In vivo role of the phosphate groove of PDK1 defined by knockin mutation

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Accepted 2 August 2005
Journal of Cell Science 118, 5023-5034 Published by The Company of Biologists 2005
doi:10.1242/jcs.02617

Summary

AGC kinases are mediators of signalling responses stimulated by agonists and are activated following phosphorylation at their T-loop residue by the 3-phosphoinositide-dependent protein kinase-1 (PDK1). Agonists stimulate the activation of the AGC kinases p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK) and serum and glucocorticoid-induced protein kinase (SGK), by inducing the phosphorylation of these enzymes at a non-catalytic regulatory site termed the hydrophobic motif. This creates a high-affinity docking site enabling PDK1 to bind and phosphorylate the T-loop of these enzymes. The site that interacts with these substrates is located on the small lobe of the catalytic domain of PDK1 and is composed of a hydrophobic groove next to a basic phosphate groove. The disruption of the hydrophobic groove ablates activation of S6K, RSK and SGK, but the role of the phosphate groove in regulating the function of PDK1 has not been explored in vivo. We generated knockin ES cells, in which both copies of the gene encoding PDK1 were altered to express a form of PDK1 that retains catalytic activity and integrity of the hydrophobic groove, but in which the phosphate groove was disrupted. The knockin ES cells were viable, mutant PDK1 was expressed at normal levels and IGF1 induced activation of protein kinase B (PKB/Akt), which is a PDK1 substrate that does not require hydrophobic motif phosphorylation to be activated. In the phosphate-groove-knockin ES cells, the activation of S6K, RSK and SGK by agonists, although markedly impaired, was not abolished. PDK1 also phosphorylates the T-loop of protein kinase C (PKC) isoforms, which stabilizes these enzymes. However, in contrast to S6K, RSK and SGK, hydrophobic motif phosphorylation of these enzymes is not thought to control their activation by PDK1. Consistent with this notion, we employed appropriate PDK1-knockin ES cells to demonstrate that the hydrophobic groove of PDK1, but not the phosphate groove, is required for the stabilization of PKC isoforms. These findings provide genetic evidence that the phosphate groove of PDK1 is required for maximal activation of isoforms of S6K, SGK and RSK, but not PKC. We also found that no live births of homozygous phosphate-groove-knockin mice are observed, indicating a key role for this regulatory motif in normal development. The knockin embryos develop to a greater extent than PDK1-knockout and hydrophobic-groove-knockin embryos, which died between E9.5-E11.5. The knockin embryos are observed until E19.5 and displayed general growth retardation and craniofacial developmental defects.

Key words: Protein kinase, Docking sites, Akt, SGK, PKC, RSK, AGC kinase

Introduction

The 3-phosphoinositide-dependent protein kinase-1 (PDK1) is an upstream T-loop kinase that phosphorylates and activates many members of the AGC family of protein kinases in response to insulin and growth factors (Mora et al., 2004). These include isoforms of PKB (Scheid and Woodgett, 2003), S6K (Volarevic and Thomas, 2001), SGK (Lang and Cohen, 2001), RSK (Frodin and Gammeltoft, 1999) and PKC (Newton, 2002). Agonists stimulate the activation of AGC kinases by promoting their interaction with PDK1, rather than by activating PDK1 directly (Mora et al., 2004). The phosphorylation of PKB by PDK1 is controlled by the second messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], generated through insulin/insulin growth factor (IGF)-induced activation of phosphoinositide 3-kinase (PI 3-kinase). PKB and PDK1 possess pleckstrin-homology (PH) domains that interact with PtdIns(3,4,5)P_3, resulting in the colocalization of these enzymes at the plasma membrane of insulin/IGF-stimulated cells (Brazil and Hemmings, 2001; Leslie et al., 2001; Scheid and Woodgett, 2001) (Fig. 1A). Consistent with this, PKB could not be activated in a knockin ES cell line expressing a mutant form of PDK1 that could not bind PtdIns(3,4,5)P_3 (McManus et al., 2004). By contrast, S6K, SGK and RSK do not bind PtdIns(3,4,5)P_3; instead, the ability of PDK1 to phosphorylate these enzymes is dependent on a docking site termed the 'phospho-hydrophobic-motif-binding pocket' (also termed the PIF pocket), located on the small lobe of the PDK1 kinase domain (Biondi et al., 2000; Biondi et al., 2001; Frodin et al., 2002; Frodin et al., 2000). This pocket enables PDK1 to interact with a non-catalytic region of S6K, SGK and RSK, termed the hydrophobic motif, lying in a Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr sequence motif (where Xaa

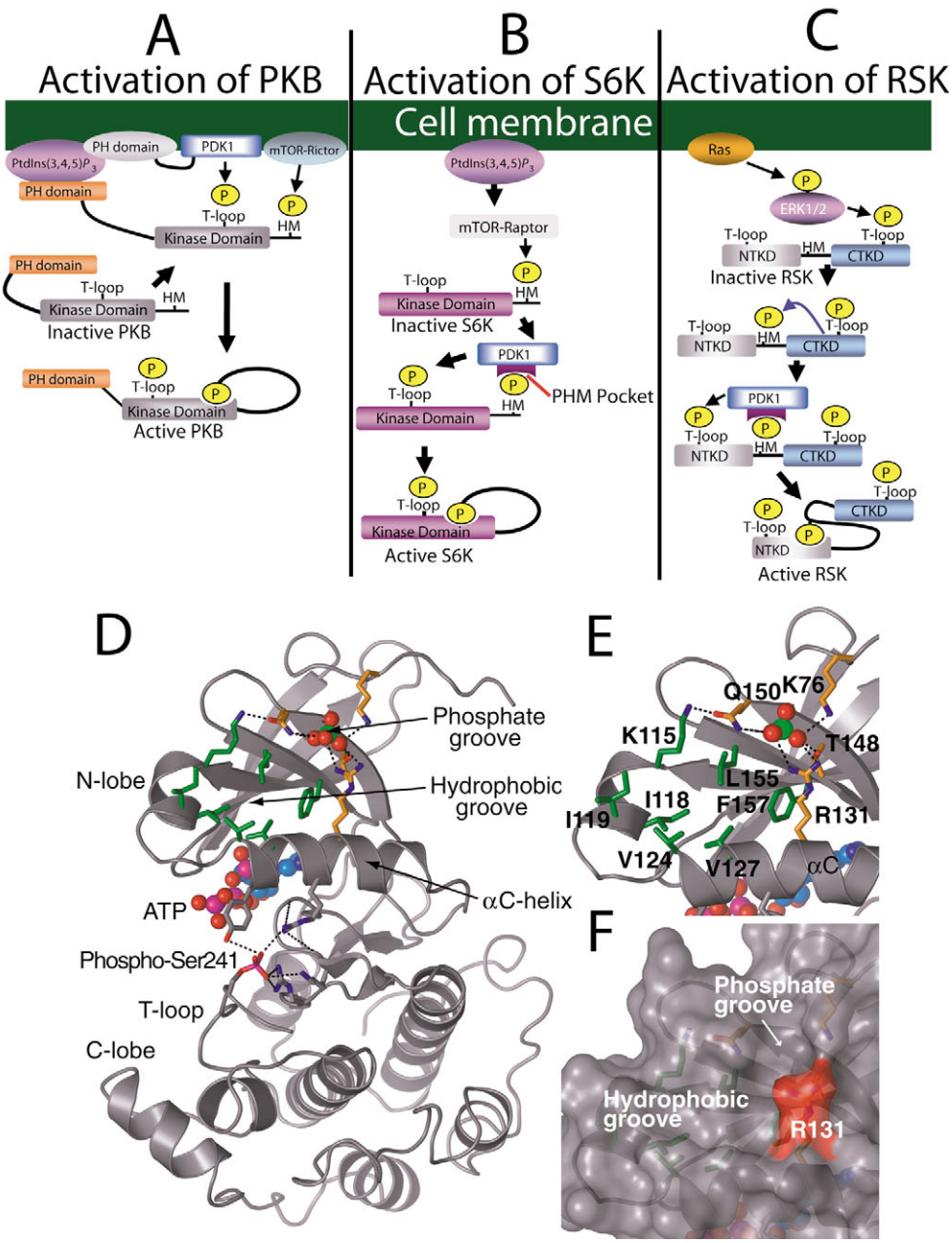


Fig. 1. Regulation of AGC kinase by PDK1 and structure of the phospho-hydrophobic-motif-binding pocket. Model described in the Introduction by which activation of PKB (A), S6K (B) and RSK (C) by PDK1 in response to extracellular agonists is controlled. Abbreviations: PH, Pleckstrin-homology; HM, hydrophobic motif; PHM, phospho-hydrophobic motif; NTKD, N-terminal kinase domain of RSK; CTKD, C-terminal kinase domain of RSK. (D) Ribbon representation of the kinase domain of PDK1 based on the structure of the catalytic domain reported previously (Biondi et al., 2002). The residues on the N-terminal lobe making up the hydrophobic groove are shown in green, whereas the residues making up the phosphate groove are in orange. ATP is shown as a ball and stick model with phosphate ions highlighted in purple and oxygen atoms highlighted in red. Locations of the T-loop phosphorylated Ser241 is indicated (E). Residues forming the hydrophobic and phosphate grooves are illustrated. The PDK1 backbone is shown as a grey ribbon. Hydrogen bonds forming with the oxygen atoms (red) of the sulphate ion (green) that lies within the phosphate groove when PDK1 is crystallized (Biondi et al., 2002) are indicated as black dotted lines. (F) Surface representation of the hydrophobic and phosphate groove. Position of the Arg131 residue that is mutated in this study is shown in red.

