INTRODUCTION

PAK4 (p21-activated kinases) are effectors of RhoGTPases, including Cdc42, Rac1 and RhoA, and play essential roles in controlling many cellular functions such as cell survival and motility [1,2]. PAK4 together with PAK5 and PAK6 belong to group II of PAKs [2]. Of these, PAK4 and PAK5 are localized at the leading edge of migrating cells, whereas PAK6 is mainly localized to the nucleus [1,3]. At the leading edge, PAK4 is a member of a multiprotein complex comprising LIMK (Lin-11/Isl-1/Mec-3 kinase), SSH1L (Slingshot 1L) and 14-3-3 proteins [4]. Activation of LIMK by PAK4 mediates phosphorylation and inactivation of cofilin, and this is part of the ‘cofilin cycle’ that facilitates actin reorganization processes required for directed cell migration [4–7]. In many cancers, PAK4 was shown to be overexpressed or mutated, and its inhibition by pharmacological means was suggested to target cancer metastases [2,8].

PAK4 requires activation loop phosphorylation at Ser974 for its activity [9,10]. Phosphorylation of this site can be mediated by autophosphorylation and by PKD1 (protein kinase D1) [10,11]. However, Ser974-phosphorylated PAK4 only acquires full activity upon binding to RhoGTPases and release of its AID (autoinhibitory domain) [12]. The mechanisms by which PAK4 is directed to the leading edge, however, are unknown.

PKD1 is down-regulated in expression or activity in invasive breast, prostate and gastric cancer [13–15]. In response to active RhoA, PKD1 negatively regulates cofilin activity at the leading edge by phosphorylating key enzymes in both of its regulatory pathways. PKD1 phosphorylates PAK4 at Ser974 mediating its activation and downstream signalling to LIMK [11]. It also phosphorylates the cofilin phosphatase SSH1L at multiple sites mediating its inactivation [16–18]. The net effect of such signalling is a shift of the cofilin pool at the leading edge to the phosphorylated inactive state, resulting in inhibition of barbed end formation and directed cell migration [19].

In the present study, we identify a previously undescribed mechanism that is required to target PAK4 to the leading edge. We show that PKD1-mediated phosphorylation of PAK4 at Ser99 generates a binding site for 14-3-3 proteins. Ser99 phosphorylation and 14-3-3 binding is necessary for localization of PAK4 to the leading edge, and mutation of this residue to alanine leads to its retention in the cytosol.

MATERIALS AND METHODS

Cell lines, antibodies and reagents

All cell lines were obtained from the A.T.C.C. (Manassas, VA, U.S.A.). HEK (human embryonic kidney)-293T and HeLa cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) with 10% (v/v) FBS. Anti-PKD1 and anti-14-3-3 antibodies were from Santa Cruz Biotechnology, anti-PAK4, anti-pSer474-PAK4, anti-cofilin and anti-LIMK antibodies were from Cell Signaling Technology, anti-FLAG (M2) and anti-PDK substrate antibody, anti-14-3-3 protein antibodies were from Cell Signaling Technology, and rabbit polyclonal antibodies were from Cell Signaling Technology. Secondary HRP (horseradish peroxidase)-linked antibodies were from Millipore. Secondary antibodies for immunofluorescence [Alexa Fluor® 568-conjugated F(ab′)2 fragment of goat anti-mouse IgG] or Alexa Fluor® 488-conjugated 546 F(ab′)2 fragment of goat anti-rabbit] were from Invitrogen. HeLaMONSTER® (Mirus) was used for transient transfection of HeLa cells, and TransIT®-293 (Mirus) was used for transient transfection of HEK-293T cells. The PKD inhibitor CID755673 was from Tocris Bioscience and the PKA4 inhibitor was from MedKoo Biosciences. Purified recombinant PKD1 was from Millipore, and PKD2 and PKD3 were both from Enzo Life Sciences.

Abbreviations used: F-actin, filamentous actin; G-actin, globular actin; HA, haemagglutinin; HEK, human embryonic kidney; LIMK, Lin-11/Isl-1/Mec-3 kinase; PAK, p21-activated kinase; PAK4.KM, kinase-dead PAK4 (PAK4.K530M); PBD, protein kinase D; PKD1.CA, constitutively active PKD1 (PKD1.S738E.S742E); PKD1.KW, kinase-dead PKD1 (PKD1.K612W); PKD2.KW, kinase-dead PKD2 (PKD2.K680W); SSH1L, Slingshot 1L; TBST, TBS with 0.2% Tween 20.

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DNA constructs

The expression plasmids for HA-tagged constitutively active PKD1 (PKD1.CA, PKD1-S738E.S742E mutation) and HA-tagged kinase-inactive PKD1 (PKD1.KW, PKD1.K612W mutation) were described previously [20]. The expression plasmids for FLATD-tagged kinase-inactive PKD2 (PKD2.KW, PKD2.K580W mutation), were generated by site-directed mutagenesis using a previously described plasmid as template [21] and the primer pair 5′-GGCCGAGCATGGACAGTCTGTTGTGCTACAGGGAAAATG—3′ and 5′-GCCAGTTGTTGCTAATGACCAACTGGC-3′. The expression plasmids for FLAG-tagged or GST-tagged (GST-fusion proteins) human wild-type, PKD30M- and S474A-mutated PKD4 were described previously [11]. To obtain S99A-mutated PKD4, site-directed mutagenesis was carried out using the QuikChange® kit (Stratagene), wild-type, PKD30M- and/or S474A-mutated PKD4 expression constructs as template, and 5′-GTGACAGCTCTCAACGCCCCTGAGGAGACAGC-3′ and 5′-GCTGCTTCTCCGAGGCGTCTCGTGTCAC-3′ as primers.

