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Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates

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3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylates and activates many kinases belonging to the AGC subfamily. PDK1 possesses a C-terminal pleckstrin homology (PH) domain that interacts with PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 and with lower affinity to PtdIns(4,5)P_2. We describe the crystal structure of the PDK1 PH domain, in the absence and presence of PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_4. The structures reveal a 'budded' PH domain fold, possessing an N-terminal extension forming an integral part of the overall fold, and display an unusually spacious ligand-binding site. Mutagenesis and lipid-binding studies were used to define the contribution of residues involved in phosphoinositide binding. Using a novel quantitative binding assay, we found that Ins(1,3,4,5,6)P_5 and InsP_6, which are present at micromolar levels in the cytosol, interact with full-length PDK1 with nanomolar affinities. Utilising the isolated PDK1 PH domain, which has reduced affinity for Ins(1,3,4,5,6)P_5/InsP_6, we perform localisation studies that suggest that these inositol phosphates serve to anchor a portion of cellular PDK1 in the cytosol, where it could activate its substrates such as p70 S6-kinase and p90 ribosomal S6 kinase that do not interact with phosphoinositides.

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Introduction

3-phosphoinositide-dependent protein kinase-1 (PDK1) plays an important role in insulin and growth factor signalling cascades (Toker and Newton, 2000; Mora *et al*, 2004). It is known to phosphorylate and activate at least 24 protein kinases that belong to the AGC (cAMP-dependent, cGMP-dependent, protein kinase C (PKC)) family of protein kinases. These comprise isoforms of protein kinase B (PKB, also known as Akt), p70 ribosomal S6-kinase (S6K), serum and glucocorticoid responsive kinase (SGK), p90 ribosomal S6 kinase (RSK) and PKC. PDK1 activates these enzymes by phosphorylating a Ser/Thr residue in their activation loop. PDK1 is a 556 amino-acid protein comprising an N-terminal Ser/Thr kinase catalytic domain (residues 71–359) and a C-terminal pleckstrin homology (PH) domain (residues 459–550) (Alessi *et al*, 1997; Stephens *et al*, 1998). The latter binds with high affinity to the second messenger phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) (Stephens *et al*, 1998; Currie *et al*, 1999), generated through insulin- and growth factor-induced activation of PI 3-kinase (Vanhaesebroeck *et al*, 2001).

Much research has shown that insulin and growth factors control PDK1 by regulating its interaction with substrates, rather than by directly activating PDK1. PKB, in contrast to other PDK1 substrates, possesses a PtdIns(3,4,5)P_3-binding PH domain. The mutual ability of PKB and PDK1 to interact with PtdIns(3,4,5)P_3 via their PH domains is likely to play a crucial role in regulating PKB activation (Anderson *et al*, 1998; Currie *et al*, 1999; Filippa *et al*, 2000). Consistent with this notion, PKB was not substantially activated in a knockin ES cell line expressing an active form of PDK1 that cannot interact with PtdIns(3,4,5)P_3 (McManus *et al*, 2004). Recent structural and cellular studies have also indicated that binding of PKB to PtdIns(3,4,5)P_3 induces a major conformational change (Mora *et al*, 2004) that is likely to be required for PKB activation by PDK1 (Alessi *et al*, 1997; Stokoe *et al*, 1997). Importantly, binding of PtdIns(3,4,5)P_3 to the PDK1 PH domain does not directly affect activity of the PDK1 kinase domain, but stimulates PKB activation by inducing the co-localisation of PKB and PDK1 (Stephens *et al*, 1998; Currie *et al*, 1999).

The other substrates that PDK1 activates, such as S6K, RSK and SGK, are phosphorylated by PDK1 in the cytosol, rather than at the plasma membrane. Moreover, these substrates do not possess PH domains or interact with phosphoinositides. Extracellular agonists regulate activation of S6K, RSK and SGK through PDK1, by inducing the phosphorylation of these enzymes at a conserved C-terminal (noncatalytic) hydrophobic motif, which creates a high-affinity docking site for interaction with a pocket located on the kinase domain of PDK1 (reviewed in Mora *et al*, 2004). This has recently been supported by a knockin cell line in which the PDK1 hydrophobic motif docking site is disrupted (Collins *et al*, 2003).

The PDK1 PH domain sequence is unusual compared to other PH domains and several computer algorithms, such as the Simple Modular Architecture Research Tool or PROSITE, fail to classify it as a PH domain. However, lipid-binding studies have firmly established that PDK1 binds PtdIns(3,4,5)P_3 through a C-terminal domain, and truncation

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or mutation of several conserved residues found in this domain abolishes PtdIns(3,4,5)P_3 binding (Anderson *et al*, 1998; Currie *et al*, 1999). In addition to binding PtdIns(3,4,5)P_3, PDK1 also binds with similar affinity to PtdIns(3,4)P_2, a potential signalling lipid generated through the dephosphorylation of PtdIns(3,4,5)P_3 by the SHIP 5-phosphatase (Stephens *et al*, 1998; Currie *et al*, 1999). Surface plasmon resonance-based lipid-binding assays revealed that PDK1 also interacts with PtdIns(4,5)P_2, the substrate for PI 3-kinase, albeit with a 15-fold lower affinity compared to PtdIns(3,4,5)P_3 (Currie *et al*, 1999). This weaker affinity for PtdIns(4,5)P_2, may be significant as the basal cellular levels of this phospholipid are 50–200-fold higher than those of PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 in insulin-stimulated cells (Batty and Downes, 1996). Consistent with this notion, quantitative electron microscopy localisation studies revealed that in unstimulated cells $\sim$10% of the cellular pool of PDK1 is associated with the plasma membrane in a manner dependent on an intact PH domain, which could be mediated through the ability of PDK1 to bind PtdIns(4,5)P_2 (Currie *et al*, 1999). Whether insulin or growth factor stimulation induces further localisation of PDK1 at the plasma membrane is controversial (Anderson *et al*, 1998; Currie *et al*, 1999). Nevertheless, a significant pool of PDK1 is required in the cytoplasm to activate its other non-lipid-binding substrates such as S6K, RSK and SGK. The mechanism by which PDK1 is anchored in the cytoplasm is not known. Here we define the molecular basis by which PDK1 interacts with its phosphoinositide ligands and show that PDK1 may be anchored in the cell cytosol through its interaction with D6-phosphorylated inositol phosphates.

Results and discussion

Structure determination of PDK1 PH domain

Constructs containing the predicted PH domain of PDK1 designed on the basis of sequence alignments with previously crystallised PH domains produced insoluble protein. To identify the boundaries of a soluble fragment of PDK1 containing the PH domain, a limited proteolysis approach was performed (described in Komander *et al*, 2004), resulting in a proteolytically stable fragment of PDK1 comprising residues 408–556. We cloned and expressed in *Escherichia coli* a fragment of PDK1 encompassing residues 409–556, which showed good stability after purification and was readily crystallisable (Komander *et al*, 2004). The protein crystals, grown in the presence or absence of phosphoinositides, diffracted to below 1.50 Å on a rotating anode generator. The single cysteine in the sequence (Cys536) was exploited to obtain experimental phases, by derivatisation with AuCN. The structure of the PDK1 PH domain in complex with Ins(1,3,4,5)P_4 was solved by a two-wavelength MAD experiment (Table I). The resulting map allowed building of residues 409–549, and showed well-defined electron density for Ins(1,3,4,5)P_4 (Figure 1A).

