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Characterisation of a plant 3-phosphoinositide-dependent protein kinase-1 homologue which contains a pleckstrin homology domain

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Abstract A plant homologue of mammalian 3-phosphoinositide-dependent protein kinase-1 (PDK1) has been identified in *Arabidopsis* and rice which displays 40% overall identity with human 3-phosphoinositide-dependent protein kinase-1. Like the mammalian 3-phosphoinositide-dependent protein kinase-1, *Arabidopsis* 3-phosphoinositide-dependent protein kinase-1 and rice 3-phosphoinositide-dependent protein kinase-1 possess a kinase domain at N-termini and a pleckstrin homology domain at their C-termini. *Arabidopsis* 3-phosphoinositide-dependent protein kinase-1 can rescue lethality in *Saccharomyces cerevisiae* caused by disruption of the genes encoding yeast 3-phosphoinositide-dependent protein kinase-1 homologues. *Arabidopsis* 3-phosphoinositide-dependent protein kinase-1 interacts via its pleckstrin homology domain with phosphatidic acid, PtdIns3P, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 and to a lesser extent with PtdIns(4,5)P_2 and PtdIns4P. *Arabidopsis* 3-phosphoinositide-dependent protein kinase-1 is able to activate human protein kinase Bα (PKB/AKT) in the presence of PtdIns(3,4,5)P_3. *Arabidopsis* 3-phosphoinositide-dependent protein kinase-1 is only the second plant protein reported to possess a pleckstrin homology domain and the first plant protein shown to bind 3-phosphoinositides.

1. Introduction

3-phosphoinositide-dependent protein kinase-1 (PDK1) was originally identified in mammalian cells as an activator of protein kinase B (PKB) [1], an enzyme that is thought to mediate many of the intracellular actions of insulin and growth factors (reviewed in [2,3]). PDK1 has subsequently been shown to phosphorylate and activate several other protein kinases [4]. Insulin and other agonists that trigger the activation of PKBα do so by switching on class I phosphoinositide (PI) 3-kinases to generate the 'second messenger' PtdIns(3,4,5)P_3, which binds to the pleckstrin homology (PH) domain of PKB, altering the conformation of PKB in such a way that it can be phosphorylated and activated by PDK1 [2,3].

PDK1 contains a PH domain that binds PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 far more tightly than does the PH domain of PKB [5,6]. Point mutations in the PH domain of PDK1 (or its deletion) that abolish interaction with inositol phospholipids greatly decrease the rate at which it phosphorylates and activates PKB in lipid vesicles containing PtdIns(3,4,5)P_3 [6]. This indicates that interaction of the PH domain of PDK1 with PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 is important for its association with PKB in lipid vesicles in vitro (and perhaps at the plasma membrane, in vivo).

The genome of *Saccharomyces cerevisiae* encodes two proteins, Pkh1 and Pkh2, whose catalytic domains display 50% amino acid sequence identity to human or *Drosophila* PDK1. Disruption of both yeast genes results in a lethal phenotype, but lethality can be rescued by expression of human PDK1 [7]. This finding, together with the observation that Pkh1 can phosphorylate human PKB at the same residue as PDK1, indicates functional similarity between mammalian PDK1 and Pkh1 of budding yeast [7]. However, unlike human PDK1, neither Pkh1 nor Pkh2 possess PH domains nor do they interact with 3-phosphoinositides (3-PIs). This may reflect the absence in yeast of genes encoding class I PI 3-kinases and the apparent absence of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 in this organism. If this hypothesis is correct, then one might have predicted that PDK1 homologues in higher plants would also lack a PH domain, because (to our knowledge) class I PI 3-kinases or PtdIns(3,4,5)P_3 have not yet been detected in plants. Indeed, so far, there is only one plant protein that is known to possess a PH domain [8]. In this paper, we identify and characterise a plant PDK1 homologue that can activate human PKB in vitro and which can rescue lethality in yeast caused by disruption of the PDK1 genes. Surprisingly, the plant PDK1, like its human counterpart, does possess a PH domain and does bind to 3-PI lipids.

2. Materials and methods

2.1. Cloning of Arabidopsis (At) PDK1 and rice PDK1

An *Arabidopsis* EST clone (NCBI accession number N96553) encoding the C-terminal 280 residues of AtPDK1 was obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, OH, USA). The 5′-end of the *Arabidopsis* cDNA was obtained by carrying out a 5′-RACE PCR reaction using an *Arabidopsis* λZAPII cDNA library (Stratagene, Cambridge, UK) as a template and an oligonucleotide derived from AtPDK1 (5′-GGAACATATG-CAGCAGTCCCGACAA-3′) and the M13 reverse primer. A 1 kb PCR product was obtained and subcloned into pCR 2.1TOPO vector (Invitrogen, Leek, Netherlands). Out of 10 individual clones sequenced, two encoded the 5′-end of the AtPDK1 cDNA. This information enabled the identification of another *Arabidopsis* EST (accession number Z35743) also encoding the 5′-end of the AtPDK1 gene. Its DNA sequence (corresponding to residues 140–400 of AtPDK1) was identical to that obtained by PCR. A full length AtPDK1 cDNA clone was isolated by PCR using the *Arabidopsis* λZAPII cDNA li-