In vitro kinase assays

Kinase assays with GST-fusion proteins were carried out by adding 250 ng of active purified PKD enzyme to 2 μg of purified GST or GST-fusion protein in a volume of 40 μl of kinase buffer (50 mM Tris/HCl, pH 7.4, 10 mM MgCl₂ and 2 mM DTT) supplemented with 100 μM ATP (non-radioactive assay) or 100 μM ATP containing 10 μCi of [γ-32P]ATP in kinase buffer (radioactive assay). The kinase reaction [30 min at room temperature (22°C)] was stopped by adding 2× Laemmli buffer.

Immunoprecipitation, PAGE, far-Western blot and immunoblotting

Cells were washed twice with 4°C PBS (140 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM KH₂PO₄, pH 7.2) and then lysed using lysis buffer I (50 mM Tris/HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl and 5 mM EDTA, pH 7.4) plus protease inhibitor cocktail (Sigma–Aldrich). After vortex-mixing and incubating for 30 min on ice, lysates were centrifuged at 16,000 g for 15 min at 4°C. To obtain the actin fraction, pellets were resolved in lysis buffer II (50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 1% Nonidet P40 and 10% glycerol) plus protease inhibitor cocktail and boiled (details described in [17]). For all other experiments, either the supernatant was collected and subjected directly to SDS/PAGE (Western blotting) or proteins of interest were immunoprecipitated by incubating for 1 h at 4°C with a specific antibody (2 μg) followed by another 30 min incubation with Protein G–Sepharose (GE Healthcare). After washing the immune complexes three times with TBS (50 mM Tris/HCl, pH 7.4, and 150 mM NaCl), 20 μl of TBS and 20 μl of 2× Laemmli buffer were added, and the samples were subjected to SDS/PAGE. Proteins were transferred on to nitrocellulose membranes and visualized by immunostaining or subjected to far-Western blotting. For far-Western blotting, nitrocellulose membranes were blocked with 5% PhosphoBLOCKER™ Blocking Reagent (Cell Biolabs) in TBST (TBST with 0.2% Tween 20) for 1 h and overlaid with His- -tagged 14-3-3ζ protein (4 μg/ml) in 5% PhosphoBLOCKER™ Blocking Reagent solution for 16 h at 4°C. Membranes were then washed three times briefly with TBST and 14-3-3ζ binding to proteins was visualized by immunostaining with an anti-His antibody.

Immunofluorescence

Cells were either seeded and transfected in eight-well ibiTreat μ-Slides (ibidi, Integrated BioDiagnostics), or grown in 6-cm-diameter plates, transfected as indicated and reseeded on to coverslips in a 24-well plate at a density of 2×10⁵ cells/well. The following day, cells were washed with PBS and then fixed with 4% (w/v) paraformaldehyde for 15 min at 37°C. Following two washes with PBS, cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature and blocked with 3% (w/v) BSA and 0.05% Tween 20 in PBS (blocking solution) for 30 min at room temperature. The samples were then incubated with primary antibody (anti-FLAG diluted 1:4000 in blocking solution) overnight at 4°C. Samples were washed five times with PBS and incubated with secondary antibody [Alexa Fluor® 568 F(ab’)] fragment of goat anti-(mouse IgG) or Alexa Fluor® 488 F(ab’), fragment of goat anti-rabbit] at a dilution of 1:1600 in blocking solution for 2 h at room temperature. F-actin (filamentous actin) and nuclei were stained together with secondary antibodies by incubating with phalloidin (Alexa Fluor® 633–phallloidin; Invitrogen) and DAPI (Sigma–Aldrich) respectively in blocking solution. Following three washes with PBS, the coverslips were mounted on to slides using Fluoromount-G (SouthernBiotech) and examined using a IX81 DSU spinning disc confocal microscope from Olympus with a ×40 objective. Images were processed using ImageJ (NIH).

Analysis of free actin filament barbed ends

Cells were transfected and reseeded on eight-well ibiTreat μ-Slides and serum-starved for 16 h. The medium was then removed, cells were washed with pre-warmed PBS, permeabilized and labelled with 0.4 μM Alexa Fluor® 594-conjugated actin in permeabilization buffer (20 mM Hepes, 138 mM KC1, 4 mM MgCl₂, 3 mM EGTA, 0.2 mg/ml saponin and 1% BSA) plus 1 mM ATP for 30 s at 37°C. The cells were fixed with 4% (w/v) paraformaldehyde in PBS at room temperature for 10 min. Samples were examined using using the IX81 DSU spinning-disc confocal microscope.

Impedance-based real-time cell migration and proliferation assays

Cells were transfected as indicated and, after 24 h, were seeded on Transwell CIM-plate 16 (motility assays) or E-plates (proliferation assays) from Roche. For proliferation assays, after attachment of cells on the E-plate, impedance was continuously monitored in real-time over a period of 16 h using the xCELLigence RTCA DP instrument (Roche). For migration assays, after attachment of cells, cell migration towards NIH 3T3-conditioned medium was continuously monitored in real-time (over 16 h) using the xCELLigence RTCA DP instrument (Roche).

