Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms

Anudharan Balendran, Gregor R. Hare, Agnieska Kieloch, Michayla R. Williams, Dario R. Alessi*

MRC Protein Phosphorylation, MSI/WTB complex, University of Dundee, Dow Street, Dundee DD1 5EH, UK

Received 5 October 2000; accepted 12 October 2000

First published online 25 October 2000

Edited by Shmuel Shaltiel

Abstract The multi-site phosphorylation of the protein kinase C (PKC) superfamily plays an important role in the regulation of these enzymes. One of the key phosphorylation sites required for the activation of all PKC isoforms lies in the T-loop of the kinase domain. Recent in vitro and transfection experiments indicate that phosphorylation of this residue can be mediated by the 3-phosphoinositide-dependent protein kinase-1 (PDK1). In this study, we demonstrate that in embryonic stem (ES) cells lacking PDK1 (PDK1−/− cells), the intracellular levels of endogenously expressed PKCα, PKCβI, PKCγ, PKCδ, PKCε, and PKC-related kinase-1 (PRK1) are vastly reduced compared to control ES cells (PDK1+/+ cells). The levels of PKCζ and PRK2 protein are only moderately reduced in the PDK1−/− ES cells. We demonstrate that in contrast to PKCζ expressed PDK1+/+ ES cells, PKCζ in ES cells lacking PDK1 is not phosphorylated at its T-loop residue. This provides the first genetic evidence that PKCζ is a physiological substrate for PDK1. In contrast, PRK2 is still partially phosphorylated at its T-loop in PDK1−/− cells, indicating the existence of a PDK1-independent mechanism for the phosphorylation of PRK2 at this residue.

1. Introduction

Members of the AGC family of protein kinases include the cAMP-dependent protein kinase (PKA), protein kinase B (PKB) and protein kinase C (PKC) isoforms. These kinases are activated by hormones and growth factors and mediate many of the cellular responses that are controlled by these agonists by phosphorylating key regulatory proteins. All AGC kinase members require phosphorylation of a Thr or Ser residue for activity that lies in a region of the kinase domain termed the T-loop [1,2]. The amino acid sequence immediately C-terminal to this site of phosphorylation is highly conserved in all AGC kinases, indicating that it may be phosphorylated by a common upstream protein kinase. Consistent with this hypothesis, the 3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylates the T-loop residue of many members of the AGC kinase family either in vitro, or when PDK1 is overexpressed in cells [2–4].

To define the cellular roles of PDK1 in activating AGC kinase members more rigorously, we have generated mouse embryonic stem (ES) cells in which both copies of the PDK1 gene have been disrupted to prevent the expression of PDK1 [5]. Although these cells were viable, we found that a number of AGC kinases activated by extracellular stimuli, namely PKB isoforms, p70 ribosomal S6 kinase (S6K1) and p90 ribosomal S6 kinase (p90Rsk), were not activated by agonists that activate these enzymes in wild type ES cells, indicating that these kinases are physiological substrates for PDK1. However, these studies also revealed that PDK1 is not rate limiting for the phosphorylation and activation of all AGC kinases, as PKA, mitogen- and stress-stimulated protein kinase-1 and the AMP-dependent protein kinase were activated normally in ES cells deficient in PDK1 [5].

The PKC superfamily consists of 12 distinct members that have been divided into four subfamilies [6]. The conventional isoforms (PKCα, PKCβ and PKCγ) are activated by diacylglycerol and calcium, the novel isoforms (PKCδ, PKCε, PKCη and PKCθ) are regulated only by diacylglycerol; while the atypical isoforms (PKCζ, PKCι and PKCλ) and related isoforms (PKC-related kinase-1 (PRK1) and PRK2) are not regulated by diacylglycerol or calcium. Like other members of the AGC family, PKC isoforms require phosphorylation of their T-loop residue for activity, a reaction that can be mediated by PDK1 in vitro or in transfection experiments [7–12]. In this study, we demonstrate that in ES cells lacking PDK1, the levels of PKCα, PKCβI, PKCγ, PKCδ, PKCε, and PRK1 are vastly reduced compared to control ES cells. Our data confirm that PKCζ is an in vivo substrate for PDK1, but indicate the existence of a PDK1-independent mechanism that may regulate T-loop phosphorylation of PRK2, as PRK2 is still partially phosphorylated at its T-loop in ES cells lacking PDK1.

2. Materials and methods

2.1. Materials

Protease inhibitor cocktail tablets were from Roche, microcystin-LR, murine leukaemia inhibitory factor (ESGRO™), KnockOut™ Dulbecco's modified Eagle's medium, KnockOut serum replacement

*Corresponding author. Fax: (44)-1382-223778.
E-mail: d.r.alessi@dundee.ac.uk

Abbreviations: ES cell, embryonic stem cell; PKB, protein kinase B; PKC, protein kinase C; PRK, PKC-related kinase; S6K1, p70 S6 kinase; Rsk, ribosomal S6 kinase; p90Rsk, p90 ribosomal S6 kinase; PDK1, 3-phosphoinositide-dependent protein kinase-1

0014-5793/00/$20.00
PII: S0014-5793(00)02162-1

and other tissue culture reagents were from Life Technologies, protamine-agarose was from Sigma, CH-Sepharose, HiTrap Q-Sepharose column and the Mono S column were from Amersham Pharmacia Biotech. The precast 4–12% and 3–8% gradient sodium dodecyl sulphate (SDS)–polyacrylamide gels were from Invitrogen. All the peptides used in this study were synthesised by Dr G. Blomberg (University of Bristol, UK). Recombinant PKCα and PKCδ were from Panvera, whilst PKCβ1, PKCβ2, PKCγ, PKCδ and PKCε were from Upstate Biotechnology. Flag epitope-tagged PKCζ and PRK2 were expressed by transient transfection in 293 cells as described previously [7].