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Abbreviations: 3-PI, 3-phosphoinositide; PKB, protein kinase B; SGK, serum and glucocorticoid-regulated protein kinase; PtdIns, phosphatidylinositol; PI 3-kinase, phosphoinositide 3-kinase; PH, pleckstrin homology

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brary as a template and the oligonucleotide derived from the AtPDK1 (5′-C**GGATCC***ATG*TTGGCAATGGAGAAAGAATTTG-3′ which places a *Bam*H1 restriction site 5′ to the initiating ATG which is underlined) and the T7 oligonucleotide. The resulting 2 kb PCR product was cloned into pCR 2.1TOPO vector and its DNA sequence was verified.

The rice PDK1 EST (accession number D40851) was obtained from the MAFF DNA Bank (Tsukuba, Ibaraki, Japan)

2.2. Expression of GST-AtPDK1 and GST-ΔPH-AtPDK1 in 293 cells

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 50 ml: Boehringer Mannheim, Lewes, UK). Buffer B: 50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 10 mM β-mercaptoethanol and 0.27 M sucrose.

The AtPDK1 encoding sequence was subcloned from the pCR 2.1TOPO vector as a *Bam*HI-*Kpn*I fragment into the *Bam*HI and *Kpn*I sites of the eukaryotic expression vector pEBG-2T [9]. In order to create AtPDK1 lacking the PH domain (GST-ΔPH-AtPDK1), a stop codon was introduced after amino acid 390 using the QuickChange mutagenesis system (Stratagene). For the expression of GST fusion proteins in 293 cells, 20 10 cm diameter dishes of 293 cells were cultured and each dish transfected with 20 μg of pEBG2T DNA encoding either GST-AtPDK1 or GST-ΔPH-AtPDK1 using a modified calcium phosphate method [10]. 36 h after transfection, the cells were lysed in 1 ml of ice-cold buffer A without any serum starvation. The lysates were pooled, centrifuged at 4°C for 10 min at $13\,000\times g$ and the supernatant incubated for 60 min on a rotating platform with 1 ml of glutathione-sepharose previously equilibrated in buffer A. The suspension was centrifuged for 1 min at $3000\times g$, the beads washed three times with 10 ml buffer A containing 0.5 M NaCl and then, a further 10 times with 10 ml buffer B. The protein was eluted from the resin at an ambient temperature by incubation with 1.0 ml buffer B containing 20 mM glutathione and the beads were removed by centrifugation through a 0.44 micron filter. The eluate was divided into aliquots, snap frozen in liquid nitrogen and stored at −80°C. 0.1 mg GST-AtPDK1 and 0.8 mg GST-ΔPH-AtPDK1 were obtained.

2.3. Strains and constructs used for complementation of yeast ΔPkh1 ΔPkh2 mutants

The strains used in this study were AYS927 and AC306 [7]. Yeast cells were grown at 30°C on YPD medium, YPGal medium or SC medium containing galactose or glucose as the carbon source, supplemented with nutrients appropriately to maintain selection for plasmids [11]. Yeast cells were transformed by the lithium acetate-DMSO method [12]. All other yeast manipulations, sporulation and tetrad analysis were carried out using standard procedures. Deletion of the entire encoding region of the yeast genes *PKH1* and *PKH2* and verification of the yeast cell genotypes by PCR were described previously [7]. Analysis of viable spores was carried out both by plating on appropriate selective media and by PCR. The complete encoding region of the AtPDK1 gene was cloned into pYES2 yeast expression vector (Invitrogen) as a *Bam*HI/*Bam*HI insert downstream of the *GAL1* promoter to yield plasmid pYES2-AtPDK1. The diploid heterozygous yeast (strain AC306) lacking a single copy of *PKH1* and *PKH2* was described previously [7].

2.4. Protein lipid overlay

To assess the phospholipid binding properties of GST-AtPDK1, a protein lipid overlay was performed [8,13]. Synthetic PIs were from Echelon (Salt Lake City, UT, USA), phosphatidic acid from Calbiochem (Nottingham, UK) and other phospholipids from Avanti Polar Lipids (Alabaster, AL, USA). Briefly, 400 pmol (2 μl) of phospholipids dissolved in a 1:2:0.8 (by volume) mixture chloroform:methanol:water were spotted onto nitrocellulose HiBond-C extra (Amersham) and allowed to dry at room temperature for 1 h. The membrane was blocked in 3% (by mass) fatty acid free BSA in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.1% Tween-20 (by volume) for 1 h in a solution containing 0.5 μg/ml of the indicated protein and incubated overnight at 4°C. The membrane was washed three times for 10 min in TBST and then incubated for 1 h with a 1/2000 dilution of anti-GST monoclonal antibody (Sigma, clone 2 mouse IgG2b). The membrane was washed as before, then incubated for 1 h with a 1/2000 dilution of anti-mouse-HRP conjugate (SAPU, Lanarkashire, UK). Finally, the membrane was washed as above and the GST fusion protein bound to the membrane by virtue of its interaction with phospholipid was detected by enhanced chemiluminescence (ECL, Amersham). GST-PDK1 was expressed in 293 cells as described [14].