Transwell assays

Cells were transfected and, after 24 h, were suspended in 0.1% BSA in serum-free medium and seeded into Transwells containing a polycarbonate membrane with pores of 8 μm (Corning). After allowing cells to migrate towards NIH 3T3-conditioned medium (for 16 h), the cells on top of the Transwell were removed. The remaining cells were fixed with 3.7% (w/v) formaldehyde for 30 min at room temperature, stained with β-gal solution [40 μM K₄Fe(CN)₆, 40 μM K₃Fe(CN)₆, 16 μM MgCl₂ and 20 μM X-Gal in PBS] and counted manually.
Statistical analysis
Results are means ± S.D. P values were acquired with Student’s t test using GraphPad Prism software, and P < 0.05 is considered statistically significant.

RESULTS
PKD1 phosphorylates PK4 at Ser^99
PK4 can autophosphorylate at Ser^94, a key residue in the activation loop of the kinase domain that contributes to activity [10]. Using a kinase-dead PK4 (PAK4.KM, PAK4.K350M), PKD1 also was shown to directly phosphorylate this residue in vitro and in vivo [11]. In vitro kinase assays with PKD1 and GST-tagged purified kinase-dead PK4 additionally mutated at Ser^94 (GST–PAK4.K350M.S474A mutant) expectedly led to a loss of PK4 phosphorylation at Ser^94. However, probing PKD-phosphorylated GST–PAK4.K350M.S474A with the pMOTIF antibody that is designed to recognize the phosphorylated PKD consensus motif [22,23] still picked up a GST–PAK4.K350M.S474A mutant, indicating additional PKD1 phosphorylation sites in PK4 (Figure 1A).

Analysis of the amino acid sequence of PK4 indicated only one additional PKD phosphorylation consensus motif (VTRSSN99) that is conserved between species and is also present in PAK5, but not in PK6 (Figure 1B). Moreover, Ser^99 phosphorylation of PK4 has been previously detected by MS [24] and was reported in phosphorylation databases such as Phosphosite (http://www.phosphosite.org). We therefore tested whether PKD1 can phosphorylate PK4 at Ser^99. We performed in vitro kinase assays with a series of bacterially expressed GST–PAK4 fusion proteins encompassing kinase-dead PK4 (K350M mutation) combined with S474A or S99A, or both, mutations (Figure 1C). In vitro kinase assays using radioactive ATP as well as non-radio labelled kinase assays and probing with anti-pSer^94-PAK4 and anti-pMOTIF antibodies suggested that PKD1 indeed can phosphorylate PK4 at both Ser^99 and Ser^94. For example, a kinase-dead and S474A-mutated PK4 was still phosphorylated by PKD1 (77% of control), whereas an S99A mutant was phosphorylated significantly less (33% of control). Only mutation of both sites fully diminished PKD1-mediated phosphorylation. Probing non-radio labelled kinase assays with anti-pMOTIF indicated that this antibody primarily recognizes PK4 phosphorylated at Ser^99. Moreover, probing with anti-pSer^94-PAK4 suggested that the phosphorylation at Ser^94 may prime for PKD1-mediated phosphorylation at Ser^94 in vitro (Figure 1C). Using PKD1-phosphorylated GST–PAK4.K350M with PKD1-phosphorylated GST–PAK4.K350M.S99A, α-pS474-PAK4 blot). Of the two other PKD family members, only PKD2 is a Ser^99 kinase, whereas PKD2 and PKD3 are Ser^94 kinases [11] (Supplementary Figure S1 at http://www.biochemj.org/bj/455/bj4550251add.htm).

We then tested whether active PKD1 mediates phosphorylation of PK4 at Ser^99 in cells. We therefore expressed a kinase-dead PK4 mutant (PAK4.KM) or a kinase-dead double mutant (PAK4.KM.S474A) in combination with PKD1.CA or PKD1.K.W. Additionally, all cells were treated with the PK4 inhibitor PF-3758309 [25] to block phosphorylations by endogenous PK4. We found that active PKD1 phosphorylated PK4.KM as well as PAK4.KM.S474A, but to a lesser extent. PKD1.K.W blocked both phosphorylations. This indicates that PKD1 can contribute to the phosphorylation of both residues in cells (Figure 1D).

Next, we tested whether Ser^99 phosphorylation can prime for Ser^94 phosphorylation in cells. We therefore first expressed PK4, PK4.S99A or PK4.S474A in HeLa cells and probed for Ser^94 phosphorylation. However, a PK4.S99A mutant showed phosphorylation at Ser^94 at a level comparable with wild-type PK4 (Figure 1E). This may be explained by previous studies demonstrating a high autophosphorylation activity of PK4 towards this residue [10]. We therefore applied the PK4 inhibitor PF-3758309 to dampen PK4 autophosphorylation. Under such conditions, PKD1.CA induced PK4 phosphorylation at Ser^94 in wild-type PK4, but significantly less in the PK4.S99A mutant (Figure 1F). In summary, our results indicate that Ser^99 phosphorylation, to some extent, can prime for PKD1-mediated Ser^94 phosphorylation, but is not necessary for PK4 autophosphorylation at this serine residue.

