Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation *in vivo*

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3-Phosphoinositide-dependent protein kinase-1 (PDK1) expressed in unstimulated 293 cells was phosphorylated at Ser-25, Ser-241, Ser-393, Ser-396 and Ser-410 and the level of phosphorylation of each site was unaffected by stimulation with insulin-like growth factor-1. Mutation of Ser-241 to Ala abolished PDK1 activity, whereas mutation of the other phosphorylation sites individually to Ala did not affect PDK1 activity. Ser-241, unlike the other phosphorylation sites on PDK1, was resistant to dephosphorylation by protein phos-phatase $2A_1$. Ser-241 lies in the activation loop of the PDK1 kinase domain between subdomains VII and VIII in the equi-valent position to the site that PDK1 phosphorylates on its protein kinase substrates. PDK1 expressed in bacteria was active and phosphorylated at Ser-241, suggesting that PDK1 can phosphorylate itself at this site, leading to its own activation.

Key words: insulin, mass spectrometry, protein kinase B, signal transduction.

INTRODUCTION

Protein kinase B (PKB, also called c-Akt) is thought to play a role in mediating some of the metabolic actions of insulin, as well as the effects of survival factors on apoptotic processes [1–3]. PKB is only active when phosphorylated at two residues, Thr-308 and Ser-473 [1]. The phosphorylation of Thr-308 only occurs *in vitro* and *in vivo* in the presence of $PtdIns(3,4,5)P_3$ or $PtdIns(3,4)P_2$ and, for this reason, the protein kinase found to phosphorylate this residue has been termed 3-phosphoinositide-dependent protein kinase-1 (PDK1) [4]. Thr-308 is located in the activation loop of the catalytic domain of PKBα, in a region that displays high homology between different AGC family members (cAMP-dependent, cGMP-dependent and PKC) [1–3]. Other members of the AGC subfamily of protein kinases, including p70 ribosomal S6 protein kinase (p70 S6K) [5], protein kinase C (PKC) isoforms [6], serum- and glucocorticoid-induced protein kinase (SGK) [7] and the cAMP-dependent protein kinase (PKA) [8], also possess residues lying in a sequence motif equivalent to Thr-308 (termed the PDK1 phosphorylation site) whose phosphorylation is required for activation. These kinases, except for PKA, also possess a consensus motif ∼ 160 residues C-terminal to the PDK1 phosphorylation site, termed the PDK2 phosphorylation site [4]. Phosphorylation of both the PDK1 and PDK2 sites is required for maximal activation of PKB or p70 S6 kinase and/or stability for PKC isoforms. Recent observations have demonstrated that PDK1 can acquire the ability to phosphorylate the PDK2 site on PKB as well as Thr-308 in the presence of a synthetic peptide derived from the C-terminus of PKC-related kinase-2 (PRK2) [9]. These observations suggest that PDK1 and PDK2 may be the same enzyme.

In this study we have investigated whether PDK1 is itself phosphorylated *in vivo* and whether phosphorylation plays a role in regulating its activity. We identify five serine residues that become phosphorylated *in vivo* and show that one of these is critical for PDK1 activity.

MATERIALS AND METHODS

Materials

Tissue culture reagents, microcystin-LR and insulin-like growth factor-1 (IGF1) were obtained from Life Technologies Inc. (Paisley, U.K.), phosphate free Dulbecco's modified minimal essential medium from ICN (Oxon., U.K.), alkylated trypsin from Promega (Southampton, U.K.), 4-vinylpyridine from Sigma–Aldrich (Poole, Dorset, U.K.), Asp-N protease, the 9E10 monoclonal antibody recognizing the Myc epitope tag, and protease inhibitor cocktail were purchased from Roche (Lewes, East Sussex, U.K.) and [32P]P_i from Amersham (Little Chalfont, Bucks., U.K.) Glutathione S-transferase (GST)–PKBα [4] and GST–SGK, lacking the N-terminal 60 amino acids, were ex-pressed in 293 cells as described previously [7].

General methods

Restriction enzyme digests, DNA ligations and other recom-binant DNA procedures were performed using standard protocols. Site-directed mutagenesis was performed using the QuikChange Kit (Stratagene) following instructions provided by the manufacturer. All DNA constructs were verified by DNA sequencing using an automated DNA sequencer. Phosphatidyl-choline and phosphatidylserine phospholipid vesicles containing $PtdIns(3,4,5)P_3$ were prepared as described previously [4].