2.5. Measurement of PKB activation by AtPDK1

This assay was carried out in two stages [1]. In the first step, GST-PKBα or GST-S473D-PKBα was incubated with GST-AtPDK1 in the presence of MgATP and phospholipid vesicles containing 0.1 mM phosphatidylserine, 0.1 mM phosphatidylcholine with or without 10 μM *sn*-1-stearoyl, 2-arachidonoyl D-PtdIns(3,4,5,)P_3. In the second stage, Mg[γ-^{32}P]ATP was added and the reaction initiated with the specific PKBα peptide substrate RPRAATF.

3. Results

3.1. Cloning of a plant PDK1 homologue

Interrogation of the NCBI EST database with the DNA sequence encoding the kinase domain of human PDK1 revealed one plant EST from *Arabidopsis* (accession number N96553) encoding part of a protein kinase most homologous to the kinase domain of human PDK1. A full length clone was then isolated from an *Arabidopsis* cDNA library (see Section 2). The open reading frame encoded a protein of 491 amino acids with a molecular mass of 54.7 kDa. A stop codon immediately 5′ to the predicted initiating ATG codon indicated that the protein was indeed full length (Fig. 1). AtPDK1 possessed 40% overall identity with human PDK1 (Fig. 2A). A BLAST search of the NCBI database revealed that human PDK1 and PDK1 homologues in different species are the closest relatives of AtPDK1. Like the mammalian PDK1, AtPDK1 possessed a kinase domain at its N-terminus, displaying 49% identity with mammalian PDK1, 45% with *Drosophila* PDK1 [14], 40% with both the *S. cerevisiae* PDK1 homologues termed Pkh1 and Pkh2 [7] and 44% with the *Schizosaccharomyces pombe* PDK1 homologue termed Ksg1 [15].

We also identified an EST that encodes the partial sequence of the rice homologue of PDK1 (accession number D40851). This cDNA encoded most of the kinase domain and the entire C-terminal domain of this protein (Fig. 2A). The rice PDK1 homologue was 80% identical to AtPDK1, indicating a high conservation of the PDK1 gene between monocotyledons and dicotyledons (Fig. 2A).

Both of the plant PDK1s also possessed a PH domain at their C-terminus, in which the most conserved residues of the PH domains were present, including the invariant tryptophan, Trp-481 (Fig. 2B).

3.2. AtPDK1 is a functional homologue of S. cerevisiae Pkh1/Pkh2

We have shown previously that *S. cerevisiae* possesses two PDK1 homologues, termed *PKH1* and *PKH2*, which share a function essential for cell viability. Thus, haploid yeast cells in which the genes corresponding to both *PKH1* and *PKH2* are deleted are inviable, but overexpression of human PDK1 in these cells restores viability [7]. In order to determine whether the AtPDK1 can function as a homologue of *PKH1* and *PKH2*, we tested if it was able to rescue lethality caused by the double deletion of *PKH1* and *PKH2* in haploid yeast cells. Diploid heterozygous yeast cells possessing a single deletion of both the *PKH1* and *PKH2* genes (strain AC306) in which the *PKH1* gene was replaced with a *HIS3* marker and the *PKH2*