is any amino acid). Phosphorylation of the Ser/Thr residue in the hydrophobic motif of S6K by mTOR in response to insulin, growth factors and nutrients (Shamji et al., 2003) enhances the binding of PDK1 to S6K, leading to its activation (Fig. 1B). In the case of RSK isoforms, a second catalytic domain is present, which is activated by ERK1/ERK2, which phosphorylates the hydrophobic motif and leads to PDK1 binding and activation (Frodin and Gammeltoft, 1999) (Fig. 1C). Activation of SGK is also triggered by its hydrophobic motif phosphorylation (Biondi et al., 2001; Kobayashi and Cohen, 1999), but the enzyme that phosphorylates the hydrophobic motif of SGK is not known. Hydrophobic motif phosphorylation of SGK1 is not thought to be regulated by PDK1, as phosphorylation of the hydrophobic motif of SGK1 is normal in PDK1$^{-/-}$ cells (Collins et al., 2003). The interaction of PKC isoforms with lipids and Ca^{2+}, as well as their cellular localization, is believed to regulate T-loop phosphorylation by PDK1. In contrast to other AGC kinases, there is not a distinct PKC hydrophobic motif kinase, rather it is the T-loop phosphorylation of PKC isoforms that enables them to autophosphorylate their own hydrophobic motif (Newton, 2002).

The structure of the catalytic domain of PDK1 revealed that the phospho-hydrophobic-motif-binding pocket was composed of a hydrophobic groove located next to a cluster of basic residues forming a phosphate groove (Biondi et al., 2002) (Fig. 1D-F). Mutagenesis of the hydrophobic groove prevented PDK1 from activating S6K, SGK and RSK in vitro, without affecting PKB activation (Biondi et al., 2001; Frodin et al., 2002). The crucial role that the hydrophobic groove of PDK1 played in vivo was emphasized in a knockin study in which disruption of this site abolished activation of S6K and RSK, but not PKB (Collins et al., 2003). To date, the role of the

phosphate groove of PDK1 has only been studied in vitro. Mutation of Arg131, located in the phosphate groove, abolished binding of PDK1 to phosphopeptides encompassing the hydrophobic motifs of S6K (Biondi et al., 2002) and RSK (Frodin et al., 2002), indicating that this site would play a key role in regulating PDK1 activity. The aim of this study was both to define the physiological role that the phosphate groove of PDK1 plays in enabling PDK1 to regulate the activation of its diverse substrates and to define its role in mouse development.

Materials and Methods

Materials

Protein G-Sepharose, Glutathione-Sepharose and Streptavidin-Sepharose High Performance were purchased from Amersham Pharmacia Biotech; protease-inhibitor cocktail tablets were from Roche; rapamycin and wortmannin were from Calbiochem; tissue culture reagents, leukocyte inhibitory factor, optiMEM®I and Lipofectamine 2000 transfection reagent were from Invitrogen; IGF1 was purchased from Biosource; TPA was from Sigma; and Ciproxin® Infusion was from Bayer.

Antibodies

The following antibodies were raised in sheep and affinity purified on the appropriate antigen: PDK1 whole protein (full-length human PDK1 protein), PDK1 mouse C-terminus (residues 540-559 of mouse PDK1, RKIQEVWRQQYQSNPDAAVQ), total GSK3α (residues 471-483 of rat GSK3α, QAPDATPTLTNSS), total GSK3β (full-length human protein), total S6K1 (residues 25-44 of rat S6K1, AGVFDIDLDQPEDAGSEDEL), S6 phospho S235-P (residues 229-242 of human S6, AKRRRLpSSLRASTS), total RSK (residues 712-734 of human p90 RSK2, RNQSPVLEPVGRSTLAQRRGIKK), RSK phospho T360-P (Thr359 in human RSK1, residues 355-364 of rat RSK1, FTSRTpPRDSP), RSK phospho S381-P (Ser380 in human RSK1, residues 376-385 of rat RSK1, FRGFSpFVATG), GST-FOXO1a (full-length human protein), PKCζ (residues 3-24 of rat PKCζ, SRTDPKMDRSGGRVRLKAHYGG) and TSC2 (residues 1791-1814 of mouse TSC2, ATPTYETGQRKRLISSVDDFTEFV). The total PKBα antibody used to immunoprecipitate and immunoblot PKBα was a mouse monoclonal antibody raised against residues 1-149 of human PKB and was purchased from Upstate (05-591). The total GAPDH antibody was a mouse monoclonal antibody and was purchased from Calbiochem (CB1001). Mouse monoclonal antibodies recognizing GST were purchased from Sigma. The following antibodies were purchased from Cell Signalling Technology and the catalogue number indicated: PKB phospho T308-P (#9275), PKB phospho S473-P (#9271), GSK3α/GSK3β phospho S21-P/S9-P (#9336), PKCζ/λ phospho T410-P/T403-P (#9378), S6K1 phospho T421-P/S424-P (#9204), S6K1 phospho T389-P (#9205), total S6 Protein (#2212), human RSK1 phospho T573-P (#9346), total ERK1/2 (#9102), ERK1/2 phospho T202/Y204-P (#9101), TSC2 phospho T1462-P (#3611), TSC2 phospho S939-P (#3615), FOXO1a/FOXO3a phospho T24-P/T32-P (#9464), FOXO1a phospho S256-P (#9461), total PRK2 (#2612), and PRK1/PRK2 phospho T778/T816-P (#2611). We found that the pan-PDK1 site antibody (#9379) recognized the phosphorylated T-loop of S6K in cell extracts. The following antibodies were purchased from Santa Cruz and the catalogue number indicated: RSK2 phospho S227-P (sc-12445-R), PKCα (Sc-208), PKCβII (Sc-210), PKCδ (Sc-213), PKCγ (Sc-211), PKCε (Sc-214) and total PRK1 (sc-7161). Secondary antibodies coupled to horseradish peroxidase (HRP) were from Pierce. Secondary antibodies coupled to IRDye800 flurophore for use with the LI-COR Odyssey infrared detection system were purchased from Rockland.

General methods and buffers

Restriction enzyme digests, DNA ligations, site-directed mutagenesis, PCR, Southern blotting and other recombinant DNA procedures were performed using standard protocols. DNA sequencing was performed by The Sequencing Service (School of Life Sciences, University of Dundee, Scotland; www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. Lysis buffer was 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (by mass) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (by vol) 2-mercaptoethanol and 'Complete' proteinase inhibitor cocktail (one tablet per 50 ml). Buffer A was 50 mM Tris-HCl pH 7.5, 0.1 mM EGTA and 0.1% (by vol) 2-mercaptoethanol. Embryo membrane digestion buffer was 50 mM KCl, 10 mM Tris, pH 7.5, 2.5 mM $MgCl_2$, 0.5% Tween 20 and 0.1 mg/ml proteinase K.

Construction of the R131M knockin targeting vector

A BAC clone containing the mouse genomic PDK1 sequence from a 129Sv mouse BAC library has been used to define the mouse intron/exon sequences of the kinase domain of PDK1 (Williams et al., 2000). This revealed that the kinase domain of PDK1 commences at exon 2 and Arg131 is located in exon 4. A pMC1-neo-pA cassette flanked by loxP sites (floxed; Gu et al., 1993) was introduced at the 5′ end of exon 3 (see Fig. 2A). The 5′ homology arm was constructed with two fragments: a *Hin*dIII fragment of 3.3 kb containing exon 2 and a 1.7 kb *Hin*dIII-*Nde*I fragment from the mouse PDK1 BAC clone. A 3.8 kb *Nde*I-*Sac*I fragment from the BAC clone containing exons 3 and 4 was used to construct the 3′ homology region and the codon encoding for Arg131 (AGA) was altered by mutagenesis to encode Met (ATG). This mutation was previously shown to prevent PDK1 from interacting with a phosphopeptide encompassing the hydrophobic motif of RSK (Frodin et al., 2002). Appropriate linkers were used to introduce an *Eco*RV restriction site at the *Hin*dIII site 3′ of exon 4. The PGK-TK-pA cassette was included at the 3′ end of the 3′ arm for negative selection. Further details of the sequence of this targeting construct are available by request. The final knockin construct was purified on a caesium chloride gradient and was linearized using *Not*I before electroporation.

ES cell targeting

E14 mouse embryonic stem (ES) cells were grown in Dulbecco's modified Eagle's medium containing high glucose supplemented with 15% foetal calf serum, 0.1 mM non-essential amino acids, antibiotics (100 units penicillin G, 100 μg/ml streptomycin), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol and 25 ng/ml murine leukaemia inhibitory factor. During the initial selection, ES cells were maintained on a feeder layer of mitotically inactivated G418-resistant primary embryonic fibroblasts, derived from MTK-Neo mice (Stewart et al., 1987). Targeting of the gene encoding PDK1 to obtain $PDK1^{131Mneo/+}$ ES cells was performed using standard procedures (Joyner, 1993). Briefly, following electroporation, cells were plated in the absence of G418 for 48 hours and then grown in the presence of 0.2 mg/ml G418 and 2 μM gancyclovir (thymidine kinase negative selection) for 10-14 days. Colonies were picked and cultured in the absence of G418 and gancyclovir. Southern blotting using an *Eco*RV digest of genomic DNA and a 1.3 kb probe that encompasses exons 5 and 6 identified targeted cell lines. 0.77% of cell lines screened were correctly targeted. Positive ES cells ($PDK1^{131Mneo/+}$) were expanded and used to generate chimaeric mice.