Ser^99 is necessary for the localization of PK4 to the leading edge
The group II PKAs PK4 and PK5 which contain the Ser^99 phosphorylation motif locate to the leading edge, whereas PK6 which does not contain this motif is a nuclear protein (Supplementary Figure S2 at http://www.biochemj.org/bj/455/bj4550251add.htm). This prompted us to test whether the phosphorylation of this residue by PKD1 can affect the cellular localization of PK4. In HeLa cells, endogenous and overexpressed PKD1 is localized to the leading edge where it co-localizes with F-actin filaments [13,17]. Similarly, a GFP-tagged version of the active PKD1 protein co-localized with F-actin filaments at the leading edge (Figures 2D–2F). Furthermore, PK4 localized to the leading edge of cells (Figure 2G), but a PK4.S99A mutant was retained in the cytosol (Figure 2H). Interestingly, the localization of active PKD1 to the leading edge was linked to PK4 phosphorylation at Ser^99, since wild-type PK4 co-localized with active PKD1 at the leading edge (Figures 2I–2K) and a PK4.S99A mutant lead to loss of PKD1 localization to the leading edge (Figures 2L–2N). This suggests that the translocation of both proteins to the leading edge is coupled to the PK4 phosphorylation status at Ser^99.

Ser^99 phosphorylation mediates binding of PK4 to 14-3-3 proteins
Phosphorylation of proteins in the PKD substrate motif has been shown to generate a binding site for 14-3-3 proteins [17,26–29]. In PK4, phosphorylation of Ser^99 within RSNpS generates an interaction of PAK4 with 14-3-3 proteins [11]. Previously, a multiprotein complex consisting of PK4, actin and 14-3-3 scaffolding proteins along with the PK4 substrate LIMK was shown to regulate ADF (actin depolymerizing factor)/cofilin activity at the leading edge [4]. We therefore determined whether the formation of such a complex is dependent on active PKD1. To test this, we introduced PKD1.CA into cells, immunoprecipitated endogenous LIMK and probed for co-immunoprecipitated endogenous PK4 and 14-3-3. We found that both proteins associated with LIMK in the presence of active PKD1 (Figure 3A). Since 14-3-3 scaffold proteins may function as the ‘connectors’ between signalling proteins of this complex, we next tested whether PK4, when phosphorylated by PKD1, can bind to 14-3-3 proteins and whether this is dependent on Ser^99 phosphorylation. We found that active PKD1 induces the interaction of PK4 with 14-3-3γ, and this is blocked when PK4 is mutated at Ser^99. These effects were obtained when PK4 or PK4.S99A were immunoprecipitated and samples were probed for co-immunoprecipitated 14-3-3γ (Figure 3B), but also vice versa when 14-3-3γ was immunoprecipitated and samples were probed for PK4 or PK4.S99A mutant (Figure 3C). Moreover, in the presence of active PKD1, both
Figure 1  Ser99 is a novel PKD phosphorylation site on PAK4

(A) In vitro kinase assays were performed with purified recombinant PKD1 and either purified GST-tagged PAK4 with only an inactivating K350M mutation (GST-PAK4.K350M) or additional mutation at Ser99 (GST-PAK4.K350M.S474A) as substrates. To analyse substrate phosphorylation, Western blots of resolved proteins were probed with anti-pSer474-PAK4 and anti-pMOTIF (recognizes a phosphorylated PKD substrate motif) antibodies. Additional blots (α-PAK4 and α-PKD1) were performed to control equal input of purified proteins. The position of the 80 kDa band is indicated. (B) Analysis of group II PAKs (PAK4, PAK5 and PAK6) for PKD phosphorylation sites other than Ser99 showed that Ser99 in PAK4 and PAK5 lies within a PKD phosphorylation consensus motif. This motif is conserved across species and its phosphorylation generates a 14-3-3 consensus motif. B. taurus, Bos taurus; H. sapiens, Homo sapiens; M. musculus, Mus musculus; R. norvegicus, Rattus norvegicus. (C) In vitro kinase assays were performed with purified recombinant PKD1 and bacterially expressed GST-tagged kinase-dead PAK4 (GST-PAK4.K350M) additionally mutated by S99A or S474A, or both, as indicated. The top panel shows a radiolabelled kinase assay, and the other panels show non-radiolabelled kinase assays. Following the kinase reactions, samples were separated by SDS/PAGE and transferred on to nitrocellulose. PKD1-mediated phosphorylation of PAK4 was determined by autoradiography (top panel) or by probing with anti-pS474-PAK4 and anti-pMOTIF antibodies. Additional staining of nitrocellulose blots was performed (α-PAK4 and α-PKD1) to control equal input of purified proteins. The position of the 80 kDa band is indicated. (D) HeLa cells were co-transfected with vector control, FLAG-tagged PAK4.KM or PAK4.KM.S474A mutant and PKD1.CA or PKD1.KW, as indicated. Immediately after transfection, samples were treated with the PAK4 inhibitor PF-3758309 (400 nM) for 16 h. Cells were lysed and PAK4 was immunoprecipitated (IP) (α-FLAG). Samples were subjected to SDS/PAGE, transferred on to nitrocellulose and analysed by immunoblotting for PKD1-mediated phosphorylation of PAK4 (α-pMOTIF) and total PAK4 (α-FLAG). Additionally, lysates were analysed by Western blotting (WB) for PKD1 expression. Molecular masses are indicated in kDa. (E) HeLa cells were transfected with vector control, FLAG-tagged PAK4 or indicated PAK4 mutants. PAK4 was immunoprecipitated (IP) from cell lysates using anti-FLAG antibody. Samples were subjected to SDS/PAGE, transferred on to nitrocellulose and analysed by immunoblotting for PAK4 activation loop phosphorylation (with anti-pS474-PAK4) and total PAK4 (α-FLAG). The position of the 58 kDa band is indicated. (F) HeLa cells were co-transfected with vector control, FLAG-tagged PAK4 or PAK4.S99A mutant and vector control or PKD1.CA, as indicated. Immediately after transfection, samples were treated with the PAK4 inhibitor PF-3758309 (400 nM) for 16 h. Cells were lysed and PAK4 was immunoprecipitated (IP) (α-FLAG). Samples were subjected to SDS/PAGE, transferred on to nitrocellulose, and analysed by immunoblotting for PAK4 activation loop phosphorylation (with anti-pS474-PAK4) and total PAK4 (α-FLAG). Additionally, lysates were analysed by Western blotting (WB) for PKD1.CA expression. Molecular masses are indicated in kDa.
when a PAK4.S99A mutant was combined with 14-3-3 phosphorylated at Ser99, we performed an isoform (results not shown, and Supplementary Figure S3 at http://www.biochemj.org/bj/455/bj4550251add.htm). Since both PKD isoforms PKD1 and PKD2 can phosphorylate PAK4 at Ser99, we next tested whether kinase-dead versions of these kinases can block PAK4-mediated decreases in cell migration. We found that both PKD1.KW and PKD2.KW mutants fully restored cell migration (Figure 4D), indicating that both kinases are redundant in their function towards PAK4.