Overall structure of the 'budded' PDK1 PH domain

The structure of the PDK1 PH domain reveals a structural variation of a standard PH domain fold (Lemmon and Ferguson, 2000), with an additional 'bud' at the N-terminus (Figures 1B–D). Residues 456–530 fold into two, almost orthogonal, β sheets, one consisting of four (β1–β4) and one of three strands (β5–β7), constituting a barrel-like structure (Figures 1B–D). On one side, the barrel is blocked by a C-terminal α helix (residues 531–549, Figures 1B and C). The other side of the barrel consists of three loops (termed variable loop (VL) 1–3 (Ferguson *et al*, 1994)), forming a bowl-like structure which is lined by positively charged residues, constituting the phosphoinositide-binding site. N-terminal to the observed standard PH domain fold, the PDK1 PH domain possesses an extension, here referred to as the 'bud'. This consists of two additional β strands, which extend the β1–β4 sheet in an antiparallel fashion, and an additional α helix, which packs against the newly formed six-stranded β sheet (Figures 1B and D). The bud packs against the β1–β4 sheets of the PH domain, through several hydrophobic contacts (Figure 1D), but also through β-sheet hydrogen bonds between strands β2′ and β1. Overall, the bud of the PDK1 PH domain buries more than 30% of the surface of the standard PH domain fold (567 Å^2 of 1749 Å^2), and a hydrophobic core is created between the α1′ helix and residues on one face of the six-stranded β sheet (Figure 1D). Residues Ile416, Phe424, Leu426, Leu456, Ile457, Met460, Leu477, Tyr485, Val487 and Leu494 from the six-stranded β sheet interact with residues Ile412, Phe430, Leu438, Leu439, Trp448, Phe451, Val452 and Leu456 from the α1′ helix and surrounding loops (Figure 1D). The presence of this additional hydrophobic core, which lies outside the standard PH domain fold, suggests that the bud is an integral part of the overall fold. This could explain the insolubility of protein obtained from initial constructs which covered the standard PH domain only (residue 458–556) (Komander *et al*, 2004). A bud-like extension of a PH domain fold as observed in PDK1 has not been described to date in any other crystallised PH domain containing protein. Although interactions of PH domains with other proteins or protein domains have been described in structures of Gβγ binding to the GRK2 PH domain (Lodowski *et al*, 2003), myotubularin-related protein phosphatase MTMR2 (Begley *et al*, 2003), or the Rac1-Tiam1-like complexes (Worthylake *et al*, 2000), none of these show the antiparallel β-sheet extension observed in the PDK1 PH domain and, therefore, do not resemble the bud on PDK1.

Interactions with the PtdIns(3,4,5)P_3 head group, Ins(1,3,4,5)P_4

The structure of the PDK1 PH domain was determined from a crystal that was grown in the presence of Ins(1,3,4,5)P_4, and well-defined ligand density could be observed in the experimental maps (Figure 1A, Table I). The PDK1 PH domain binds Ins(1,3,4,5)P_4 in a location equivalent to that described for other PH domains (Baraldi *et al*, 1999; Ferguson *et al*, 2000; Lietzke *et al*, 2000; Thomas *et al*, 2002), that is, within a shallow but positively charged pocket formed by VL1-3, at the open end of the β barrel (Figure 1). The phosphoinositide-binding site is lined by positively charged residues that contact the phosphates of Ins(1,3,4,5)P_4, and a total of 11 hydrogen bonds are possible between the PDK1 PH domain and Ins(1,3,4,5)P_4 (Figure 2A). Arg472 contacts the phosphate at the D1 position (D1-phosphate) with two hydrogen bonds. The D3-phosphate is contacted by Arg474 (two hydrogen bonds) and Lys465. Lys495 is in close proximity (4.4 Å) to the D3-phosphate. The D4-phosphate forms up to four hydrogen bonds to Lys465, Tyr486, and Arg521. Finally, the D5-phosphate makes one hydrogen bond to Lys467 (Figures

Table I Data collection and refinement statistics

	PDK1 PH domain –Ins(1,3,4,5,)P_4		PDK1 PH domain uncomplexed	PDK1 PH domain–PtdIns(3,4,5)P_3
Beamline	BM-14 λ_1	BM-14 λ_2	Rotating anode	Rotating anode
Wavelength (Å)	0.861	1.040	1.54	1.54
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Unit cell (Å)	$a=35.40$ $b=58.92$ $c=36.58$ $\beta=101.48°$	$a=35.42$ $b=58.94$ $c=36.58$ $\beta=101.47°$	$a=42.69$ $b=65.28$ $c=101.12$ $\beta=97.48°$	$a=36.26$ $b=58.94$ $c=36.44$ $\beta=102.34°$
Resolution (Å)	25.0–1.50 (1.58–1.50)	25.0–1.80 (1.86–1.80)	30.0–1.45 (1.50–1.45)	30.0–1.45 (1.50–1.45)
Observed reflections	85433	33500	422700	73746
Unique reflections	23341	13556	95063	25810
Redundancy	3.7 (3.3)	2.5 (2.1)	4.5 (3.0)	2.9 (2.7)
Completeness (%)	98.6 (99.5)	95.5 (95.6)	97.5 (80.1)	99.7 (100)
R_{merge}	0.076 (0.439)	0.043 (0.181)	0.048 (0.397)	0.054 (0.372)
$I/\sigma I$	17.7 (3.2)	17.3 (6.4)	13.2 (2.7)	8.8 (2.5)
Reflections in test set	617		934	545
R_{cryst}	0.150		0.171	0.185
R_{free}	0.204		0.211	0.237
Phasing statistics				
⟨FOM⟩	0.44 (25.0–2.5 Å)			
Phasing power	1.11 (25.0–2.5 Å)			
Number of groups				
Protein residues	143		573	143
Water	217		715	163
Ligand atoms	28		—	33
SO_4^{2-} atoms	—		50	—
Glycerol atoms	6		24	—
Wilson *B* ($Å^2$)	14.5		19.5	23.0
⟨*B*⟩ protein ($Å^2$)	14.6		18.6	30.3
⟨*B*⟩ water ($Å^2$)	27.6		35.8	45.4
⟨*B*⟩ ligand ($Å^2$)	14.6		—	36.2
R.m.s.d. from ideal geometry				
Bond length (Å)	0.020		0.015	0.016
Bond angles (deg)	1.8		1.6	1.7
Main chain *B* ($Å^2$)	1.6		1.5	1.7

Values between brackets are for the highest resolution shell. All measured data were included in structure refinement.

2A and 4A). Neither the D2- nor the D6-hydroxy groups show any direct hydrogen bonds to the protein (Figure 2A).

