3-kinase (PI3K) inhibitor, inhibits HGF-induced PAK4 kinase activation, relocalisation, and cell rounding. However, the isolated C-terminal kinase domain of PAK4 can induce cell rounding in the presence of LY294002, suggesting that the N-terminal region acts as a negative regulator of PAK4 activity. These results indicate that HGF stimulates PAK4 through PI3K, and that PAK4 could contribute to HGF-induced changes in actin organisation and cell-substratum adhesion.

Movies available on-line.

Key words: p21-activated kinase, Phosphoinositide 3-kinase, PAK4, HGF, Actin, Cytoskeleton, Focal complexes

Introduction

The p21-activated kinases (PAKs) interact with Rac/Cdc42 and are involved in Rac/Cdc42-mediated actin organisation during cell adhesion and migration (Daniels and Bokoch, 1999). All six mammalian PAK isoforms share a highly conserved C-terminal kinase domain and an N-terminal Cdc42/Rac-interactive binding (CRIB) domain (Burbelo et al., 1995), also known as the GTPase-binding domain (GBD) (Symons et al., 1996). PAKs can be categorised into two subgroups based on sequence homology (Jaffer and Chernoff, 2002). The Group I PAKs (PAK1, -2 and -3) share approximately 75% sequence homology throughout the protein (Manser et al., 1995; Pawson and Scott, 1997; Teo et al., 1995), whereas the Group II PAKs (PAK4, -5 and -6) have highly related kinase domains but lower homology in the rest of the protein (Dan et al., 2002; Yang et al., 2001). PAK1, -2 and -3 bind to both Cdc42 and Rac, and this interaction is believed to induce a conformational change, which relieves auto-inhibition and stimulates kinase activity above a low basal level (Sells et al., 1997; Zhao et al., 1998). In contrast, PAK4 and PAK6 interact preferentially with Cdc42, but their kinase activity is not stimulated by this association (Abo et al., 1998; Lee et al., 2002). PAK1, -2 and -3 contain an SH3-binding site N-terminal to the GBD domain, which is responsible for binding to the adapter protein Nck (Bokoch et al., 1996). In addition, there is an SH3-binding site between the GBD domain and the C-terminal kinase domain that mediates association with PIX, a putative exchange factor for Rac (Manser et al., 1998). In contrast, PAK4 has no SH3-binding sites N-terminal to the GBD domain and does not bind Nck or PIX, but has seven putative SH3-binding sites between the GBD and kinase domains (Abo et al., 1998).

Both Group I and Group II PAKs can induce actin rearrangement in cells (Daniels and Bokoch, 1999; Jaffer and Chernoff, 2002; Sells and Chernoff, 1997). For example, PAK1 can induce lamellipodium formation and has been localised to sites of membrane ruffling in fibroblasts (Dharmawardhane et al., 1997; Sells et al., 1997). PAK4 has been shown to be involved in Cdc42-induced filopodium extension in endothelial cells (Abo et al., 1998) and fibroblasts (Qu et al., 2001), and PAK5 can induce neurite extension (Dan et al., 2002). PAKs are also implicated in the regulation of cell:substrate adhesion: expression of constitutively activated PAK1, 2 and 4 has been reported to induce cell rounding (Manser et al., 1997; Qu et al., 2001; Zeng et al., 2000). In addition, PAK1 has been localised to focal adhesions (Manser et al., 1997; Zhao et al., 1998) and co-immunoprecipitated with paxillin, a focal adhesion component (Turner et al., 1999). Effects of PAK1 are cell-type-dependent. However, as in fibroblasts, expression of an activated PAK1 mutant enhances the cell migration rate (Sells et al., 1999), whereas in endothelial cells the same mutant increases cell contractility and reduces cell migration (Kiosses et al., 1999).

HGF is a multifunctional cytokine that can act as a motility-inducing factor for epithelial cells (Stoker et al., 1987; Weidner et al., 1993) through the c-Met tyrosine kinase receptor (Bottaro et al., 1991; Naldini et al., 1991). HGF-stimulated epithelial cells lose cell:cell contacts, take on a more fibroblastic-like morphology and become more migratory. PAK1 is activated by HGF (Royal et al., 2000), as well as by other pro-migratory stimuli including PDGF (Dechert et al., 2001) and heregulin (Adam et al., 1998). In contrast, the activation of group II PAKs by growth factors has not been studied. We show here that PAK4 kinase activity is stimulated by HGF in MDCK cells, and that activated PAK4 induces a loss of cell:substratum adhesion in an HGF-dependent manner. Furthermore, both activity and subcellular localisation of PAK4 during HGF signalling are regulated by PI3K.

Materials and Methods

Cell culture and microinjection

A sub-clone of MDCK cells (Ridley et al., 1995) was grown in DMEM containing 10% bovine FCS. Cells for microinjection were seeded in 15 mm wells at 10^4 cells per well on 13 mm circular glass coverslips. Three days after seeding, cells were transferred to DMEM containing 0.2% FCS for 1 hour. Cells were then injected at the edge or middle of colonies. Cells were microinjected with one of the following plasmids at a concentration of 100 ng/μl (unless otherwise indicated): pSRα-HA-PAK4 (wild-type PAK4); pSRα-HA-PAK4ΔGBD (activated PAK4); pSRα-HA-PAK4Δ (aa 324-591, kinase domain of PAK4); pSRα-HA-PAK4M350 (kinase dead PAK4); pSRα-HA-PAK4ΔM350 (aa 324-591, kinase-inactive kinase domain) (Abo et al., 1998); 25 ng/μl pCMV-Flag-V12Cdc42. Following microinjection, the cells were incubated for 3 hours. Cells then either remained in DMEM with 0.2% FCS or were stimulated with HGF (10 ng/ml; R and D systems, Abingdon, UK) for up to 20 hours. For kinase inhibition studies microinjected cells were routinely pre-incubated for 30 minutes with 20 μM LY294002 (Calbiochem, Nottingham, UK) before further incubation in the absence or presence of HGF. In all cases, expressed proteins were detected by immunofluorescence.

Time-lapse video microscopy

MDCK cells were microinjected with pSRα-HA-PAK4ΔGBD at a concentration of 100 ng/μl. After 3 hours, cells were transferred to DMEM with 0.2% FCS and 10 ng/ml HGF and then placed in an incubator mounted on the stage of a Zeiss Axiovert 135 microscope, maintained at 37°C in a humidified atmosphere containing 10% CO_2. Cell images were collected by a KPM1E/K-S10 CCD camera (Hitachi Denshi, Japan) every 2 minutes for 20 hours using Tempus software (Kinetic Imaging, Liverpool, UK). Cells expressing PAK4 were subsequently identified by immunofluorescence.