Ser99 phosphorylation affects cell motility

At the leading edge, PAK4 has been shown to activate LIMK, but also to inactivate SSH1L [4]. The net effect of such signalling is an increase in (inactive) pSer1-cofilin and decreased directed cell migration [11]. Consequently, ectopic introduction of wild-type PAK4 decreased directed cell migration, whereas a PAK4.S99A mutant that does not localize to the leading edge did not significantly decrease directed cell migration when compared with the vector control (Figure 4A). The effects observed were not caused by differences in cell proliferation, since ectopic expression of neither wild-type PAK4 nor the PAK4.S99A mutant had an impact on cell proliferation in the period of analysis (Figure 4B). The calculated doubling of the cell index in the logarithmic growth phase were 5.2 ± 0.24 h for vector control, 4.84 ± 0.19 h for wild-type PAK4 and 4.97 ± 0.21 h for PAK4.S99A mutant. Taken together, these data suggest that blocking PAK4 localization to the leading edge can favour directed cell migration. The negative effects of wild-type PAK4 on directed cell migration are blocked when cells are treated with the PKD inhibitor CID755673 (Figure 4C). Inhibiting PKD1 led to a reversion of PAK4-mediated inhibitory effects on cell migration (compare PAK4-expressing cells in the absence and presence of CID755673). These results are also consistent with previous reports showing that introduction of active PKD1 into motile cells decreases directed cell migration by inhibiting cofilin-induced free actin barbed end formation [11,17,18] (Supplementary Figures S4 and S5 at http://www.biochemj.org/bj/455/bj4550251add.htm). Since both PKD isoforms PKD1 and PKD2 can phosphorylate PAK4 at Ser99, we next tested whether kinase-dead versions of these kinases can block PAK4-mediated decreases in cell migration. We found that both PKD1.KW and PKD2.KW mutants fully restored cell migration (Figure 4D), indicating that both kinases are redundant in their function towards PAK4.

Ser99 phosphorylation affects PAK4 localization and downstream signalling at the leading edge

Since the PAK4.S99A mutant did not localize to the cell periphery (Figure 3D), we next analysed whether this affects downstream signalling towards cofilin at the leading edge. First, we determined F-actin structures in cells expressing wild-type PAK4 or PAK4.S99A. Cells expressing the S99A mutant showed a more motile phenotype as judged by organization of the F-actin structures (Figure 5A) and this correlated with our findings on directed cell migration (Figure 4A). However, when we quantified cofilin phosphorylation in cells expressing vector control, PAK4 or PAK4.S99A, we found only marginal differences between the two versions of PAK4 (Figure 5B). In control transfected cells 17.5 ± 5.7 % of the total cofilin was phosphorylated at Ser1. In cells expressing wild-type PAK4, cofilin phosphorylation was at 52.3 ± 2.2 %, and in cells expressing PAK4.S99A, we detected slightly decreased phospho-cofilin levels at 42.8 ± 3.2 %. This correlated with data on LIMK activity shown in Figure 4(C), and indicates that the S99A mutated form of the kinase is functional. However, when we isolated the cytosolic and F-actin fractions, we found that, whereas PAK4 occurs in both fractions, PAK4.S99A is exclusively localized to the cytosol (Figure 5C).

![Figure 2](http://www.biochemj.org/bj/455/bj4550251add.htm) Ser99 is necessary for the localization of PAK4 to the leading edge

(A)-(F) HeLa cells were transfected with GFP vector control (A-C) or GFP-PKD1.CA (D-F). At 24 h after transfection, cells were fixed and F-actin was stained with phalloidin. In the overlay in (F), two areas are enhanced (labelled 1 and 2). Both show areas of PKD1.CA and F-actin co-localization at the leading edge of cells. (G–N) HeLa cells were transfected with either wild-type FLAG-tagged PAK4 (PAK4) or FLAG-tagged PAK4.S99A and either empty vector (G and H) or GFP-tagged PKD1.CA (I–N). Samples were subjected to immunofluorescence analysis in which PAK4 was detected by using anti-FLAG antibody and Alexa Fluor® 568-conjugated secondary antibody. Scale bars, 10 μm.