Figure 2 shows a comparison of the structures of the PDK1, DAPP1 (Ferguson *et al*, 2000) and PKBα (Thomas *et al*, 2002) PH domains in complex with Ins(1,3,4,5)P_4. DAPP1 binds Ins(1,3,4,5)P_4 in a similar fashion to GRP1 (Ferguson *et al*, 2000; Lietzke *et al*, 2000) and Btk (Baraldi *et al*, 1999). The orientation of Ins(1,3,4,5)P_4 in PDK1 is identical to that found in DAPP1/Btk/GRP1 (Figure 2A and B), while in PKBα the Ins(1,3,4,5)P_4 molecule is rotated by 60° (Figure 2C) (Thomas *et al*, 2001). However, in all PH domain Ins(1,3,4,5)P_4 complex structures described to date, Ins(1,3,4,5)P_4 interacts with both protein side chains as well as the backbone, whereas in PDK1 Ins(1,3,4,5)P_4 interacts with protein side chains only. This results in a significantly reduced number of protein–ligand hydrogen bonds in the PDK1 PH domain Ins(1,3,4,5)P_4 complex (a total of 11), compared to the PKBα (15 hydrogen bonds), DAPP1 (15 hydrogen bonds), Grp1 (16 hydrogen bonds) and Btk (16 hydrogen bonds) PH domain–Ins(1,3,4,5)P_4 complexes (Figure 2). A major difference between the PH domain–Ins(1,3,4,5)P_4 complexes is the position of VL1 (Figure 2D). In the structures of the DAPP1/GRP1/Btk PH domains, VL1 envelopes the D5-phosphate group, which in the case of DAPP1 forms four backbone hydrogen bonds with VL1. In the case of the PDK1 PH domain, the D5-phosphate forms only a single hydrogen bond to Lys467, the residue preceding VL1, and no further direct interactions between VL1 and the inositol headgroup are observed (Figure 2A). In the case of PKBα, due to different orientation of Ins(1,3,4,5)P_4, VL1 interacts with the D1-phosphate, leaving the D5-phosphate solvent exposed (Figure 2C). For DAPP1/GRP1/Btk and PKBα, backbone hydrogen bonds between the D2-hydroxyl groups are observed. In the case of PDK1, there is no direct interaction of the D2-hydroxyl group with the protein; instead, we observe it binding to the protein via an ordered water molecule. Strikingly, in the PDK1 Ins(1,3,4,5)P_4-binding pocket, a layer of five-ordered water molecules (*B* factors 10.7–23.4 $Å^2$) separate Ins(1,3,4,5)P_4 from the protein. The water molecules mediate a number of hydrogen bonds from Ins(1,3,4,5)P_4 to protein side chains and backbone (Figure 2A). One of the water molecules is consistently found in the other PH domain–Ins(1,3,4,5)P_4 complexes, in a similar position and contacting the D3-phosphate (coloured yellow, Figure 2). The presence of the four additional ordered water molecules is surprising, as this would appear to be entropically unfavourable. Overall, the PDK1 PH domain phosphoinositide pocket is significantly more spacious than

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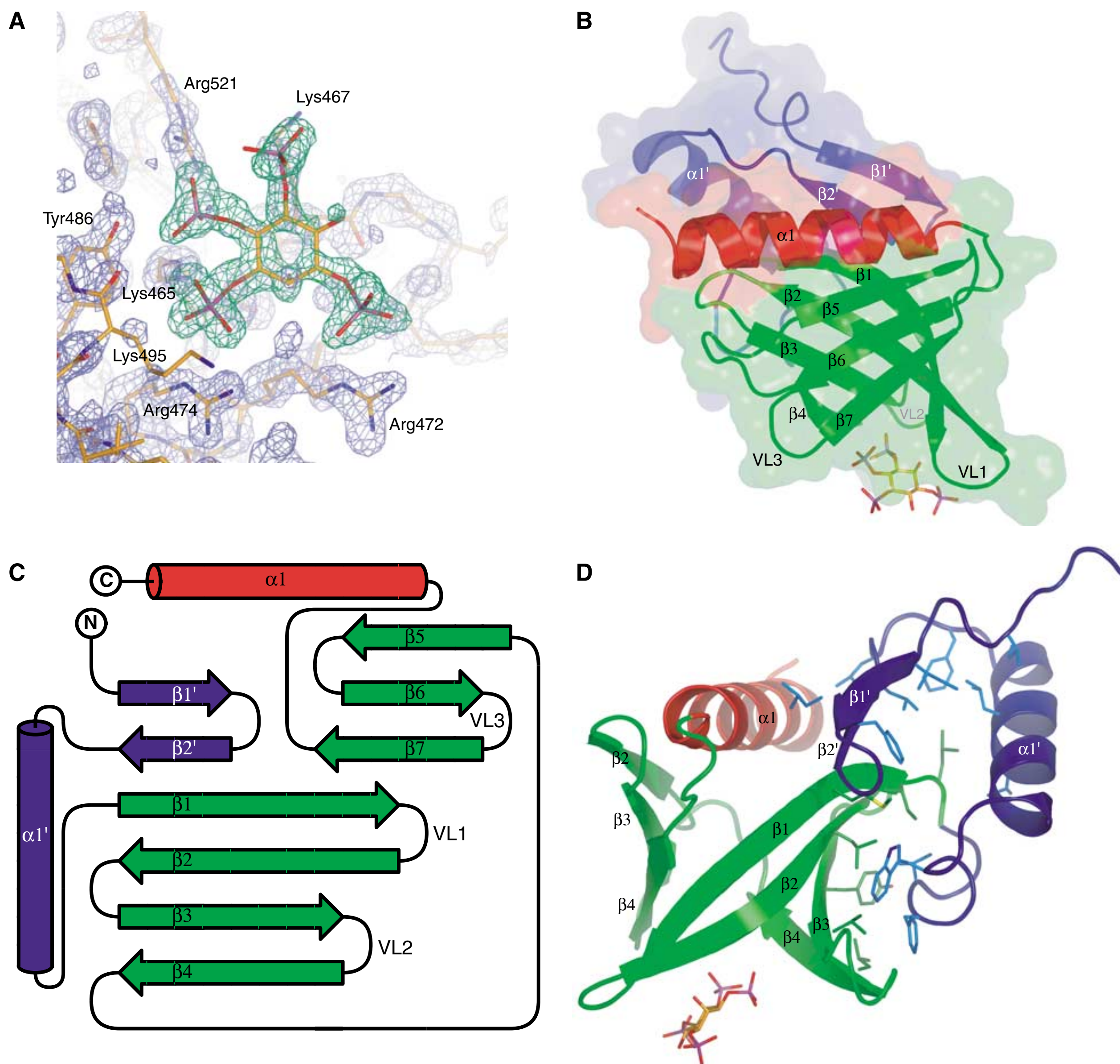

Figure 1 (**A**) Electron density maps calculated from experimental phases after solvent flattening, contoured at 0.6σ. Blue electron density covers the protein, the density for the phosphoinositide ligand is shown in green, for clarity. The refined, final model is shown as stick representation. (**B**) Ribbon representation of the PDK1 PH domain (residues 409–549). The standard PH domain fold is coloured green and red (β strands and the C-terminal α helix, respectively). The extension on the N-terminus is coloured blue. The Ins(1,3,4,5)P_4 ligand is shown as a stick representation. A coloured, semitransparent surface covers the protein molecule. (**C**) Topology diagram of the PH domain of PDK1, coloured according to B. (**D**) Hydrophobic interactions of the six-stranded β sheet with the N-terminal α1′ helix. Hydrophobic residues on the six-stranded β sheet and on the α1′ helix are shown as a stick representation, as is the Ins(1,3,4,5)P_4 ligand.

that of other Ins(1,3,4,5)P_4-binding PH domains. Perhaps, this more spacious binding pocket explains the observed ability of the PDK1 PH domain to bind different stereoisomers of phosphoinositides (Stephens *et al*, 1998).