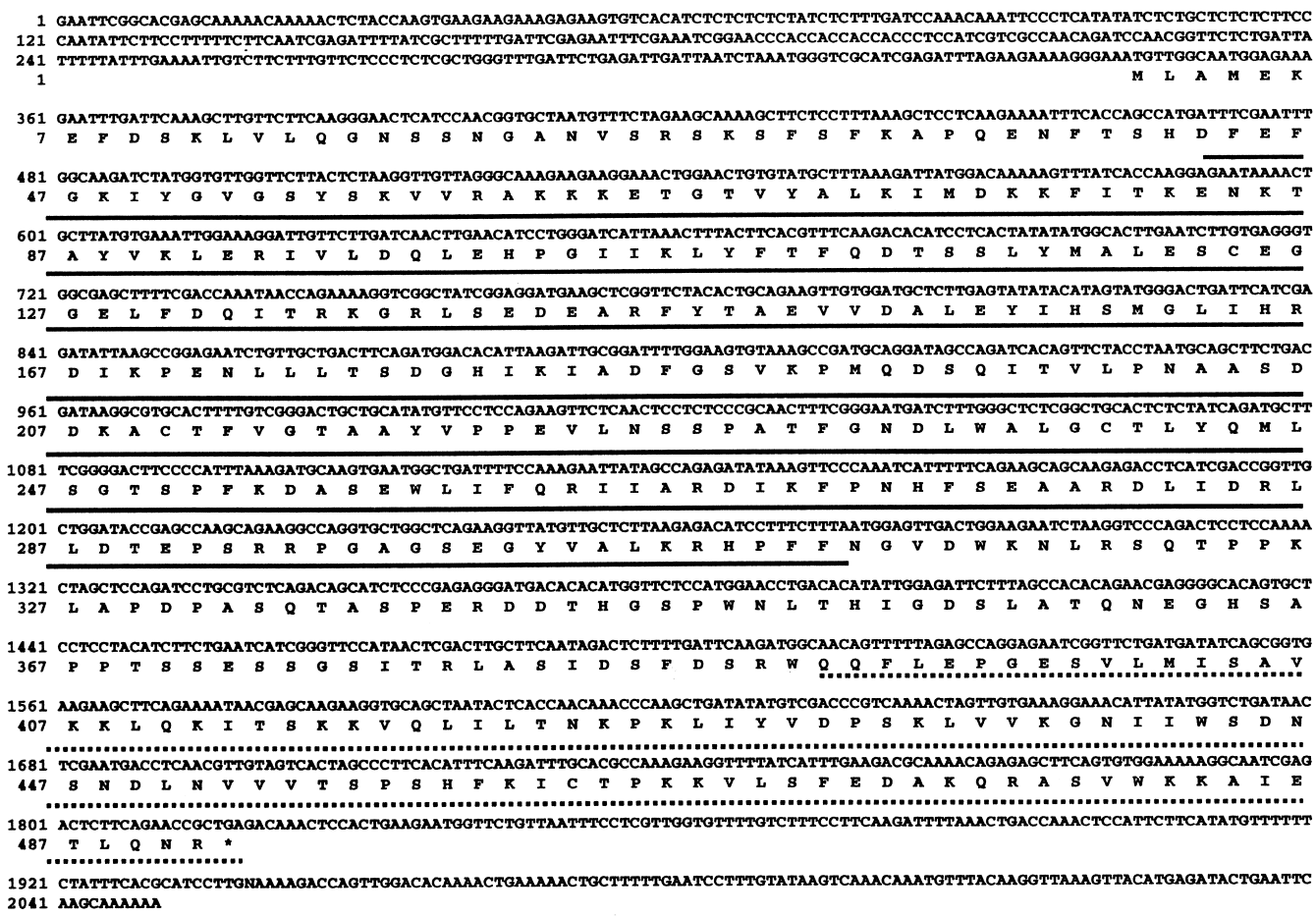

Fig. 1. Nucleotide and deduced amino acid sequence of AtPDK1. The underlined residues correspond to the kinase domain and the broken line the PH domain. The stop codon is denoted with an asterisk. The NCBI accession number for AtPDK1 is AF132742.

gene was replaced with a *TRP1* marker were transformed with an AtPDK1 expression construct in the pYES2 vector or an empty YES vector as a control and the sporulation was induced. This vector expresses AtPDK1 under a galactose inducible promoter and also contains a *URA3* marker that results in the vector being lost from cells grown on medium containing 5-fluoro-orotic acid (5-FOA) [16]. These yeasts were germinated on medium containing galactose, where all spores dissected were able to grow. Genotypes of the resultant haploid strains were analysed both by plating on appropriate selective media and by PCR. Several sets of representative spores, containing all four possible genotypes (wild-type, *PKH1 pkh2Δ*, *pkh1Δ PKH2* and *pkh1Δ Pkh2Δ*) were plated on medium containing either glucose, galactose or galactose and 5-FOA. On glucose medium (where AtPDK1 is not expressed), all the tetrads selected yielded three viable and one non-viable spore (Fig. 3A). None of the viable haploid cells were Trp^+ and His^+ and none was deleted in both the Pkh1 and Pkh2 genes. By contrast, when cells were grown on galactose containing medium which induces the expression of AtPDK1, all four spores were viable, including cells that were Trp^+ and His^+ (Fig. 3B). PCR analysis confirmed that both the *PKH1* and *PKH2* genes were deleted in these cells (data not shown). However, if the spores were grown on medium containing galactose and 5-FOA which induces the loss of the pYES2-AtPDK1 expression vector, only three viable spores were obtained, none of which was deleted in both the *PKH1* and *PKH2* genes (Fig. 3C). In the control experiments in which the AC306 yeast strain was transformed with the empty pYES2 vector, no viable haploid cells lacking both*PKH1* and *PKH2* were isolated following sporulation (data not shown). These results demonstrate that AtPDK1 is able to phosphorylate the same essential substrates in yeast cells as *PKH1* and *PKH2*.

3.3. AtPDK1 interacts with 3-PIs

AtPDK1 was expressed in human 293 cells as a fusion to glutathione S-transferase (GST). After purification from cell lysates by affinity chromatography on glutathione-sepharose, a single Coomassie blue staining band was observed on SDS-PAGE that migrated with the expected molecular mass (78 kDa). An AtPDK1 mutant containing only the first 390 amino acids which lacks the PH domain (GST-ΔPH-AtPDK1) was also expressed.