2.2. Antibodies

The characterisation of the phospho-specific antibody recognising PRK2 phosphorylated at its T-loop (Thr816) termed T816-P and the phospho-specific antibody recognising PKCζ phosphorylated at its T-loop (Thr410), termed T410-P, has been described previously [7]. Antibodies recognising PRK2 and PKCζ were raised in sheep against the peptide KKVKPPFIPTIRGREDVSNFDDEFT (corresponding to residues 927–951 of the human PRK2) and peptide TPDDEDAIKRIDQSEFEGFEYINIP (corresponding to residues 560–583 of the mouse PKCζ), respectively. The antibodies were affinity-purified on CH-Sepharose covalently coupled to the peptide antigen used to raise the antibody, and are available from Upstate Biotechnology. The antibodies recognising the following PKC isoforms were purchased as indicated: PKCα (UBI, catalogue number 05-154), PKCβ (Transduction Laboratories, catalogue number P17720), PKCβI (Santa Cruz, catalogue number Sc-209), PKCβII (Santa Cruz, catalogue number Sc-210), PKCγ (Santa Cruz, catalogue number Sc-211), PKCδ (Santa Cruz, catalogue number Sc-213), PKCε (Transduction Laboratories, catalogue number P14820) and PRK1 (Santa Cruz, catalogue number SC-1842). Secondary antibodies coupled to horseradish peroxidase used for immunoblotting were from Pierce.

2.3. Buffer solutions

Buffer A: 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, 1 μM microcystin-LR, 0.1% (by volume) β-mercaptoethanol and 'complete' proteinase inhibitor cocktail (one tablet per 25 ml Roche-Boehringer Mannheim, Lewes, UK). Buffer B: 50 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose and 0.1% (by volume) β-mercaptoethanol. Buffer C: 50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, and 0.1% (by volume) β-mercaptoethanol. SDS sample buffer: 50 mM Tris–HCl pH 6.8, 2% (by mass) SDS, 10% (by volume) glycerol and 1% (by volume) β-mercaptoethanol.

2.4. Cell Culture and lysis

PDK1+/+ and PDK1−/− ES cells were cultured to confluence on gelatinised 10 cm diameter dishes in KnockOut™ Dulbecco's modified Eagle's medium supplemented with 10% KnockOut™ serum replacement, 0.1 mM non-essential amino acids, antibiotics (100 U penicillin G, 100 mg/ml streptomycin), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol and 1000 U/ml ESGRO™ (murine leukaemia inhibitory factor to prevent differentiation of the cells). Unless indicated otherwise, the cells were then lysed in 0.5 ml of ice cold buffer A, and centrifuged at 4°C for 10 min at 16 000×*g*. The supernatants were 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. Where indicated, cells were also lysed in 0.5 ml of SDS sample buffer and the DNA was fragmented by sonicating the lysates in a water-bath sonicator (Decon FS2006) for 15 min at maximum output. The lysates were passed through a 25GA microlance3 needle (Becton Dickinson) 10 times and centrifuged at room temperature for 10 min at 16 000×*g*. The supernatant (10–20 μl) was used for immunoblotting.

2.5. Purification of PKCζ and PRK2 from ES cells on protamine-agarose

Cell lysates (500 μg of protein) were incubated with protamine-agarose resin (10 μl) previously equilibrated in buffer A, for 2 h at 4°C on a shaking platform. The beads were washed twice with buffer A containing 0.5 M NaCl and twice with buffer C. The beads were immediately resuspended in one volume of 2-fold concentrated SDS sample buffer and subjected to SDS–polyacrylamide gel electrophoresis, followed by immunoblotting with the indicated antibodies.

2.6. Purification of PRK2 and PKCζ from ES cells on Mono Q and Mono S Sepharose

Cell lysates (40 mg of protein) derived from PDK1+/+ and PDK1−/− ES cells were applied to a HiTrap Q-Sepharose column (5 ml) equilibrated in buffer B. The column was washed sequentially with 15 ml of buffer B, buffer B containing 0.1 M NaCl, and buffer B containing 0.4 M NaCl. Immunoblotting revealed that PRK2 and PKCζ were present in the 0.4 M eluate, which was dialysed in a Slide-A-Lyzer (Pierce) for 2 h with two changes of buffer B and then chromatographed on a 1 ml Mono S HR 5/5 column equilibrated in buffer B. The column was washed with 10 ml of buffer B and then developed with a 30 ml non-linear salt gradient to 1 M NaCl at a flow rate of 1 ml/min and fractions of 1 ml were collected. The non-linear gradient was achieved by increasing the salt concentration from 0 to 0.1 M NaCl (fractions 1–8), 0.1–0.3 M NaCl (fractions 9–16), 0.3–0.7 M NaCl (fractions 17–24), 0.7–1.0 M NaCl (fractions 25–27) and 1.0 M NaCl (fractions 28–30).