Interactions of the PDK1 PH domain with diC4-PtdIns(3,4,5)P_3

It is possible that some of the water molecules close to the D1-phosphate are replaced by the glycerol backbone upon PtdIns(3,4,5)P_3 binding. To investigate this, the PDK1 PH domain was co-crystallised with a PtdIns(3,4,5)P_3 analogue, containing two C4 acyl chains (diC4-PtdIns(3,4,5)P_3). Crystals were obtained under similar conditions as for the Ins(1,3,4,5)P_4 complex, and were of the same space group with similar unit cell dimensions (Table I). After refinement of the protein model, well defined $|F_o|-|F_c|$ density was visible for the inositol ring, the phosphates and the glycerol backbone (Figure 3A). A partial model for diC4-PtdIns(3,4,5)P_3 was then included in the refinement (Table I). Analysis of the resulting model shows that while Arg472 coordinates the free oxygen atoms on the D1-phosphate, the oxygen atom involved in the ester bond to the glycerol does not make any significant contact with the protein. Of the lipid moiety, only the glycerol backbone is

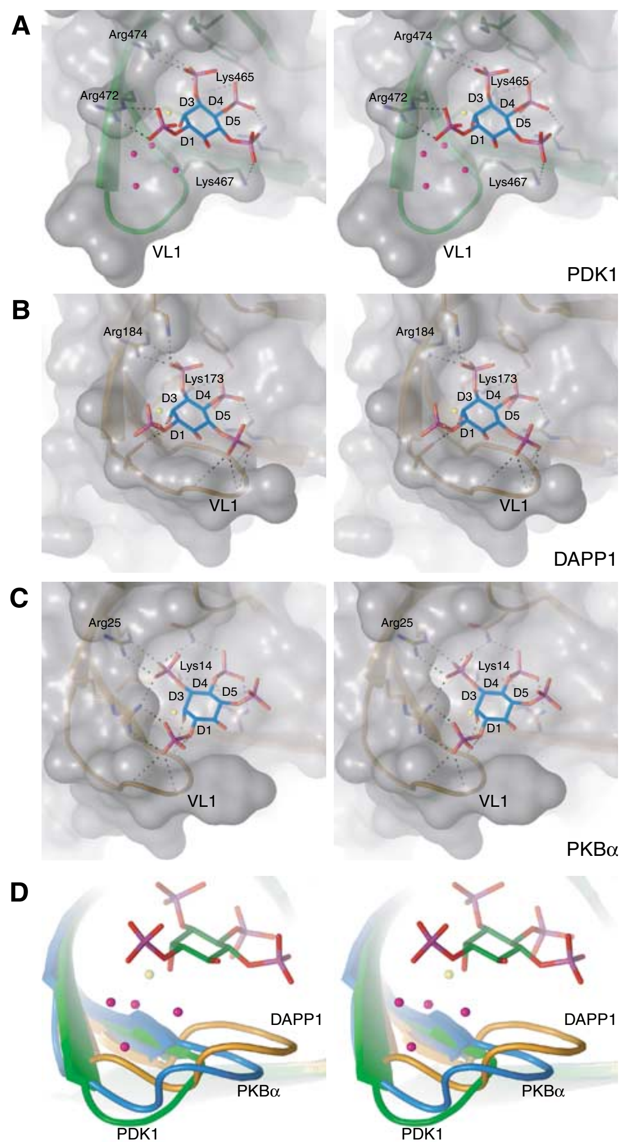

Figure 2 Comparison of $Ins(1,3,4,5)P_4$ binding to the PH domains of PDK1, DAPP1 and PKBα. Interactions of $Ins(1,3,4,5)P_4$ (marine) with protein residues (green in PDK1, orange in DAPP1/PKBα) in the phosphoinositide-binding site are shown in a stereo representation. Hydrogen bonds are indicated as black dotted lines, and conserved water molecules are shown as yellow spheres. (**A**) Protein–ligand interactions of PDK1 PH domain with $Ins(1,3,4,5)P_4$. A layer of ordered water molecules (coloured in magenta) separates the ligand from VL1, only one of which is conserved in other PH domain structures (coloured in gold). (**B**) $Ins(1,3,4,5)P_4$ binding to DAPP1 (pdb-id 1fao; Ferguson *et al*, 2000). The D5-phosphate is closely enveloped by VL1. (**C**) $Ins(1,3,4,5)P_4$ binding to PKBα (pdb-id 1h10; Thomas *et al*, 2001). The ligand is rotated and VL1 contacts the D1-phosphate, while the D5-phosphate is solvent exposed. (**D**) Overlay of the structures of the PDK1 (green), DAPP1 (orange) and PKBα (blue) PH domains. The $Ins(1,3,4,5)P_4$ ligand and the layer of ordered water molecules of PDK1 are shown, and coloured according to (A).

ordered in the crystal structure (Figure 3A). The glycerol backbone projects away from the surface of the protein and does not display any other interactions.

Currently, the only other example of a protein domain in complex with an ordered phosphoinositide is the PtdIns(3)P-binding phox homology (PX) domain of the $p40^{phox}$ subunit of NADPH oxidase (pdb-id 1h6h; Bravo *et al*, 2001). In Figure 3, the structures of the PDK1 PH domain–diC4–$PtdIns(3,4,5)P_3$ and the PX domain–diC4–PtdIns(3)P complexes are compared after superposition on the inositol ring. Despite possessing completely different folds, the binding arrangements by which these proteins interact with the D1-phosphodiester are strikingly similar. The two oxygen atoms not involved in the two phosphoester linkages will carry most of the $-1e$ negative charge, and it is these oxygen atoms that interact with basic residues in the underlying binding pocket on the surface of the proteins. In the PDK1 PH domain, the guanidinium group of Arg472 hydrogen bonds both these oxygen atoms, while in the PX domain each oxygen atom interacts separately with a basic residue (Arg60/Lys92). Consequently, the oxygen atom involved in the phosphoester bond to the glycerol projects away from the binding pocket and in neither structure interacts with the protein. However, in contrast to the PDK1 PH domain diC4–$PtdIns(3,4,5)P_3$ complex, the glycerol backbone in the PX domain–diC4–PtdIns(3)P complex displays hydrophobic interactions with an exposed tyrosine side chain (Tyr94), which is not observed in the PDK1 $PtdIns(3,4,5)P_3$ complex (compare Figure 3A and B).

To investigate whether the PDK1 PH domain undergoes ligand-induced conformational change when it binds $PtdIns(3,4,5)P_3$, the PDK1 PH domain structure was solved in the absence of any ligand (Table I). However, all four independent molecules in the asymmetric unit show one molecule of sulphate, presumably originating from the 0.2 M ammonium sulphate containing crystallisation buffer, at the position equivalent to the D3-phosphate in the $Ins(1,3,4,5)P_4$ structure (Supplementary Figure 1). Crystal structures of other noncomplexed PH domains, including Grp1 (Ferguson *et al*, 2000) and PKBα (Milburn *et al*, 2003), also contain a phosphate/sulphate at equivalent positions. The uncomplexed structures of PDK1 superimpose well onto the $Ins(1,3,4,5)P_4$ and $PtdIns(3,4,5)P_3$ complexes (pairwise with an average RMSD of up to 0.6 Å, Supplementary Figure 1). No significant conformational changes are visible even in the VLs that are involved in ligand binding, with the exception of Lys467, which occupies the position of the inositol ring in the absence of ligand but rotates to a position to contact the D5-phosphate upon ligand binding. This suggests that the PDK1 PH domain, like most other PH domains analysed to date, assumes the same conformation in the presence or absence of phosphoinositides. In contrast, there is evidence that the PKBα PH domain undergoes a conformational change when it binds to $PtdIns(3,4,5)P_3$ (Mora *et al*, 2004), which is thought to enhance the rate at which PKB can be activated by PDK1 (Vanhaesebroeck and Alessi, 2000).