32P-labelling of 293 cells transfected with Myc-PDK1

293 cells were transfected with a pCMV5-encoded DNA con-struct expressing either the wild-type Myc-PDK1 or a catalytically inactive Myc-PDK1 mutant (in which both Lys-111 and Asp-223 were changed to Ala) [10] using a modified calcium phosphate procedure [11]. Thirty-six hours after transfection, the

cells were washed with phosphate-free Dulbecco's modified minimal essential medium, incubated for 4 h with [^{32}P]P$_i$ (1 mCi/ml), then stimulated with IGF1 (100 ng/ml) for 5 min. The cells were lysed with 1.0 ml of ice-cold buffer A [50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 % (v/v) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium glycero-phosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 μM microcystin-LR, 0.27 M sucrose, 0.1 % (v/v) 2-mercaptoethanol and proteinase inhibitor cocktail (one tablet per 50 ml)]. The lysates were centrifuged at 4 °C for 10 min at 13 000 g and the supernatants were incubated for 30 min on a shaking platform with 20 μl of Protein G–Sepharose coupled to 20 μg of 9E10 immunoprecipitating antibody. The Protein G–Sepharose-antibody–PDK1 complex was washed eight times with 1.0 ml of buffer A containing 0.5 M NaCl, and twice with 50 mM Tris/HCl, pH 7.5/0.1 mM EGTA/0.1 % (v/v) 2-mercaptoethanol (buffer B). The immunoprecipitated protein was alkylated with 4-vinylpyridine, and subjected to SDS/PAGE. After autoradiography, the ^{32}P-labelled band corresponding to Myc-PDK1 was excised and PDK1 was eluted from the gel, digested with trypsin and analysed by chromatography on a C$_{18}$ column, as described previously for PKBα immunoprecipitated from ^{32}P-labelled cells [11].

Expression of GST–PDK1 mutants in 293 cells

In order to express the mutant forms of PDK1 used in this study, appropriate site-directed mutagenesis was carried out using the full-length wild-type PDK1 cDNA subcloned into the pEBG2T vector (which expresses PDK1 as a GST fusion protein in 293 cells [10]). To prepare each GST–PDK1 mutant, twenty 10 cm diameter dishes of 293 cells were transfected with 20 μg of DNA, and the GST fusion proteins were expressed and purified as described previously [10]. Between 0.5 and 1.0 mg of each GST fusion protein was obtained by this procedure and each protein was more than 90 % homogeneous, as judged by SDS/PAGE (results not shown).

Expression of wild-type and mutant GST–PDK1 in *Escherichia coli*

A cDNA construct encoding for GST fused to residues 52–556 of PDK1 was generated by subcloning PDK1 as a *Bam*HI/*Sal*I fragment into the same sites of the pGEX-4T3 vector (Pharmacia). These constructs were transformed into BL21 DE3 (pLysS) *E. coli* cells and the bacteria were grown at 37 °C to a density at which the absorbance at 600 nm was 0.6. The temperature was reduced to 28 °C and isopropyl-β-D-thiopyranoside was added to 30 μM. The bacteria were grown for a further 16 h before the cells were lysed. The GST fusion proteins were purified as described for the purification of GST–mitogen-activated protein kinase [12]. Bacterially expressed GST–PDK1 was 20 % undegraded, with the remainder consisting of N-terminal proteolytic fragments fused to GST, as judged by SDS/PAGE.

Phosphopeptide sequence analysis

PDK1 phosphopeptides were analysed by matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) MS on a PerSeptive Biosystems Elite-STR mass spectrometer using alpha-cyanocinnamic acid as the matrix. Spectra were obtained in both the linear and reflector mode. The sequence identities of the peptides were also confirmed by Edman sequencing on an Applied Biosystems 476A sequenator. The sites of phosphorylation were determined by solid-phase Edman degradation of the peptide coupled to Sequelon-AA membrane (Milligen; Bedford, MA,

U.S.A.) as described previously [13]. Peptide P3 was sub-digested with 0.2 μg of Asp-N protease in 20 mM ammonium bicarbonate containing 0.1 % (w/w) n-octyl-glucoside (Calbiochem; Nottingham, U.K.) for 6 h at 30 °C to yield a peptide termed D3. The identities of the digestion products were confirmed by MALDI–TOF MS and the digested material was coupled to Sequelon-AA for solid-phase sequence analysis.

