***In vivo* role of the PIF-binding docking site of PDK1 defined by knock-in mutation**

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PKB/Akt, S6K, SGK and RSK are mediators of responses triggered by insulin and growth factors and are activated following phosphorylation by 3-phosphoinositide-dependent protein kinase-1 (PDK1). To investigate the importance of a substrate-docking site in the kinase domain of PDK1 termed the 'PIF-pocket', we generated embryonic stem (ES) cells in which both copies of the PDK1 gene were altered by knock-in mutation to express a form of PDK1 retaining catalytic activity, in which the PIF-pocket site was disrupted. The knock-in ES cells were viable, mutant PDK1 was expressed at normal levels and insulin-like growth factor 1 induced normal activation of PKB and phosphorylation of the PKB substrates GSK3 and FKHR. In contrast, S6K, RSK and SGK were not activated, nor were physiological substrates of S6K and RSK phosphorylated. These experiments establish the importance of the PIF-pocket in governing the activation of S6K, RSK, SGK, but not PKB, *in vivo*. They also illustrate the power of knock-in technology to probe the physiological roles of docking interactions in regulating the specificity of signal transduction pathways.

Keywords: Akt/docking sites/protein kinase/RSK/SGK

Introduction

Protein kinases mediate many of the diverse cellular processes regulated by extracellular agonists such as growth factors and insulin. The major function of these enzymes is to phosphorylate their substrates efficiently in a regulated manner. Based mainly on *in vitro* binding and overexpression studies in cells, there is mounting evidence that many protein kinases rely on residues, termed docking sites, lying outside the catalytic centre, to bring the kinase and substrate together (Holland and Cooper, 1999; Tanoue *et al.*, 2001; Biondi and Nebreda, 2003). These docking sites are predicted to play important roles in controlling the overall specificity and function of signal transduction pathways.

The 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylates and activates a group of related protein enzymes termed AGC kinases, which mediate many of the physiological responses triggered by growth

factors and hormones. These include isoforms of protein kinase B (also known as Akt) (Brazil and Hemmings, 2001; Scheid and Woodgett, 2001), p70 ribosomal S6 kinase (S6K) (Avruch *et al.*, 2001; Volarevic and Thomas, 2001), p90 ribosomal S6 kinase (RSK) (Frodin and Gammeltoft, 1999) and the serum- and glucocorticoid-induced-protein kinase (SGK) (Lang and Cohen, 2001). PDK1 activates its substrates by phosphorylating these enzymes at their activation loop (reviewed in Toker and Newton, 2000; Alessi, 2001). A key interest has been to address the mechanism by which PDK1 can recognize, phosphorylate and activate its many substrates *in vivo*. The conclusions drawn from previous work indicate that the phosphorylation of PKB by PDK1 is dependent upon prior activation of the phosphoinositide 3-kinase (PI-3-kinase) and the production of the second messenger, phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃]. This binds to the pleckstrin homology (PH) domain of both PDK1 and PKB, co-localizing these enzymes at the plasma membrane, allowing PDK1 to activate PKB (Brazil and Hemmings, 2001; Leslie *et al.*, 2001; Scheid and Woodgett, 2001). However, unlike PKB, other PDK1 substrates do not bind PtdIns(3,4,5)P₃ as they lack PH domains and, moreover, the phosphorylation of these enzymes by PDK1 is not stimulated by PtdIns(3,4,5)P₃. Instead, recent studies indicate that the ability of PDK1 to phosphorylate S6K, SGK and RSK is dependent on a docking site termed the 'PIF-pocket', located on the small lobe of the PDK1 kinase domain (Biondi *et al.*, 2000, 2001; Frodin *et al.*, 2000, 2002). The PIF-pocket enables PDK1 to interact with a C-terminal non-catalytic region of these enzymes, known as the hydrophobic motif. This lies in a Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr motif, which is conserved in all PDK1 substrates. Phosphorylation of the Ser/Thr residue in the hydrophobic motif greatly enhances the binding of PDK1 to S6K, SGK and RSK, and thus enhances the activation of these enzymes by PDK1. Activation of PI-3-kinase triggers the phosphorylation of the hydrophobic motif of S6K and SGK. Recent studies indicate that mammalian target of rapamycin (mTOR) complexed to other proteins may directly phosphorylate the hydrophobic motif of S6K (Hara *et al.*, 2002; Kim *et al.*, 2002), although the kinases that phosphorylate SGK at this site are unknown. In the case of RSK isoforms, phosphorylation by the ERK1/ERK2 MAP kinases activates a second catalytic domain that is present in this class of AGC kinase, which then phosphorylates the hydrophobic motif (Frodin and Gammeltoft, 1999).

The recent elucidation of the crystal structure of the catalytic domain of PDK1 revealed that the PIF-pocket is composed of a hydrophobic groove located next to a cluster of basic residues (Biondi *et al.*, 2002). Mutagenesis of the basic residues in the PIF-pocket indicates that they form a binding site for the phosphorylated Ser/Thr