Mutations of the lipid-binding site: functional analysis

Figure 4A shows a summary of the ligand–protein hydrogen bonds between $PtdIns(3,4,5)P_3$ and residues of the PDK1 PH domain. To determine the contribution of the interactions to the affinity for phosphoinositides, these residues were mutated in full-length PDK1 and expressed as GST-fusion proteins in HEK-293 cells. The ability of these mutants to bind $PtdIns(3,4,5)P_3$, $PtdIns(3,4)P_2$ and $PtdIns(4,5)P_2$ was first tested with a qualitative protein-phospholipid overlay assay (Dowler *et al*, 2002) (Figure 4B). As reported previously,

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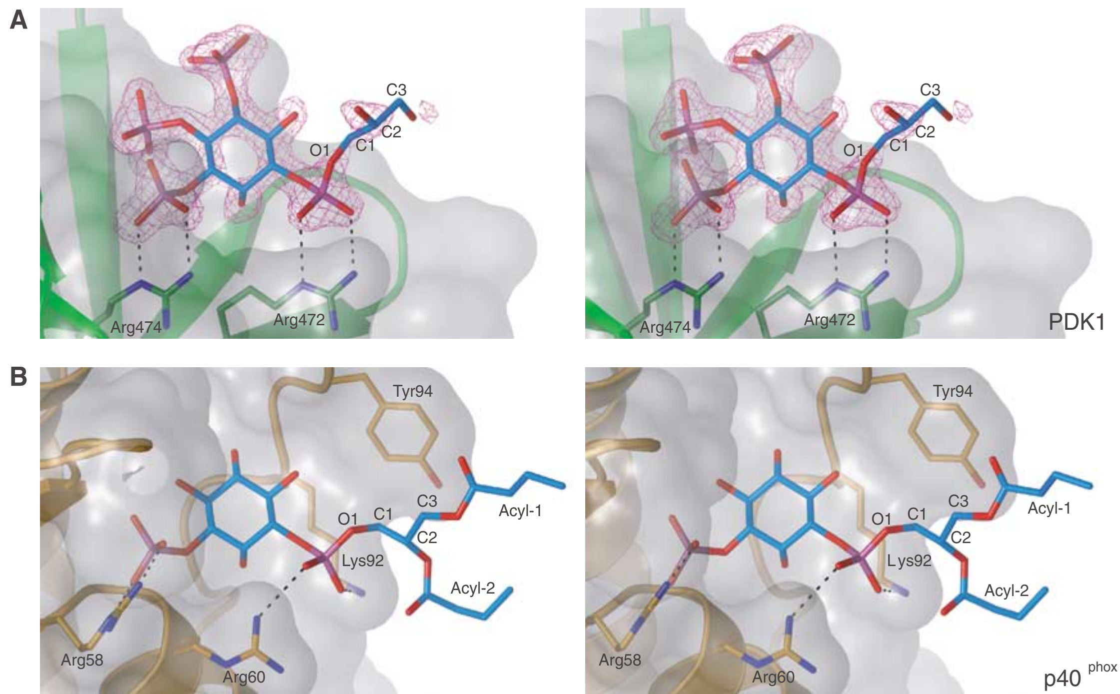

Figure 3 Analogy of PH and PX domain binding to phosphoinositides. The protein and ligand molecules are coloured as in Figure 2. Under the semitransparent surface, the conserved Arg residues contacting the D1- and the D3-phosphates are drawn as stick representation. (**A**) Stereo representation of the PH domain of PDK1 interacting with diC4-PtdIns(3,4,5)P_3. A $|F_o|-|F_c|$ simulated annealing omit map, contoured at 2.25σ, is shown in purple, covering the ligand molecule. (**B**) PtdIns(3)P binding to the PX-domain of p40^{phox} (pdb-id 1h6h; Bravo *et al*, 2001). Lys94 and Arg60 contacting the D1-phosphate are drawn as stick representation, as is Tyr94 from the membrane attachment loop.

wild-type PDK1 interacts strongly with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, and weakly with PtdIns(4,5)P_2 (Currie *et al*, 1999) (Figure 4B). Only two of the mutants (PDK1[K465E] and PDK1[R472A/R474A]) failed to bind phosphoinositides (Figure 4B). Lys465 and Arg474 of PDK1 are equivalent to Lys14 and Arg25 of PKBα, and Lys173 and Arg184 of DAPP1, where mutation of these residues was previously shown to inhibit the binding to PtdIns(3,4,5)P_3 (Dowler *et al*, 2000; Thomas *et al*, 2002) (Figure 2). Three mutations (PDK1[R474A], PDK1[R474A/K467A] and PDK1[K465A]) significantly reduced binding (Figure 4B). The individual mutation of Arg472, Tyr486, Lys 495 or Arg521 had little or moderate effects on phosphoinositide binding.

Of particular interest was the effect of mutation of Lys467 on the ability of PDK1 to bind D5-phosphorylated phosphoinositides, as Lys467 is the only residue to contact the D5-phosphate (Figures 2 and 4). In order to assess accurately the contribution of Lys467 to ligand binding, we employed a novel time-resolved fluorescence resonance energy transfer (TR-FRET) assay described in Materials and methods, which quantitatively measures binding of PDK1 to phosphoinositides (Figure 5). In agreement with previous surface plasmon resonance (SPR) studies (Currie *et al*, 1999), in the TR-FRET assays, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 were found to bind PDK1 with significantly higher affinity than PtdIns(4,5)P_2 (Figure 5). Affinities obtained are in close agreement for TR-FRET and SPR for both PtdIns(3,4,5)P_3 (4.0 and 1.6 nM), and PtdIns(3,4)P_2 (23 and 5.2 nM), respectively. In contrast, for PtdIns(4,5)P_2, a lower affinity of 700 nM was measured by TR-FRET compared to 24 nM that was calculated from previous SPR studies (Currie *et al*, 1999). We next used the TR-FRET assay to investigate how mutation of Lys467 affected binding to PtdIns(4,5)P_2. Mutation of Lys467 to Ala resulted in an ∼eight-fold reduction in affinity for PtdIns(4,5)P_2, whereas the more conservative replacement of Lys467 to Arg maintained high-affinity PtdIns(4,5)P_2 binding. Interestingly, in agreement with the structural data, the PDK1[K467A] mutant interacted with six- to eight-fold lower affinity to all D5-phosphorylated inositides including PtdIns(3,4,5)P_3 (Figure 5). In comparison, affinities for PtdIns(3,4)P_2 were reduced by two-fold.

Binding of PDK1 to inositol phosphates

The structures of the PDK1 PH domain show that the phosphoinositide-binding site of PDK1 is unusually spacious, and, in contrast to other PtdIns(3,4,5)P_3-binding PH domains, additional space is present around the D2- and D6-hydroxyl groups, which potentially could accommodate further phosphate groups (Figure 3). Physiologically, only inositol phosphates but not phosphoinositides are known to be phosphorylated at the D2 and/or D6 position (Michell, 1997; Irvine and Schell, 2001). Ins(1,3,4,5,6)P_5 and InsP_6 are present at high micromolar levels within the cell, at higher levels than Ins(1,3,4,5)P_4 or Ins(1,4,5)P_3 (Orchiston *et al*, 2004). These observations prompted us to analyse the binding of inositol phosphates to full-length PDK1 employing the TR-FRET assay, which can be used for this purpose.