RESULTS

Mapping the phosphorylation sites of PDK1 in transiently transfected 293 cells

In order to establish whether PDK1 was phosphorylated in cells, ^{32}P-labelled 293 cells expressing Myc-PDK1 were stimulated with or without 100 ng/ml IGF1 for 5 min, the cells were lysed and the Myc-PDK1 was immunoprecipitated and electro-phoresed on an SDS-polyacrylamide gel. Autoradiography of the gel revealed that Myc-PDK1 was equally heavily phosphorylated in samples derived from the unstimulated and IGF1-stimulated cells (Figure 1A). In order to map the phosphorylation sites, the ^{32}P-labelled PDK1 was digested with trypsin, and the resulting peptides were separated by chromato-graphy on a C$_{18}$ column. Four major ^{32}P-labelled peptides were recovered from both the unstimulated (Figure 1B) or IGF1-stimulated (Figure 1C) cells, termed P1, P2, P3 and P4. IGF1 did
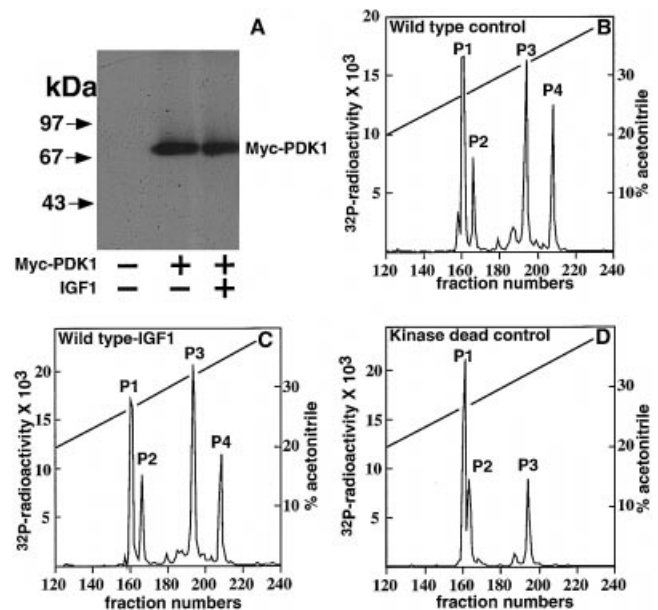
Figure 1   Tryptic peptide map of ^{32}P-labelled PDK1

293 cells transiently transfected with DNA constructs encoding wild-type Myc-PDK1 or the empty vector as a control were ^{32}P-labelled and treated for 5 min without or with 100 ng/ml IGF1. The ^{32}P-labelled PDK1 protein was immunoprecipitated from the lysates using a Myc antibody, treated with 4-vinylpyridine, electrophoresed on a 10% polyacrylamide gel, and the ^{32}P-labelled PDK1 was visualized by autoradiography (**A**). The ^{32}P-labelled PDK1 was excised from the gel, digested with trypsin and chromatographed on a Vydac 218TP54 C$_{18}$ column (Separations Group, Hesperia, CA, U.S.A.) equilibrated with 0.1 % (v/v) trifluoroacetic acid, and the columns were developed with a linear acetonitrile gradient (diagonal line). The flow rate was 0.8 ml/min and fractions of 0.4 ml were collected (**B** and **C**). The four major ^{32}P-labelled peptides eluting at 26.5% (termed P1), 28% (termed P2), 32% (termed P3) and 34.5% (termed P4) are indicated. (**D**) As above, except that cells were transfected with a catalytically inactive Myc-PDK. No ^{32}P-labelled peptides are present in the fractions that are not shown. Similar results were obtained in three separate experiments for (**B**) and (**C**) and two experiments for (**D**).
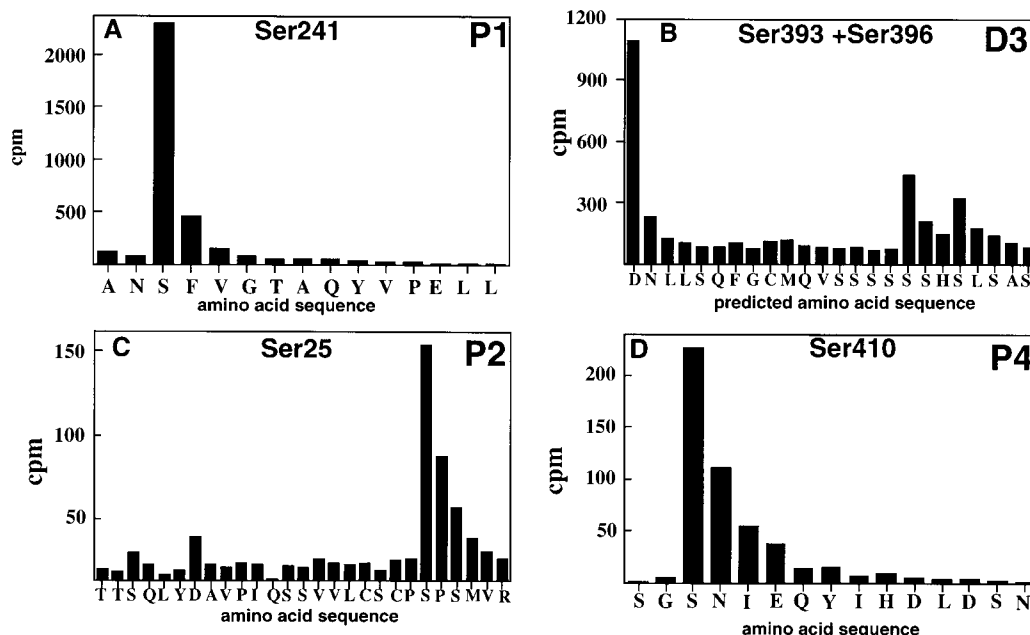
Figure 2   Identification of the phosphorylation sites in ^{32}P-labelled PDK1 peptides