Fig. 1. ES cell knock-in strategy. (A) Diagram illustrating the targeting knock-in construct, the 5' end of the PDK1 gene and the allele modification generated. The black boxes represent exons and the black triangles loxP sites. The position of the 3' probe used to genotype targeted knock-in cells in (B) is shown. The positions of the PCR primers used to genotype the Cre recombinase-mediated excision of the neomycin cassette are indicated by arrows. The position of Leu155/Glu155 in exon 4 is represented by an asterisk. The position of the novel *EcoRV* restriction site is marked. + = wild-type allele; 155Eneo = the targeted knock-in allele with the neomycin cassette still present; 155E = the targeted knock-in allele with the neomycin cassette removed. (B) Genomic DNA purified from the indicated ES cell lines was digested with *EcoRV*, electrophoresed on a 1% agarose gel, transferred to nitrocellulose and the membrane incubated with the ³²P-labelled 3' probe. The wild-type allele generates a 17 kb fragment whereas the targeted knock-in allele generates a 7.2 kb fragment in this analysis. (C) Genomic DNA purified from the indicated ES cell lines was used as a template for PCR with the P1 and P2 primers. The wild-type allele (+) generates a 200 bp product, whereas a 330 bp product is obtained with the targeted allele in which the neomycin cassette is excised (155E). (D) Genomic DNA purified from the indicated ES cell lines was subjected to PCR using primers 5'-gctccaaggagatcagtagacag and 5'-ggtagtcgcaggcctgtgctg to generate a 460 bp product that encompasses the 155 mutation region on exon 4. The resultant PCR products were ligated into the pCR-Topo 2.1 vector, transformed into *E.coli* and clones sequenced. The numbers of the wild-type Leu155 and knock-in Glu155 sequences obtained for each cell line are indicated.

residues of the hydrophobic motif of S6K, SGK and RSK, explaining why phosphorylation of the hydrophobic motif greatly enhances the ability of PDK1 to interact with and activate these substrates (Biondi *et al.*, 2002; Frodin *et al.*, 2002). Mutation of a central residue in the hydrophobic groove of the PIF-pocket, Leu155, to a glutamate prevented PDK1 from interacting with or phosphorylating S6K or SGK *in vitro* but, strikingly, did not affect the ability of PDK1 to activate PKB in the presence of PtdIns(3,4,5)P₃ (Biondi *et al.*, 2001). The location of Leu155 in the structure of PDK1 is illustrated in Supplementary figure 1 available at *The EMBO Journal* Online. These findings initially suggested that the PIF-pocket was required for the activation of S6K, SGK and RSK, but was not rate limiting for the activation of PKB by PDK1. However, this conclusion was challenged recently by Woodgett and colleagues (Scheid *et al.*, 2002), who showed that, in overexpression studies, the PDK1[L155E] mutant poorly activated a membrane-targeted form of PKB lacking its PH domain. Moreover, these authors reported that the mutation of the hydrophobic motif phosphorylation site (Ser473) of PKB α to an acidic residue promoted phosphorylation of the activation loop (Thr308) of membrane-localized PKB α (Scheid *et al.*,

2002). These results suggested, in contrast to the conclusions drawn from our *in vitro* work, that *in vivo*, the PIF-pocket of PDK1 might indeed be required for efficient activation of PKB.

These studies illustrate that assessing the role of docking site interactions in mediating the specificity of protein kinases is dependent on the approach employed. *In vivo*, the correct concentration of kinase and substrate expressed, as well as their localization and interaction with endogenous scaffolding or other proteins, will greatly influence the docking interactions that take place. These conditions are not easily replicated during *in vitro* or overexpression studies. Moreover, the interpretation of experiments is complicated further in overexpression studies if the endogenous kinase is still present in the cells in which mutant forms of this enzyme are transfected. In this study, we wished to establish the *in vivo* importance of the PIF-binding pocket of PDK1 in regulating the specificity of activation of AGC kinases. To overcome the potential problems outlined above, we decided to perform a knock-in mutation in embryonic stem (ES) cells in which Leu155 in both copies of the endogenous PDK1 gene was changed to glutamate, in order to disrupt the function of the PIF-pocket of PDK1. Here we describe how this affects

Fig. 2. Expression and activity of PDK1 in knock-in ES cells. The indicated ES cells were cultured to 80% confluence and lysed. PDK1 was immunoprecipitated from the cell lysate and assayed with the indicated peptide as described in Materials and methods. The results shown are the average $\pm$ SEM of three separate dishes of cells with each assay performed in duplicate. The cell lysates were also immunoblotted with PDK1 antibody 1 (raised against the C-terminal 20 residues of mouse PDK1) or PDK1 antibody 2 (raised against recombinant human PDK1 protein). The lysates were also incubated with Sepharose conjugated to PIF to affinity purify PDK1 as described in Materials and methods. The washed resin was then immunoblotted for PDK1 using PDK1 antibody 1. Similar results were obtained in two separate experiments. It should be noted that PDK1 in ES cells, as observed in other cell lines, is detected as two bands on immunoblot analysis (Balendran *et al.*, 1999a; Williams *et al.*, 2000).

the activation of the signalling pathways that are controlled by PDK1.

Results

Generation of PDK1^{155E/155E} knock-in ES cells

A targeting construct was generated to replace the wild-type exon 4 of the PDK1 gene, which encodes Leu155, with a mutant form of exon 4 encoding glutamate at this position (see Materials and methods and Figure 1). Heterozygous cells (PDK1^{155E/+}) were retargeted with the same construct to obtain homozygous cells expressing the mutant exon in both alleles (termed PDK1^{155E/155E}). Southern blotting, PCR analysis and genomic DNA sequencing confirmed that replacement of the wild-type with the mutant exon had occurred (Figure 1). In the strategy deployed, the neomycin selection marker located in the intron between exons 2 and 3 was excised using the Cre-LoxP system, as we have found previously that a neomycin cassette in this intron significantly reduced PDK1 expression in both ES cells and mice (Lawlor *et al.*, 2002). The PDK1^{155E/155E} ES cells appeared morphologically indistinguishable and proliferated at a rate similar to PDK1^{+/+} and PDK1^{-/-} ES cells (data not shown).