Interestingly, Ins(1,3,4,5,6)P_5 and InsP_6, as well as Ins(1,3,4,5)P_4, were found to bind PDK1 with high affinity, with K_i values of 20–60 nM (Figure 6A). In contrast, Ins(1,4,5)P_3 binding to PDK1 could not be detected. The hydrogen bonds to the D1-phosphate (Figures 2–4) appear to play a crucial role in mediating binding of inositol phosphates to PDK1, as Ins(3,4,5,6)P_4 had a five-fold lower

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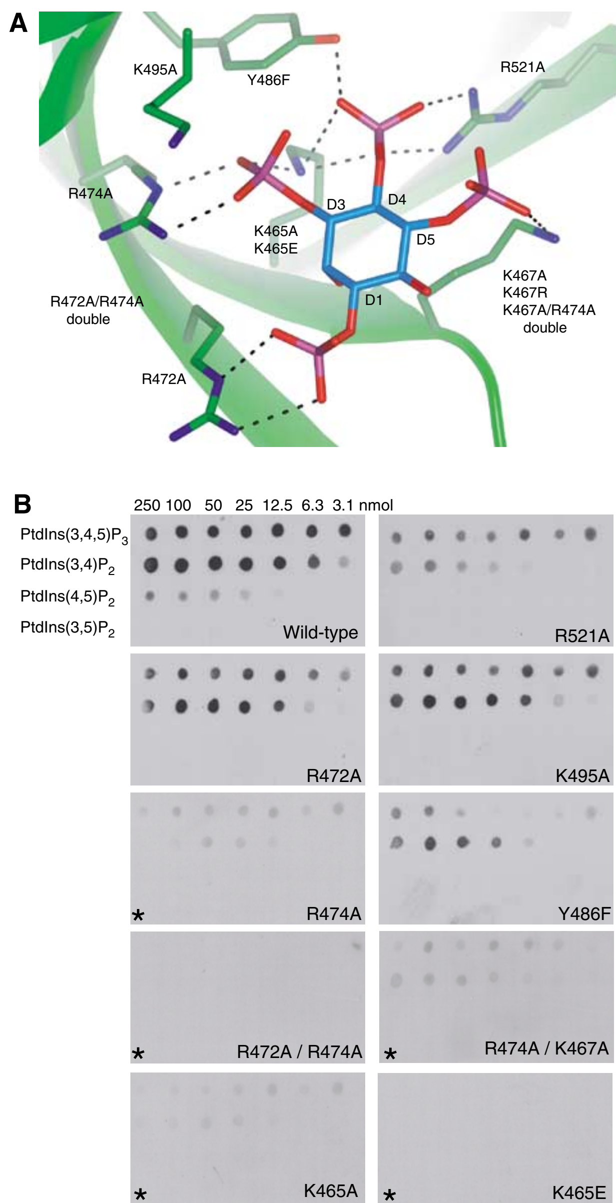
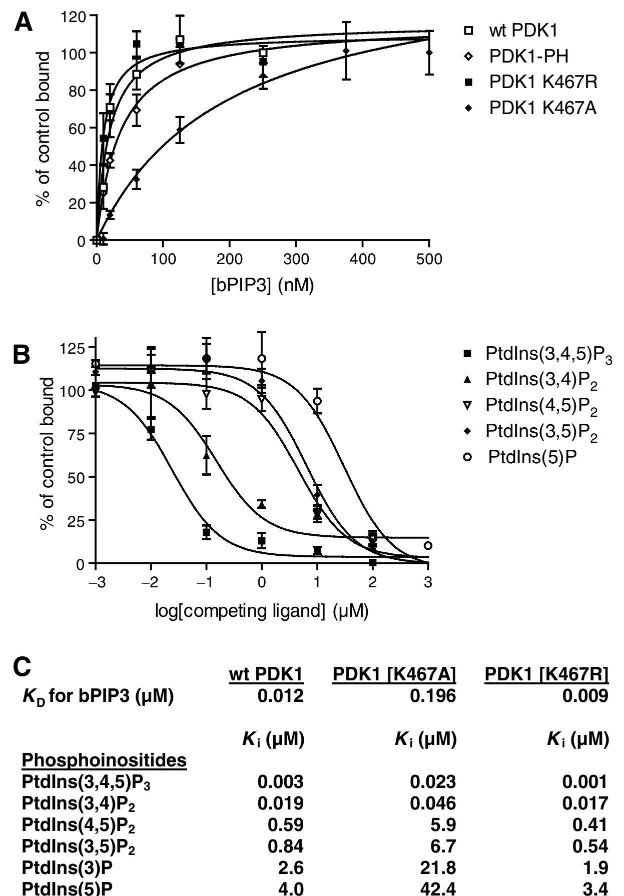


Figure 4 Phosphoinositide-binding properties of wild type and mutant PDK1. (**A**) Representation of the Ins(1,3,4,5)P_4-binding pocket on the PDK1 PH domain. The residues that make contacts with Ins(1,3,4,5)P_4 and that are mutated in this study are labelled. (**B**) The ability of the indicated wild type and mutant forms of PDK1 to interact with phosphoinositides was analysed using a protein-lipid overlay assay. Serial dilutions of the indicated phosphoinositides (250, 100, 50, 25, 12.5, 6.3, 3.1 and 1.6 pmol) were spotted onto nitrocellulose membranes, which were then incubated with the purified GST-PDK1 species. The membranes were washed, and the GST-PDK1 bound to the membrane by virtue of their interaction with lipid was detected using a GST antibody (Dowler *et al*, 2002). A representative of at least two separate experiments is shown. (*) indicates a long exposure of the film to detect weak binding.



	wt PDK1	PDK1 [K467A]	PDK1 [K467R]
K_D for bPIP3 (μM)	0.012	0.196	0.009
	K_i (μM)	K_i (μM)	K_i (μM)
Phosphoinositides			
PtdIns(3,4,5)P_3	0.003	0.023	0.001
PtdIns(3,4)P_2	0.019	0.046	0.017
PtdIns(4,5)P_2	0.59	5.9	0.41
PtdIns(3,5)P_2	0.84	6.7	0.54
PtdIns(3)P	2.6	21.8	1.9
PtdIns(5)P	4.0	42.4	3.4

Figure 5 TR-FRET measurements of phosphoinositide binding to PDK1. Affinities of wild-type and mutant PDK1 for binding to phosphoinositides was measured by the TR-FRET assay, as described in Materials and methods. (**A**) Saturation-binding isotherms of the indicated forms of PDK1 binding to biotinylated-PtdIns(3,4,5)P_3 (bPIP3). (**B**) The PDK1-bPIP3 complexes formed at 80 nM bPIP3 were incubated with nonlabelled phosphoinositides and the displacement of bound bPIP3 measured. The data in (A) and (B) are presented as an average of three independent experiments$\pm$s.d. (**C**) The K_D values were calculated from (A). IC_{50} values obtained from (B) were used to calculate K_i values using the Cheng and Prusoff equation (Cheng and Prusoff, 1973). The K_D value for bPIP3 for the wild type and indicated mutant of PDK1 is also stated.

affinity compared to Ins(1,3,4,5,6)P_5 (Figure 6A). This is also consistent with our notion of Arg472 interacting with the delocalised negative charge on the D1-phosphate (Figure 3). Irvine and colleagues have suggested that binding studies using $InsP_6$ should be performed in the presence of Mg^{2+} as the counter ion as the physiological form of $InsP_6$ is complexed to Mg^{2+} (RF Irvine, unpublished observation). Binding of inositol lipids and inositol phosphates to PDK1 was not affected by the inclusion of 1 mM Mg^{2+} (data not shown).

Evidence for physiological interaction of PDK1 with D6-phosphorylated inositol phosphates

Initially, we attempted to design a mutant of PDK1 that inhibited binding of D6-phosphorylated inositol phosphates without affecting binding of phosphoinositides. Unfortunately, all the point mutants of PDK1 that we generated, which reduced binding of $InsP_6$, also affected binding to PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2. We also attempted to co-crystallise the PDK1 PH domain with Ins(1,3,4,5,6)P_5 or $InsP_6$, without success. This prompted us to compare the affinities of the isolated PH domain and full-length PDK1 for phosphoinositides and D6-phosphorylated inositol phosphates.