Immunofluorescence

Mouse anti-HA antibody was obtained from Berkeley Antibody Company (Richmond, CA), rabbit anti-HA antibody from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-paxillin antibody from Transduction Laboratories (Lexington, KY) and mouse anti-flag antibody from Sigma. FITC-conjugated goat anti-mouse IgG secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PS) and TRITC-conjugated goat anti-rabbit IgG from Southern Biotechnology (Birmingham, AL). TRITC-conjugated phalloidin was obtained from Sigma. Cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature and then permeabilised with 0.2% Triton X-100 in PBS for 5 minutes. Primary and secondary antibodies were diluted in PBS containing 0.5% bovine serum albumin and all incubations were for 1 hour at room temperature. For detection of expressed proteins, cells were incubated with a 1:100 dilution of mouse anti-HA antibody, a 1:200 dilution of rabbit anti-HA antibody, or a 1:200 dilution of mouse anti-flag antibody. Following incubation with the primary antibody, cells were washed six times in PBS and then incubated with a 1:400 dilution of FITC-conjugated goat anti-mouse IgG, a 1:200 dilution of TRITC-conjugated goat anti-rabbit IgG and/or TRITC-conjugated phalloidin. Images of cells were obtained using a Zeiss LSM510 confocal laser-scanning microscope (Welwyn Garden City, UK), using the accompanying LSM 510 software, and were processed in Adobe PhotoShop 4.0.

Antibody production and immunoblotting

A rabbit polyclonal anti-PAK4 antibody was raised against a peptide derived from the PAK4 kinase domain, PRRK[SL]VGTPYMAPE. The antibody was then affinity-purified against this peptide. An anti-PAK1 (C19) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); C19 is known to crossreact with PAK2 and PAK3. Mouse anti-HA antibody was obtained from Berkeley Antibody Company (Richmond, CA). MDCK cells were lysed for 10 minutes in lysis buffer (0.5% NP-40, 30 mM sodium pyrophosphate, 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin and 1 μg/ml aprotinin). Lysates were clarified by centrifugation at 14,000 ***g*** for 10 minutes. Equal amounts of protein were electrophoresed on 10% SDS-polyacrylamide gels then transferred to nitrocellulose membranes (Schleicher and Schell). Membranes were blocked in 5% nonfat dried milk in PBS then incubated for 16 hours at 4°C with either a 1:1000 dilution of rabbit anti-PAK4 antibody, a 1:1000 dilution of C19 anti-PAK1 antibody or a 1:1000 dilution of mouse anti-HA in 0.5% nonfat dried milk/PBS. Membranes were then incubated for 1 hour at room temperature with a 1:2000 dilution of horseradish-peroxidase-conjugated donkey anti-rabbit or anti-mouse antibody in 0.5% nonfat milk: PBS (Amersham Pharmacia, Little Chalfont, UK). Blots were developed by enhanced chemiluminescence (ECL, Amersham Pharmacia).

Kinase assay

MDCK cells were transiently transfected with HA-PAK4wt, HA-PAK4ΔGBD or PAK4Δ using Fugene transfection reagent (Roche, Roche Molecular Biochemicals, IN). Cells were incubated for 24 hours in DMEM with 10% FCS prior to 4 hours starvation in DMEM with 0.2% FCS. Cells were then stimulated for up to 30 minutes with HGF (10-100 ng/ml; R and D systems, Abingdon, UK). For LY294002 experiments cells were pre-incubated (20 μM, Calbiochem, Nottingham, UK) for 30 minutes prior to stimulation with HGF. Following stimulation, cells were harvested in lysis buffer (0.5% NP-40, 30 mM sodium pyrophosphate, 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin and 1 μg/ml aprotinin). Lysates were clarified by centrifugation at 14,000 ***g*** for 10 minutes. Cell lysates were then pre-cleared twice with mouse anti-myc antibody 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA), and protein G-Sepharose (Amersham Pharmacia, Little Chalfont, UK). A small amount of lysate was removed from each sample for SDS-PAGE anaylsis. The pre-cleared lysates were then mixed with anti-HA antibody (Berkeley Antibody Company, Richmond, CA) overnight at 4°C followed by a 1 hour incubation with protein G-Sepharose at 4°C. The immune complexes were washed twice with lysis buffer, once with Wash buffer 1 (0.5 M LiCl and 20 mM Tris pH 8.0) and twice with kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT) then incubated in kinase buffer containing 30 μM ATP and 3 μCi of [γ-^{32}P]ATP together with Histone H1 (Roche) for 30 minutes

at 30°C. The reaction was stopped by adding 6× SDS loading buffer. As a control a kinase assay was also performed on cell lysates from untransfected cells, which had been immunoprecipitated with protein G-Sepharose beads coupled to anti-HA antibody. Proteins were resolved by SDS-PAGE and any phosphorylation was visualised by autoradiography.

Results

PAK4 is expressed in MDCK cells

A PAK4-specific antibody was used to demonstrate PAK4 expression in MDCK cells (Fig. 1A, lane 1, 68 kDa). PAK5 and PAK6 are similar in sequence to PAK4 in the region used to generate this antibody (Yang et al., 2001) and could possibly be recognised by it. However, no proteins with the predicted sizes of PAK5 (80 kDa) or PAK6 (75 kDa) were detected with this antibody (Fig. 1A), and it only recognised one protein on western blots of 2D electrophoresis gels of MDCK lysates (data not shown). This indicates either that PAK5/6 are not expressed in MDCK cells, or that the antibody does not crossreact with them. As PAK5 is reported to be brain-specific (Dan et al., 2002), we would not expect to see it in MDCK cell lysates. An anti-PAK1 antibody known to crossreact with PAK2 and PAK3 (C19; Santa Cruz Biotechnology) detected two further PAK isoforms in MDCK cell lysates, which we predict are PAK1 and PAK2 (Fig. 1A, lane 2, 65 kDa and 62 kDa, respectively) as PAK3 (65 kDa) is reported to be brain-specific (Manser et al., 1995).

Activated PAK4 induces cell rounding in HGF-stimulated MDCK cells

To analyse the morphological responses induced by PAK4, we transiently expressed wild-type PAK4 (PAK4wt) and various PAK4 mutants in MDCK cells. PAK4 lacking the GBD domain (PAK4ΔGBD) has increased kinase activity compared with wild-type PAK4 (Abo et al., 1998) (Fig. 1B). The kinase domain alone of PAK4 also has elevated kinase activity when expressed in MDCK cells (Fig. 1B). Exogenous PAK4 protein expression was readily detectable by immunofluorescence up to 24 hours after microinjection of expression vectors (Fig. 2 and data not shown), indicating that PAK4 overexpression is not toxic to the cells.

Expression of PAK4ΔGBD for up to 23 hours in the absence of HGF had no discernible effect on cell morphology (Fig. 2ii, A). In contrast, in the presence of HGF the majority of cells expressing PAK4ΔGBD rounded up (Fig. 2ii, B). This response was titratable, and at lower levels of expression PAK4ΔGBD was unable to induce cell rounding (data not shown). For PAK4ΔGBD to induce cell rounding, it was not essential for HGF to be present for the whole timecourse, but cells needed to be stimulated with HGF for a minimum of 15-30 minutes. This is similar to the length of HGF stimulation required to induce discernible scattering (data not shown). PAK4-induced cell rounding required an activated protein, as expression of PAK4wt at comparable levels had no effect on cell morphology either in the absence or presence of HGF (data not shown). This is consistent with previous reports where overexpression of PAK4wt had no effect on the actin cytoskeleton or cell morphology (Dan et al., 2001; Qu et al., 2001). MDCK cells expressing activated PAK1 or PAK3 were able to round up in the absence of HGF stimulation, thus cell rounding was not strictly dependent on the morphological changes that occur during HGF stimulation (data not shown). Although it has been previously reported that an activated PAK4 mutant can induce cell rounding in fibroblasts (Qu et al., 2001), this is the first demonstration of a growth-factor-dependent response to PAK4.