PAK4 and 14-3-3γ co-localized at the leading edge, whereas when a PAK4.S99A mutant was combined with 14-3-3γ, both did not localize to the leading edge (Figure 3D). The effects observed were not 14-3-3 isoform-specific since similar results were obtained when 14-3-3ζ was expressed instead of the γ isoform (results not shown, and Supplementary Figure S3 at http://www.biochemj.org/bj/455/bj4550251add.htm).

In order to test whether 14-3-3 binds directly to PAK4 phosphorylated at Ser99, we performed an in vitro kinase assay in which we phosphorylated wild-type PAK4 or the PAK4.S99A mutant with recombinant PKD1. We then performed a far-Western blot to detect binding of recombinant 14-3-3 protein. We found that 14-3-3 can directly bind to PAK4, when phosphorylated by PKD1 (Figure 3E). Recombinant 14-3-3 also bound to autophosphorylated PKD1, as shown previously by Hausser et al. [31].

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Figure 3  Ser\textsuperscript{99} phosphorylation mediates binding of PAK4 to 14-3-3 proteins

(A) HEK-293T cells were transfected with HA-tagged PKD1.CA. Endogenous LIMK was immunoprecipitated (IP) (α-LIMK). Following SDS/PAGE and transfer on to nitrocellulose, samples were analysed for co-immunoprecipitated endogenous PAK4 (α-PAK4) or 14-3-3 (α-14-3-3) and then probed for LIMK (α-LIMK). Additionally, lysates were analysed by Western blotting for PKD1.CA expression (input control). (B) HEK-293T cells were co-transfected with FLAG-tagged PAK4 or PAK4.S99A mutant combined with vector or HA-tagged 14-3-3γ and vector or PKD1.CA, as indicated. PAK4 was immunoprecipitated (IP) from cell lysates using an anti-FLAG antibody. Samples were subjected to SDS/PAGE, transferred on to nitrocellulose and analysed by immunoblotting for co-immunoprecipitated 14-3-3γ (α-HA). Samples were re-probed for total PAK4 (α-PAK4). Additionally, lysates were analysed by Western blotting for PKD1 expression (α-PKD1) and 14-3-3γ (α-HA) expression. (C) HEK-293T cells were co-transfected with FLAG-tagged PAK4 or PAK4.S99A mutant combined with vector or HA-tagged 14-3-3γ and PKD1.CA, as indicated. 14-3-3γ was immunoprecipitated (IP) from cell lysates using anti-HA antibody. Samples were subjected to SDS/PAGE, transferred on to nitrocellulose, and analysed by immunoblotting for co-immunoprecipitated PAK4 (α-FLAG). Samples were re-probed for total 14-3-3γ (α-HA). Additionally, lysates were analysed by Western blotting for PKD1 expression (α-FLAG) and PAK4 (α-FLAG) expression. (D) HeLa cells were co-transfected with FLAG-tagged PAK4 or PAK4.S99A mutant, HA-tagged 14-3-3γ and PKD1.CA, as indicated. Samples were subjected to immunofluorescence analysis in which PAK4 was detected by using anti-FLAG antibody and Alexa Fluor\textsuperscript{®} 647-conjugated secondary antibody, and 14-3-3γ was detected using an anti-HA antibody and Alexa Fluor\textsuperscript{®} 568-conjugated secondary antibody. Scale bars, 10 μm. (E) In vitro kinase (IVK) assays were performed with purified recombinant PKD1 and bacterially expressed GST–PAK4.KM or GST–PAK4.KM.S99A, as indicated. Following the kinase reactions, samples were separated by SDS/PAGE and transferred on to nitrocellulose. A far-Western blot was performed with PKD1 protein (α-His) and purified His\textsubscript{6}-tagged 14-3-3ζ. Staining was α-His. Molecular masses are indicated in kDa in (A)–(C) and (E).

Moreover, localization of PAK4 or mutant correlated with coflin phosphorylation. This indicates that the mechanism by which Ser\textsuperscript{99} phosphorylation affects PAK4 function is that it regulates its function at the lamellipodium. Indeed, expression of wild-type PAK4 led to increased coflin phosphorylation at the lamellipodium and cytosol (Figure 5D, top row), whereas expression of a PAK4.S99A mutant that is not localized at the leading edge also led to coflin phosphorylation but only in the cytosol (Figure 5D, bottom row).
Figure 4  Ser$^{99}$ phosphorylation affects cell motility, but not cell proliferation