2.7. Immunoblotting

For blots of total ES cell lysate, 40 μg of protein was used. For blots of the protamine-agarose pulls down, 10 μl of beads that had been incubated with 500 μg of cell lysate protein was used. For blots of the Mono S column eluate, 30 μl of each fraction was used. These samples were subjected to SDS–polyacrylamide gel electrophoresis, and transferred to nitrocellulose. For experiments in which PKC isoforms were being immunoblotted, the membranes were incubated in 50 mM Tris–HCl pH 7.5, 0.15 M NaCl, 0.5% (by volume) Tween (TBS-Tween) and 10% (by mass) skimmed milk for 2 h at room temperature in the presence of the indicated antibody concentrations: PKCα (0.2 μg/ml), PKCβI (0.4 μg/ml), PKCβII (0.4 μg/ml), PKCγ (0.4 μg/ml), PKCδ (0.4 μg/ml), PKCε (2.5 μg/ml), PKCζ (0.4 μg/ml), PRK1 (0.4 μg/ml) and PRK2 (0.4 μg/ml). Immunoblotting with the phospho-specific antibodies (0.5 μg/ml) unless otherwise indicated was carried out in the presence of 10 μg/ml non-phosphorylated peptide corresponding to the antigen used to raise the antibody in TBS-Tween containing 5% (by mass) fraction-V bovine serum albumin for 16 h at 4°C. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent (Amersham/Pharmacia). Densitometric analysis of the bands on the autorads was carried out using the NIH Image software (http://rsb.info.hih.gov/nih-image/).

2.8. Northern blot analysis

Total RNA was purified from PDK1−/− and PDK1+/+ ES cells using Trizol reagent (Gibco) and mRNA subsequently isolated using an Oligotex mRNA Mini kit (Qiagen). mRNA samples were mixed with an equal volume of glyoxal sample buffer (BMA) and incubated at 65°C for 15 min prior to loading on a 1.25% SeaKem Gold agarose gel (BMA). mRNA isolated from 125 μg of total RNA was loaded in each lane. Electrophoresis was carried out in 1×MOPS buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, 10 mM EGTA, pH 7.0). Gels were blotted onto Hybond N+ filters (Amersham) and the nucleic acids fixed on the membranes according to standard procedures. The filters were incubated in 20 mM Tris pH 8.0 for 15 min at 65°C, to remove the glyoxal, and prehybridised for 1 h at 65°C in ExpressHyb (Clontech). Probes were labelled by random priming and added to ExpressHyb at 1–2×10^6 cps/ml and the incubation was carried out overnight. Filters were then washed in 2×SSC (30 mM sodium citrate and 0.3 M NaCl) containing 0.05% SDS at ambient temperature for 40 min and in 0.1×SSC containing 0.1% SDS for 30 min at 50°C. Blots were exposed for 3–12 h using Hyperfilm MP (Amersham). After probing, the filters were stripped using boiling 1% SDS which was allowed to cool. They were then prehybridised and probed with a β-actin probe to confirm similar loading of mRNA in each gel lane. The mouse PRK1 probe corresponding to amino acids 807–946 was obtained from EST clone 1380826 (accession number AI035847 obtained from the I.M.A.G.E. consortium [13]) by digestion with *Eco*RI and *Not*I. The mouse PCKδ probe corresponding to amino acids 1–76 was obtained from EST clone 1230613 (accession number AA879873 obtained from the I.M.A.G.E. consortium [13]) by digestion with *Eco*RI and *Not*I. The β-actin probe was from Clontech.

3. Results

3.1. Reduced expression of PKC isoforms in PDK1−/− ES cells

Cell lysates derived from PDK1+/+ or PDK1−/− ES cells were immunoblotted with antibodies that recognise specific isoforms of PKC and the specificity of each antibody was verified using a panel of recombinant PKC isoforms (Fig. 1). The cells were lysed either in a buffer containing Triton X-100 or in SDS sample buffer to ensure solubilisation of particulate PKC isoforms. All eight PKC isoforms that were immunoblotted were expressed at detectable levels in the control PDK1+/+ ES cells. Although the expression of PKCζ and PRK2 is similar in PDK1+/+ and PDK1−/− ES cells, the levels of PKCα, PKCβII, PKCγ, PKCδ, PKCε and PRK1 were vastly reduced in ES cells lacking PDK1 compared to control PDK1+/+ ES cells. The expression of PKCε and PRK1 was virtually undetectable in PDK1−/− ES cells, whilst PKCα, PKCβII, PKCγ and PKCδ were expressed at about 5-fold lower levels in PDK1−/− cells compared to the control PDK1+/+ ES cells (Fig. 1). PKCβII may be the most abundant splice variant of the PKCβ isoform in ES cells as we were unable to detect expression of PKCβI in PDK1+/+ ES cells (data not shown). As a control, we also immunoblotted