We studied the interaction between AtPDK1 and PI lipids using 'protein lipid overlay'[8,13]. Phospholipids and PIs were spotted on to a nitrocellulose membrane and incubated with GST-AtPDK1, GST-ΔPH-AtPDK1 and human GST-PDK1. The membrane was then washed and immunoblotted using a GST antibody to detect the GST PDK1 fusion proteins bound to the membrane by virtue of their interaction with lipid. As observed previously, using a different type of assay, human GST-PDK1 binds strongly to both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, but not to other lipids tested. In contrast,

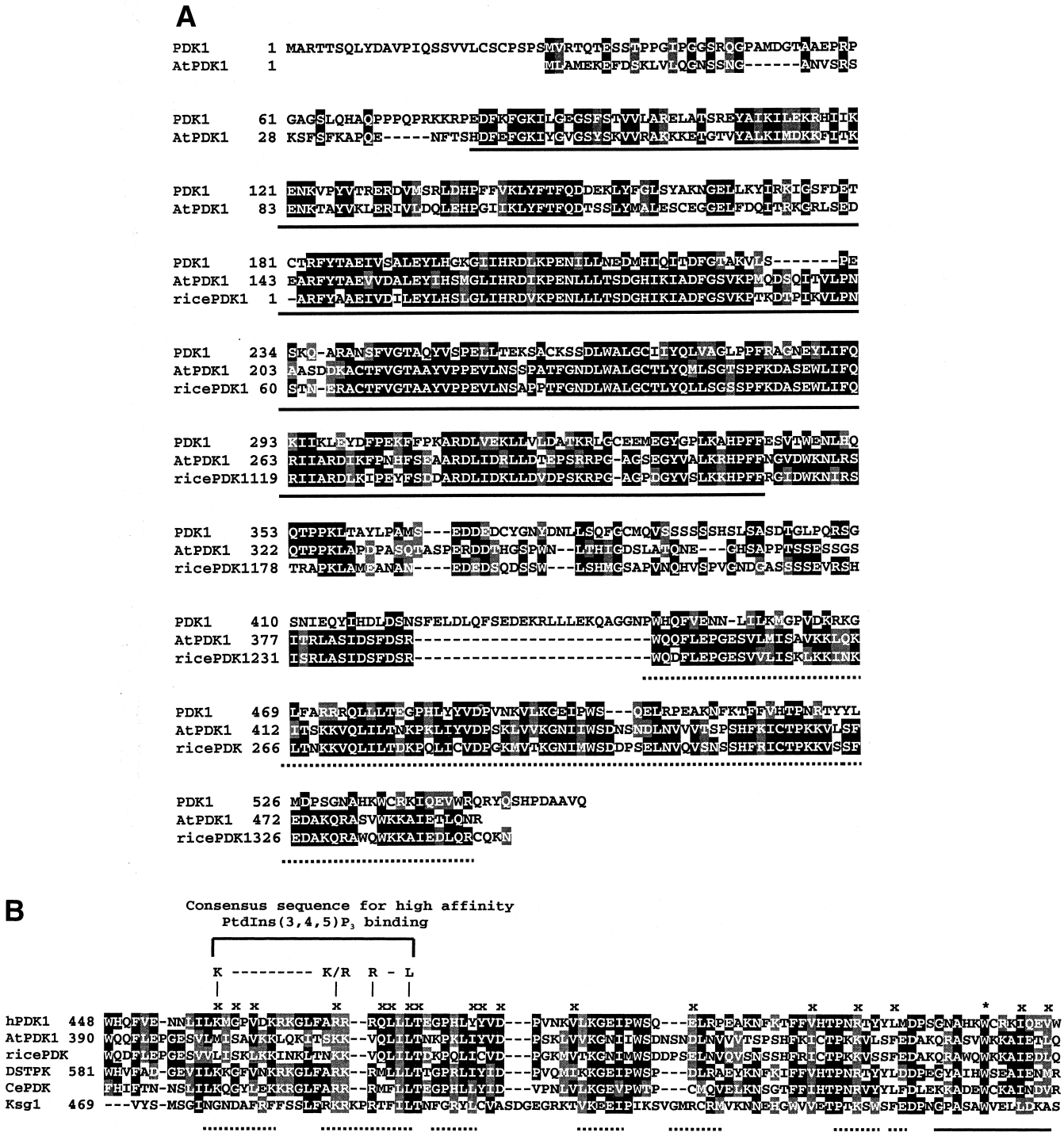

Fig. 2. Alignment of the amino acid sequences of human, *Arabidopsis* and rice PDK1. (A) The alignment was carried out using the Clustal W program [25]. Identities are shaded in black and homologies in grey. The NCBI accession numbers for human PDK1 is AF017995, for AtPDK1 is AF132742 and for the partial rice PDK1 sequence is AF132743. The underlined residues correspond to the kinase domains and the broken line to the PH domain. (B) Evidence that plant PDK1 possesses a PH domain. Sequence alignment of the C-terminal region of AtPDK1 with the PH domains of PDK1 from human, *Drosophila* (NCBI accession number Y07908), *C. elegans* (EMBL accession number H33H01) and *S. pombe* (NCBI, accession number X99280). The alignment was carried out using the Clustal W program [25]. Identities are shaded in black and similar residues in grey. The regions of the PH domain predicted to form β-strands are indicated with dotted lines and that predicted to form an α-helix by a solid line. Residues conserved in all PH domain family members are indicated with an X. The conserved tryptophan residue (equivalent to Trp-535 in human PDK1) found in all PH domains is marked with an asterisk. The four key residues, required for a high affinity interaction with PtdIns(3,4,5)P_3 [21] are indicated, the first and third are missing in plant PDK1. Numbering is based on the sequences in the data base. No numbering is used for the rice and *C. elegans* PDK1 in which the full length protein sequence is currently not known.