(A and B) HeLa cells were transfected with control vector, wild-type PAK4 or PAK4.S99A mutant, and reseeded in Transwell CIM-Plate 16 plates (chemotaxis assays, A), or E-plates (proliferation assays, B). After attachment of cells, cell migration or proliferation was continuously monitored in real-time using the xCELLigence RTCA DP instrument. Results are means ± S.D. for four experiments. Protein expression was controlled by Western blot ($\alpha$-FLAG for PAK4 expression, $\alpha$-β-actin for loading control). (C and D) HeLa cells were transfected with control vector, wild-type PAK4 or PAK4.S99A mutant, and treated with either DMSO (control) or the PKD inhibitor C1D755673 (20 μM, during time of the assay) as indicated (C), or were additionally transfected with PKD1.KW or PKD2.KW as indicated (D). Transwell assays were performed to assess cell migration towards NIH 3T3-conditioned medium at 16 h. In (C), protein expression was controlled by Western blot ($\alpha$-FLAG for PAK4 expression, $\alpha$-β-actin for loading control). Additionally, samples were analysed by Western blot for expression and activity of LIMK ($\alpha$-LIMK, $\alpha$-pT508-LIMK). In (D), protein expression was controlled by Western blot for PAK4 ($\alpha$-FLAG), PKD1 ($\alpha$-PKD1), PKD2 ($\alpha$-PKD2) or $\beta$-actin (for loading control). *P < 0.05 compared with vector control (first bar); ** P < 0.05 compared with wild-type PAK4 (second bar). Molecular masses are indicated in kDa.

This was confirmed further by performing barbed end formation [G-actin (globular actin) incorporation] assays, in which we show that PAK4 completely blocks barbed end formation, whereas cells expressing PAK4.S99A can still form barbed ends and show G-actin incorporation (Figure 5E). Taken together, this indicates that the ability of PAK4 to induce cofilin phosphorylation at Ser$^{3}$ is not affected by its mutation at Ser$^{99}$, but that alone the lack of localization to the leading edge may be responsible for its effects on directed cell migration.

In summary, with Ser$^{99}$, we describe a novel PKD1 phosphorylation site in PAK4 that regulates PAK4 localization and complex formation with LIMK and 14-3-3 proteins. We suggest that lack of phosphorylation of PAK4 at Ser$^{99}$ prevents inactivation of cofilin at the leading edge, thus affecting directed cell migration (Figure 6).
Figure 5  Loss of Ser99 phosphorylation decreases cofilin phosphorylation at the leading edge

(A) HeLa cells were transfected with wild-type PAK4 or PAK4.S99A mutant. At 24 h after transfection, cells were fixed and PAK4 was stained by immunofluorescence (α-FLAG). F-actin was stained with phalloidin. The inset shows an area at the leading edge of cells. Scale bars, 20 μm. (B) HeLa cells were transfected with control vector, wild-type PAK4 or PAK4.S99A mutant. Total cell lysates (n = 5 experiments) were analysed by Western blotting for cofilin phosphorylation (anti-pSer3-cofilin antibody) or total cofilin (anti-cofilin). The percentage of Ser3-phosphorylated cofilin and unphosphorylated cofilin was calculated as depicted. (C) HeLa cells were transfected with control vector, wild-type PAK4 or PAK4.S99A mutant. Cytosolic and F-actin fractions were prepared and analysed by Western blotting (WB) for the presence of PAK4 (α-FLAG), or the presence of endogenous cofilin or pSer3-cofilin. Molecular masses are indicated in kDa. (D) HeLa cells were transfected with wild-type PAK4 or PAK4.S99A mutant as indicated. Samples were subjected to immunofluorescence analysis in which PAK4-expressing cells were stained by immunofluorescence using primary antibodies directed against their tag as well as Alexa Fluor® 488-conjugated secondary antibody. Scale bar, 20 μm.
Phosphorylation at Ser474 is probably independent of PKD1—However, in cells with PAK4 autophosphorylation activity, and in vivo mediated phosphorylation of Ser99 (Figure 1E).

Cell migration [11]. In the present study, with Ser99, we have decreased actin reorganization at the leading edge and directed such signalling are an increase in the phospho-cofilin pool and towards its substrate LIMK [11]. Functional consequences of PKD1-mediated activation loop phosphorylation at Ser474 migration.

Overexpression of wild-type PAK4 decreased directed cell migration, whereas an S99A mutant that was not localized at the leading edge showed migration behaviour similar to wild-type cells (Figures 4A, 4C and 4D).

Actin reorganization at the leading edge, a key process in directed cell migration, is facilitated by the cofilin cycle [35]. PKD1 can regulate activities of both LIMK and SSH1L, thereby being a key regulator of cofilin activity [11,17,18,36]. Activation of LIMK by PK4 mediates phosphorylation and inactivation of cofilin [4–7]. Although showing significant differences in their effects on cell migration, the expression of PAK4.S99A, similarly to that described for wild-type PAK4, increased cofilin phosphorylation (Figure 5B). This is not surprising since Ser99 is located neither in the p21-Rho-binding domain (amino acids 10–66), nor in the protein kinase domain (amino acids 322–572), suggesting that binding to RhoGTPases can still occur and that the kinase activity is intact. However, we found that both PAK4 and PAK4.S99A localized in the cytosol, but that only wild-type PAK4 localized to the F-actin fraction (Figure 5C). In addition, we found that cofilin in PAK4.S99A-expressing cells is mainly phosphorylated in the cytosol and that in wild-type PAK4-expressing cells, phosphorylation occurs in the cytosol and the F-actin fraction. Consequently, expression of wild-type PAK4 led to increased cofilin phosphorylation at the lamellipodia and cytosol, whereas expression of a PAK4.S99A mutant that is not localized at the leading edge also led to cofilin phosphorylation, but only in the cytosol (Figure 5D). To demonstrate decreased cofilin activity when cells express PAK4, we performed G-actin incorporation (barbed end) assays. Cofilin-initiated G-actin incorporation at the cell periphery was blocked when cells were expressing wild-type PAK4, but not when PAK4.S99A was expressed (Figure 5E). This indicates that the ability of PAK4 to induce cofilin phosphorylation at Ser1 is not affected by its mutation at Ser99, but that alone the lack of localization to the leading edge may be responsible for its effects on directed cell migration.