Microinjection of positively targeted ES cells into blastocysts

In order to obtain blastocysts for microinjection, CB6f1 (a hybrid of BALB/c and C57Bl/6) female mice were induced to superovulate by

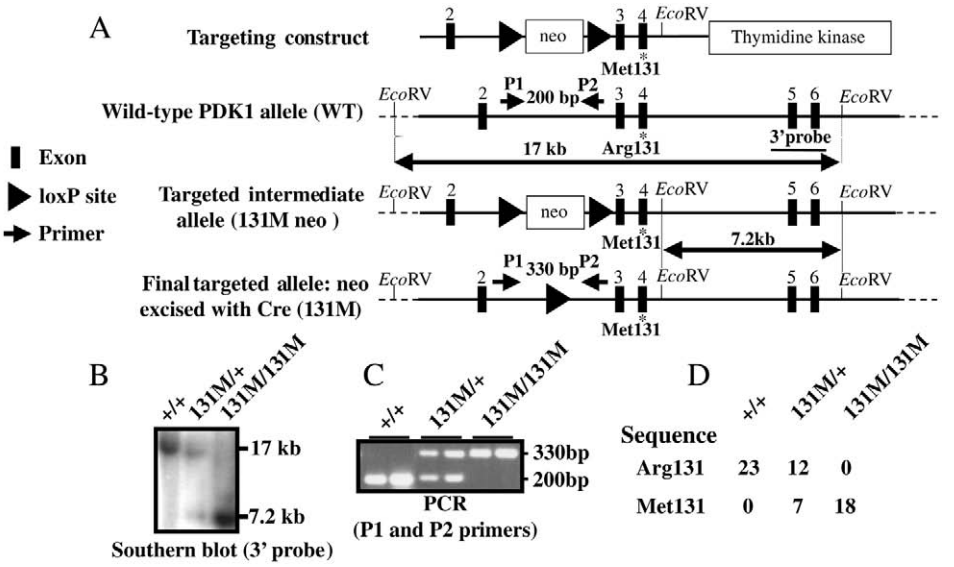


Fig. 2. ES cell knockin strategy. (A) Diagram illustrating the targeting knockin construct, the 5′ end of the gene for PDK1 and the allele modification generated. The position of the 3′ probe used to genotype targeted knockin cells in B is shown. The position of the PCR primers used to genotype the Cre recombinase-mediated excision of the neomycin cassette is indicated by arrows. The position of Arg131/Met131 in exon 4 is represented by an asterisk. The position of the introduced novel *Eco*RV restriction site is marked. (+), wild-type allele; (131Mneo), targeted knockin allele with the neomycin cassette still present; (131M), targeted knockin allele with the neomycin cassette removed. (B) Genomic DNA purified from the indicated ES cell lines was digested with *Eco*RV, electrophoresed on a 1% agarose gel, transferred to nitrocellulose and the membrane incubated with the ^{32}P-labelled 3′ probe. The wild-type allele generates a 17 kb fragment whereas the targeted knockin allele generates a 7.2 kb fragment in this analysis. (C) Genomic DNA purified from the indicated ES cell lines or embryos 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 (131M). (D) Genomic DNA purified from the indicated ES cell lines was subjected to PCR using primers 5′-GCCTCCAAGGAGATCAGTACACAG and 5′-GGTAGTCGCAGGGCCTGTGCTG to generate a 460 bp product that encompasses the 131 mutation region on exon 4. The resultant PCR products were ligated into the pCR-Topo 2.1 vector, transformed into *Escherichia coli* and clones sequenced. The numbers of the wild type Arg131 and knockin Met131 sequences obtained for each cell line is indicated.

the injection of pregnant mare serum gonadotropin (PMSG), which has follicle-stimulating hormone activity. This was followed 48 hours later by the injection of human chorionic gonadotropin (HCG), which has lutenizing hormone (LH) activity, thus mimicking the LH surge that normally brings about ovulation. These females were then mated with C57Bl/6 males. Blastocysts were removed at 2.5 days post coitus (dpc) and cultured overnight. The following morning (3.5 dpc) each expanded blastocyst was injected with 10-15 PDK1$^{131Mneo/+}$ ES cells. Around 40 blastocysts were injected on each round of microinjection. Following microinjection, the blastocysts were returned to culture medium for 2 hours, prior to transfer to CD1 pseudopregnant females. The offspring were born ~17 days following re-implantation. These animals were chimaeras, having developed from both the injected ES cells and the recipient blastocyst. Subsequent matings were continued with C57Bl/6 mice. The Neomycin cassette was excised in the PDK1$^{131Mneo/+}$ mice by mating with the Bal1 mouse line, which expresses the Cre recombinase enzyme in all tissues (Betz et al., 1996). Neomycin excision was identified using the Primers P1 (5′-CTATGCTGTGTTACTTCTTGGAGCACAG) and P2 (5′-AATAG-CCAGGGCTACACAGAGAAACCTTTC) as described in Fig. 2. PCR yields a product of 200 bp for the wild-type allele and 330 bp for the 131M allele in which the Neo has been excised (PDK1$^{131M/+}$) (Fig. 2C).

Analysis of phenotype of PDK1$^{131M/131M}$ embryos

Heterozygous PDK1$^{131M/+}$ mice were intercrossed and the day of plugging was designated E0.5. Pregnant females were sacrificed and embryos were dissected from the uterus in PBS. Embryos were then photographed using a Leica M275 microscope before being fixed overnight in 4% PFA in PBS, then stored in PBS with 0.02% sodium azide. Genotyping was performed on embryonic membranes that were incubated overnight in embryo membrane digestion buffer at 65°C and subjected to PCR with the P1 and P2 primers (Fig. 2A,C).

Generation and isolation of PDK1$^{131M/131M}$-knockin ES cells

Female PDK1$^{131M/+}$ mice were induced to superovulate by the injection of PMSG. This was followed 48 hours later by the injection of HCG. These mice were then mated with male PDK1$^{131M/+}$ mice. Blastocysts were removed at 2.5 dpc and cultured on 24-well plates on a feeder layer of MEFs for 1-2 weeks to allow the ES cells to grow. Wells were trypsinized and 80% of the aliquot frozen in two batches while the remaining 20% was used to grow cells for DNA preparation. Cells were analysed by Southern blotting with the 3′ probe and by PCR using P1 and P2. 20% of the cell lines were found to possess the PDK1$^{131M/131M}$ genotype.

Cell culture, stimulation and cell lysis

PDK1$^{+/+}$, PDK1$^{131M/131M}$ and PDK1$^{-/-}$ ES cells were grown on gelatinized tissue culture plastic in KnockOut DMEM containing 10% KnockOut SR supplemented with 0.1 mM non-essential amino acids, antibiotics (100 units penicillin G, 100 μg/ml streptomycin), antimycotic (1 μg/ml Ciproxin Infusion), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol and 25 ng/ml murine leukaemia inhibitory factor. The ES cells were cultured to 80% confluence on 15 cm diameter dishes and incubated for 4 hours in KnockOut DMEM lacking serum. The cells were then stimulated with the indicated agonists as described in the figure legends. The cells were lysed in 0.4 ml of ice-cold lysis buffer and centrifuged at 4°C for 15 minutes at 13,000 ***g***. The supernatants were aliquoted, frozen in liquid nitrogen and stored at –80°C until use. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard.

Immunoprecipitation and assay of protein kinases

500 μg ES cell lysate protein was used to immunoprecipitate and assay PKBα, S6K1 and RSK isoforms. The lysates were incubated at 4°C for 1 hour on a shaking platform with 5 μg of each antibody

coupled to 10 μl of protein G-Sepharose. The immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.5 M NaCl, and once with 1 ml of buffer A. The standard assay (50 μl) contained: washed Protein G-Sepharose immunoprecipitate, 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 2.5 μM PKI (TTYADFIASGRTGRRNAIHD, peptide inhibitor of cyclic-AMP-dependent protein kinase), 10 mM magnesium acetate, 0.1 mM [γ^{32}P]ATP (~200 cpm/pmol) and Crosstide (GRPRTSSFAEG). For PKB and S6K assays, 100 μM Crosstide was used; for RSK assays, 30 μM Crosstide was used (Alessi et al., 1996). The assays were carried out for 30 minutes at 30°C, the assay tubes being agitated continuously to keep the immunoprecipitate in suspension, then terminated and analysed as described previously (Alessi et al., 1995). PDK1 was immunoprecipitated from 1 mg of ES cell lysate protein with the anti-mouse PDK1 C-terminal antibody and the immunoprecipitates were washed and assayed as above, except that T308tide (KTFCGTPEYLAPEVRR, 1 mM) was used as the substrate (Biondi et al., 2000). In all assays, 1 mUnit of activity was that amount of enzyme that catalysed the phosphorylation of 1 pmol of substrate in 1 minute.