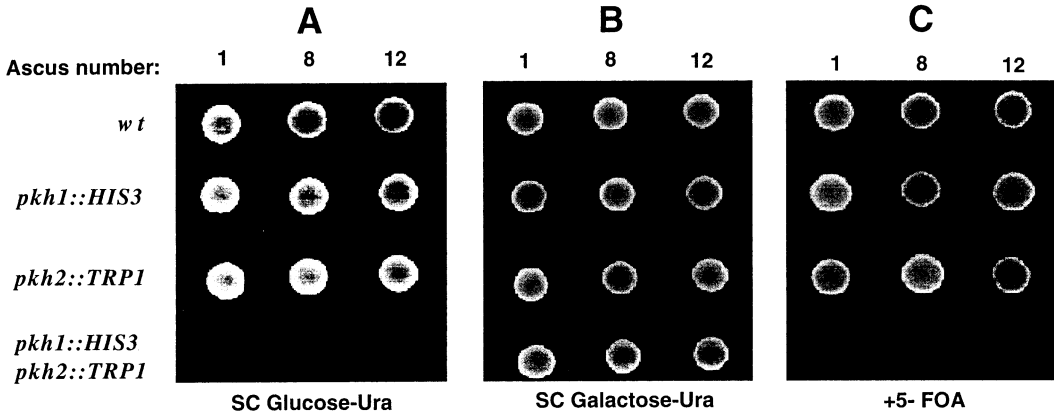

Fig. 3. AtPDK1 rescues lethality caused by loss of the PDK1 genes in *S. cerevisiae*. The strain AC306 (*MAT*a/*MAT*α *PKH1*/*pkh1Δ::HIS3 PKH2*/*pkh2Δ::TRP1*) that was transformed with plasmid pYES2-AtPDK1 (which is marked with *URA3*) was induced to sporulate and the resultant haploid cells were analysed by growth on medium containing glucose, which represses AtPDK1 expression (A), or medium containing galactose, which induces the expression of AtPDK1 (B), or on medium containing 5-FOA which selects for loss of the plasmid (C). The phenotype of each spore is indicated and was determined by growth on appropriate medium lacking histidine and/or tryptophan and by PCR analysis.

GST-AtPDK1 was much more promiscuous in its lipid binding properties and interacted with phosphatidic acid and PtdIns3P as well as PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 and also to a lesser extent with PtdIns(4,5)P_2 and PtdIns4P (Fig. 4). However, GST-AtPDK1 did not bind to other acidic phospholipids, such as phosphatidylserine, phosphatidylglycerol, PtdIns5P, PtdIns(3,5)P_2 or the zwitterionic phospholipids phosphatidylcholine and phosphatidylethanolamine. The interaction of AtPDK1 with lipids was mediated by the PH domain because the GST-ΔPH-AtPDK1 mutant did not interact with any lipid tested (Fig. 4).

3.4. *AtPDK1 can activate PKBα*

AtPDK1 activity was measured by its ability to activate wild-type GST-PKBα or a mutant of PKBα in which Ser-473 is changed to an Asp (GST-S473D-PKBα). As expected, this activation of the PKB forms required the presence of lipid vesicles containing PtdIns(3,4,5)P_3 that binds to PKBα and permits it to become phosphorylated and activated by PDK1 (Fig. 5). These findings demonstrate that AtPDK1 is capable of inducing the phosphorylation of human PKBα at Thr-308. Addition of PtdIns3-P and/or phosphatidic acid to lipid vesicles containing PtdIns(3,4,5)P_3 did not lead to an increase in the rate at which AtPDK1 phosphorylated and activated GST-473D-PKBα (data not shown). AtPDK1 was over 100-fold less effective than mammalian PDK1 in phosphorylating GST-473D-PKBα or GST-PKBα.