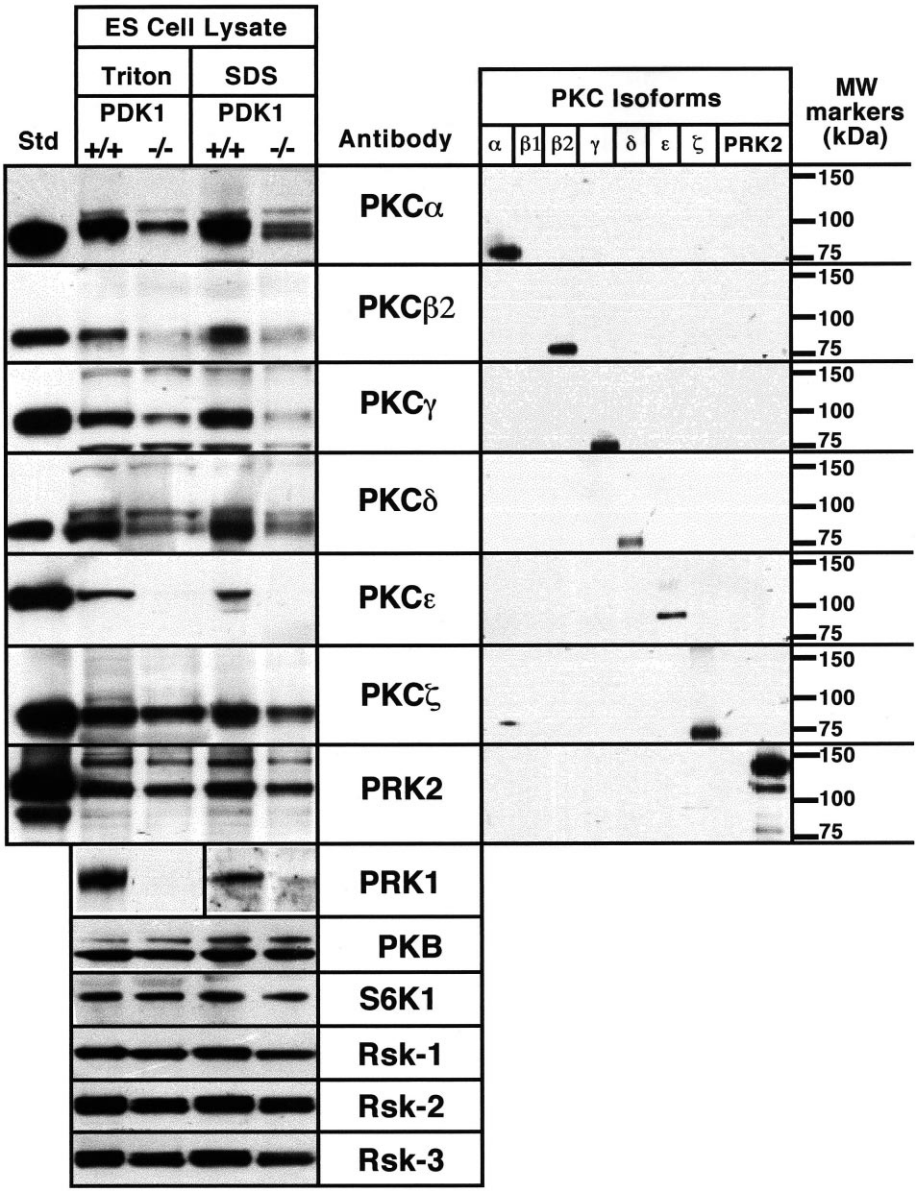

Fig. 1. Immunoblotting of PKC isoforms in PDK1−/− and PDK1+/+ cells. PDK1−/− and PDK1+/+ ES cells cultured in the presence of 10% serum were lysed in the same volume of either buffer A (which contains Triton) or in SDS sample buffer as described in Section 2. Aliquots of the Triton lysate (30 μg of protein) and the equivalent volumes of the SDS containing lysates, as well as indicated recombinant PKC isoforms (10 ng), were subjected to SDS–polyacrylamide gel electrophoresis on a 3–8% gradient Tris acetate gel and immunoblotted with the indicated PKC isoform-specific antibodies. In order to detect PRK1 in extracts of Triton-lysed cells, the PDK1−/− and PDK1+/+ ES cell lysates were incubated with protamine-agarose to bind PRK1, the beads were washed and immunoblotted with the PRK1 antibody as described in Section 2. As a control, the cell lysates were immunoblotted for other AGC kinases (PKB, S6K1 and p90Rsk isoforms) previously shown to be expressed at similar levels in PDK1−/− and PDK1+/+ cells. Similar results were obtained in at least four independent experiments.

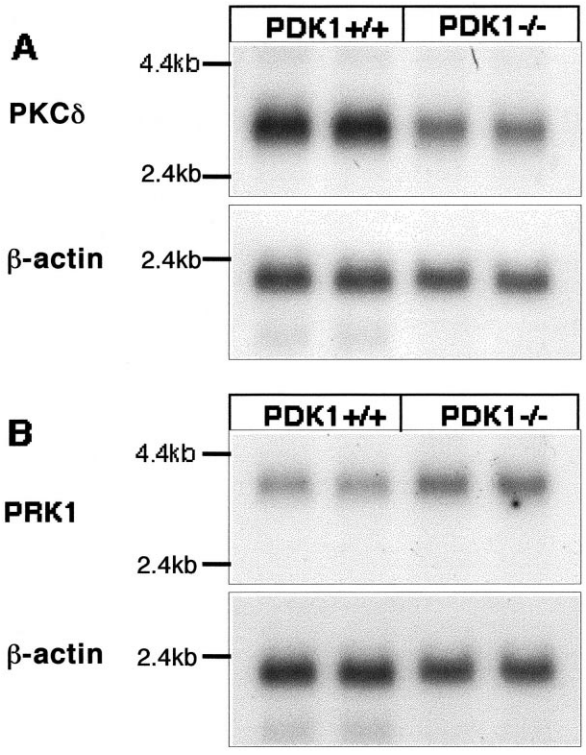

Fig. 2. Northern blotting of PKCδ and PRK1 in PDK1−/− and PDK1+/+ cells. Partial cDNA for mouse PKCδ (A) and PRK1 (B) was labelled with ^{32}P, using random primers, and used to probe a Northern blot containing mRNA isolated from PDK1−/− and PDK1+/+ ES cells. The blots were washed and autoradiographed. The PKCδ probe was observed to hybridise with a 3 kb message and the PRK1 probe hybridised with a 3.5 kb message. The blots were also probed with a β-actin probe as a loading control.