Expression of GST-PDK1 in 293 cells and GST-SGK1 in ES cells

GST-PDK1 and GST-PDK1(R131M) was expressed in 293 cells and affinity purified on glutathione-Sepaharose as described previously (Alessi et al., 1997). Constructs encoding the expression of GST-SGK1 or GST-SGK1(S422D) lacking the first 60 N-terminal residues have been described previously (Kobayashi and Cohen, 1999). The 60 N-terminal amino acids of SGK encode polyubiquitination sites and, unless these are removed, SGK cannot be expressed at significant levels (Brickley et al., 2002). ES cells were grown to 90% confluence on 15 cm tissue culture dishes in Dulbecco's modified Eagle's medium containing high glucose supplemented with 15% foetal calf serum, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol and 25 ng/ml murine leukaemia inhibitory factor. After washing cells twice in PBS, cells were then fed with 11 ml of DMEM (with no LIF, serum or antibiotics). 22 μg of GST-SGK1 ebg2T plasmid was premixed for 20 minutes with 25 μl Lipofectamine 2000 in 1 ml of optiMEM I and was added dropwise to each dish and the cells were then incubated for 5 hours. Media was then made up to contain 15% foetal calf serum, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol and 25 ng/ml murine leukaemia inhibitory factor. 24 hours post-transfection, the cells were stimulated as described in the figure legends and lysed in 0.4 ml lysis buffer and the lysates centrifuged at 13000 ***g*** for 15 minutes at 4°C. After immunobloting the lysates with anti-GST antibody to verify expression levels of SGK, the supernatants containing similar levels of SGK were incubated for 1 hour on a shaking platform with 20 μl of glutathione-Sepharose equilibrated previously in lysis buffer. The suspension was centrifuged for 2 minutes at 13000 ***g*** and the beads were washed twice with lysis buffer containing 0.5 M NaCl and once with buffer A. The purified SGK was either assayed for SGK1 activity employing the same assay and peptide substrate (100 μM Crosstide) as described above or immunoblotted with the anti-GST antibody after resuspension in SDS sample buffer.

Affinity purification of PDK1 on HM-PRK2 Sepharose

Streptavidin-Sepharose High Performance (0.5 ml) equilibrated in lysis buffer was incubated with 25 μg of Biotinylated HM-PRK2 peptide (Biotin-C_6spacer: REPRILSEEEQEMFRDFDYIADWC) on a shaking platform for 30 minutes and the beads washed with lysis buffer to remove any unconjugated peptide. 0.5 mg of ES cell lysate protein was incubated with 10 μl of the conjugated HM-PRK2 peptide-Sepharose on a shaking platform for 1 hour at 4°C, which was washed three times with 1 ml of lysis buffer. The beads were resuspended in a volume of 15 μl of lysis buffer to which 5 μl of 4× SDS Sample Buffer was added. The samples were then immunoblotted for PDK1 as described below.

Immunoblotting

Unless stated otherwise, 25 μg of protein lysate in SDS Sample Buffer was subjected to SDS/polyacrylamide gel electrophoresis for immunoblotting. Exceptions were: ERK total, 10 μg; ERK-P, 5 μg; GAPDH, 5 μg; S6 total, 10 μg; and PDK1 total, 40 μg. Proteins were transferred to nitrocellulose. For phospho-specific antibody blots, the nitrocellulose membranes were immunoblotted at 4°C for 16 hours using the indicated antibodies (2 μg/ml for the sheep antibodies or 500-fold dilution for commercial antibodies) in the presence of 10 μg/ml of the de-phosphopeptide antigen used to raise the antibody for sheep-raised phospho-specific antibodies. For total blots, the nitrocellulose membranes were immunoblotted at 4°C for 16 hours using the indicated antibodies (1 μg/ml for the sheep and rabbit-raised antibodies and 1000-fold dilution for the mouse monoclonal PKBα and GAPDH antibodies). PKCε was used at 2 μg/ml. The blots were incubated in 50 mM Tris/HCl pH 7.5, 0.15 M NaCl, 0.2% (by vol) Tween containing either 5% (by mass) skimmed milk for sheep-raised antibodies or 0.5-1% BSA (by mass) for rabbit and mouse-raised antibodies. Detection was performed using HRP-conjugated secondary antibodies and the enhanced chemiluminescence reagent or, for the PKC isoform and indicated PKB blots, IRDye800 flurophore-conjugated antibody and using the Odyssey infrared detection system (LI-COR Biosciences). Band intensity was quantitated using LI-COR software.

Results

Generation of PDK1$^{131M/131M}$-knockin ES cells

A targeting construct was generated as described in the Materials and Methods to replace the wild-type exon 4 of the gene encoding PDK1 with a mutant form of exon 4 encoding for Met at position Arg131 (Fig. 2A). This mutation was previously shown to prevent PDK1 from interacting with a phosphopeptide encompassing the hydrophobic motif of RSK as well as the hydrophobic motif of PKC-related kinase-2 (PRK2), which possesses an acidic Asp residue in place of a phosphorylated Ser/Thr residue (Frodin et al., 2002). In addition, a Neomycin cassette flanked with Cre recombinase-removable loxP sites was present in the targeting construct to permit selection of targeted ES cells. Wild-type ES cells were targeted with this construct and heterozygous ES cells, termed PDK1$^{131Mneo/+}$, were identified by Southern blotting (Fig. 2B). PDK1$^{131Mneo/+}$ ES cells were injected into blastocysts and were re-implanted into pseudopregnant females. Chimaeric offspring were further bred to obtain heterozygous PDK1$^{131Mneo/+}$ mice that were identified by PCR analysis. The PDK1$^{131Mneo/+}$ mice were mated with the Cre-expressing mouse strain Bal1 (Betz et al., 1996) to remove the neomycin selection marker as we have previously found that an intronic neomycin cassette in the gene for PDK1 significantly reduced PDK1 expression in both ES cells and mice (Lawlor et al., 2002). The resulting PDK1$^{131M/+}$ mice were mated with each other, day E2.5 blastocysts were isolated and ES cells from these were propagated as described in the Materials and Methods. Using this approach, we were able to isolate littermate wild-type PDK1$^{+/+}$ and homozygous knockin PDK1$^{131M/131M}$ ES cells. PCR analysis (Fig. 2C) and genomic DNA sequencing (Fig. 2D) confirmed that replacement of the

wild-type exon with the mutant exon had occurred. The PDK1$^{131M/131M}$ ES cells were morphologically indistinguishable and proliferated at a similar rate to PDK1$^{+/+}$ ES cells (data not shown).

PDK1 is normally expressed in knockin ES cells

Employing two different PDK1 antibodies, we found that PDK1 is expressed at similar levels in PDK1$^{131M/131M}$ and PDK1$^{+/+}$ ES cells (Fig. 3A). We also immunoprecipitated PDK1 from ES cell lysates and assayed its activity using the T308tide peptide substrate that does not interact with the phospho-hydrophobic-motif-binding pocket (Biondi et al., 2000). The activity of PDK1 isolated from the PDK1$^{131M/131M}$-knockin ES cells was similar to that measured from PDK1$^{+/+}$ wild-type cells. We also expressed recombinant wild-type GST-PDK1 and mutant GST-PDK1(R131M) and found that these displayed identical specific activities (Fig. 3B). In order to confirm disruption of the phosphate groove of PDK1 in the PDK1$^{131M/131M}$-knockin ES cells, cell lysates were incubated with streptavidin-Sepharose conjugated to a biotinylated peptide that encompasses the hydrophobic motif of PRK2 that interacts strongly with the phospho-hydrophobic-motif-binding pocket of PDK1 (Balendran et al., 1999; Biondi et al., 2000). Using this approach, PDK1 could be affinity purified from wild-type PDK1$^{+/+}$ ES cells, but not from PDK1$^{131M/131M}$ ES cells, confirming disruption of the phosphate groove in knockin cells (Fig. 3A, lower panel).

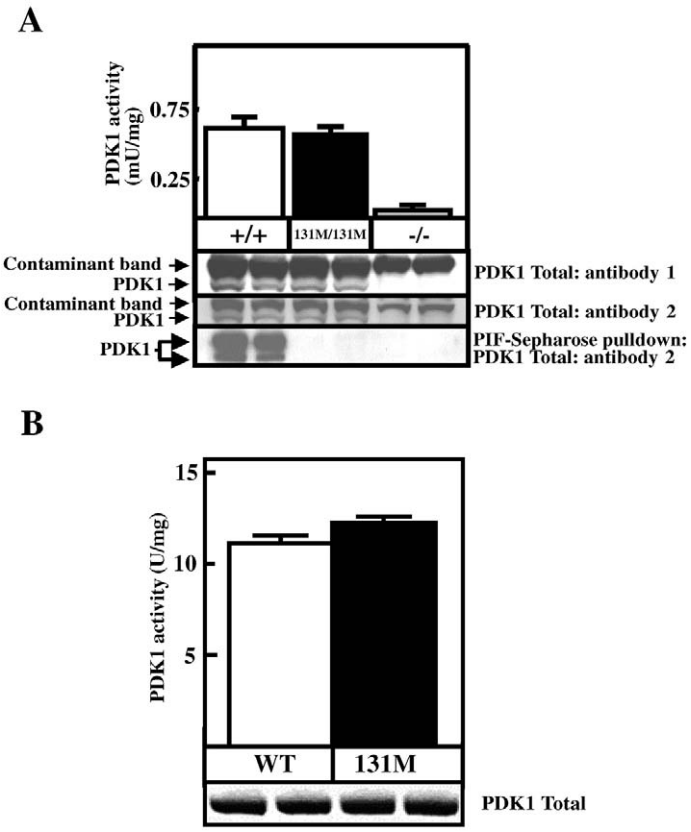


Fig. 3. Expression and activity of PDK1 in knockin ES cells. (A) The wild-type PDK1$^{+/+}$, knockin PDK1$^{131M/131M}$ and knockout PDK1$^{-/-}$ ES cells (Williams et al., 2000) were cultured to 80% confluence and lysed. PDK1 was immunoprecipitated from the cell lysate and assayed with the T308tide peptide as described in the Materials and Methods. The results shown are the average specific activity ±s.e.m. of three separate dishes of cells with assays for each dish 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 the recombinant human PDK1 protein). The lysates were also incubated with Sepharose conjugated to the HM-PRK2 peptide in order to affinity purify PDK1 and bound PDK1 visualized by immunoblot analysis as described in the Materials and Methods. Similar results were obtained in three separate experiments. It should be noted that PDK1, as observed in other cell lines, is detected as two bands on immunoblot analysis for reasons that are not yet known (Alessi et al., 1997; Balendran et al., 1999). (B) Equal amounts of recombinant wild-type GST-PDK1 and mutant GST-PDK1(R131M) were assayed with the T308tide peptide and levels of PDK1 present in each assay was assessed by Coomassie staining of a polyacrylamide gel. The assays were performed in triplicate and results presented as the average ±s.e.m.