4. Discussion

Mammalian PDK1 phosphorylates and activates a group of the AGC subfamily of protein kinases including PKB isoforms, p70 S6K, PKC isoforms and SGK [4]. We have identified in *Arabidopsis* a protein kinase which is more highly related in sequence to PDK1 than to any other known protein kinase. Like the PDK1 homologues found in mammals, *Drosophila* and *S. cerevisiae*, plant PDK1 possesses a PH domain at its C-terminus and phosphorylates and activates PKBα in

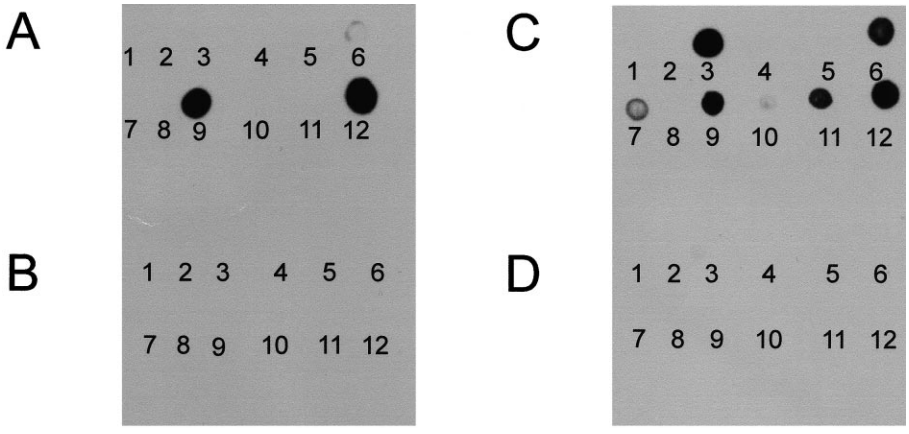

Fig. 4. The phospholipid binding properties of the *Arabidopsis* PDK1 and human PDK1. The ability of the *Arabidopsis* and human PDK1 GST fusion proteins to bind a variety of phospholipids (see below) was analysed using a protein lipid overlay. The indicated phospholipids were spotted onto a nitrocellulose membrane which was then incubated with human GST-PDK1 (A), human GST-ΔPH-PDK1 (B), GST-AtPDK1 (C), GST-ΔPH-ATPDK1 (D). The membranes were washed and the GST fusion proteins detected using a GST antibody. A representative experiment of three is shown. Lipid 1, phosphatidylcholine; Lipid 2, phosphatidylethanolamine; Lipid 3, phosphatidic acid; Lipid 4, phosphatidylserine; Lipid 5, phosphatidylinositol; Lipid 6, PtdIns3P, Lipid 7, PtdIns4P, Lipid 8, PtdIns5P; Lipid 9, PtdIns(3,4)P_2, Lipid 10, PtdIns(3,5)P_2, Lipid 11, PtdIns(4,5)P_2 and Lipid 12, PtdIns(3,4,5)P_3.

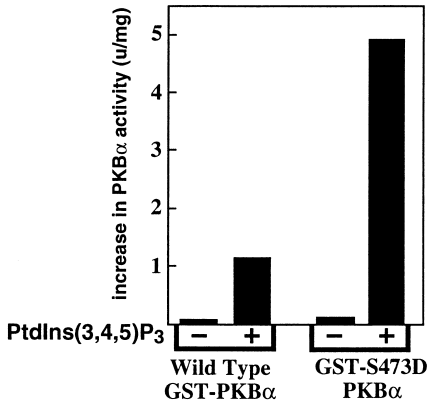

Fig. 5. *Arabidopsis* PDK1 activates human PKBα as a substrate. GST-PKBα, GST-473D-PKBα was incubated for 30 min at 30°C with either AtPDK1, or in buffer and 100 μM ATP as a control, in the presence or absence of phospholipid vesicles containing 100 μM phosphatidylcholine, 100 μM phosphatidylserine and 10 μM *sn*-1-stearoyl-2-arachidonyl-D-PtdIns(3,4,5)P_3. Reactions were terminated by adjusting the mixtures to a final concentration of 1% (by volume) Triton X-100 to dissolve the lipid vesicles and the resulting increase in the specific activity (U/mg) of GST-PKBα was then determined in the presence of [γ-^{32}P]ATP, as described previously [1].

the presence of PtdIns(3,4,5)P_3. However, the rate at which AtPDK1 phosphorylates PKBα is much lower than that observed with human PDK1 and this may reflect a significant difference in substrate specificity between the plant and human PDK1 isoforms. The most convincing functional evidence that AtPDK1 is a homologue of mammalian PDK1 is that it is able to rescue the lethal phenotype in *S. cerevisiae* caused by deletion of the *PKH1* and *PKH2* genes. This indicates that AtPDK1, like human PDK1, must be able to phosphorylate one or more protein substrates in *S. cerevisiae* thought to comprise Ypk1, Ykr2, Pkc1 and Sch9 [7] whose phosphorylation by Pkh1 and Pkh2 is essential for cell viability.