PDK1−/− and PDK1+/+ cell lysates for other AGC kinases (PKB, S6K1 and p90Rsk isoforms) which, as reported previously [5], were expressed at similar levels in PDK1−/− and PDK1+/+ cell lines.

3.2. Analysis of PKCδ and PRK1 mRNA levels in PDK1−/− and PDK1+/+ cells

The level of expression of PKCδ and PRK1 mRNA in PDK1−/− and PDK1+/+ ES cells was investigated by Northern blot analysis. PKCδ was expressed as a 3 kb transcript (Fig. 2A) whilst PRK1 was expressed as a 3.5 kb transcript similar to the sizes reported previously for these transcripts in other cell/tissue types [14,15]. PKCδ mRNA levels were moderately (∼50%) lower in the PDK1−/− ES cells compared to the PDK1+/+ ES cells (Fig. 2A). In our view, this small difference in mRNA expression of PKCδ is unlikely to account for the ∼5-fold difference in expression of PKCδ protein in the PDK1−/− cells compared to the PDK1+/+ ES cells. PRK1 mRNA was expressed at identical levels in PDK1−/− and PDK1+/+ ES cells (Fig. 2B). The levels of mRNA encoding PKCβ2 and PKCε in PDK1−/− or PDK1+/+ ES cells may be very low as we were unable to detect expression of these PKC isoforms in Northern blotting experiments (data not shown).

3.3. PKCζ is not phosphorylated at Thr410 in PDK1−/− ES cells

We have previously raised phospho-specific antibodies that recognise PKCζ and PRK2 phosphorylated at their activation loop, termed T410-P and T816-P, respectively [7]. In Fig. 3A, we demonstrate that the T816-P antibody also recognised PKCζ, with far higher sensitivity than the T410-P antibody [7]. The specificity of the T816-P antibody for PKCζ phosphorylated at Thr410 was established by the demonstration that this antibody failed to recognise a mutant form of PKCζ in which Thr410 was changed to an Ala (Fig. 1A) and by the finding that its recognition of PKCζ was abolished by preincubating the T816-P antibody with the phosphopeptide immunogen, but not the non-phosphorylated form of this peptide (data not shown). For this reason, we employed the T816-P rather than the T410-P antibody to measure phosphorylation of the endogenous PKCζ at its T-loop in ES cells.

It was not possible to detect phosphorylation of endogenous PKCζ by immunoblotting the total ES cell lysates with the T816-P antibody. We therefore used two approaches to purify sufficient PKCζ from PDK1−/− and PDK1+/+ ES cells to be able to assess its T-loop phosphorylation. The first approach was to adsorb PKCζ from the cell lysates onto protamine-agarose, with which PKCζ interacts with very high affinity. After washing with 0.5 M NaCl, the beads were then immunoblotted with the T816-P antibody as well as with an antibody that recognises total PKCζ protein, which runs with a molecular mass of 73 kDa distinct from that of PRK2 (130 kDa) (Fig. 3). Although similar amounts of PKCζ were purified on protamine-agarose from both PDK1−/− and PDK1+/+ ES cell extracts, the T816-P antibody only recognised PKCζ purified from PDK1+/+ cells and not from the PDK1−/− cells (Fig. 3B).

The second approach used to purify PKCζ was to fractionate cell lysates by batchwise chromatography on Q-Sepharose, followed by gradient elution from Mono S. PKCζ purified from PDK1+/+ and PDK1−/− ES cell lysates eluted as a broad peak from Mono S at ∼0.5 M NaCl (see Fig. 3C). The fractions in which PKCζ eluted were immunoblotted with the T816-P antibody which recognised only PKCζ derived from control PDK1+/+ but not from PDK1−/− cell lysates. These findings strongly indicate that PKCζ is not phosphorylated at its T-loop residue in PDK1−/− cells.

3.4. Evidence that PRK2 is partially phosphorylated at its T-loop residue in PDK1−/− ES cells

As the T816-P antibody did not recognise PRK2 in immunoblots of total ES cell lysates, we purified PRK2 from PDK1−/− and PDK1+/+ ES cell extracts after their adsorption to protamine-agarose (Fig. 4A) and following its batchwise elution from Q-Sepharose and its chromatography on Mono S (Fig. 4B). Immunoblotting of partially purified PRK2 with the T816-P antibody revealed that it was significantly phosphorylated at its T-loop in PDK1−/− ES cells, albeit to a ∼3-fold lower level than that observed in PDK1+/+ ES cells (Fig. 4). Incubation of the T816-P antibody with the phosphorylated phosphopeptide immunogen used to raise the antibody abolished its ability to recognise PRK2 purified from both PDK1−/− and PDK1+/+ ES cells (Fig. 4A,B), but did not affect the recognition of a number of non-specific bands recognised by the T816-P antibody in the Mono Q column fractions (Fig. 4B).