PKB is activated in knockin ES cells

In order to assess PKBα activation, serum-starved PDK1$^{+/+}$ and PDK1$^{131M/131M}$ ES cells were stimulated with IGF1 in the presence or absence of the PI 3-kinase inhibitor wortmannin. PKBα activity was assayed following its immunoprecipitation, and PKBα phosphorylation at Thr308 and Ser473 was measured by immunoblot analysis with phosphospecific antibodies. In both PDK1$^{+/+}$ and PDK1$^{131M/131M}$ ES cells, IGF1 induced a marked activation and phosphorylation of Thr308 and Ser473 that was inhibited by wortmannin (Fig. 4, upper panel). The activation of PKB and phosphorylation of Thr308 was slightly reduced in IGF1-stimulated PDK1$^{131M/131M}$ ES cells. Quantitative analysis of Thr308 phosphorylation employing LI-COR infrared analysis in two additional experiments confirmed a slight reduction of Thr308 phosphorylation in the knockin cells (Fig. 4, lower panel). IGF1 induced the phosphorylation of the PKB substrates glycogen synthase kinase-3 (GSK3) and tuberous sclerosis complex-2 (TSC2), as well as the FOXO-1a forkhead transcription factor, in both PDK1$^{+/+}$ and PDK1$^{131M/131M}$ ES cells, albeit to a slightly reduced extent than observed in wild-type cells (Fig. 4).

Activation of S6K is impaired in knockin ES cells

As observed previously (Collins et al., 2003; McManus et al., 2004; Williams et al., 2000), the basal activity of S6K in serum-starved wild-type ES cells is relatively high and is further stimulated twofold by IGF1 (Fig. 5). Earlier work has indicated that the basal S6K activity in serum-starved ES cells is dependent on amino acids, as removal of these abolished S6K activity, whereas restoring amino acids restored activity (McManus et al., 2004). In the serum-starved PDK1$^{131M/131M}$ ES cells, the basal S6K activity is ninefold lower than that observed in PDK1$^{+/+}$ ES cells; however, IGF1 still induced a fourfold activation of S6K (Fig. 5). Treatment of PDK1$^{+/+}$ and PDK1$^{131M/131M}$ ES cells with the mTOR inhibitor rapamycin reduced S6K activity to undetectable levels, whereas treatment with the PI 3-kinase inhibitor wortmannin only moderately reduced S6K activity to similar levels seen in serum-starved cells, consistent with the notion that the basal S6K activity is

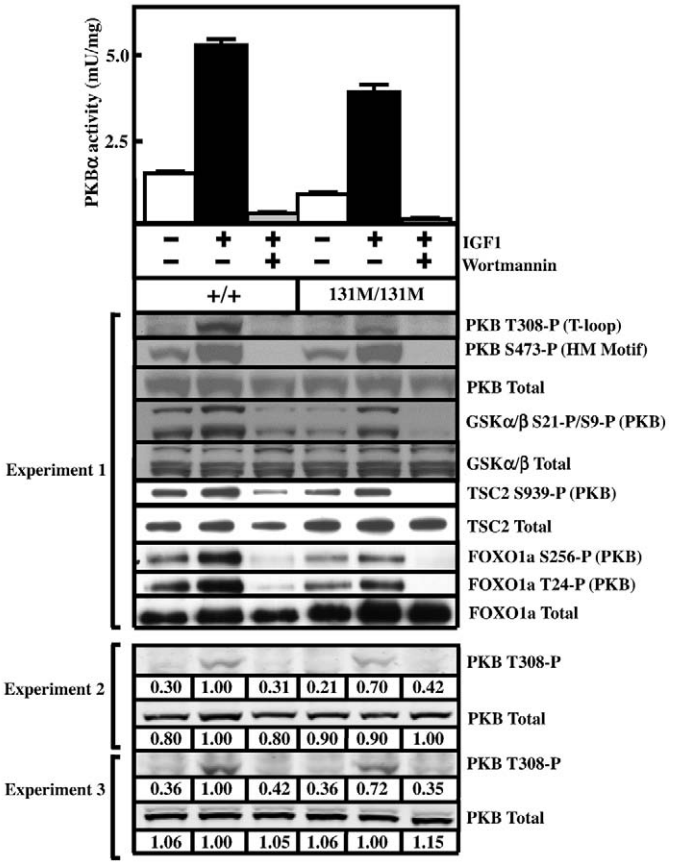


Fig. 4. Activation of PKBα and phosphorylation of PKB substrates in knockin cells. The wild-type and knockin ES cells were cultured to 80% confluence. Cells were deprived of serum for 4 hours and either left untreated or incubated in the presence or absence of 100 nM wortmannin for 10 minutes and then stimulated with 20 ng/ml IGF1 for 15 minutes. The cells were lysed and PKBα immunoprecipitated and assayed. The results shown are the average specific activity ±s.e.m. of three separate dishes of cells with assays for each dish performed in duplicate. The ES cell lysates were also immunoblotted with the indicated antibodies. For the TSC2 and FOXO1a blots, the ES cells were treated as above except IGF1 stimulation was performed for 30 minutes. Cells were lysed and lysates immunoblotted with the indicated antibodies. Similar results were obtained in two separate experiments. In the lower panel, the results of two further experiments are shown in which quantitation of the immunoblots was performed using the LI-COR Odyssey infrared imaging system as described in the Materials and Methods. The results are plotted relative to the values of the IGF1-stimulated sample in PDK1$^{+/+}$ cells, which is given a value of 1.0. Abbreviation: HM, hydrophobic motif.

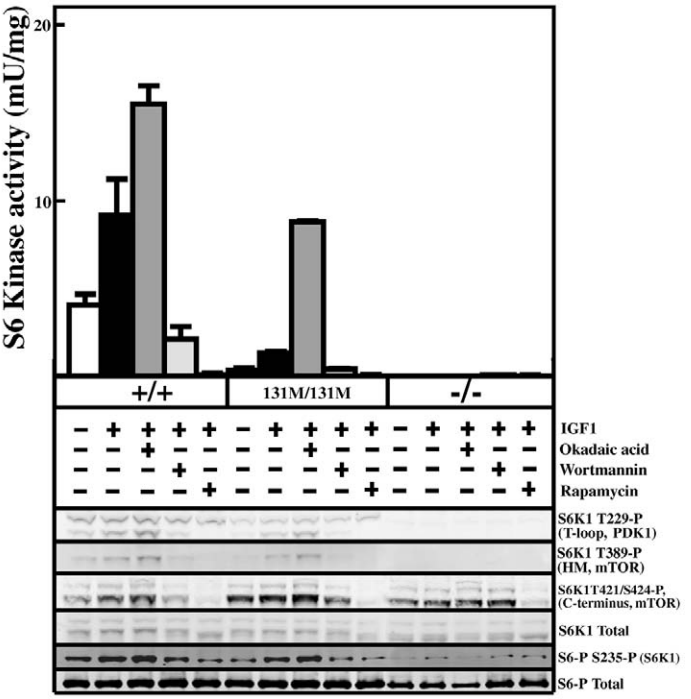


Fig. 5. S6K1 activation is impaired in knockin cells. The indicated ES cells were cultured to 80% confluence. Cells were deprived of serum for 4 hours and either left untreated or incubated in the presence or absence of 500 nM okadaic acid for 30 minutes, 100 nM wortmannin for 10 minutes or in the presence or absence of 100 nM rapamycin for 30 minutes, and then stimulated with 20 ng/ml IGF1 for 30 minutes. The cells were lysed and S6K1 immunoprecipitated and assayed. The results shown are the average specific activity ±s.e.m. of two separate dishes of cells with assays for each dish performed in duplicate. The ES cell lysates were also immunoblotted with the indicated antibodies. Thr389 is the phosphorylated S6K hydrophobic motif residue. S6K phosphorylates the S6 protein (S6-P) at Ser235. Similar results were obtained in three separate experiments. Abbreviation: HM, hydrophobic motif.

regulated by a PI 3-kinase-independent nutrient pathway (Fig. 5). Phosphorylation of S6K at both the T-loop (Thr229) (measured using a pan-PDK1 phospho antibody) and the hydrophobic motif (Thr389) mirrored the specific activity of S6K that was measured in the immunoprecipitation assay (Fig. 5). We also monitored phosphorylation of the S6K substrate S6 protein at Ser235 and found that it was reduced in the PDK1$^{131M/131M}$ ES cells compared with the control cells. IGF1 also induces the phosphorylation of S6K at C-terminal Ser/Thr-Pro residues. In IGF1-stimulated PDK1$^{131M/131M}$ ES cells, the phosphorylation of two of these sites (Thr421 and Ser424) was not reduced and even slightly increased.