In order to identify the site(s) of phosphorylation, an aliquot of each of the four major ^{32}P-labelled peptides, derived from ^{32}P-labelled PDK1 (Figure 1) of each peptide, was coupled covalently to a Sequelon arylamine membrane and analysed on an Applied Biosystems 470A sequencer using the modified programme [13]. ^{32}P-radioactivity was measured after each cycle of Edman degradation. In combination with phosphoamino acid analysis, MALDI-TOF MS and gas-phase Edman sequencing (see Results section); this enabled the identification of the site(s) of phosphorylation in each of the peptides.

not increase the phosphorylation of any of the four sites nor did it induce any novel phosphorylation site in PDK1, but, in parallel experiments, IGF1 did induce maximal PKBα activation after 5 min and its phosphorylation at Thr-308 and Ser-473 (results not shown). A catalytically inactive mutant of Myc-PDK1 expressed in 293 cells was phosphorylated at sites P1, P2 and P3, but P4 was absent (Figure 1D).

PDK1 was isolated from both unstimulated and IGF1-stimulated cells and the ^{32}P-labelled tryptic peptides P1, P2, P3 and P4 were isolated by HPLC. Phosphoamino acid analysis revealed that all four peptides contained phosphoserine but no phosphothreonine or phosphotyrosine (results not shown). When peptide P1 was subjected to solid-phase sequencing, ^{32}P-radio-activity was released after the third cycle of Edman degradation (Figure 2A). Its molecular mass, determined by MALDI-TOF MS (2134.01), was identical with that expected for the tryptic phosphopeptide comprising residues 239–257 and phosphory-lated at Ser-241. This was confirmed by gas-phase Edman sequencing of this peptide (Figure 2A). Ser-241 lies in the T-loop of the kinase domain between subdomains VII and VIII in an equivalent position to the site that PDK1 phosphorylates on its protein kinase substrates (e.g. Thr-308 of PKBα).

^{32}P-radioactivity was released from peptide P2 after 22 cycles of Edman degradation (Figure 2B). Its identity was established by MALDI-TOF MS, which revealed that the molecular mass (3158.48) was identical with that expected for the tryptic phospho-peptide comprising residues 4–30 and phosphorylated at Ser-25. This was confirmed by gas-phase Edman sequencing of this peptide (Figure 2B). Ser-25 lies N-terminal to the kinase catalytic domain and is followed by a Pro residue.

MALDI-TOF MS of peptide P3, revealed that its molecular mass was identical with that expected for the 50-residue tryptic phosphopeptide comprising residues 358–407 and phosphoryl-

ated at one residue. This peptide was subjected to solid-phase sequence analysis and no release of ^{32}P was observed after 23 cycles of Edman degradation, suggesting that the phosphoryl-ation site of this peptide was at the C-terminal end. In order to map this site the peptide was subdigested with the Asp-N protease. The digest, termed peptide D3, was then subjected to solid-phase sequence analysis, which revealed a burst of counts at cycles 1, 17 and 20 (Figure 1C). The burst of radioactivity at cycle 1 is caused by the coupling of a large percentage of the peptide to the Sequelon arylamine membrane through its N-terminal Asp residue. The ^{32}P-radioactivity released at cycles 17 and 21 suggested that the peptide corresponded to residues 377–400 of PDK1 phosphorylated at either Ser-393 or Ser-396 or both of these residues. The mass of this peptide determined by MALDI-TOF MS revealed that the molecular mass (2644.02) was virtually identical with that expected (2644.14) for this peptide phosphorylated at one position. This suggests that pep-tide D3 is a mixture of peptides in which either Ser-393 or Ser-396 is phosphorylated. Ser-393 and Ser-396 lie in a Ser-rich region of PDK1 that lies in the 'linker' region between the kinase domain and the pleckstrin homology (PH) domain.