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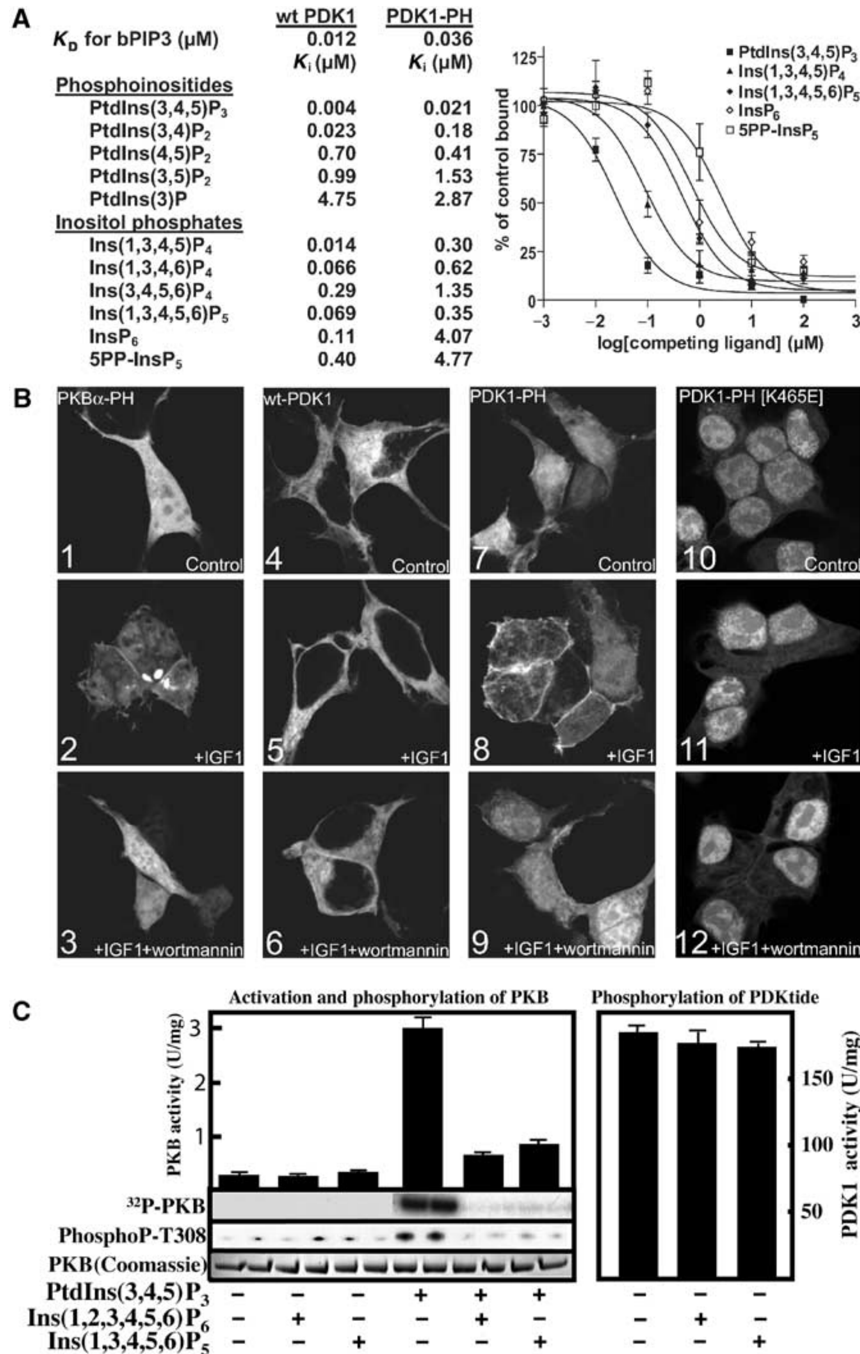

Figure 6 Interaction of PDK1 with inositol phosphates. (**A**) Affinities of wild-type PDK1 and its isolated PH domain (residues 409–556), for the indicated phosphoinositides and inositol phosphates were measured by the TR-FRET assay using the approach described in Figure 5A and B. The K_D value for biotinylated-PtdIns(3,4,5)P_3 (bPIP3) is also stated. (**B**) HEK-293 cells were transiently transfected with DNA constructs encoding the GFP-tagged PKBα PH domain (residues 1–123, panels 1–3), wild-type PDK1 (panels 4–6), the isolated PDK1 PH domain (residues 409–556, panels 7–9) and the non-PtdIns(3,4,5)P3-binding isolated PDK1[K465E] PH domain (residues 409–556, panels 10–12). At 10 h post-transfection, the cells were serum starved for 16 h and either left unstimulated, stimulated with IGF1 for 10 min, or treated with 100 nM wortmannin for 20 min and then stimulated with IGF1 for 10 min. The cells were then fixed and imaged using a Zeiss LSM 510 META confocal microscope with an optical section thickness of 0.5 μm. The cells shown are representative images obtained in three separate experiments. (**C**) Wild-type GST PKBα was incubated with PDK1, magnesium, γ[^{32}P]ATP in the presence (+) or absence of 100 μM phosphatidylserine (PS), 100 μM phosphatidylcholine (PC) vesicles containing 10 μM PtdIns(3,4,5)P_3 and/or 300 μM of the indicated inositol phosphates, and the activation of PKBα was assessed as described previously (Alessi *et al*, 1997). In samples in which PtdIns(3,4,5)P_3 was omitted, the PC/PS vesicles were still added. Phosphorylation of the PKB was determined following electrophoresis on a 4–12% gradient polyacrylamide gel, either by immunoblotting with the phosphospecific Thr308 antibody or by autoradiography of the Coomassie Blue-stained PKBα band. PDK1 activity was assayed as above, employing the PDKtide peptide substrate rather than by measuring PKB activation and phosphorylation. Experiments were performed in quadruplicate and similar results obtained in two to four separate experiments.

Surprisingly, we observed that the isolated PH domain of PDK1, although it still interacted with PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 with high affinity, possessed 40-fold reduced affinity for $InsP_6$ and five-fold lower affinity for Ins(1,3,4,5,6)P_5 (Figure 6A). Inspection of the structure of the PH domain–Ins(1,3,4,5)P_4 complex suggests that a

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D6-phosphate would be solvent exposed and unable to interact with the PH domain. However, in the context of the full-length PDK1, the D6-phosphate may well form interactions with non-PH domain regions of the protein, explaining the observed higher affinity of full-length PDK1 for D6-phosphorylated inositol phosphates. Clearly, further structural studies are required to investigate this specific interaction and to understand the alignment of the PH domain and the kinase domain in context of the full-length PDK1 protein.