We attempted to assay PKCζ and PRK2 activity in PDK1+/+ and PDK1−/− ES cells following the immunopre-

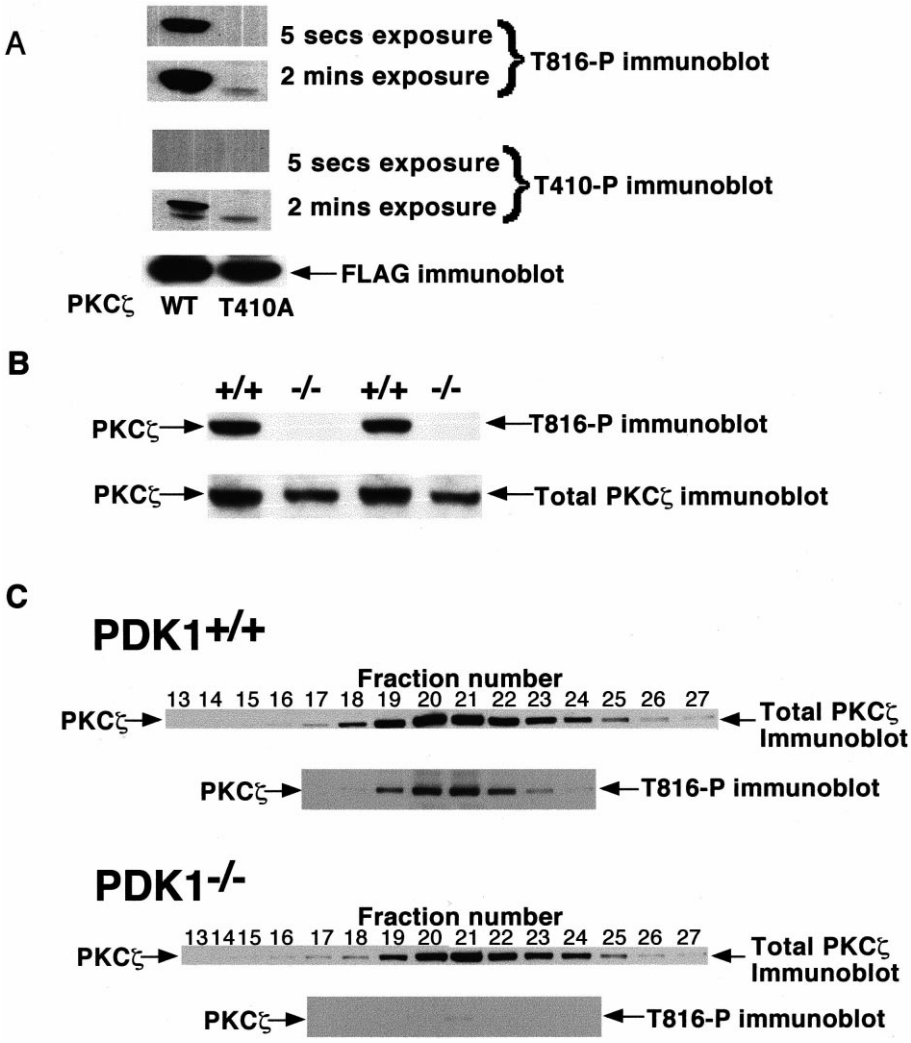

Fig. 3. PKCζ is not phosphorylated at Thr410 in PDK1−/− ES cells. (A) 293 cells were transfected with constructs expressing Flag epitope-tagged wild type PKCζ (WT) or the a mutant PKCζ in which Thr410 was changed to Ala (T410A) as described previously [7]. 36 h post-transfection, the cells were lysed in buffer A, and the lysates subjected to immunoblotting with the T410-P or T816-P antibody. Short (5 s) and long (2 min) exposures of the blots were carried out so that the relative sensitivity of the T410-P and T816-P antibodies towards PKCζ could be assessed. (B) PDK1−/− and PDK1+/+ ES cell lysates were incubated with protamine-agarose to bind PKCζ, the beads were washed and immunoblotted with the T816-P antibody and an antibody that recognises PKCζ (termed total PKCζ) as described in Section 2. PKCζ migrated with an apparent molecular mass of 73 kDa and comigrated with recombinant Flag epitope-tagged PKCζ (data not shown). (C) PDK1−/− and PDK1+/+ ES cell lysates were subjected to batchwise chromatography on Q-Sepharose, followed by a NaCl gradient elution from Mono S as described in Section 2. The indicated column fractions (30 μl) were immunoblotted with the T816-P antibody and the total PKCζ antibody. Similar results were obtained in three separate experiments.

cipitation of these enzymes from either ES cell lysates or from Mono S column fractions in which ES cell lysates had been fractionated. Although we were able to demonstrate that these antibodies immunoprecipitated PKCζ and PRK2, we were unable to detect any significant kinase activity in the immunoprecipitates towards myelin basic protein, protamine sulphate and the peptide substrates selectide and PRK2tide (data not shown) that we [7] and others [8,9,11,12] have previously used to assay PKCζ and PRK2 expressed in transient transfection experiments. We were also unable to measure any significant activity of PKCζ and PRK2 following the immunoprecipitation of these kinases from PDK1+/+ or PDK1−/− ES cells stimulated with IGF1, serum or lysophosphatidic acid (data not shown).

4. Discussion

Conventional and novel PKC isoforms are activated by a series of ordered phosphorylation events that are predicted to convert these enzymes from a 'nascent form' to a 'mature form' that still exists in an inactive conformation but which can then become activated following interaction with diacylglycerol [16,17]. The first phosphorylation event for conventional PKC isoforms occurs at the T-loop residue, and is catalysed by PDK1 [10,12]. This induces a conformational change that enables these PKC isoforms to phosphorylate themselves at two conserved residues lying at the carboxyl-terminus. One of these is termed the 'turn motif' (Thr638 in PKCα) and the other the 'hydrophobic motif' (Ser657 in