^{32}P-radioactivity was released from peptide P4 after the third cycle of Edman degradation (Figure 2D). Its identity was established by MALDI-TOF MS, which revealed that the molecular mass (3495.72) was virtually identical with that expected (3495.52) for the tryptic phosphopeptide comprising residues 408–435 and phosphorylated at Ser-410. This was confirmed by gas-phase Edman sequencing of this peptide (Figure 2D). Ser-410 also lies in the 'linker' region between the catalytic and PH domains.

Ser-241 is likely to be stoichiometrically phosphorylated in 293 cells, as we were unable to detect the unphosphorylated form of the tryptic peptide containing Ser-241 by MALDI-TOF MS of
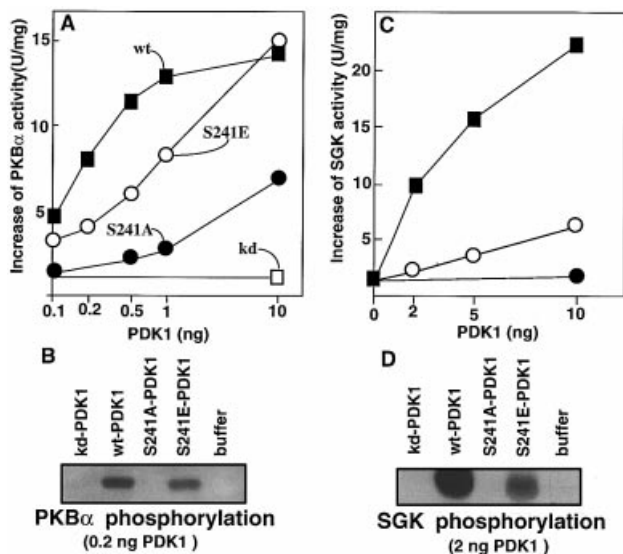
Figure 3   Effect of mutation of Ser-241 on PDK1 activity

(**A**) GST–PKBα was incubated for 30 min at 30 °C with the indicated amounts of wild-type (wt) GST–PDK1 (■), catalytically inactive (kd) GST–PDK1 (□), GST–S241A-PDK1 (●) or GST–S241E-PDK1 (○), in the presence of MgATP and phospholipid vesicles containing 100 μM phosphatidylcholine, 100 μM phosphatidylserine and 10 μM sn-1-stearoyl-2-arachidonyl-D-PtdIns(3,4,5)P_3. Reactions were terminated and the resulting increase in the specific activity (units/mg) (**A**) or phosphorylation (**B**) of GST–PKBα was then determined [10]. (**C** and **D**) GST–SGK (lacking the N-terminal 60 amino acids) was incubated for 30 min at 30 °C with the indicated amounts of wild-type and mutant PDK1 proteins in the presence of MgATP and the increase in specific activity (**C**) and phosphorylation (**D**) of GST–SGK was measured as described previously [7]. The results shown are the average of three separate experiments, with each determination carried out in triplicate.

PDK1 isolated from 293 cells (results not shown). In contrast, we were able to detect the dephosphorylated tryptic peptides corresponding to Ser-25, Ser-393, Ser-396 and Ser-410 of PDK1, indicating that these residues were not stoichiometrically phosphorylated in 293 cells. It is not possible to calculate the relative ratios of the phosphorylated versus the dephosphorylated forms of these peptides, as the detection of a phosphorylated peptide by MALDI-TOF MS is vastly less sensitive than for the equivalent dephosphorylated peptide. It is also not possible to infer the relative degree of phosphorylation of each residue on PDK1 from the tryptic peptide mapping (Figure 1) as the turnover of each residue *in vivo* occurs at a different rate.