PDK1 is normally expressed in knock-in ES cells and does not bind PIF

Employing two different PDK1 antibodies, we found that PDK1 is expressed at similar levels in PDK1^{155E/155E} and PDK1^{+/+} ES cells (Figure 2). We also incubated ES cell lysates with Sepharose conjugated to the PIF peptide that interacts strongly with the PIF-pocket of wild-type PDK1 (Balendran *et al.*, 1999a; Biondi *et al.*, 2000). PDK1 could be affinity purified from wild-type PDK1^{+/+} ES cells with PIF-Sepharose, but not from PDK1^{155E/155E} ES cells,

Fig. 3. Activation of PKB α in PDK1^{155E/155E} knock-in cells. The indicated ES cells were deprived of serum for 4 h, incubated in the presence or absence of 100 nM wortmannin for 10 min and then either left unstimulated or stimulated with 20 ng/ml IGF1 for 15 min. The cells were lysed, and PKB α was immunoprecipitated and assayed. The results shown are the average $\pm$ SEM for three dishes of cells each assayed in duplicate. The ES cell lysates were also immunoblotted with the indicated antibodies. Similar results were obtained in four separate experiments.

confirming that the PIF-pocket was disrupted in the knock-in cell line. The PDK1[L155E] mutant is known to possess an $\sim$ 3-fold higher specific activity than wild-type PDK1 towards a peptide substrate that encompasses the activation loop of PKB α termed T308tide (Biondi *et al.*, 2000). Consistent with this, PDK1 immunoprecipitated from PDK1^{155E/155E} ES cells possessed a 3-fold higher specific activity towards this peptide substrate than PDK1 derived from wild-type PDK1^{+/+} cells (Figure 2). PDKtide, a substrate generated by fusing PIF to T308tide, is phosphorylated more efficiently by PDK1 than T308tide (Biondi *et al.*, 2000). PDK1 immunoprecipitated from wild-type PDK1^{+/+} ES cells phosphorylated PDKtide more efficiently than T308tide, whereas PDK1 from PDK1^{155E/155E} ES cells phosphorylated both peptide substrates with comparable efficiency, consistent with its inability to bind PIF.

PKB is activated normally in PDK1^{155E/155E} ES cells

We next tested whether PKB could be activated in the PDK1^{155E/155E} ES cells. The cells were deprived of serum and stimulated with insulin-like growth factor 1 (IGF1) in the presence or absence of the PI-3-kinase inhibitor wortmannin, and PKB α was immunoprecipitated and assayed. The basal PKB α activity in unstimulated PDK1^{155E/155E} ES cells was similar to that found in PDK1^{+/+} ES cells, and IGF1 induced an $\sim$ 4-fold activation in both cell lines, which was inhibited by wortmannin (Figure 3). PKB α was not activated by IGF1 in PDK1^{-/-} ES cells, as reported previously (Williams *et al.*, 2000). IGF1 induced phosphorylation of PKB α at the site of PDK1 phosphorylation (Thr308) as well as the hydrophobic motif (Ser473), in both wild-type PDK1^{+/+} and PDK1^{155E/155E} ES cells. Consistent with PKB α being activated in the PDK1^{155E/155E} ES cells, the PKB substrates GSK3 α and GSK3 β are phosphorylated at the expected sites in IGF1-stimulated cells (Ser21 in GSK3 α and Ser9 in GSK3 β), and phosphorylation was inhibited by

Fig. 4. S6K1 is not activated in PDK1^{155E/155E} knock-in cells. (A) The indicated ES cells were deprived of serum for 4 h, incubated in the presence or absence of 100 nM rapamycin or 500 nM okadaic acid for 30 min, and then either left unstimulated or stimulated with 20 ng/ml IGF1 for 30 min. The cells were lysed, and S6K1 was immunoprecipitated and assayed. The results shown are the average $\pm$ SEM for three dishes of cells each assayed in duplicate. The ES cell lysates were also immunoblotted with the indicated antibodies. Thr389 is the hydrophobic motif residue. (B) The indicated ES cell lines were transfected with a DNA construct encoding the expression of wild-type GST-S6K. At 24 h post-transfection, the ES cells were deprived of serum for 4 h, incubated in the presence or absence of 100 nM rapamycin for 30 min and then either left unstimulated or stimulated with 20 ng/ml IGF1 for 30 min. The cells were lysed, and GST-S6K was affinity purified from the cell lysate on glutathione-Sepharose and immunoblotted with the indicated antibodies. Thr252 is the T-loop residue phosphorylated by PDK1. Similar results were obtained in two separate experiments.

wortmannin (Figure 3). No phosphorylation of GSK3 α and GSK3 β was observed in the PDK1^{-/-} ES cells as reported previously (Williams *et al.*, 2000).