As phosphorylation of the hydrophobic motif of S6K is regulated by PKB, which is activated in PDK1$^{131M/131M}$ ES cells (Fig. 4), we were interested to explore whether the reduction of Thr389 phosphorylation of S6K in the PDK1$^{131M/131M}$-knockin cells was the result of increased dephosphorylation of this residue, as was previously proposed to occur in the absence of T-loop phosphorylation (Collins et al., 2003). We therefore treated ES cell lines with the protein phosphatase inhibitor okadaic acid and found that it markedly stimulated S6K activity as well as phosphorylation of Thr389 in the IGF1-stimulated PDK1$^{131M/131M}$ cells, but not the PDK1$^{-/-}$ ES cells, which lack PKB activity (Williams et al., 2000) (Fig. 5).

Reduced activation of SGK1 in knockin ES cells

As we have not previously been able to measure endogenous SGK activity in ES cells (Collins et al., 2003), we transfected PDK1$^{+/+}$ and PDK1$^{131M/131M}$ ES cells with a construct

encoding wild-type SGK1, and measured SGK1 activity as well as hydrophobic motif phosphorylation at Ser422, in unstimulated and IGF1-treated cells in the presence or absence or wortmannin (Fig. 6A). In PDK1$^{+/+}$ ES cells, IGF1 increased activity approximately threefold without significantly enhancing the high basal level of Ser422 phosphorylation, similar to what has been observed before in ES cells (Collins et al., 2003). In PDK1$^{131M/131M}$ ES cells, the basal SGK1 activity was nearly tenfold lower than control cells and IGF1 stimulated SGK1 activity to a level that was approximately fourfold lower than that observed in PDK1$^{+/+}$ ES cells. Wortmannin blocked IGF1-induced SGK1 activation in both the control and knockin ES cells. It had been shown previously that an SGK1 mutant in which the hydrophobic residue is changed to Asp [SGK1(S422D)] is constitutively active when expressed in cells (Kobayashi and Cohen, 1999) owing to its high affinity for the phospho-hydrophobic-motif-binding pocket of PDK1 (Biondi et al., 2001). When expressed in PDK1$^{131M/131M}$-knockin ES cells, the SGK1(S422D) mutant possessed more comparable activity to that observed in the wild-type cells (Fig. 6B).

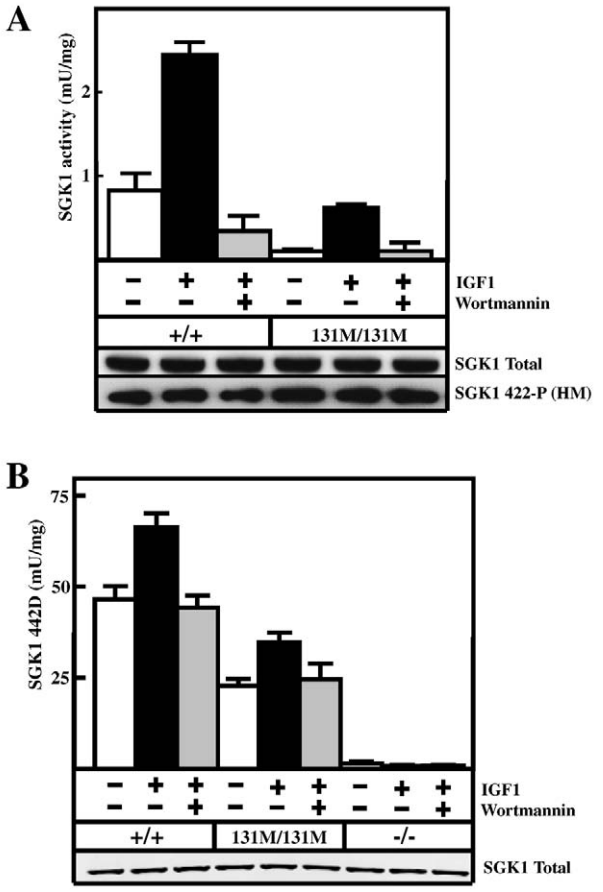


Fig. 6. SGK1 activation is impaired in knockin cells**.** The indicated ES cells lines were transfected with a DNA construct encoding GST-SGK1 (A) or GST-SGK1(S422D) (B). At 24 hours post-transfection, the ES cells were deprived of serum for 5 hours, incubated in the presence or absence of 100 nM wortmannin for 10 minutes and then either left unstimulated or stimulated with 50 ng/ml IGF1 for 20 minutes. 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 ±s.e.m. for three dishes of cells each assayed in duplicate. 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 the S6K T389 phosphospecific antibody that also crossreacts with the phosphorylated hydrophobic motif of SGK (Lizcano et al., 2002). Similar results were obtained in two separate experiments. Abbreviations: HM, hydrophobic motif.

Activation of RSK is reduced in knockin ES cells

RSK activity was measured after immunoprecipitation from serum-starved ES cells with an antibody that immunoprecipitates all RSK isoforms. In PDK1$^{+/+}$ ES cells, RSK activity was stimulated approximately twofold by the phorbol ester TPA (Fig. 7), which activates RSK through the

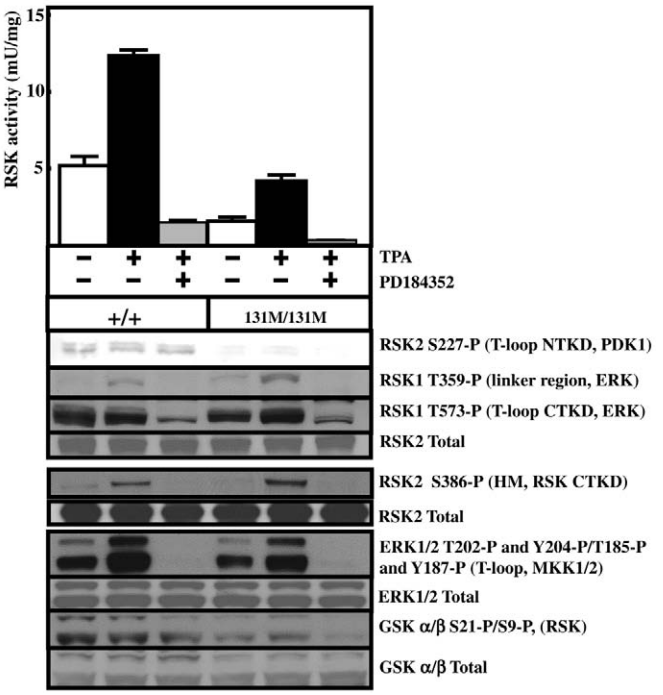


Fig. 7. RSK isoform activation is impaired in knockin cells. The indicated ES cells were cultured to 80% confluence. Cells were deprived of serum for 4 hours and were either left untreated or incubated in the presence or absence of 2 μM PD 184352 for 30 minutes and then either left unstimulated or stimulated with 0.4 μg/ml TPA for 15 minutes. The cells were lysed and RSK isoforms immunoprecipitated with an antibody that recognizes all isoforms and assayed. The results shown are the average specific activity ±s.e.m. of three separate dishes of cells with assays for each dish performed in duplicate. The ES cell lysates were also immunoblotted with the indicated antibodies. Ser227 (numbering based on human RSK2) is phosphorylated by PDK1, Thr359 and Thr573 (numbering based on human RSK1) are phosphorylated by ERK and Ser386 (numbering based on human RSK2) is the hydrophobic motif (HM) phosphorylation residue and is phosphorylated by the C-terminal RSK kinase domain. The RSK2 Ser386-P blot was performed on RSK2 immunoprecipitated from 0.5 mg of ES cell lysate. Similar results were obtained in two separate experiments. Abbreviations: NTKD, N-terminal kinase domain of RSK; CTKD, C-terminal kinase domain of RSK.

ERK mitogen-activated protein (MAP) kinase pathway. Treatment of ES cells with the PD184352 MEK inhibitor (Sebolt-Leopold et al., 1999) prevented ERK phosphorylation and RSK activation. In unstimulated PDK1$^{131M/131M}$ ES cells, RSK activity was considerably reduced, to a level similar to that observed for PDK1$^{+/+}$ ES cells treated with PD184352 (Fig. 7). Stimulation of PDK1$^{131M/131M}$ ES cells with TPA increased RSK activity approximately threefold, whereas treatment with PD184352 reduced RSK activity to below basal levels. Reduced activation of RSK in the PDK1$^{131M/131M}$ ES cells is not a result of lack of TPA-induced ERK activation, as TPA stimulated ERK phosphorylation to the same extent as in PDK1$^{+/+}$ ES cells. Moreover, phosphorylation of RSK at three ERK-dependent phosphorylation sites (Thr359, Thr573, Ser386) was not inhibited and was even moderately enhanced in the PDK1$^{131M/131M}$-knockin ES cells (Fig. 7). Instead, the reduction of basal and TPA-stimulated RSK activity in the PDK1$^{131M/131M}$ ES cells correlated with lower phosphorylation of RSK at the T-loop site (Ser227), assessed with a phosphospecific antibody. Phosphorylation of GSK3α/GSK3β, a physiological substrate of RSK (Frame and Cohen, 2001), was also reduced in the PDK1$^{131M/131M}$ ES cells.