The removal of the GBD domain of PAK4 is presumably required to make the kinase sufficiently active to stimulate this response. Cdc42 binding is unable to mimic the effect of GBD

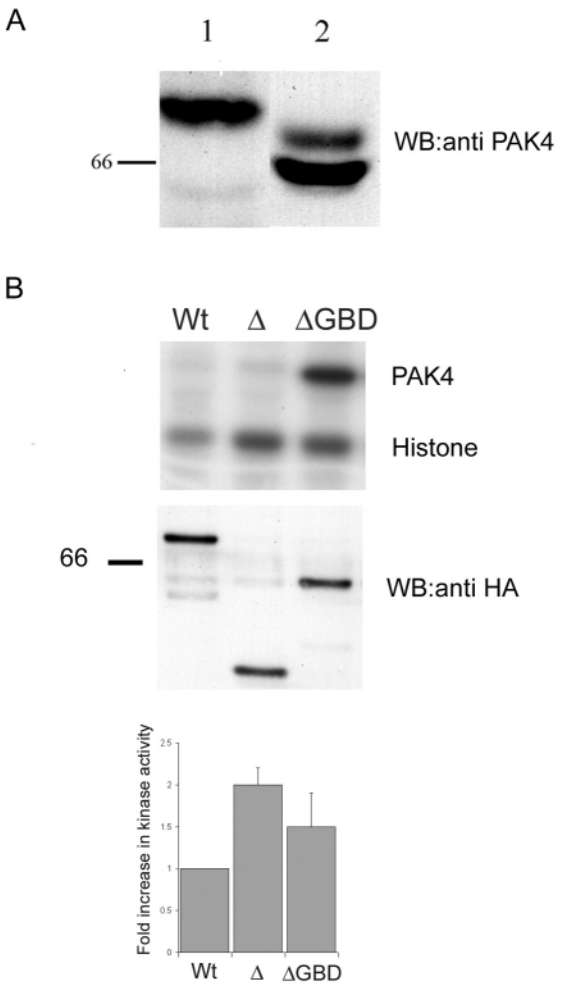
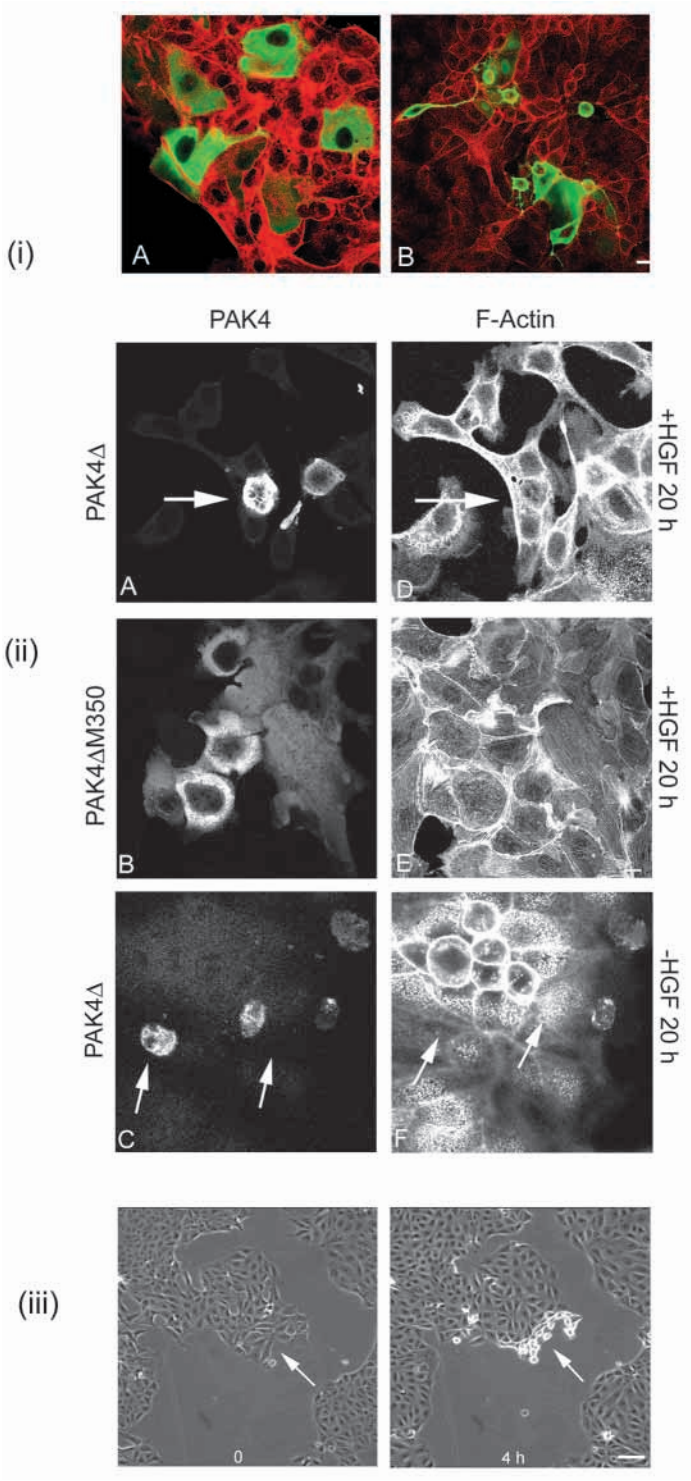


Fig. 1. PAK4 expression and activity in MDCK cells. (A) Analysis of endogenous PAK isoform expression in MDCK cell lysates. A western blot of MDCK cell lysates was probed with antibodies against PAK4 (lane 1) and PAK1, -2 and -3 (lane 2). (B) Kinase activity of PAK4. MDCK cells were transfected with HA-PAK4wt (Wt), HA-PAK4Δ (Δ) and HA-PAK4ΔGBD (ΔGBD) and incubated for 24 hours in 0.2% FCS. PAK4 was immunoprecipitated from cell lysates with anti-HA mAb and its kinase activity assayed in the presence of Histone H1 and [γ-^{32}P]ATP. Substrate phosphorylation was analysed after SDS-PAGE and autoradiography. Phosphorylation of Histone (arrow) and autophosphorylation (arrowhead) is indicated. Retained cell lysates were subjected to western blotting with the anti-HA mAb to show expression levels of exogenous PAK4 proteins. Autoradiographs and western blots were quantified using Kinetic Imaging software and the level of kinase activity normalised to protein expression levels. The results shown are the means±s.e.m. of three independent experiments.

domain deletion, as co-expression of PAK4wt and V12Cdc42 did not induce cell rounding (data not shown). This is consistent with the observation that Cdc42 binding does not elevate the kinase activity of PAK4 (Abo et al., 1998).

PAK4-induced MDCK cell rounding is kinase dependent

PAK1-induced changes in cell morphology and actin organisation can be either kinase-dependent (Frost et al., 1998; Zhao et al., 1998) or kinase-independent (Sells et al., 1999; Sells et al., 1997). PAK1-induced fibroblast cell rounding requires a functional kinase domain (Frost et al., 1998), and so we investigated whether PAK4-induced rounding of MDCK cells also required a functional kinase domain. Expression of the kinase domain alone of PAK4 (PAK4Δ) induced cell rounding in the presence of HGF (Fig. 2ii, A), but expression of the inactive kinase domain PAK4ΔM350 [(Abo et al., 1998) and data not shown] did not (Fig. 2ii, B). This confirms that PAK4-induced cell rounding also requires kinase activity. Interestingly, expression of PAK4Δ induced cell rounding in the absence of HGF (Fig. 2ii, C). This indicates that the N-terminal region of PAK4 negatively regulates PAK4 activity and suggests that this region normally negatively regulates the kinase domain of PAK4wt and PAK4ΔGBD in unstimulated MDCK cells.