Role of the PDK1 phosphorylation sites in regulating PDK1 activity

The serine residues identified above were mutated to either Ala or Glu and the resulting mutant PDK1 proteins were expressed as GST fusion proteins in 293 cells. All the mutants were expressed to the same level and purified to an equal degree of purity as the wild-type PDK1 (results not shown). The ability of wild-type and mutant PDK1 proteins to activate and phosphorylate PKBα in the presence of lipid vesicles containing PtdIns(3,4,5)P_3 was then measured (Figure 3). We also assessed the rate at which the mutants of GST–PDK1 phosphorylated and activated SGK in the absence of phospholipids (Figure 4). SGK is a member of the AGC subfamily of protein kinase whose kinase domain is most related to that of PKBα. However, unlike PKBα, SGK does not possess a PH domain. SGK is phosphorylated by PDK1 at the residue equivalent to Thr-308 of
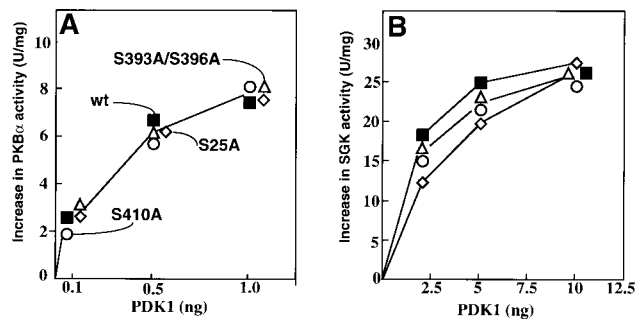
Figure 4   Phosphorylation of Ser-25, Ser-393, Ser-396 and Ser-410 is not required for PDK1 activity

The increase in the specific activities of GST–PKBα (**A**) or GST–SGK (**B**) following incubation with the indicated amounts of wild type (wt) GST–PDK1 (■), GST–S25A-PDK1 (◇), GST–S393A/396A-PDK1 (△), or GST–S410A-PDK1 (○) was determined as described in the legend to Figure 3. The results shown are the averages of two separate experiments with each determination carried out in triplicate.

PKBα, resulting in activation of this kinase [7]. The PDK1 mutant in which Ser-241 was changed to Ala (GST–S241A-PDK1) phosphorylated and activated PKBα at a 50-fold lower initial rate than wild-type GST–PDK1 (Figures 3A and 3B). GST–S241A-PDK1 did not significantly activate or phosphorylate SGK even at the highest concentrations tested (Figure 3C and 3D). In contrast, the PDK1 mutant, in which Ser-241 was changed to Glu, phosphorylated and activated PKBα at a 10-fold higher rate than the GST–S241A-PDK1 mutant (Figures 3A and 3B), and was also capable of phosphorylating and activating SGK (Figures 3C and 3D), although at a much slower rate than that observed for the wild-type PDK1.

In contrast, GST–S25A-PDK1, GST–PDK1 in which both Ser-393 and Ser-396 were mutated to Ala (GST–S393A/396A-PDK1) and GST–S410A-PDK1, phosphorylated and activated PKBα (Figure 4A) and SGK (Figure 4B) at a similar rate to the wild-type GST–PDK1.

PDK1 is also able to phosphorylate PKBα at Ser-473 in a PtdIns(3,4,5)P_3-dependent manner when it is complexed to a peptide comprising the 24 C-terminal amino acids of PRK2 [9]. The GST–S25A-PDK1, GST–S393A/396A-PDK1 and GST–S410A-PDK1 mutants were also capable of phosphorylating Ser-473 of PKBα in the presence the PRK2 C-terminal peptide,
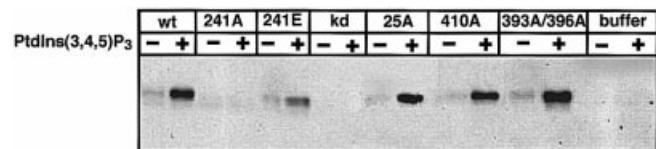
Figure 5   Effect of phosphorylation-site mutants on the rate at which PDK1 phosphorylates PKBα at Ser-473 in the presence of the PRK2 C-terminal peptide

The indicated GST–PDK1 proteins (20 ng) were incubated in the presence (+) or absence (−) of 100 μM of the PRK2 C-terminal peptide (corresponding to residues 969–984 of human PRK2 [9]). After standing for 1 h at 4 °C, GST–PKBα, MgATP and phospholipid vesicles containing 100 μM phosphatidylcholine, 100 μM phosphatidylserine and 10 μM sn-1-stearoyl-2-arachidonyl-D-PtdIns(3,4,5)P_3 were added. After 1 h at 30 °C, the reactions were terminated by making the solutions 1% in SDS. The samples were then subjected to SDS/PAGE and immunoblotted using a phospho-specific antibody which recognises PKBα phosphorylated at Ser-473. Similar results were obtained in two separate experiments.

and PtdIns(3,4,5)P_3 (Figure 5). In contrast, the GST–S241A-PDK1 mutant was unable to phosphorylate PKBα at Ser-473, whereas GST–S241E-PDK1 phosphorylated PKBα at this residue but to a lower extent than wild-type GST–PDK1.