S6K is not activated in PDK1^{155E/155E} ES cells

To determine whether S6K1 was activated, serum-starved ES cells were stimulated with IGF1 in the presence or absence of the mTOR inhibitor rapamycin, which abolishes S6K activation *in vivo* (Raught *et al.*, 2001). Following immunoprecipitation of S6K1, a significant basal S6K activity was observed in wild-type PDK1^{+/+} ES cells, which was increased a further 2.5-fold by stimulation with IGF1 (Figure 4A). As expected, rapamycin reduced S6K activity to undetectable levels. Strikingly, although S6K1 was expressed at the same level in PDK1^{+/+} and PDK1^{155E/155E} ES cells, no measurable S6K1 activity was found in PDK1^{155E/155E} or PDK1^{-/-} ES cells, even after stimulation with IGF1. To confirm the lack of S6K1 activity in the PDK1^{155E/155E} ES cells, phosphospecific antibodies recognizing sites on ribosomal protein S6 phosphorylated by S6K1 were employed. In PDK1^{+/+} ES cells, IGF1 induced the phosphorylation of the S6 protein and this was inhibited by rapamycin. In contrast, the S6 protein, although expressed normally, was not phosphorylated detectably in IGF1-treated PDK1^{155E/155E} ES cells (Figure 4A). Furthermore, using phosphospecific antibodies that recognize the phosphorylated hydrophobic motif of S6K1 (Thr389), it was found that IGF1-induced phosphorylation of this residue was attenuated in the

Fig. 5. RSK isoforms are not activated in PDK1^{155E/155E} knock-in cells. The indicated ES cells were deprived of serum for 4 h, incubated in the presence or absence of 2 μ M PD 184352 for 1 h and then either left unstimulated or stimulated with 0.4 μ g/ml TPA for 15 min. The cells were lysed, and RSK isoforms were immunoprecipitated with an antibody that recognizes all isoforms and assayed. The results shown are the average $\pm$ SEM for two dishes of cells each assayed in triplicate. The ES cell lysates were also immunoblotted with the indicated antibodies. Ser227 is phosphorylated by PDK1, Thr360 and Thr573 are phosphorylated by ERK, and Ser380 is phosphorylated by the C-terminal kinase domain of RSK (CT-KD). Similar results were obtained in two separate experiments.

PDK1^{155E/155E} ES cells (Figure 4A) but, as reported previously (Williams *et al.*, 2000), abolished in the PDK1^{-/-} ES cells. To investigate whether inhibiting protein phosphatases potentiated phosphorylation of the hydrophobic motif of S6K, the ES cell lines were treated with the protein phosphatase inhibitor okadaic acid. We found that okadaic acid markedly stimulated phosphorylation of the hydrophobic motif of S6K in the IGF1-stimulated PDK1^{+/+} and PDK1^{155E/155E}, but not the PDK1^{-/-} ES cells (Figure 4A). This observation will be considered further in the Discussion.

As phosphospecific antibodies that recognize S6K phosphorylated at its T-loop residue (Thr252) are not sufficiently sensitive to recognize the endogenous enzyme, S6K was overexpressed in the different ES cell lines and immunoblotted with a previously characterized S6K T-loop phosphospecific antibody (Biondi *et al.*, 2001) that recognizes overexpressed S6K (Figure 4B). We found that in wild-type PDK1^{+/+} ES cells, S6K is phosphorylated at its T-loop residue and that this phosphorylation is increased with IGF1 and reduced with wortmannin. Consistent with the lack of endogenous S6K activity in the PDK1^{155E/155E} or PDK1^{-/-} ES cells, we found that overexpressed S6K was not phosphorylated under any condition at its T-loop in these cell types (Figure 4B).

RSK isoforms are inactive in PDK1^{155E/155E} ES cells

ES cells were deprived of serum and then stimulated with the phorbol ester 12-*o*-tetradecanoylphorbol-13-acetate (TPA), which induces the activation of ERK and RSK in ES cells (Williams *et al.*, 2000). Following immunopre-

Fig. 6. SGK1 is inactive in PDK1^{155E/155E} knock-in cells. (A) The indicated ES cells lines were transfected with a DNA construct encoding GST-SGK1. At 44 h post-transfection, the ES cells were deprived of serum for 4 h, incubated in the presence or absence of 100 nM wortmannin for 10 min and then either left unstimulated or stimulated with 20 ng/ml IGF1 for 20 min. The cells were lysed, and GST-SGK1 was affinity purified from the cell lysate on glutathione-Sepharose and assayed. The results shown are the average $\pm$ SEM for three dishes of cells each assayed in triplicate. The purified GST-SGK1 was immunoblotted with the anti-GST antibody (SGK1-Total) to ensure that similar amounts of enzyme were assayed for each condition, as well as with a phospho-antibody recognizing Ser422, the hydrophobic motif. (B) As (A), except that the indicated ES cells lines were transfected with a construct encoding expression of GST-SGK1[S422D]. Similar results were obtained in two separate experiments.

precipitation with an antibody recognizing all RSK isoforms, it was shown that in wild-type PDK1^{+/+} ES cells, TPA induced activation of RSK (Figure 5). As expected PD 184352, an inhibitor of MKK1 activation (Sebolt-Leopold *et al.*, 1999), prevented TPA-induced ERK phosphorylation at its T-loop and RSK activation. In the PDK1^{155E/155E} ES cells, RSK2 was expressed at the same level as in wild-type ES cells, but only low basal RSK activity could be detected in unstimulated cells, which was significantly lower than the basal RSK activity found in unstimulated PDK1^{+/+} ES cells (Figure 5). Stimulation of PDK1^{155E/155E} ES cells with TPA did not increase RSK activity (Figure 5). As reported previously (Williams *et al.*, 2000), no detectable RSK activity was observed in