Activated PAK4 expression reduces cell-substratum adhesion

To investigate the time course of PAK4-induced responses, PAK4-expressing cells were followed by time-lapse video microscopy. As expected, PAK4ΔGBD-expressing cells in the absence of HGF did not round up (unless entering mitotic division) or detach from the substratum, and cells injected with control IgG or expressing PAK4wt exhibited a normal motile response to HGF and did not round up (data not shown). In addition, we found no evidence of HGF-dependent cell rounding in uninjected cells. However, cells expressing PAK4ΔGBD (Fig. 2iii; Movie 1, see http://jcs.biologists.org/supplemental) started to round up as early as 5 hours post injection, after 2 hours in the presence of HGF. By 4 hours, in the presence of HGF, the majority of cells were almost completely rounded and some had begun to detach from the substratum. Interestingly, although cells detached from the substratum they retained cell-cell contacts with each other and with uninjected cells in the surrounding colony. This observation accounts for the detection of rounded cells up to 23 hours after injection (Fig. 2i, B). PAK4ΔGBD-expressing cells remained rounded but did not bleb for up to 24 hours, indicating that they were not apoptotic.

The video time-lapse observations indicated that cell-

Fig. 2. Activated PAK4 induces cell rounding. (i) MDCK cells were microinjected with a plasmid encoding PAK4ΔGBD and incubated for 3 hours in 0.2% FCS prior to a further incubation in 0.2% FCS for 20 hours (A) or stimulation with HGF for 20 hours (B). Cells were then fixed and stained for HA-tagged PAK4 (green) and F-actin (red). Bar, 10 μm. Apical sections (at the level of F-actin-containing microvilli) are shown of PAK4ΔGBD expression (B) in order to visualise clearly any rounded-up PAK4-expressing cells (arrows). In this plane non-expressing rounded cells are primarily mitotic cells. (ii) MDCK cells were microinjected with a plasmid encoding the kinase domain of PAK4, PAK4Δ (A,C), or a kinase-inactive PAK4 kinase domain, PAK4ΔM350 (B), and incubated for 3 hours in 0.2% FCS prior to stimulation for 20 hours with HGF (A,B) or further incubation in the absence of HGF (C). Cells were fixed and stained for HA-tagged PAK4 (A-C) and F-actin (D-F). Bar, 10 μm. (iii) Selected frames (time=0 or 4 hours) are shown from a time-lapse video recording (Movie 1) of MDCK cells expressing activated PAK4 (PAKΔGBD) (arrows) and stimulated at t=0 with HGF. Bar, 20 μm.

substratum adhesions are altered by PAK4ΔGBD as early as 2 hours after HGF stimulation. HGF normally induces a decrease in actin stress fibres and large focal adhesions and an increase in smaller peripheral focal complexes in MDCK cells [(Dowrick et al., 1991) data not shown]. This change in the nature of cell-substratum adhesions can be detected by following paxillin localisation. Paxillin-containing peripheral focal complexes were clearly observed in PAK4wt-expressing cells (Fig. 3D) at 3 hours after HGF stimulation. However, in PAK4ΔGBD-expressing cells (Fig. 3C) there was a significant reduction in the number of paxillin-containing peripheral focal complexes. This could only be visualised in cells expressing very low levels of PAK4ΔGBD where the spreading edge had not been completely retracted. These results suggest that PAK4-induced cell rounding is either due to an increase in focal complex turnover or to a decrease in focal complex formation.

Activated PAK4 is localised to the cell periphery

Time-lapse microscopy and paxillin localisation in PAK4ΔGBD-expressing cells suggests that induction of cell rounding occurs approximately 2-3 hours after HGF stimulation. We therefore investigated the localisation of PAK4ΔGBD and PAK4wt at this time point after microinjection, with or without short-term HGF stimulation. PAK1 is known to be localised in lamellipodia and focal adhesions (Dharmawardhane et al., 1997; Sells et al., 2000).

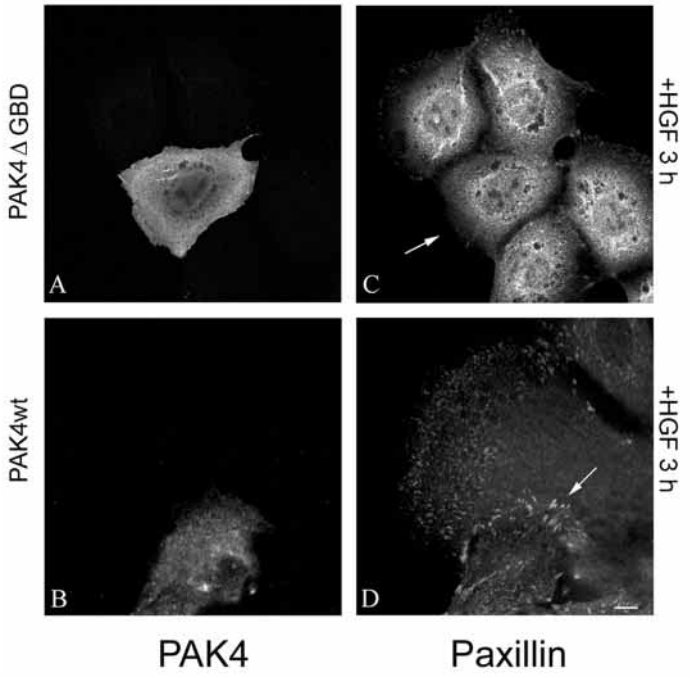


Fig. 3. PAK4 induces loss of paxillin-associated focal complexes. MDCK cells were microinjected with a plasmid encoding either PAKΔGBD or PAK4wt and incubated for 3 hours in 0.2% FCS before stimulation for 3 hours with HGF. Cells were then fixed and stained for HA-tagged PAK4 (A,B) and paxillin (C,D). Arrows indicate the absence of paxillin-associated focal complexes in a PAKΔGBD-expressing cell (C) and the presence of paxillin-associated focal complexes in a cell expressing PAK4wt (D). Bar, 10 μm.