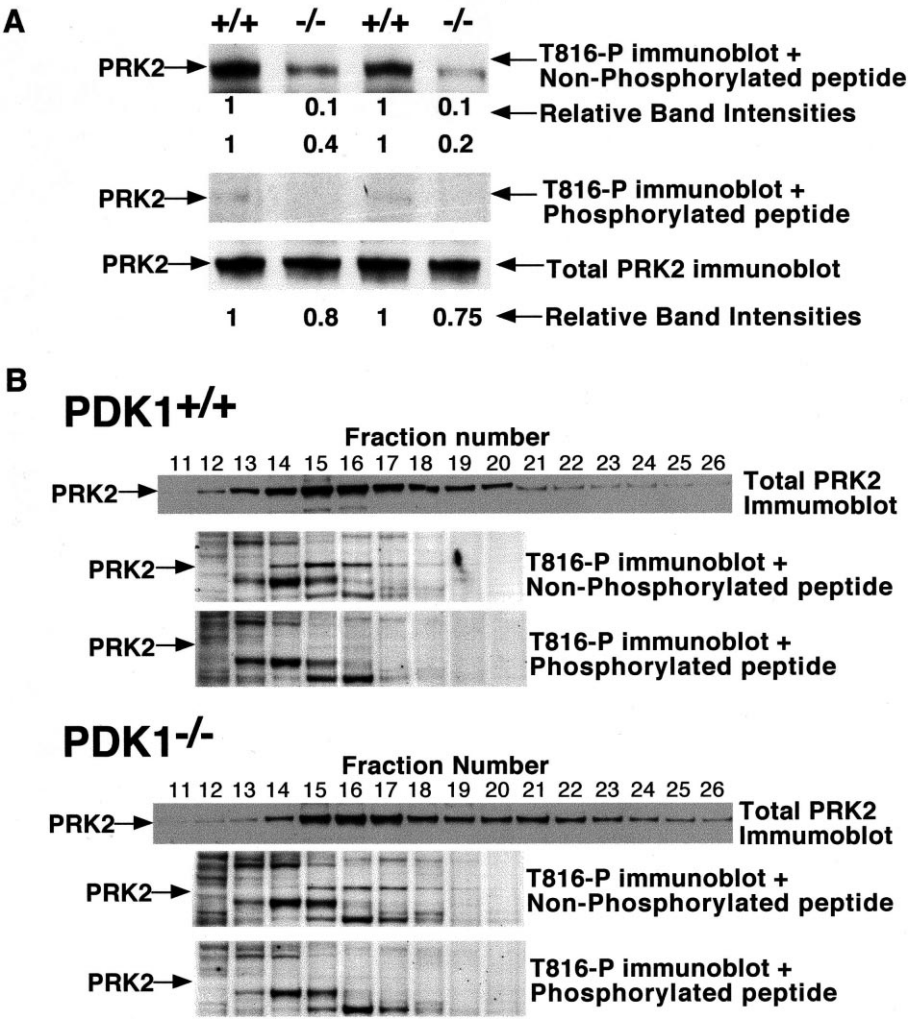

Fig. 4. PRK2 is partially phosphorylated at Thr816 in PDK1−/− ES cells. PDK1−/− and PDK1+/+ ES cell lysates were incubated with protamine-agarose to bind PRK2. The beads were washed and immunoblotted with the T816-P antibody and an antibody that recognises PRK2 (termed total PRK2) as described in Section 2. PRK2 migrated with an apparent molecular mass of 130 kDa and comigrated with recombinant Flag epitope-tagged PRK2 (data not shown). Densitometric analysis of the autorads to determine the relative intensity of PRK2 band recognised by the T816-P antibody was carried out using the NIH Image software. (B) PDK1−/− and PDK1+/+ ES cell lysates were subjected to batchwise chromatography on Q-Sepharose followed by a NaCl gradient elution from Mono S as described in Section 2. The indicated column fractions (30 μl) were immunoblotted with the T816-P antibody and the total PRK2 antibody. Similar results were obtained in three separate experiments.

PKCα) [18]. Novel PKC isoforms are also phosphorylated at their T-loop residue by PDK1 [12] but, while the phosphorylation of the 'turn motif' (Ser643 in PKCδ) is mediated by autophosphorylation, the hydrophobic motif (Ser662 in PKCδ) is thought to be phosphorylated by a distinct kinase(s) [19,20]. Moreover, phosphorylation of the T-loop of novel PKC isoforms protects the hydrophobic motif from becoming dephosphorylated in cells [19] and therefore phosphorylation of the hydrophobic motif of novel PKC isoforms is also dependent upon phosphorylation of these enzymes at their T-loop residue.

The observation made in this study that the levels of conventional and novel PKC isoforms are vastly reduced in ES cells lacking PDK1 (Fig. 1) indicates that the phosphorylation of the nascent forms of conventional and novel PKC isoforms by PDK1 plays an important role in stabilising these enzymes. It has been suggested that the primary role that phosphorylation of the hydrophobic motif of conventional PKC isoforms plays is to stabilise these enzymes. The evidence for this is based on the finding that mutation of the phosphorylated Ser residue in the hydrophobic motif of conventional PKC isoforms to Ala significantly decreases the in vitro thermostability of these enzymes [21,22]. As discussed above, the lack of phosphorylation of the T-loop of conventional and novel isoforms of PKC in PDK1−/− ES cells would also be expected to prevent the phosphorylation of the hydrophobic motif of these enzymes. It is therefore possible that it is the lack of phosphorylation of the hydrophobic motif of PKC isoforms rather than that of the T-loop itself that accounts for the instability of these enzymes in PDK1−/− ES cells. The finding that there is no detectable expression of PKCε and that the expression of PKCδ is greatly reduced also demonstrates that phosphorylation of novel PKC isoforms by PDK1 also plays an important role in stabilising these enzymes, which has previously not been reported. It should also be noted that the expression of PKCδ mRNA in PDK1−/− cells is also moderately (∼50%) reduced (Fig. 2A), indicating that PDK1 may play a minor role in regulat-

ing the transcription of the PKCδ gene or stabilisation of the PKCδ mRNA.