Incubation of ^{32}P-labelled PDK1 derived from unstimulated 293 cells with 100 m-units/ml protein phosphatase 2A$_1$ (PP2A$_1$) had no effect on the activity of PDK1, but led to the complete dephosphorylation of peptides P2, P3 and P4 (Ser-25, Ser-393, Ser-396 and Ser-410). Peptide P1 (Ser-241) was not significantly dephosphorylated under these conditions, indicating that it is not accessible to PP2A$_1$ (results not shown).

PDK1 expressed in bacteria phosphorylates itself on Ser-241

In order to establish whether PDK1 was capable of phosphorylating itself at Ser-241, we expressed wild-type GST–PDK1 in *E. coli*, with the aim of establishing whether it was phosphorylated at Ser-241. Bacterially expressed PDK1 was significantly proteolysed, with only 20 % of the purified material corresponding to the full-length protein. However, allowing for proteolysis, the specific activity of the wild-type GST-PDK1 towards PKBα was comparable with that of PDK1 expressed in 293 cells. GST–PDK1 expressed in bacteria and 293 cells was treated with 4-vinylpyridine to alkylate cysteine residues, electrophoresed on an SDS polyacrylamide gel and stained with KCl to locate the unproteolysed protein (2 μg). This was then excised from the gel, digested with trypsin and the resulting peptides were separated by chromatography on a C$_{18}$ column. Under the conditions used, the tryptic peptide phosphorylated at Ser-241 was eluted at fraction 159 (peptide P1 in Figure 1). Mass spectrometric analysis of this fraction, derived from either the wild-type PDK1 expressed in bacteria or 293 cells, revealed the presence of a peptide of mass 2134.01, identical with that expected for the tryptic phosphopeptide comprising residues 239–257 and phosphorylated at Ser-241. Only a small amount of the non-phosphorylated tryptic peptide 239–257 was detected in a slightly later-eluting fraction (162), indicating that the majority of the bacterially expressed protein was phosphorylated at Ser-241 (results not shown). Also, incubation of bacterially expressed GST–PDK1 with [γ^{32}P]MgATP *in vitro* resulted in a low level of autophosphorylation of PDK1. In order to identify the site(s) of autophosphorylation the ^{32}P-labelled *E. coli*-expressed GST–PDK1 was digested with trypsin, and the resulting peptides were separated by chromatography on a C$_{18}$ column. Only a single major ^{32}P-labelled peptide was eluted from the column at fraction 159, corresponding to the peptide 239–257 phosphorylated at Ser-241 (results not shown).

As a catalytically inactive PDK1 expressed in 293 cells was not phosphorylated at Ser-410 (Figure 1C) this initially suggested that this residue may be a site of autophosphorylation. However, mass spectrometric analysis of HPLC fractions, derived from trypsin-digested *E. coli*-expressed PDK1, around the region in which this peptide was eluted (fraction 207), revealed only the presence of the dephosphorylated peptide, indicating that bacterially expressed PDK1 cannot phosphorylate itself at this residue. Furthermore, incubation of *E. coli*-expressed PDK1 with [γ^{32}P]MgATP *in vitro* did not result in phosphorylation of this residue (results not shown).

DISCUSSION

In this study we have identified five serine residues on PDK1 which are phosphorylated in cells. Four of these sites (Ser-25, Ser-393, Ser-396 and Ser-410) are present in the non-catalytic and non-PH domain region of PDK1 and are not conserved in the PDK1 homologues found in *Drosophila* [10], *Arabidopsis*

PDK1	236-QARANS FVGTAQYVSPE LT KSACKSS DLWALGCI
DSTPK61	372-RHRKAS FVGTAQYVSPE LQNGPITPAAD LWALGCI
C.elegans PDK1-1a	264-TARRT T FVGTA YVSPE LA GDVGPQTD W LGCI
C.elegans PDK1-2	-TGRRSS FVGTAF V P PE LTGSEMSPSS DLWA SVT
KSG	266-QAHSRS FVGTAR YVSPE LS KIAGTAS D W A GCI
PKH1	291-YAKSKS FVGTAE YVSPE LN NYTDSRCD W A GCI
PKH2	343-STRSKS FVGTAE YVSPE LN SFTDYRCD W A GCIL
AtPDK1	206-DDKACT FVGTAA YVPPE LN SSPATFGND LWALGCT
ricePDK1	-NERACT FVGTAA YVPPE LN SAPPTFGND LWALGCT
PKA	192-KGRTW T CGTPE Y LSKGYNKAVD W WALGV I