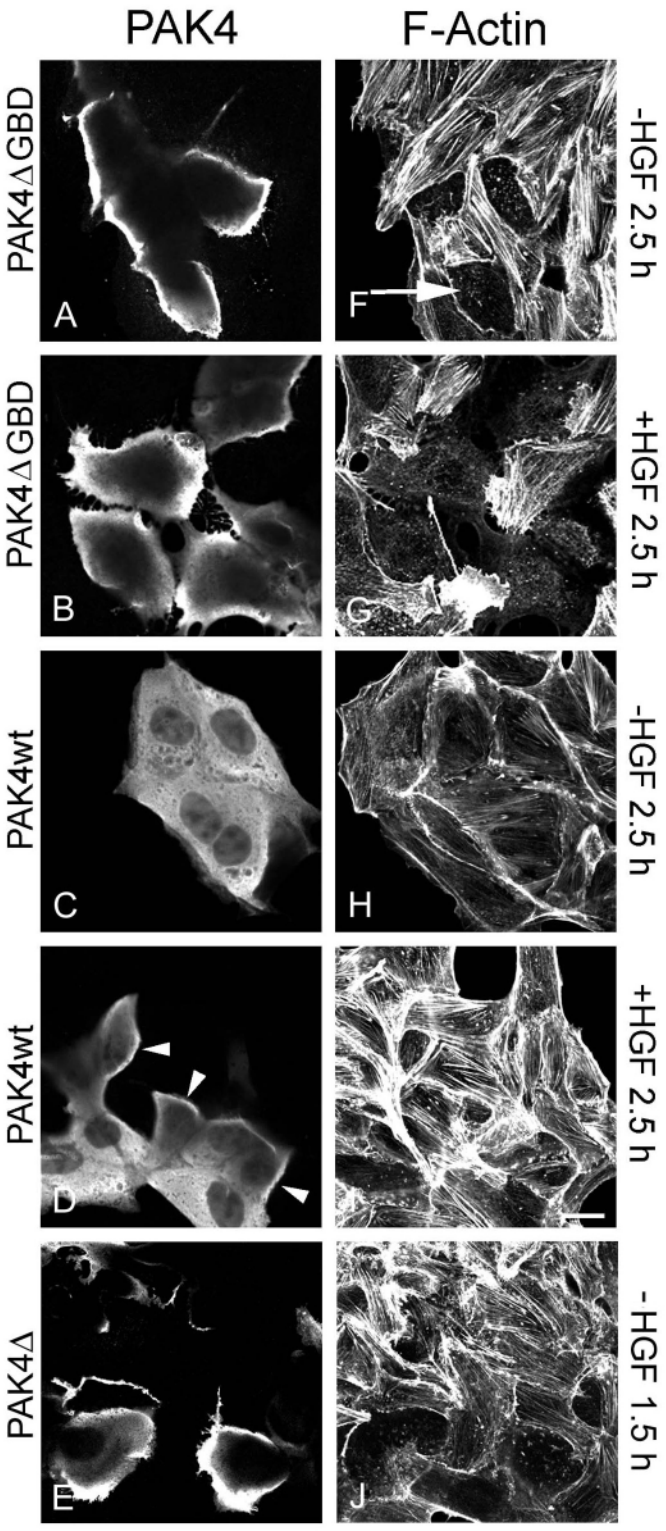


Fig. 4. PAK4 is localised at the cell periphery. MDCK cells were microinjected with plasmids encoding PAK4ΔGBD or PAK4wt and incubated for 5.5 hours in 0.2% FCS (A,C) or 3 hours in 0.2% FCS prior to stimulation with HGF for 2.5 hours (B,D). (E) MDCK cells were microinjected with a plasmid encoding PAK4Δ and incubated for 4.5 hours in 0.2% FCS. Cells were then fixed and stained for HA-tagged PAK4 and F-actin. Bar, 10 μm. The arrow (F) indicates a PAK4ΔGBD-expressing cell; arrowheads (D) indicate PAK4wt localisation at the periphery.

Table 1. HGF induces PAK4 relocalisation

Cells	Cells with peripheral localisation (%)	
	Total	Edge cells
PAK4ΔGBD –HGF	93	95
PAK4ΔGBD –HGF+LY	81	89
PAK4wt –HGF	14	14
PAK4wt +HGF	30	56
PAK4wt +HGF+LY	11	14

Colonies of MDCK cells in 0.2% FCS were microinjected with a plasmid encoding PAK4wt at a concentration of 30 ng/μl or PAK4ΔGBD at a concentration of 100 ng/μl and incubated for 3 hours followed by a further 2.5 hours incubation in the absence or presence of HGF. LY294002 was added 2.5 hours post injection for 30 minutes before a further incubation for 2.5 hours in the absence or presence of HGF. Cells were then fixed and stained for PAK4 expression and F-actin. Using confocal microscopy, cells were then scored for the localisation of PAK4 proteins. A total of 60-100 cells were counted over two experiments.

However, in the majority of both unstimulated and HGF-stimulated cells, PAK4ΔGBD was localised at the periphery (Fig. 4A,B; Table 1) 5.5 hours post-injection, but localisation was not restricted to the lamellipodium. In a small number of cells the distribution was diffusely cytoplasmic and PAK4ΔGBD was occasionally localised to the nucleus (data not shown), as observed in PAE cells (Abo et al., 1998). Whether PAK4 plays a role in the nucleus is not known, but it is interesting that PAK6 can also localise to the nucleus (Yang et al., 2001). However, we found no evidence for PAK4 localisation in focal adhesions.

HGF induced relocalisation of PAK4wt to the cell periphery (Fig. 4D) in a proportion of cells, which was particularly significant in cells at the edge of the colonies (Table 1). At higher expression levels, PAK4wt exhibited a diffuse cytoplasmic staining in the absence or presence of HGF (data not shown; there are currently no antibodies available that detect endogenous PAK4 by immunofluorescence). PAK4Δ exhibited a peripheral localisation similar to that of PAK4ΔGBD either in the absence (Fig. 4E) or presence of HGF (data not shown), whereas PAKΔM350 was diffusely localised (Fig. 2ii, B and data not shown). This indicates that the region of the protein involved in localisation of PAK4 to the cell periphery is within the kinase domain and not the regulatory domain and that this localisation is dependent on the kinase activity. Our results suggest that PAK4 subcellular localisation is growth-factor-regulated. This may explain why PAK4wt and PAK4ΔGBD were reported to have a diffuse cytoplasmic localisation in PAE cells and fibroblasts (Abo et al., 1998; Callow et al., 2002; Qu et al., 2001).

Activated PAK4 causes stress fibre disassembly

HGF stimulation of MDCK cells is known to induce initially a loss of actin stress fibres and associated focal adhesions [data not shown (Dowrick et al., 1991)]. Expression of PAK4ΔGBD caused a decrease in actin stress fibres in the presence or absence of HGF (Fig. 4F), with 75% of PAK4ΔGBD-expressing cells lacking actin stress fibres, 5.5 hours post-injection, compared with 12.5% of uninjected cells. Furthermore, expression of PAK4Δ induced an even more dramatic loss: 91.3% of PAK4Δ-expressing cells had no actin stress fibres 5.5 hours post-injection (Fig. 4J). Consistent with these data, it has recently been reported that expression of a different highly activated PAK4 mutant in fibroblasts causes disruption of actin stress fibres and cell rounding (Qu et al., 2001).

However after 23 hours of expression in the absence of HGF, PAK4ΔGBD-expressing cells appeared similar to uninfected cells and contained normal levels of actin stress fibres (Fig. 2i and data not shown). In addition, after prolonged PAK4ΔGBD expression in the absence of HGF, PAK4ΔGBD was no longer localised to the cell periphery suggesting that peripheral localisation of activated PAK4 is a transient event (Fig. 2i). Indeed, after 23 hours in the presence of HGF, PAK4wt expressed at low levels assumed a diffuse cytoplasmic localisation (data not shown). As peripheral localisation and stress fibre disassembly are both transient events and correlate with kinase activity, these results suggest that PAK4ΔGBD activity is downregulated in the absence of HGF.