Phosphorylation of the PKD substrate motifs can generate a 14-3-3 protein consensus motif (Figure 1B). For example, PKD1-mediated phosphorylation of SSH1L at Ser787 has been shown to lead to binding of 14-3-3 [17]. Similarly, PKD1 phosphorylation of PAK4 at Ser99 created a 14-3-3-binding motif (Figures 3B and 3C) and mediated direct binding of 14-3-3 in far-Western blot assays (Figure 3E). Although both PKD1-phosphorylated PAK4 and PKD1 bind 14-3-3 directly, this does not prove the formation of a complex between these three proteins. It was shown previously that PAK4 at the leading edge, along with LIMK, interacts with SSH1L and 14-3-3 to form a multiprotein complex which regulates the activity of cofilin [4]. We found that PKD1 may contribute to such complex formation, since both PAK4 and 14-3-3 associated with LIMK in the presence of active PKD1 (Figure 3A). The mechanisms by which such a complex is formed are not yet fully elucidated. One possibility of how 14-3-3 proteins could contribute to complex formation is due to their ability to form dimers or multimers [37]. Moreover, the binding of 14-3-3 proteins to PKD1, as well as to LIMK1-phosphorylated cofilin, has been described previously [31,38], suggesting that 14-3-3 may function as scaffold to keep this complex at the leading edge. However, our data do not exclude additional levels of regulation of the PAK4–SSH1L–LIMK complex through 14-3-3 proteins or other scaffolding proteins.

To conclude, in the present study, we identified an additional mechanism by which PAK4 function and directed cell migration can be regulated. We describe PKD1-mediated phosphorylation of Ser99 as a requirement for complex formation of PAK4 with 14-3-3 proteins, a mechanism that targets a PAK4–LIMK complex to the leading edge and facilitates cofilin phosphorylation at this location (Figure 6).
AUTHOR CONTRIBUTION

Ligia Bastea, Heike Döppler, Sarah Pearce and Peter Storz conceived and designed the experiments. Ligia Bastea, Heike Döppler, Sarah Pearce, Nisha Durand and Peter Storz performed the experiments. Ligia Bastea, Heike Döppler, Sarah Pearce, Samantha Spratley and Peter Storz analysed the data. Peter Storz wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Protein kinase D-mediated phosphorylation at Ser\textsuperscript{99} regulates localization of p21-activated kinase 4

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Figure S1 Ser\textsuperscript{99} is phosphorylated by PKD2, but not PKD3

In vitro kinase assays were performed with purified recombinant PKD2 or PKD3 and either purified GST-tagged PAK4 with only an inactivating K350M mutation (GST–PAK4.K350M), additional mutation at Ser\textsuperscript{99} (GST–PAK4.K350M.S99A) or additional mutation at Ser\textsuperscript{99} and S474 (GST–PAK4.K350M.S99A.S474A). To analyse substrate phosphorylation, Western blots of resolved proteins were probed with anti-pMOTIF antibody (recognizes a phosphorylated PKD substrate motif). Additional blots (α–PAK4 and α–PKD2 or α–PKD3) were performed to control input of purified proteins. The position of the 80 kDa band is indicated.

Figure S2 Localization of endogenous PAK4, PAK5 and PAK6

HeLa cells (10\textsuperscript{4} cells, ibiTreat μ-slide) were subjected to indirect immunofluorescence analysis. Samples were labelled with anti-PAK4 (Abcam ab62509; dilution 1:200), anti-PAK5 (Abcam ab62510; dilution 1:200) or anti-PAK6 (Abcam ab62511; dilution 1:200) antibody and secondary Alexa Fluor® 546-conjugated anti-rabbit antibodies (dilution 1:800). Additionally, nuclei of cells were stained with DAPI. Scale bars, 50 μm.
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Figure S3 14-3-3ζ interacts with PAK4 in the presence of active PKD1

Cells were co-transfected with FLAG-tagged PAK4, vector control or PKD1.CA and HA-tagged vector control or 14-3-3ζ as indicated. PAK4 was immunoprecipitated (IP) (α-FLAG) and samples were analysed for co-immunoprecipitated 14-3-3ζ (α-HA). Blots were stripped and analysed for total PAK4 (α-FLAG). Control blots were performed on lysates to determine expression of active PKD1 (α-PKD1) and 14-3-3ζ (α-HA). Molecular masses are indicated in kDa.

Figure S4 Active PKD1 blocks directed cell migration

HeLa cells (5×10⁵ cells, 6-cm-diameter dish) were transfected with control vector or PKD1.CA. After 3 h of attachment, cell migration towards NIH 3T3-conditioned medium over 14 h was monitored continuously in real-time using Transwell CIM-Plate 16 and the xCELLigence RTCA DP instrument. Results are means ± S.D. for four experiments. Inset shows control blots of cell lysates probed for expression of active PKD1 (α-HA). Staining for β-actin (α-β-actin) served as a leading control. Molecular masses are indicated in kDa.

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