Figure 6   Alignment of amino acid sequences surrounding the activation loops in PDK1 homologues and PKA

(**A**) Identities are shaded in black and homologies in grey. The NCBI accession number for human PDK1 is AF017995 [10], for *Drosophila* PDK1 (DSTPK61) is Y07908 [10] and for *S. pombe* PDK1 (KSG) is X99280 [16]. The accession numbers for the *S. cerevisiae* PDK1 homologues Pkh1 and Pkh2 are S69657 and Q12236 respectively [15], for *Arabidopsis* PDK1 (AtPDK1) is AF132742 [14]; for the rice PDK1 sequence is AF132743 [14]. There appear to be two PDK1 isoforms in *C. elegans*, PDK1-1a [17] GenBank accession number AF017995, and the other termed PDK1-2 (NCBI accession number AF100305). The accession number for the human PKA is NP002721. The underlined residues correspond to the residue Ser-241 in the human PDK1 sequence. No numbering is given for the *C. elegans* PDK1-2 and rice PDK1 sequences as the full-length sequence of these is not known.

[14], *Saccharomyces cerevisiae* [15], *Saccharomyces pombe* [16] or *Caenorhabditis elegans* [17]. Mutation of these residues to Ala or the removal of phosphate from these residues using a protein phosphatase had no effect on PDK1 activity assayed by phosphorylation and activation of PKBα and SGK. Nor did the mutation of these residues individually to Ala prevent PDK1 from phosphorylating PKBα at Ser-473 in the presence of the PRK2 C-terminal peptide. Thus phosphorylation of PDK1 at these residues is not required for the activity of PDK1. Ser-25 is followed by a proline residue and is likely to be phosphorylated by a proline-directed protein kinase. Neither the catalytically inactive PDK1 mutant expressed in 293 cells (Figure 1D), nor the wild-type PDK1 expressed in *E. coli*, is phosphorylated on Ser-410. This indicates that the kinase that phosphorylates this residue in 293 cells does not recognise catalytically inactive PDK1, or that bacterially expressed PDK1 is not in the correct conformation to phosphorylate itself at this residue.

In contrast to the other phosphorylation sites in PDK1, Ser-241 and the residues surrounding this site are highly conserved in all the known PDK1 homologues in other species (Figure 6). Mutation of Ser-241 to Ala drastically reduces the activity of PDK1 towards PKBα and SGK, and towards Ser-473 of PKBα in the presence of the PRK2 C-terminal peptide. Ser-241 lies in the equivalent position in the T-loop of the kinase domain to the residues on other protein kinases that are phosphorylated by PDK1 (e.g. Thr-308 of PKBα). This raised the possibility that PDK1 might be able to phosphorylate itself at this residue, leading to its own activation. That this is indeed the case is supported by the finding that bacterially expressed PDK1 possesses a high activity and is phosphorylated at Ser-241.

Ser-241 of PDK1 is similar in many respects to the stable phosphorylation site in the T-loop of PKA (Thr-197) [18]. This residue lies in an equivalent position in the kinase domain to Ser-241 of PDK1 (see Figure 6), and is resistant to dephosphorylation by protein phosphatases, because it is buried inside the protein [8,18]. Ser-241 of PDK1 is also not dephosphorylated following incubation with high concentrations of PP2A$_1$, and PDK1 purified from rabbit skeletal muscle could not be inactivated by incubation with PP2A or protein phosphatase-1 [10]. Thus, Ser-241 of PDK1 also appears to be inaccessible to protein

phosphatases. PKA expressed in bacteria is phosphorylated at Thr-197, demonstrating that PKA can also phosphorylate itself at this residue. However, in mammalian cells, there is now some evidence that Thr-197 may not necessarily be phosphorylated by an autophosphorylation reaction, but rather by PDK1 [8]. By analogy, it cannot be ruled out that in mammalian cells other kinases distinct from PDK1 may also be able to phosphorylate Ser-241 of PDK1, even though PDK1 has the intrinsic ability to phosphorylate itself at this residue.

Importantly, we show that IGF1-stimulation of cells does not increase phosphorylation of PDK1 at Ser-241 or induce any novel phosphorylation of PDK1, suggesting that PDK1 is not a target for IGF1-stimulated protein kinases. However, as the phosphorylation of PDK1 at Ser-241 is essential for its activity, it cannot be ruled out that the activity of PDK1 in certain cellular situations may be increased by the phosphorylation of this residue, or inhibited by its dephosphorylation.

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