Atypical and related PKC isoforms are activated by phosphorylation of their T-loop, but instead of possessing a phosphorylatable Ser/Thr in their hydrophobic motif, they contain an acidic residue. Recent studies, based on in vitro and overexpression experimentation, suggest that PDK1 mediates the phosphorylation of the T-loop motif of PKCζ, PRK1 and PRK2 [7–9,11,12]. The finding in this paper, that PKCζ isolated from PDK1−/− ES cells is not phosphorylated at its T-loop residue, provides the first genetic evidence that PKCζ is indeed a physiological substrate for PDK1.

Unexpectedly, PRK2 derived from PDK1−/− cells was phosphorylated at its T-loop residue, albeit at a significantly lower level than that observed for PRK2 purified from PDK1+/+ ES cells (Fig. 4). Thus, in addition to being phosphorylated at its T-loop by PDK1, another kinase (or perhaps PRK2 itself) could phosphorylate this site in ES cells. The observation that expression of PRK1 in PDK1−/− cells was virtually abolished (Fig. 1) although the mRNA levels of PRK1 were identical in PDK1−/− and PDK1+/+ cells (Fig. 2B) was very surprising, especially as the levels of its closely related isoform PRK2 are similar in these cells (Fig. 1). It is possible that phosphorylation of the T-loop residue of both PRK1 and PRK2 is required to stabilise these enzymes but only PRK2 and not PRK1 is capable of undergoing a limited T-loop phosphorylation in PDK1−/− cells.

In summary, this study provides the first genetic evidence that PDK1 regulates the stability of multiple PKC isoforms in vivo. This work supports previous biochemical studies indicating that phosphorylation of PKC isoforms plays an important role in stabilising these enzymes.

Acknowledgements: We thank J. Leitch for preparation of sheep antibodies, Nick Helps for DNA sequencing and Maria Deak and David Campbell for obtaining mouse EST clones encoding PKC isoforms. This work was supported by the Diabetes UK (D.R.A.) and the UK Medical Research Council (D.R.A.).

References

[1] Chan, T.O., Rittenhouse, S.E. and Tsichlis, P.N. (1999) Annu. Rev. Biochem. 68, 965–1014.
[2] Peterson, R.T. and Schreiber, S.L. (1999) Curr. Biol. 9, R521–R524.
[3] Belham, C., Wu, S. and Avruch, J. (1999) Curr. Biol. 9, R93–R96.
[4] Vanhaesebroeck, B. and Alessi, D.R. (2000) Biochem. J. 346, 561–576.
[5] Williams, M.R., Arthur, J.S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P. and Alessi, D.R. (2000) Curr. Biol. 10, 439–448.
[6] Mellor, H. and Parker, P.J. (1998) Biochem. J. 332, 281–292.
[7] Balendran, A., Biondi, R.M., Cheung, P.C., Casamayor, A., Deak, M. and Alessi, D.R. (2000) J. Biol. Chem. 275, 20806–20813.
[8] Chou, M.M. et al. (1998) Curr. Biol. 8, 1069–1077.
[9] Dong, L.Q., Landa, L.R., Wick, M.J., Zhu, L., Mukai, H., Ono, Y. and Liu, F. (2000) Proc. Natl. Acad. Sci. USA 97, 5089–5094.
[10] Dutil, E.M., Toker, A. and Newton, A.C. (1998) Curr. Biol. 8, 1366–1375.
[11] Flynn, P., Mellor, H., Casamassima, A. and Parker, P.J. (2000) J. Biol. Chem. 275, 11064–11070.
[12] Le Good, J.A., Ziegler, W.H., Parekh, D.B., Alessi, D.R., Cohen, P. and Parker, P.J. (1998) Science 281, 2042–2045.
[13] Lennon, G., Auffray, C., Polymeropoulos, M. and Soares, M.B. (1996) Genomics 33, 151–152.
[14] Mukai, H. and Ono, Y. (1994) Biochem. Biophys. Res. Commun. 199, 897–904.
[15] Mizuno, K., Kubo, K., Saido, T.C., Akita, Y., Osada, S., Kuroki, T., Ohno, S. and Suzuki, K. (1991) Eur. J. Biochem. 202, 931–940.
[16] Parekh, D.B., Ziegler, W. and Parker, P.J. (2000) EMBO J. 19, 496–503.
[17] Dutil, E.M. and Newton, A.C. (2000) J. Biol. Chem. 275, 10697–10701.
[18] Behn-Krappa, A. and Newton, A.C. (1999) Curr. Biol. 9, 728–737.
[19] Parekh, D., Ziegler, W., Yonezawa, K., Hara, K. and Parker, P.J. (1999) J. Biol. Chem. 274, 34758–34764.
[20] Ziegler, W.H., Parekh, D.B., Le Good, J.A., Whelan, R.D., Kelly, J.J., Frech, M., Hemmings, B.A. and Parker, P.J. (1999) Curr. Biol. 9, 522–529.
[21] Bornancin, F. and Parker, P.J. (1997) J. Biol. Chem. 272, 3544–3549.
[22] Edwards, A.S. and Newton, A.C. (1997) J. Biol. Chem. 272, 18382–18390.