Levels of PKC isoforms in the PDK1$^{131M/131M}$-knockin ES cell line

As outlined in the Introduction, phosphorylation of PKC isoforms by PDK1 plays an important role in stabilizing these enzymes. To study the importance of the phosphate groove of PDK1 in stabilizing PKC isoforms, we assessed the levels of these enzymes in the PDK1$^{131M/131M}$ ES cells. As a control, we also employed hydrophobic-groove-knockin ES cells (PDK1$^{155E/155E}$) (Collins et al., 2003) and PDK1-knockout ES cells (PDK1$^{-/-}$) (Williams et al., 2000). The levels of PKC isoforms were assessed by quantitative immunoblot analysis employing a LI-COR Odyssey infrared imaging system. In the PDK1$^{131M/131M}$ ES cells the levels of conventional and novel PKC isoforms that require hydrophobic motif phosphorylation for activation (PKCα, PKCβII, PKCγ, PKCδ and PKCε) were more similar to those found in the PDK1$^{+/+}$ cells than in the PDK1$^{155E/155E}$ and PDK1$^{-/-}$ cells, where the levels of these PKC isoforms were markedly reduced (Fig. 8A). The levels of atypical PKC isoforms (PKCζ, PRK1 and PRK2) that possess an acidic residue at the hydrophobic motif rather than a phosphorylatable Ser/Thr are moderately reduced in the PDK1$^{131M/131M}$ ES cells, albeit to level lower than that observed in PDK1$^{155E/155E}$ and PDK1$^{-/-}$ cells. We also measured T-loop phosphorylation of PKCζ, PRK1 and PRK2 and found that the phosphorylation was reduced to a lesser extent in the PDK1$^{131M/131M}$ ES cells compared with PDK1$^{155E/155E}$ and PDK1$^{-/-}$ cells (Fig. 8A).

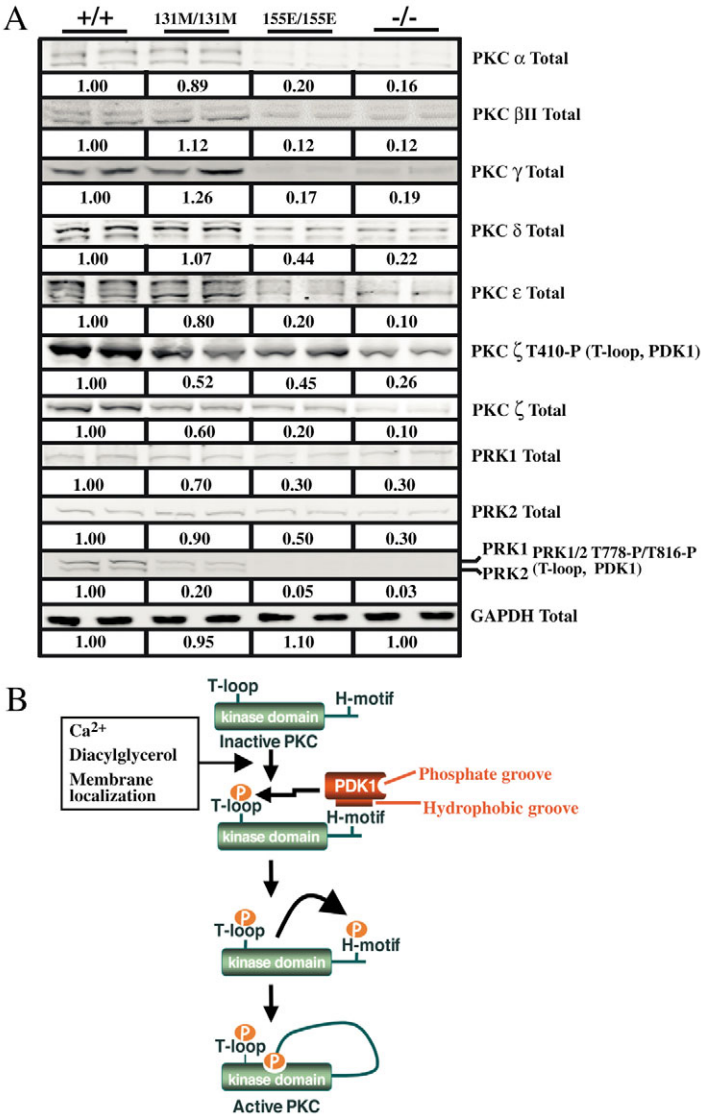
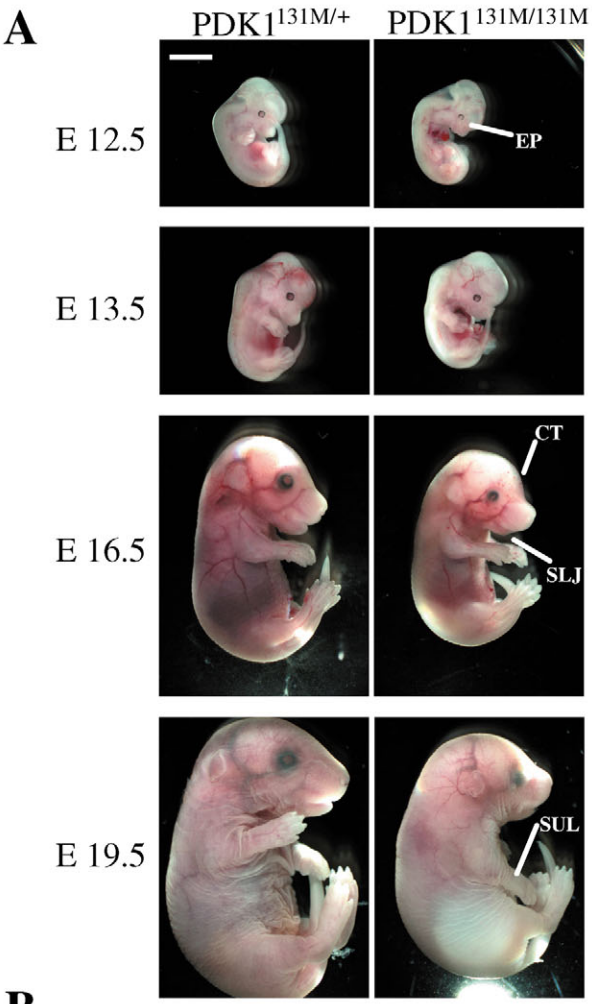


Fig. 8. Immunoblotting of PKC isoforms in ES cells. (A) The indicated wild-type, knockin and knockout PDK1 ES cells lines were cultured to 80% confluence, washed twice in PBS and lysed. Lysates were subjected to SDS polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies. Quantitation of the immunoblots, indicated immediately below each blot, was performed using the LI-COR Odyssey infrared imaging system as described in the Materials and Methods. The value of expression of each PKC isoform detected in the control PDK1$^{+/+}$ cells is taken as 1.0 and the expression of the isoforms in other cells is expressed relative to this. As a loading control, the cell lysates were immunoblotted for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Similar results were obtained in two independent experiments. (B) Diagram illustrating a potential mechanism of activation for the conventional and novel PKC isoforms. Allosteric modification of inactive PKC by diacylglycerol and/or Ca^{2+} and membrane localization allows PDK1 to dock with the hydrophobic motif of PKC via the PDK1 hydrophobic groove, independently of the PDK1 phosphate groove. Once docked, PDK1 then phosphorylates the T-loop of PKC. This enables PKC isoforms to autophosphorylate their hydrophobic motif and become active.

Embryonic lethality of knockin mice

The PDK1$^{131M/+}$ heterozygous mice were healthy, of normal size and displayed no obvious phenotypes. In an attempt to generate complete PDK1$^{131M/131M}$-knockin mice, matings were set up between heterozygous PDK1$^{131M/+}$ mice. No live PDK1$^{131M/131M}$ postnatal mice were ever recovered; however, out of 61 progeny analysed, one was found to be a stillborn

PDK1$^{131M/131M}$ mouse. The PDK1$^{131M/+}$ heterozygous mice were born at a mendelian frequency that was moderately lower than expected (Fig. 9B), indicating that development of these mice is moderately impaired. We next analysed the embryos from days E8.5 to E19.5 of development derived from PDK1$^{131M/+}$ matings, which revealed the presence of PDK1$^{131M/131M}$ embryos until E19.5, albeit at reduced mendelian distribution at E16.5 and E19.5 (Fig. 9B). PDK1$^{131M/131M}$ embryos did not display a marked phenotype at early stages (E8.5; data not shown). At later stages of development (E12.5-E19.5), some distinguishing features of the PDK1$^{131M/131M}$ embryos were observed (Fig. 9A). These include a slight size reduction (from E12.5), abnormal eye pigmentation (from E12.5) and a reduction of cranial tissues, giving a sharper frontal profile and shorter lower jaw at E16.5. Later still (E19.5), upper limbs have a slack posture characteristic of disuse.