PDK1^{-/-} ES cells, even in TPA-stimulated cells. Using phosphospecific antibodies, we also assessed the phosphorylation of endogenously expressed RSK at its T-loop residue (Ser227), two sites phosphorylated by ERK1/ERK2 (Thr360 and Thr573), as well as the hydrophobic motif phosphorylation site (Ser380) that is phosphorylated by the C-terminal RSK kinase domain (Dalby *et al.*, 1998). We found that in PDK1^{+/+} ES cells, TPA increased phosphorylation of the T-loop of RSK and this was reduced with PD 184352. Consistent with the lack activation of RSK in the PDK1^{155E/155E} and PDK1^{-/-} ES cells, we found that endogenously expressed RSK in these cell lines was not phosphorylated at the T-loop residue. In contrast, in both unstimulated and TPA-treated PDK1^{+/+}, PDK1^{155E/155E} and PDK1^{-/-} ES cells, ERK1/ERK2 were phosphorylated to a similar extent at the T-loop and RSK was phosphorylated similarly at Thr360 and Thr574 (Figure 5). Consistent with the notion that PDK1 does not play a role in the phosphorylation of the hydrophobic motif of RSK, and that this is mediated through activation of the C-terminal kinase domain by ERK1/ERK2, we found that TPA induced similar phosphorylation of the hydrophobic motif of RSK in PDK1^{+/+}, PDK1^{155E/155E} and PDK1^{-/-} ES cells (Figure 5).

Once activated, RSK phosphorylates GSK3 α and GSK3 β at the same sites as PKB (Frame and Cohen, 2001). In the PDK1^{+/+} ES cells, TPA stimulated GSK3 phosphorylation, which was inhibited by PD 184352, indicating that this is mediated through RSK (Figure 5). Consistent with inactivity of RSK isoforms in the PDK1^{155E/155E} ES cells, no significant phosphorylation of GSK3 α and GSK3 β in response to TPA stimulation was observed in these cells. Similarly, in the PDK1^{-/-} ES cells, TPA failed to induce the phosphorylation of GSK3 (Figure 5).

SGK1 is not activated in PDK1^{155E/155E} and PDK1^{-/-} ES cells

The activity state of SGK1 in PDK1^{+/+} and PDK1^{-/-} ES cells had not been investigated previously. As we were unable to measure activation of SGK isoforms in ES cells (M.Williams and D.R.Alessi, unpublished data), we decided to transfect PDK1^{+/+}, PDK1^{155E/155E} and PDK1^{-/-} ES cells with wild-type SGK1 and measure the activity of SGK1 in unstimulated and IGF1-treated cells in the presence or absence of wortmannin. Consistent with results obtained in other cells (Kobayashi and Cohen, 1999; Park *et al.*, 1999), IGF1 induced ~3-fold activation of SGK1 in PDK1^{+/+} ES cells, which was inhibited by wortmannin (Figure 6A). In contrast, no detectable SGK1 activity was observed in the PDK1^{155E/155E} ES cells, indicating that the PIF-pocket of PDK1 is essential for the activation of SGK. As expected, SGK1 isolated from IGF1-stimulated PDK1^{-/-} ES cells was also devoid of activity. We also monitored SGK1 phosphorylation at its hydrophobic motif (Ser422) and found that SGK1 was phosphorylated at this residue to the same extent in PDK1^{+/+}, PDK1^{155E/155E} and PDK1^{-/-} ES cells (Figure 6A). This indicates that PDK1 does not influence the phosphorylation of this site. It had been shown previously that the SGK1 mutant in which the hydrophobic residue is changed to aspartate (SGK1[S422D]) is constitutively active when expressed in cells (Kobayashi and Cohen,

Fig. 7. Phosphorylation of FKHR at Thr24, Ser256 and Ser319 in PDK1^{155E/155E} knock-in cells. The indicated ES cell lines were deprived of serum for 4 h, incubated in the presence or absence of 100 nM wortmannin for 10 min and then either left unstimulated or stimulated with 20 ng/ml IGF1 for 30 min. The cells were lysed, and FKHR was immunoprecipitated and immunoblotted with the indicated antibodies. For the blotting of the Ser319 site, two different batches of wild-type PDK1^{+/+} ES cell lines were employed that consistently gave marginally different responses to IGF1. Similar results were obtained in two separate experiments in which each stimulation was performed in triplicate.

1999; Park *et al.*, 1999) due to this mutant's higher affinity for the PIF-pocket of PDK1 than wild-type SGK1 (Biondi *et al.*, 2001). The high activity of the SGK1[S422D] mutant is therefore dependent on the constitutive phosphorylation by PDK1 of the SGK T-loop residue. Consistent with findings in other cell types, the SGK1[S422D] mutant expressed in PDK1^{+/+} ES cells possessed a high activity, which was not reduced by treatment with wortmannin (Figure 6B). In contrast, the SGK1[S422D] mutant did not possess significant activity when expressed in PDK1^{155E/155E} or PDK1^{-/-} ES cells, further emphasizing the key role of the PIF-pocket of PDK1 in regulating the T-loop phosphorylation of SGK (Figure 6B).

FKHR is phosphorylated at a putative SGK1 phosphorylation site in PDK1^{155E/155E} ES cells

The forkhead transcription factor member, FKHR, is regulated by insulin and growth factors, which induce its phosphorylation at multiple residues, leading to nuclear exclusion (Tran *et al.*, 2003). Several of these phosphorylation sites (Thr24, Ser256 and Ser319) lie in a PKB consensus phosphorylation motif, and there is compelling evidence that FKHR is directly phosphorylated by PKB (Brunet *et al.*, 1999; Rena *et al.*, 1999). Consistent with this, in IGF1-stimulated PDK1^{-/-} ES cells, FKHR is not phosphorylated at Thr24, Ser256 and Ser319 (Rena *et al.*, 2002). Greenberg and colleagues have also provided evidence that in addition to PKB, SGK1 might also phosphorylate the forkhead family of transcription factors (Brunet *et al.*, 2001). Their data indicated that SGK1 and PKB phosphorylated Thr24 with similar efficiency, whereas PKB preferentially phosphorylated Ser256 and SGK1 preferentially phosphorylated Ser319. As SGK1 is not activated in PDK1^{155E/155E} ES cells, we investigated whether IGF1 was able to induce phosphorylation of endogenous FKHR at Thr24, Ser256 and Ser319 in these cells. We immunoprecipitated endogenous FKHR from unstimulated and IGF1-treated cells with or without wortmannin pre-treatment and immunoblotted the immunoprecipitates with phosphospecific antibodies specifically recognizing Thr24, Ser256 and Ser319 (Rena *et al.*,