Two related proteins in *Arabidopsis* termed AtS6k1 [17] (also termed Atpk1 [18] and cATPK19 [19]) and AtS6k2 [17] (also termed atpk2 [18] and cATPK6 [19]) have been identified that are highly homologous to the mammalian p70 S6 kinase, but no other members of the AGC subfamily of protein kinases (including PKA, PKB and PKC isoforms) have been identified so far in plants. When expressed in plant cells, AtS6k2 can phosphorylate the S6 ribosomal protein in vitro and there is limited evidence that AtS6k1 and AtS6k2 may also phosphorylate the S6 protein in vivo [17]. Both the AtS6k1 and AtS6k2 proteins possess a good consensus sequence for phosphorylation by PDK1 [4] in the T-loop of the kinase domain (Ser-290-AtS6k1, Ser-296-AtS6k2). It seems that AtS6k1 and AtS6k2 will prove to be substrates for AtPDK1 in vivo. The signals that activate AtS6k1 and AtS6k2 in plants are unknown, though it has been reported that the phytohormone cytokinin increases S6 phosphorylation in detached pumpkin cotyledons [20]. Therefore, it is possible that plant hormones can activate a signalling pathway that can induce AtPDK1 to phosphorylate and activate AtS6k1 and AtS6k2.

We have shown that the PH domain of AtPDK1 interacts rather promiscuously with PtdIns 3P, PtdIns (4,5)P_2, PtdIns 4P, PtdIns(3,4)P_2 and phosphatidic acid. Our studies indicate that the interaction of AtPDK1 with PtdIns(3,4,5)P_3 was markedly weaker than that of the human PDK1 with this phospholipid as measured by surface plasmon resonance (data not shown). This observation is likely to be explained by recent work [21] that has identified four key conserved amino acid residues lying at the N-terminal region of the PH domain required for high affinity binding of PtdIns(3,4,5)P_3 lying in a motif K-X_{12-14}-R/K-X-R-Hyd, where X is any amino acid and Hyd is a hydrophobic amino acid (see Fig. 2B). All proteins that bind with a high affinity to PtdIns(3,4,5)P_3 such as PKB, human PDK1, GRP1 and Bruton tyrosine kinase possess this motif and mutation of the conserved residues abolishes the interaction of these proteins with PtdIns(3,4,5)P_3. The key residues of this PtdIns(3,4,5)P_3 binding motif are conserved in the human PDK1, *Drosophila* PDK1 and *C. elegans* PDK1 homologues, but two out of the four residues are not conserved in the plant PDK1 and one of the residues is not conserved in *S. pombe* PDK1 (Fig. 2). This is consistent with the presence of class I PI 3-kinases and a role for PtdIns(3,4,5)P_3 binding to the PH domain of PDK1 in mammals, *Drosophila* and *C. elegans*, but not in plants or *S. pombe* which do not appear to possess class I PI 3-kinases. In plants and *S. pombe*, it is likely that other PI(s) interact(s) with the PH domain of PDK1, perhaps localising it to a particular site in the cell or enabling it to phosphorylate downstream of kinase substrates in response to extracellular signals.

Thus far, no homologues of the class I PI 3-kinase have been found in plants and no PtdIns(3,4,5)P_3 has been detected in any plant tissue [22]. Plant cells do possess high levels of PtdIns3P, PtdIns(4,5)P_2, PtdIns4P, as well as PtdIns(3,4)P_2, PtdIns(3,5)P_2 and phosphatidic acid, but the roles that these lipids play in signalling pathways are unknown [22]. No agonist has thus far been demonstrated to increase the levels of 3-PIs in plant cells, although the rate at which PtdIns3P and PtdIns(3,4)P_2 turn over in plant cells is relatively high, suggesting that these lipids may have a signalling role [22]. PtdIns(3,4)P_2 could be synthesised by the phosphorylation of PtdIns4P at the 3-position of the inositol ring by a PtdIns4P-3 kinase as occurs in activated platelets [23] or via a 4-kinase acting directly on PtdIns3P. Phosphatidic acid is produced in plants as in mammalian cells through the phospholipase D-catalysed hydrolysis of phospholipids. In plants, phosphatidic acid has been implicated in many processes including senescence, aging, stress responses and pathogenic attack [22,24]. Future studies will be required to establish whether the interaction of AtPDK1 with phosphatidic acid is of physiological importance.

Apart from plant PDK1, only one other plant protein, a PI-4 kinase, has been found to contain a PH domain. The plant PI 4-kinase PH domain interacts with PtdIns(4,5)P_2, PtdIns4P and phosphatidic acid, but not with 3-PIs [8]. *S. cerevisiae* does not possess homologues of class I PI 3-kinases and, consistent with this, the Pkh1 and Pkh2 proteins do not possess a PH domain and do not interact with 3-PIs [7]. While this work was in progress, a PDK1 homologue which is essential for cell viability, termed ksg1, was identified in *S. pombe* [15], which like the plant PDK1 homologue possesses a PH domain. No class I PI 3-kinase and neither PtdIns(3,4)P_2 or PtdIns(3,4,5)$_3$ have been detected in this organism. It thus appears in both plants and *S. pombe* phospholipds other than PtdIns(3,4)P_2 or Ptd(3,4,5)$_3$ are likely to

play key roles in regulating the activity and/or cellular location of PDK1.

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