B

Stage	PDK1$^{131M/+}$X PDK1$^{131M/+}$ % progeny (numbers in brackets)			Total
	PDK1$^{+/+}$	PDK1$^{131M/+}$	PDK1$^{131M/131M}$	
E12.5	20 (1)	80 (8)	20 (1)	10
E13.5	14 (1)	43 (3)	43 (3)	7
E16.5	28 (5)	56 (10)	16 (3)	18
E19.5	38 (3)	50 (4)	12 (1)	8
Neonate	25 (2)	63 (5)	12 (1) Stillborn	8
4 weeks post natal	46 (28)	54 (33)	0 (0)	61

Fig. 9. Phenotypes of PDK1$^{131M/131M}$ embryos. (A) Representative PDK1$^{131M/+}$ or PDK1$^{131M/131M}$ embryos at the indicated embryonic stage were dissected in PBS and imaged on a Leica M275 microscope and wholemount photographs were taken. In all images, embryos are shown at the same magnification. Abbreviations: EP, eye pigment; CT, cranial tissue, SLJ, short lower jaw; SUL, slack upper limb. Bar, 2560 μM. (B) PDK1$^{+/131M}$ mice were crossed and the resulting proportion of progeny at each stage is indicated. Genotyping was performed as described in the Materials and Methods.

Discussion

Although no live PDK1$^{131M/131M}$ mice were observed, the PDK1$^{131M/131M}$-knockin embryos develop to a vastly greater extent compared with PDK1-knockout or other PDK1-knockin embryos that have been generated to date. PDK1$^{-/-}$ embryos died at E9.5, displaying multiple profound abnormalities including lack of forebrain, heart, dorsal root ganglia, somites and failure to turn or develop a placenta (Lawlor et al., 2002). PDK1-knockin embryos containing a mutation in the PH domain preventing binding of PDK1 to PtdIns(3,4,5)P_3 died at E10.5, also with a reduction in forebrain development and lack of dorsal root ganglia, as well as vascular agenesis in the head and extra-embryonic membranes and poor placental development (McManus et al., 2004). The PDK1$^{155E/155E}$ hydrophobic-groove-knockin embryos survived until E11.5, again displaying a reduction in forebrain size along with twisting of the posterior portion of the embryo. In these mice however, placental development appeared normal, and heart, somites and normal circulation were observed (McManus et al., 2004).

By contrast, PDK1$^{131M/131M}$ embryos survived almost to birth but nevertheless some overt defects in craniofacial development and limb posture are evident and are suggestive of defects in cranial neural crest development and in the functioning of the peripheral nervous system. These findings, together with lack of dorsal root ganglia reported in PDK1$^{-/-}$ and PtdIns(3,4,5)P_3-binding-deficient PDK1-knockin embryos (McManus et al., 2004), suggest a role for PDK1 substrates in the formation and/or survival of neural-crest-derived tissues. All of these PDK1 mutant phenotypes display a reduction in forebrain size (although this is less evident in PDK1$^{131M/131M}$ embryos). This is a characteristic feature of 'flat-top' mutant mice that have a mutation in mTOR that leads to a reduction in its kinase activity and hence phosphorylation of S6K (Hentges et al., 1999; Hentges et al., 2001). As attenuation or loss of S6K activation is observed in all PDK1 mutants, this comparison suggests that forebrain defects in these animals are attributable to reduced S6K activity.

The results of this study demonstrate that the phosphate groove of PDK1 plays an important role in enabling PDK1 to activate S6K, SGK and RSK maximally. Both the basal and stimulated activities of these enzymes were markedly reduced in the PDK1$^{131M/131M}$-knockin ES cells (Figs 5-7). Importantly,

agonists still induced a substantial activation of S6K, SGK and RSK in the knockin cells. This is in contrast to the PDK1$^{155E/155E}$ hydrophobic-groove-knockin ES cells, in which S6K and RSK are completely inactive and not stimulated by agonists (Collins et al., 2003). In addition to phosphorylation of the hydrophobic motif, activation of S6K is accompanied by phosphorylation of other residues including C-terminal Ser/Thr-Pro residues. Phosphorylation of these residues is regulated by mTOR and plays a role in enabling S6K to be activated (Isotani et al., 1999). Indeed, mutation of these residues to Glu has been shown to facilitate activation of S6K by PDK1 in vitro (Pullen et al., 1998) and activation of S6K in cells (Dennis et al., 1998; Han et al., 1995). PDK1 does not regulate the phosphorylation of two of these residues (Thr421 and Ser424) as, in PDK1$^{-/-}$ ES cells, IGF1 still induced normal phosphorylation of these residues (McManus et al., 2004). Consistent with this, IGF1-induced phosphorylation of Thr421/Ser424 is not affected in PDK1$^{131M/131M}$-knockin ES cells, and even increased under conditions in which phosphorylation of the T-loop and hydrophobic motif of S6K are markedly reduced (Fig. 5). It is possible that phosphorylation of the C-terminal Ser/Thr-Pro residues of S6K induces a conformational change that exposes the hydrophobic motif and enables PDK1 to bind S6K through its hydrophobic groove independently of the phosphate groove. Consistent with this observation, the PDK1(R131M) phosphate groove mutant (Frodin et al., 2002), unlike the PDK1(L155E) mutant (Biondi et al., 2001), was still capable of phosphorylating S6K in vitro, but to a lower extent than wild-type PDK1. It is also conceivable that phosphorylation of the hydrophobic motif itself could induce a conformational change, facilitating its direct interaction with the hydrophobic groove of PDK1. The finding that stimulation of the phosphorylation of the hydrophobic motif of S6K by treatment with the phosphatase inhibitor okadaic acid markedly enhanced S6K activity in the PDK1$^{131M/131M}$-knockin cells (Fig. 5) supports this notion. Moreover, the finding that mutation of the hydrophobic motif phosphorylation site of SGK to an acidic residue also markedly enhanced SGK activity in PDK1$^{131M/131M}$-knockin cells (Fig. 6B) indicates that phosphorylation of the hydrophobic motif promotes the interaction of SGK with PDK1 in a phosphate-groove-independent manner, probably mediated through the hydrophobic groove of PDK1. Similar arguments also apply to RSK, which is also phosphorylated at sites other than the hydrophobic motif upon activation (Dalby et al., 1998; Frodin and Gammeltoft, 1999).

Previous work has suggested that phosphorylation of PKC isoforms by PDK1 plays an important role in stabilizing these enzymes (Newton, 2002). Studies by Newton and colleagues (Gao et al., 2001) showed that the hydrophobic motif of PKCβII is important for the activation of this enzyme by PDK1, indicating a role for the phospho-hydrophobic-motif-binding pocket of PDK1 in regulating PKC isoforms. Consistent with this, we have found that the levels of PKC isoforms were markedly decreased in PDK1$^{155E/155E}$ hydrophobic-groove-knockin ES cells (McManus et al., 2004). As outlined in the Introduction, T-loop phosphorylation of PKC isoforms by PDK1 results in the hydrophobic motifs of these enzymes becoming autophosphorylated. If this model is correct, the mechanism by which PDK1 recognizes PKC isoforms must be distinct from that of S6K, SGK and RSK, in that PDK1-mediated activation of PKC isoforms will not be triggered by phosphorylation of the hydrophobic motif. It is likely that the binding of PKC isoforms to diacylglycerol/phorbol esters at membranes, together with Ca^{2+} binding, induces conformational changes that promote phosphorylation and hence activation by PDK1. The finding that the levels of PKC isoforms are not reduced in the PDK1$^{131M/131M}$ phosphate-groove-knockin ES cells, in contrast to PDK1$^{155E/155E}$ hydrophobic-groove-knockin ES cells, suggests that PDK1 docks to the unphosphorylated hydrophobic motif of PKC isoforms through its hydrophobic groove independently of the phosphate-binding pocket. A model for the activation of PKC isoforms by PDK1 based on these observations is presented in Fig. 8B.

In summary, our studies define the crucial role that the phosphate groove of PDK1 plays in enabling PDK1 to activate S6K, SGK and RSK maximally in vivo, but also provide evidence for a phosphate-groove-independent mechanism in regulating activation of these enzymes. Our results also provide further insight into the mechanism by which PDK1 regulates the phosphorylation of conventional and novel PKC isoforms, suggesting that the hydrophobic groove rather than the phosphate groove is a key determinant enabling PDK1 to phosphorylate these enzymes. This study also provides a further example of how knockin technology can be exploited to define the physiological importance of a domain on a signalling protein.

We thank David Komander for generating Fig. 1; Pamela Halley for help with embryo dissection; Ricardo Biondi, Morten Frodin and Katy Schmidt for discussion; Agnieszka Kieloch for tissue culture assistance; Laura Armit for help in generating knockin mice and ES cells. We also thank the Sequencing Service (School of Life Sciences, University of Dundee; www.dnaseq.co.uk) for DNA sequencing, and the antibody purification team [Division of Signal Transduction Therapy (DSTT), University of Dundee] coordinated by Hilary McLauchlan and James Hastie for generation and purification of antibodies. B.J.C. was the recipient of a 4-year Wellcome Trust Studentship and K.G.S. is an MRC Senior Research Fellow (Grant Number G9900177). We thank the Association for International Cancer Research (D.R.A.), Diabetes UK (D.R.A.) the Medical Research Council (D.R.A.), the Moffat Charitable Trust (D.R.A.) and the pharmaceutical companies supporting the Division of Signal Transduction Therapy Unit (AstraZeneca, Boehringer-Ingelheim, GlaxoSmithKline, Merck & Co., Merck KGaA and Pfizer) for financial support.

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