1999). In Figure 7, we demonstrate that IGF1 induced similar phosphorylation of FKHR at all three sites in both the PDK1^{+/+} and PDK1^{155E/155E} ES cells, which was inhibited by wortmannin. These findings indicate that SGK1 is not rate limiting for the IGF1-induced phosphorylation of FKHR at Thr24, Ser256 and Ser319 in ES cells. Consistent with previous results (Rena *et al.*, 2002), IGF1 failed to induce phosphorylation of FKHR at any of these residues in IGF1-stimulated PDK1^{-/-} ES cells.

Discussion

To overcome the inherent problems associated with assessing protein-docking interactions using overexpression approaches in cells, we decided to employ a more rigorous knock-in strategy to define the *in vivo* role of the PIF-pocket of PDK1 in regulating the activation of AGC kinases. We generated the PDK1[L155E] knock-in cell line, as our previous work showed that this mutation abolished the binding of PDK1 to S6K1 as well as to peptides that encompass the hydrophobic motif of AGC kinases (Biondi *et al.*, 2000, 2001). In the PDK1^{155E/155E} knock-in cells, PDK1 was expressed at the same level as in control PDK1^{+/+} ES cells, possessed the expected catalytic activity towards peptide substrates and did not bind detectably to PIF-Sepharose (Figure 2). We showed that IGF1 stimulation of PDK1^{155E/155E} ES cells induced normal activation of PKB α and phosphorylation of the activation loop (Thr308), which is mediated by PDK1 (Figure 3). In marked contrast, S6K (Figure 4), RSK (Figure 5) and SGK1 (Figure 6) possess either no detectable or very low basal activity in serum-starved cells and, consistent with lack of T-loop phosphorylation of these enzymes, IGF1 (S6K and SGK) or TPA (RSK) failed to stimulate their activity. This provides the first genetic evidence that the PIF-pocket of PDK1 is required for the activation of these enzymes *in vivo*. This shows that although PDK1 is fully active in the PDK1^{155E/155E} knock-in cells, if it is unable to 'dock' with S6K, RSK and SGK, these enzymes cannot be activated. This illustrates the key role that substrate-docking sites play in regulating protein kinase function.

PKB and RSK phosphorylate the same sites on GSK3 α and GSK3 β (reviewed in Frame and Cohen, 2001). In ES cells, IGF1 only activates PKB and not the ERK/RSK pathway, whereas TPA stimulates RSK and not PKB (Williams *et al.*, 2000). Consistent with PKB, but not RSK, being activated in PDK1^{155E/155E} ES cells, GSK3 isoforms are phosphorylated following IGF1, but not after TPA stimulation (compare Figures 3 and 5). Moreover, the S6 protein, a substrate of S6K, is not phosphorylated in IGF1-stimulated PDK1^{155E/155E} ES cells, confirming that S6K is not activated in these cells. Taken together, these data provide further evidence that the PIF-pocket of PDK1 is essential for the activation of S6K and RSK *in vivo*. However, as PKB α is activated normally in PDK1^{155E/155E} ES cells, the PIF-pocket cannot be required *in vivo* for the activation of PKB. It is likely that the mutual binding of PKB and PDK1 through their PH domains to PtdIns(3,4,5)P₃ and/or PtdIns(3,4)P₂ at the plasma membrane is the critical event in allowing PDK1 to phosphorylate and activate PKB (Figure 8).

Fig. 8. Summary of the mechanism of activation of PKB, S6K and SGK by PDK1. PKB is activated following its recruitment to the plasma membrane where it is phosphorylated at Thr308 by PDK1 and at Ser473 by a distinct unknown hydrophobic motif kinase, termed PDK2. The mutual binding of PKB and PDK1 through their PH domains co-localizes PDK1 and PKB. Once PKB is phosphorylated at Thr308, a hydrophobic motif-binding site is formed in the catalytic domain, resulting in the intramolecular binding of the PKB hydrophobic motif phosphorylated at Ser473 to this site. This is the step that leads to the maximal activation of PKB. In contrast, for S6K and SGK, it is the phosphorylation of these enzymes at their hydrophobic motif that enables PDK1 to interact through its PIF-pocket and hence phosphorylate the T-loop of these substrates. PI-3-kinase regulates the phosphorylation of S6K and SGK at their hydrophobic motif. Phosphorylation of the T-loop of S6K and SGK is predicted to promote the formation of a binding site within the kinase domain of these enzymes, for their own phosphorylated hydrophobic motif, leading to activation. We propose that this protects dephosphorylation of the hydrophobic motif by protein phosphatases. In PDK1^{L155E/155E} cells, S6K is not phosphorylated at its T-loop and therefore the hydrophobic motif phosphorylation site will remain exposed and is thus more likely to be dephosphorylated.