PAK4 is activated by HGF

Our results suggest that PAK4 kinase activity is required for cell rounding and localisation and that it is specifically regulated by HGF. A previous report indicated that PAK1 kinase activity could be stimulated by HGF in MDCK cells (Royal and Park, 1995). To determine whether HGF can activate PAK4 kinase activity, MDCK cells were transiently transfected with an expression vector encoding HA-tagged PAK4wt, at levels where exogenous PAK4wt expression were no more than two-fold higher than endogenous PAK. PAK4wt was immunopurified from stimulated and unstimulated cell lysates, and kinase activity was measured using Histone H1 as a substrate. PAK4 kinase activity was very low in starved cells, which suggests that in MDCK cells PAK4 is not constitutively activated. PAK4 became autophosphorylated and had elevated kinase activity 5 minutes after stimulation with HGF. Kinase activity was maximal (2.5-fold) after 15 minutes, and then decreased at 30 minutes (Fig. 5A). A similar 2.5-fold activation of kinase activity by HGF has also been reported for SGK1 (serum- and glucocorticoid-inducible kinase 1) and PAK1 (Royal et al., 2000; Shelly and Herrera, 2002). This relatively low fold-activation of kinases is probably due to only a proportion of cells in epithelial colonies responding to HGF stimulation. As the HGF receptor Met is localised on the basolateral surface of polarized MDCK cells (Crepaldi et al., 1994), polarised cells at the centre of colonies would be expected to respond very weakly, if at all, to apically applied HGF.

It is unlikely that a co-precipitating kinase is responsible for the phosphorylation of Histone H1, as no other proteins in the anti-HA immunoprecipitates became detectably labelled with ^{32}P. In addition, Histone H1 phosphorylation was reduced in kinase assays with kinase-defective HA-tagged PAK4ΔM350. Consistent with a previous report (Callow et al., 2002), this mutant retained a low level of kinase activity, but this was lower than that of PAK4wt (no HGF) or the control (Fig. 5D). Immunoprecipitates of endogenous PAK4 also demonstrated HGF-stimulated kinase activity towards Histone H1; however, in this case we cannot rule out the presence of another kinase in the immunoprecipitates (data not shown).

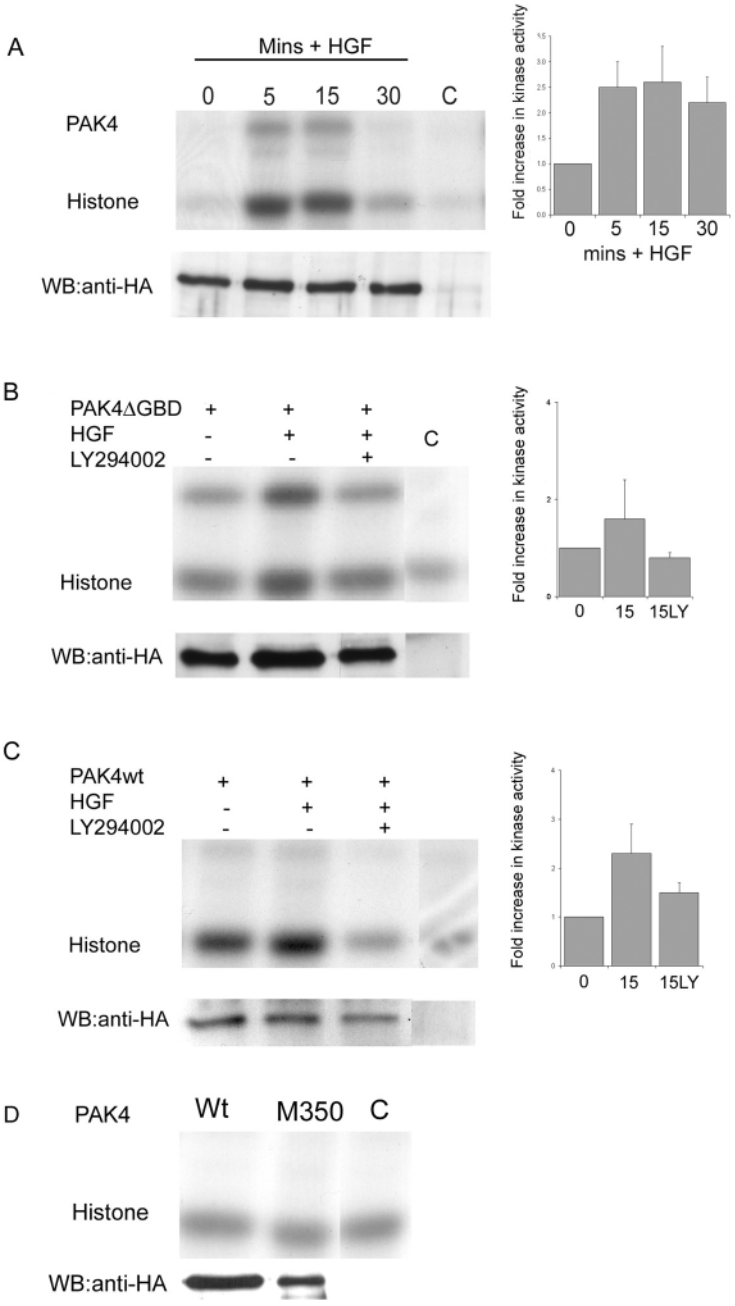


Fig. 5. PAK4 kinase activity is elevated by HGF. (A) MDCK cells were transfected with HA-PAK4wt and serum-starved for 4 hours prior to stimulation with HGF for the indicated times. PAK4 was immunoprecipitated from cell lysates with anti-HA mAb and its kinase activity assayed in the presence of Histone H1 and [γ-^{32}P]ATP. Phosphorylation of Histone is indicated. As a control (C) in each experiment (A-D), a kinase assay was also performed on protein G-Sepharose beads coupled to anti-HA mAb, which had been incubated with untransfected cell lysate. Autoradiographs and western blots were quantified using Kinetic Imaging Software and the level of kinase activity normalised to protein expression levels. (B,C) MDCK cells were transfected with HA-PAK4ΔGBD or HA-PAK4wt and serum-starved for 4 hours prior to stimulation with HGF for 15 minutes. LY294002 was added to cultures after 3.5 hours of serum starvation. PAK4 was immunoprecipitated from cell lysates with anti-HA mAb and its kinase activity assayed in the presence of Histone H1 and [γ-^{32}P]ATP. Autoradiographs and western blots were quantified as above. Time in the presence of HGF in minutes is indicated; LY, LY294002. The results shown are mean±s.e.m. of three independent experiments. (D) MDCK cells were transfected with HA-PAK4wt and HA-PAK4M350 and starved for 4 hours. PAK4 proteins were immunoprecipitated from cell lysates with anti-HA mAb and their kinase activity assayed in the presence of Histone H1 and [γ-^{32}P]ATP.

PAK4-induced rounding is prevented by PI3K inhibitors

PI3K is required for the response of MDCK cells to HGF (Potempa and Ridley, 1998; Royal and Park, 1995). Pre-incubation with LY294002 (a PI3K inhibitor) inhibits HGF-induced changes to the actin cytoskeleton and intercellular junctions that are required for cell migration (Potempa and Ridley, 1998; Royal and Park, 1995). PAK4ΔGBD-induced cell rounding was inhibited by LY294002 (Fig. 6A) and 20 nM wortmanin (data not shown). During long-term stimulation with HGF, in the presence of LY294002, PAK4ΔGBD-expressing cells remained well spread, their morphology was indistinguishable from uninjected cells and stress fibres were readily detected. Furthermore the diffuse cytoplasmic localisation of PAK4ΔGBD was similar to that observed for long-term expression of PAK4ΔGBD in the absence of HGF (Fig. 2i). To determine when PI3K activity was required during the response to HGF/PAK4ΔGBD, we added LY294002 at different time points. If LY294002 was added 5 minutes after HGF stimulation the scattering response was reduced but not completely inhibited and PAK4ΔGBD expression induced a lower level of cell rounding, whereas LY294002 addition 15 minutes after HGF had no inhibitory effect on either response (data not shown). As PI3K inhibition requires time to allow the LY294002 not only to enter cells but also to bind to the ATP-binding site, these results imply that PI3K activity is required during the first 30 minutes of HGF stimulation for PAK4ΔGBD to induce a morphological response. This is consistent with PI3K being required for PAK4 activation, which peaks during the first 30 minutes after HGF addition (Fig. 5A).

In contrast to its effect on PAK4ΔGBD-induced rounding, LY294002 was unable to block PAK4Δ-induced cell rounding (Fig. 6B). The LY294002 was clearly active as it inhibited HGF-induced scattering (data not shown). As PAK4Δ is constitutively active and lacks the N-terminal regulatory region of PAK4, these results indicate that PI3K acts upstream of PAK4 to regulate its activity, rather than downstream or on a parallel pathway to PAK4.

PAK4wt localisation is regulated by PI3K

We have shown that localisation of PAK4wt at the cell periphery is dependent on HGF (Fig. 6D) whilst PAK4ΔGBD is localised at the cell periphery independently of HGF (Fig. 6A,B). Although LY294002 inhibits PAK4ΔGBD-induced cell rounding, the transient peripheral localisation of PAK4ΔGBD was not significantly inhibited by LY294002 (Fig. 6C). There was a slight reduction in the percentage of PAK4ΔGBD-

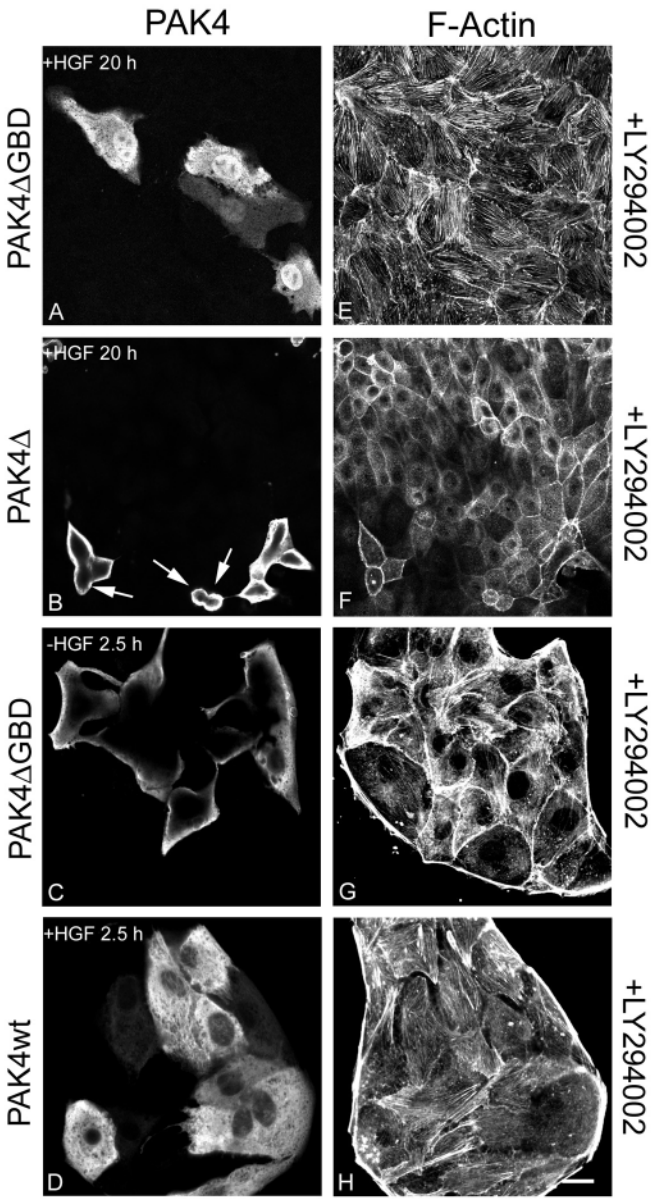


Fig. 6. PAK4-induced rounding and PAK4wt localisation is prevented by PI 3K inhibitors (A) Cells were microinjected with a plasmid encoding PAK4ΔGBD and incubated for 2.5 hours in 0.2% FCS. Cells were then incubated for 30 minutes with LY294002. Cells were then incubated for 20 hours in the presence of HGF and LY294002. (B) Cells were microinjected with a plasmid encoding PAK4Δ and incubated for 2.5 hours in 0.2% FCS. Cells were then incubated for 30 minutes with LY294002, prior to further incubation in 0.2% FCS plus LY294002 for 20 hours. (C) Cells were microinjected with a plasmid encoding PAK4ΔGBD and incubated for 2 hours in 0.2% FCS. Cells were then incubated for 30 minutes with LY294002 prior to a further incubation in 0.2% FCS plus LY294002 for 2.5 hours. (D) Cells were microinjected with a plasmid encoding PAK4wt and incubated for 2 hours in 0.2% FCS. Cells were then incubated for 30 minutes with LY294002 prior to stimulation for 2.5 hours with HGF and LY294002. All the cells were fixed and stained for HA-tagged PAK4 and F-actin (E-H). Basal sections (at the level of stress fibres) of PAK4ΔGBD-expressing cells are shown, while apical sections (at the level of F-actin-containing microvilli) of PAK4Δ-expressing cells are shown in order to visualise clearly the rounded-up cells (arrows). Bar, 10 μm.

expressing cells with a peripheral localisation (Table 1), but no inhibition of stress fibre disassembly (data not shown). In contrast, LY294002 inhibited the HGF-dependent localisation of PAK4wt at the cell periphery (Table 1; Fig. 6D). This suggests that HGF-stimulated responses are PI3K-dependent, whereas the transient responses to PAK4ΔGBD in unstimulated cells are PI3K-independent.

PAK4 kinase activity is inhibited by LY294002

We have demonstrated that PAK4ΔGBD-induced cell rounding requires an active kinase domain and can be inhibited by the PI3K inhibitor, LY294002. We therefore investigated the effect of LY294002 on PAK4 kinase activity. HGF stimulated PAK4ΔGBD kinase activity was partially inhibited by LY294002 (Fig. 5B). Similarly, in the presence of LY294002 there was a reduction in the activation of PAK4wt kinase activity by HGF (Fig. 5C).

Discussion

We have investigated the function and regulation of PAK4 in MDCK epithelial cells. PAK4 kinase activity is stimulated by HGF, and activated PAK4 induces cell rounding and loss of focal complexes only when MDCK cells are stimulated with HGF. In addition, PAK4 activation correlates with re-localisation to the cell periphery and HGF-induced PAK4 kinase activation and localisation are PI3K-dependent.