Recently, Woodgett and colleagues (Scheid *et al.*, 2002) concluded that the PIF-pocket of PDK1 was required for the activation of PKB. This was based on the finding that a membrane-targeted mutant of PKB α that lacked its PH domain (Δ PH-PKB α) was poorly activated by overexpression of PDK1[L155E] in cells. We demonstrated previously that the PDK1[L155E] mutant, although capable of activating full-length PKB in the presence of PtdIns(3,4,5)P₃ *in vitro* with the same efficiency as wild-type PDK1, could not phosphorylate Δ PH-PKB α *in vitro* (Biondi *et al.*, 2001). Moreover, the PIF peptide inhibited phosphorylation of Δ PH-PKB α , but not full-length PKB α , by wild-type PDK1 (Biondi *et al.*, 2001). These observations indicate that removal of the PH domain of PKB converts it into a form that, like S6K and SGK, is recognized and activated by PDK1 using a PIF-pocket-dependent mechanism. In our opinion, this is most likely to explain why the membrane-targeted Δ PH-PKB α was activated inefficiently by overexpression of the PDK1[L155E] mutant in the previous study (Scheid *et al.*, 2002). It would also explain why the overexpression of membrane-targeted Δ PH-PKB α [S473D] was phosphorylated at Thr308 in unstimulated cells (Scheid *et al.*, 2002). Indeed, S6K1 (Weng *et al.*, 1998; Balendran *et al.*,

1999b), SGK1 (Kobayashi and Cohen, 1999; Park *et al.*, 1999) and RSK2 (Frodin *et al.*, 2000), in which the hydrophobic motif is altered to an acidic residue, are also constitutively phosphorylated at their activation loop when overexpressed in unstimulated cells. Scheid *et al.* (2002) also reported that a full-length PKB α [S473D] was phosphorylated more rapidly at Thr308 than wild-type PKB α when induced to translocate to the plasma membrane. These results could be reconciled with the findings in this study, if phosphorylation of Ser473 enhanced activation of PKB by PDK1 through a PIF-pocket-independent mechanism. This might be via another PKB-binding or scaffolding protein such as CTMP, which has been reported to interact with the hydrophobic motif of PKB (Maira *et al.*, 2001) and potentially could control accessibility to PDK1. Relevant to this suggestion, a splice variant of PKB γ that lacks a hydrophobic motif is poorly activated by insulin or pervanadate in cells (Brodbeck *et al.*, 2001).

The binding of PIF to PDK1 stimulates ~3-fold the rate at which PDK1 phosphorylates the T308tide peptide, indicating that occupancy of the PIF-pocket activates PDK1 (Biondi *et al.*, 2000). Thus the binding of the hydrophobic motif of S6K or RSK to the PIF-pocket of

PDK1 not only enables PDK1 to dock with these substrates but also stimulates the rate at which PDK1 phosphorylates the activation loop of these enzymes (Frodin and Gammeltoft, 1999; Biondi *et al.*, 2001; Frodin *et al.*, 2002). Mutation of Leu155 to glutamate increases the basal activity of PDK1 towards the T308tide ~3-fold, indicating that this mutation mimics the activating effect of PIF binding to PDK1 (Biondi *et al.*, 2000). Consistent with this observation, PDK1 immunoprecipitated from PDK1^{155E/155E} ES cells also possessed ~3-fold higher specific activity towards the T308tide substrate than wild-type PDK1 (Figure 2). Although PDK1 is more active in the PDK1^{155E/155E} ES cells than wild-type ES cells, this does not promote the activation or phosphorylation of the activation loop of PKB α (Figure 3). Moreover, as S6K1 (Figure 4), RSK (Figure 5) and SGK1 (Figure 6) are inactive in PDK1^{155E/155E} ES cells, this indicates that the ability of PDK1 to dock with S6K1, RSK and SGK1 is necessary for their activation. There has been interest in developing PDK1 inhibitors for the treatment of cancers in which the PI-3-kinase pathway is constitutively active, as PKB and other PDK1-activated AGC kinases are thought to be important mediators of cell proliferation and survival (Lawlor and Alessi, 2001). Our results suggest that a drug that binds to the PIF-pocket of PDK1 would not inhibit the activation of PKB. Thus the development of drugs that bind to the PIF-pocket may not represent a good therapeutic target for cancer, as current data indicate that it would be essential to inhibit PKB activation to prevent growth and survival of many cancer cells (Lawlor and Alessi, 2001).

Recent findings indicate that PKB plays an important role in promoting the phosphorylation of the hydrophobic motif of S6K1 (Thr389) by phosphorylating the tuberous sclerosis complex-2 (TSC2) protein (reviewed in McManus and Alessi, 2002). Consistent with these reports, S6K is not phosphorylated at its hydrophobic motif in IGF1-stimulated PDK^{-/-} ES cells (Williams *et al.*, 2000). Conversely, it would be expected that S6K would be phosphorylated normally at its hydrophobic motif in IGF1-stimulated PDK1^{155E/155E} ES cells, since PKB is still activated. However, in PDK1^{155E/155E} ES cells, phosphorylation of the hydrophobic motif of S6K1 was greatly reduced compared with wild-type ES cells following IGF1 stimulation (Figure 4A). This could be accounted for if phosphorylation of the activation loop of S6K1 by PDK1 promoted phosphorylation of the hydrophobic motif. Recent structural studies of the kinase domain of PKB β demonstrate that phosphorylation of the activation loop of PKB β stabilizes the α C-helix in the small lobe of the PKB β catalytic domain, generating a binding pocket for its own phosphorylated hydrophobic motif (Yang *et al.*, 2002a,b). Interestingly, this pocket in PKB β is located at the same position, and is structurally very similar to the PIF-pocket on PDK1, which consists of a hydrophobic groove located next to a phosphate-binding pocket. This intramolecular binding of the hydrophobic motif of PKB β to the pocket on the kinase domain also stimulates the activity of PKB β (Yang *et al.*, 2002a,b). Sequence alignments indicate that all AGC kinase members that are activated by PDK1 are likely to possess equivalent hydrophobic grooves and phosphate-binding sites in the small lobe of their kinase domains, whose expected role is