Although it has previously been shown that PAK1 kinase activity is regulated by PI3K downstream of heregulin (Adam et al., 1998), and downstream of Ras during cell transformation (Tang et al., 1999), this is the first time that PI3K has been shown to influence not only kinase activity but also subcellular localisation of a group II PAK. Rounding induced by the PAK4 kinase domain alone (PAK4Δ) is insensitive to PI3K inhibitors, implying that the N-terminal region of PAK4 acts as an autoinhibitor of kinase activity.

The fact that inhibition of PI3K did not perturb the transient HGF-independent localisation of PAK4ΔGBD at the cell periphery and loss of stress fibres, but did inhibit the longer-term HGF-regulated PAK4ΔGBD-induced cell rounding, suggests that PAK4 is regulated at multiple levels. As described for group I PAKs, the N-terminal region of PAK4 may be subject to regulation both through protein-protein interaction and phosphorylation (Daniels and Bokoch, 1999; Zhao et al., 2000a). We predict that, like group I PAKs, PAK4 normally exists in a folded inactive state, mediated in part by the GBD region of the protein binding to the kinase domain. PAK4ΔGBD presumably exists in a partially unfolded active conformation, as it lacks the GBD domain. However, it can still be stimulated by HGF through PI3K, leading to further activation and thereby cell rounding. This stimulation via PI3K could further reduce autoinhibition by the N-terminal region, or directly increase the kinase activity. In addition, we propose that PAK4ΔGBD is downregulated following long-term expression in unstimulated cells, which explains the reappearance of stress fibres and loss of peripheral localisation, despite continuous expression of PAK4ΔGBD. PAK4Δ is insensitive to this downregulation as it lacks the regulatory N-terminal domain.

It is possible that PAK4 is regulated by phosphorylation

downstream of PI3K. Little is known about the regulation of mammalian PAK kinase activity by phosphorylation, although PAK1 has multiple autophosphorylation sites (Chong et al., 2001). However, recent data suggest that PAK1 is a substrate for 3-phosphoinositide-dependent kinase-1 (PDK1) phosphorylation (King et al., 2000) and that Akt can stimulate its kinase activity independently of Rac and Cdc42 (Tang et al., 2000). In addition, *Dictyostelium* PAKa phosphorylation by Akt is thought to mediate kinase activation in response to cAMP stimulation (Chung and Firtel, 1999).

We, like others (Abo et al., 1998; Dan et al., 2001; Qu et al., 2001), were unable to elicit any morphological effects by expressing PAK4wt alone. Indeed, most of the reported morphological effects of PAK1 have been identified through expression of an activated protein (Daniels et al., 1999; Manser et al., 1997), probably because wild-type proteins are rapidly turned off after stimulation. However, by using a mildly activated protein we have been able to study activation of PAK4 by HGF. Expression of PAK4ΔGBD in fibroblasts and macrophages fails to cause cell rounding in either unstimulated or growth-factor-stimulated cells (C.M.W. and A.J.R., unpublished), although in endothelial (PAE) cells wild-type PAK4 cooperates with Cdc42 to induce filopodium extension (Abo et al., 1998; Qu et al., 2001), and in fibroblasts constitutively active PAK4 transiently induces filopodia (Qu et al., 2001). Together, these results suggest that the response to PAK4 is cell-type specific.

Our data indicate that activated Cdc42-V12 does not stimulate PAK4wt to induce cell rounding, consistent with previous observations that binding of Cdc42 to PAK4 does not stimulate kinase activity (Abo et al., 1998; Lee et al., 2002). However, recent evidence suggests that expression of Cdc42-V12 leads to increased PAK4 phosphorylation (Callow et al., 2002). It is possible that Cdc42 affects PAK4 phosphorylation in cells by bringing it into the vicinity of other kinases. Indeed, in endothelial cells re-localisation of PAK4 to the Golgi by Cdc42-V12 is required for induction of filopodia (Abo et al., 1998). It will therefore be interesting to investigate whether Cdc42 affects the localization of PAK4 in MDCK cells.

Previous reports of activated PAK1-induced cell rounding have inferred that the loss of cell-substratum adhesions (and subsequent cell rounding) are a consequence of de-regulated turnover of focal adhesions (Manser et al., 1997). It is probable that the progressive decrease in substratum adhesion induced by PAK4ΔGBD also reflects a failure to make or retain focal complexes in MDCK cells responding to HGF. There is considerable evidence to suggest that PAKs are involved in cell-substratum adhesion. Adhesion to fibronectin strongly stimulates PAK1 kinase activity (Price et al., 1998) and PAK3 can bind to and phosphorylate the focal adhesion/complex component paxillin (Hashimoto et al., 2001). It has recently been suggested that PAK1-induced loss of focal complexes could be achieved through its interaction with a complex of proteins including the PAK-interacting exchange factor PIX and a paxillin-interacting GIT1 family member (de Curtis, 2001; Zhao et al., 2000b). However, PAK4 does not interact with PIX (Abo et al., 1998), and we have been unable to demonstrate an association between paxillin and PAK4 by co-immunoprecipitation (data not shown). In addition, kinase-inactive PAK1 has been reported to localise to focal complexes (Manser et al., 1997), but we did not observe any localisation to focal complexes of PAK4wt, kinase-inactive PAK4 or PAKΔGBD (data not shown). It is therefore likely that PAK4 affects focal complexes via a different mechanism to PAK1, and indeed the respective localisations of PAK1 and PAK4 in HGF-stimulated cells imply that they play distinct roles. Whereas PAK1 was reported to localise within lamellipodia (Royal et al., 2000), activated PAK4 is localised to the cell periphery but not specifically to lamellipodia. To complement our studies and those of others using exogenously expressed PAKs, it will be important in the future to devise methods to inhibit each PAK selectively, in order to elucidate their respective contributions to morphological responses.

To date, the only substrate for PAK4 identified is LIMK1, which can also be phosphorylated by PAK1 (Dan et al., 2001). Activated LIMK1 in turn induces phosphorylation of cofilin, an actin filament-depolymerising protein that is inactivated by LIMK1 phosphorylation (Arber et al., 1998). Increased cofilin phosphorylation should therefore lead to an increase in polymerised actin in cells. However, expression of dominant-negative LIMK1 and cofilin mutants affects the rounding response to PAK4, suggesting that it might in part contribute to PAK4 morphological changes (Dan et al., 2001). How cofilin phosphorylation contributes to PAK4-induced responses is unclear, as cofilin is generally believed to act in the lamellipodia to promote actin filament turnover (Carlier et al., 1999). PAK4 undoubtedly has additional substrates in addition to LIMK1, which will contribute to PAK4 responses.

In conclusion, we have demonstrated for the first time growth-factor-induced activation of a group II PAK, and investigated the signalling pathways regulating PAK4 that lead to cell rounding. We have identified PI3K as a key regulator of PAK4 responses and localisation. As both PAK4 and PAK1 are activated by HGF but have different intracellular localisations, they may act together to mediate HGF-induced dissolution of stress fibres and reorganisation of cell-substratum adhesions, which are essential for initiation of cell migration.

We are grateful to Alan Entwistle for assistance with time-lapse videomicroscopy.

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