to bind to their own phosphorylated hydrophobic motif (Biondi *et al.*, 2002). Frodin *et al.* (2002) recently have performed elegant mutagenesis studies that support the existence of such a hydrophobic motif-binding pocket in the kinase domains of RSK, S6K and SGK. Moreover, they have provided evidence that occupancy of these pockets plays an important role in activating these AGC kinases. Based on these recent results, we propose that although S6K1 is still capable of becoming phosphorylated at its hydrophobic motif through PKB-mediated TSC2 phosphorylation in PDK1^{155E/155E} ES cells, this does not occur to a normal extent because S6K does not become phosphorylated at its activation loop. When the activation loop of S6K1 is not phosphorylated, we suggest that the α C-helix in the N-terminal lobe of the S6K1 kinase domain will be destabilized and the PIF-like hydrophobic motif-binding pocket will not be formed, as observed for PKB β (Yang *et al.*, 2002a,b). Thus the phosphorylated hydrophobic motif of S6K1, instead of being buried in its own catalytic domain, becomes exposed and susceptible to dephosphorylation by protein phosphatases. Consistent with this notion, treatment of the PDK1^{155E/155E} ES cells in which PKB is active with the protein phosphatase inhibitor okadaic acid greatly promoted the phosphorylation of the hydrophobic motif of S6K in IGF1-treated cells. In contrast, in PDK1^{-/-} ES cells, in which PKB is inactive, IGF1 stimulation in the presence of okadaic acid failed to induce detectable hydrophobic motif phosphorylation of S6K (Figure 4A). These results provide strong evidence to support the role of PKB in regulating the hydrophobic motif phosphorylation of S6K. This model of how S6K1 is regulated compared with PKB is outlined in Figure 8. Consistent with this hypothesis, a mutant of S6K1 in which the activation loop threonine is changed to alanine is poorly phosphorylated at its hydrophobic motif in insulin/IGF1-stimulated cells (Weng *et al.*, 1998; Balendran *et al.*, 1999b). In addition, mutation to alanine of the site in the activation loop of PKC δ that is phosphorylated by PDK1, inhibited phosphorylation of the hydrophobic motif of this enzyme (Parekh *et al.*, 1999). It has also been shown recently that in two *Drosophila* cell lines, knock-down of PKB by double-stranded RNAi prevented activation and phosphorylation of S6K at its hydrophobic motif phosphorylation site (Lizcano *et al.*, 2003).

Much of the work to define the cellular roles of AGC kinases has relied on the overexpression of constitutively active or dominant-negative mutants of these enzymes. These approaches could lead to misleading results, since AGC kinases possess similar substrate specificities, and the overexpression of active mutants of these protein kinases in cells could induce phosphorylation of proteins that are normally phosphorylated by other AGC kinase members. Moreover, dominant-negative mutants of AGC kinases could exert their effects by binding to PDK1, thereby preventing PDK1 from activating other AGC kinases. To overcome the potential drawbacks with such overexpression approaches, we have exploited PDK1^{-/-} ES cells to study these problems. These cells have proved very useful in conjunction with pharmacological inhibitors of PI-3-kinase, mTOR and ERK activation in defining roles of the PDK1/AGC kinase pathway in several processes (Sapkota *et al.*, 2001; Wang *et al.*, 2001;

Rena *et al.*, 2002; Greene *et al.*, 2003). Generation of PDK1^{155E/155E} ES cells described in this study in which PKB, but not other AGC kinases, is active provides an additional reagent with which to probe the specific roles of the diverse branches of the AGC kinase pathway in mediating cellular responses.

Currently, there is no pharmacological inhibitor that allows discrimination between substrates for PKB and SGK1. The PDK1^{155E/155E} ES cells in which PKB, but not SGK, is activated represent the first model system to verify whether phosphorylations are mediated by endogenous PKB or SGK1. We demonstrate that endogenous FKHR immunoprecipitated from PDK1^{155E/155E} ES cells is still phosphorylated at Thr24 and Ser319, suggested to be phosphorylated by SGK in cells (Brunet *et al.*, 2001). As PKB is the only AGC kinase activated downstream of PI-3-kinase in the PDK1^{155E/155E} ES cells, this observation strongly indicates that SGK1 is not necessary for the phosphorylation of these sites and that PKB mediates the phosphorylation of these residues *in vivo*. However, we cannot yet exclude the possibility that SGK1 could play a role in mediating the phosphorylation of FKHR in other cell types or the phosphorylation of other isoforms of FKHR.

To our knowledge, this is the first report in which a knock-in mutation has been employed to disrupt a substrate-docking site on a protein kinase. The results presented in this study indicate that this is a powerful approach to investigate the physiological roles of kinase–substrate docking interactions in regulating the specificity of signal transduction pathway activation. Furthermore, the knock-in cell lines and/or mice generated will provide useful insights in combination with pharmacological inhibitors to probe the function of specific branches of downstream signalling pathways without having to rely on overexpression of dominant-negative and constitutively active mutants.

Materials and methods

The materials and methods used in this study are described in the Supplementary data available at *The EMBO Journal* Online.

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