We next studied the cellular location of green fluorescent protein (GFP)-tagged full-length PDK1 compared to the isolated PH domain of PDK1. As observed previously (Currie *et al*, 1999), full-length PDK1 was mainly cytosolic, with only a small fraction being at the membrane, and was excluded from the nucleus in unstimulated HEK-293 cells. PDK1 did not markedly translocate to the plasma membrane following stimulation of cells with the PI 3-kinase agonist IGF1. Similarly, localisation of full-length PDK1 was not affected by the PI 3-kinase inhibitor wortmannin (Figure 6B, panels 4–6). In parallel experiments, we observed IGF1-induced plasma membrane translocation of the PtdIns(3,4,5)P_3-binding PH domain of PKBα, which was inhibited by wortmannin (Figure 6B, panels 1–3). The isolated PDK1 PH domain was localised in the cytoplasm and nucleus of unstimulated cells, and, in contrast to full-length PDK1, IGF1 induced a marked translocation to the plasma membrane that was inhibited by wortmannin (Figure 6B, panels 7–9). A non-phosphoinositide-binding isolated PDK1[K465E] PH domain mutant was localised throughout the cell and did not translocate to the plasma membrane upon IGF1 stimulation (Figure 6B, panels 10–12). Control Western blotting demonstrated the absence of proteolytic degradation of the expressed constructs (data not shown). Taken together, these findings are consistent with a model in which full-length PDK1 is anchored in the cytosol through high-affinity binding to D6-phosphorylated inositol phosphates. Similar observations have been made in *Dictyostelium discoideum,* in which Luo *et al* (2003) followed the localisation of the PH domain of Crac (PH_{Crac}). Crac shows high affinity for diphosphoinositol pentakisphosphate (PP-InsP_5, the isomer was not stated), and appears mostly cytosolic, as is PDK1 in our study. Following cAMP stimulation, which causes PtdIns(3,4,5)P_3 accumulation in *D. discoideum*, the movement of PH_{Crac} is potentiated in mutants lacking inositol hexakisphosphate kinase, the enzyme responsible for PP-InsP_5 synthesis. It should be noted that levels of inositol diphosphates in *D. discoideum* are about 100 times those of mammalian tissues, but still only about half those of InsP_6 (Luo *et al*, 2003). PDK1 was found to interact with 5PP-InsP_5, with a significantly lower affinity (K_i 400 nM) than Ins(1,3,4,5,6)P_5 and InsP_6 (Figure 6A). As the cellular concentration of 5PP-InsP_5 is much lower than Ins(1,3,4,5,6)P_5 and InsP_6, it is unlikely that 5PP-InsP_5 is a physiological ligand for PDK1.

The PKBα PH domain shows rapid translocation to the plasma membrane (Figure 6B, panels 1–3) upon IGF1 stimulation. PKBα PH domain and PDK1 show similar affinities for phosphoinositides, but PKBα PH domain shows significantly lower affinity towards higher inositol phosphates (Frech *et al*, 1997). Indeed, InsP_6 binds PDK1 with 80-fold higher affinity than PKBα PH domain. As such, the competition between cytosol-localised inositol phosphates and membrane-localised phosphoinositides greatly favours phosphoinositide binding in the case of the PKBα PH domain, resulting in a marked translocation of PKBα PH domain to the plasma membrane in IGF1 treated cells (Figure 6B, panels 1–3). Consistent with the notion that high-affinity binding to inositol phosphates maintains PDK1 located in the cytoplasm, the isolated PH domain of PDK1, which displays high-affinity interactions with phosphoinositides, but has an affinity (4.1 μM) similar to that of PKBα for InsP_6 (8.7 μM, (Frech *et al*, 1997)), is seen to translocate in a fashion analogous to the PKBα PH domain. Our data do not rule out the possibility that other ligands/factors could interact with full-length PDK1, contributing towards its cytosolic or membrane localisation. In this regard, it has recently been reported that PDK1 can be stabilised at the cell membrane by interaction with Grb14, which binds the insulin receptor through an SH2 domain (King and Newton, 2004).

In many studies of PH domain-containing proteins, the lipid-binding and localisation properties of the PH domain are assessed employing the isolated PH domain rather than the full-length protein (Irvine, 2004). Our data emphasise a pitfall of this approach, as the isolated PH domain of PDK1 has quite distinct inositol phosphate-binding properties and localisation pattern, compared to the full-length protein. Recently, it was shown that the isolated PH domain of phospholipase Cδ4 (PLCδ4) was localised at the plasma membrane, whereas the full-length protein localises at intracellular membranes, and also showed differences in PtdIns(4,5)P_2 versus Ins(1,4,5)P_3 binding (Lee *et al*, 2004). Thus, in order to determine the key localisation signals of lipid-binding proteins, we would recommend comparing the lipid-binding and localisation properties of the isolated lipid-binding domain with that of the full-length protein.

We next investigated whether Ins(1,3,4,5,6)P_5 and InsP_6 could compete with PtdIns(3,4,5)P_3 in regulating the activation of PKB by PDK1 in an *in vitro* assay. Addition of 300 μM InsP_6 or Ins(1,3,4,5,6)P_5 inhibited the activation and phosphorylation of PKB in the presence of PtdIns(3,4,5)P_3, by over 80% (Figure 6C). InsP_6 or Ins(1,3,4,5,6)P_5 did not directly inhibit PDK1 catalytic activity, as the phosphorylation of the PDKtide peptide substrate by PDK1 was not affected by D6-phosphorylated inositol phosphates (Figure 6C). It has also been reported that increasing extracellular Ins(1,3,4,5,6)P_5 in cells lowered PKB activation, through an undefined mechanism (Piccolo *et al*, 2004). The data presented here indicate that these effects could be mediated, at least in part, through binding of PDK1 to Ins(1,3,4,5,6)P_5 and anchoring it in the cytosol. It is also possible that Ins(1,3,4,5,6)P_5 might bind to the PH domain of PKB *in vivo*, and thus affect its translocation to the plasma membrane.

Several other PtdIns(3,4,5)P_3-binding PH domains including Grp1 and Btk have been shown to interact *in vitro* with InsP_6 and Ins(1,3,4,5,6)P_5. Btk interacts with both InsP_6 and Ins(1,3,4,5,6)P_5 with K_i of 90 nM (Kojima *et al*, 1997), whereas Grp1 binds to InsP_6 and Ins(1,3,4,5,6)P_5 with K_D's of 6 and 0.3 μM, respectively (Kavran *et al*, 1998). PtdIns(3,4,5)P_3 recruits Grp1 and Btk to the plasma membrane, where they are thought to exert their physiological effects. Thus, if it occurred *in vivo*, inositol phosphate binding to these PH domain-containing proteins would inhibit their function. In the case of PDK1, cytosolic localisation is important in enabling PDK1 to activate substrates that do not

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bind lipids, such as SGK or S6K. Our data indicate that the micromolar levels of Ins(1,3,4,5,6)P_5 and $InsP_6$ found in cells would be sufficient to act as physiological ligands of PDK1, and could compete with PtdIns(3,4,5)P_3, PtdIns(3,4)P_2 and PtdIns(4,5)P_2 for binding to PDK1. Such a mechanism could ensure that sufficient PDK1 was distributed in both the cytosol and plasma membrane to enable PDK1 to fulfil its cellular functions. Thus, where the binding of Btk and Grp1 to inositol phosphates would act as a brake in signalling, for PDK1 this binding would accelerate activation of cytoplasmic substrates. In this respect, PDK1 is unique among PH domain-containing proteins described so far.

Conclusions

Here we describe the unusual structure of the PDK1 PH domain. It possesses several unique features not previously observed in other PH domains, including the N-terminal bud which forms an integral part of the overall PH domain structure. The PDK1 PH domain in complex with a PtdIns(3,4,5)P_3 analogue shows a surprising analogy in the phosphoinositide-binding mode of the PDK1 PH domain and the PX domain of $p40^{phox}$. We define the relative importance of the residues in the ligand-binding site for enabling PDK1 to bind phosphoinositides. In particular, we identify Lys467 as the residue in PDK1 responsible for binding the D5-phosphorylated ligands and thus permitting interaction with PtdIns(4,5)P_2. Moreover, the PDK1 PH domain possesses a much more spacious ligand-binding site, which we demonstrate enables PDK1 to interact with Ins(1,3,4,5,6)P_5 and $InsP_6$ with high affinity. We perform studies that support the notion that these ligands could function to anchor PDK1 in the cytosol, where it is needed to activate its nonlipid-binding substrates. Overall, our results provide a molecular explanation for how PDK1 could be localised both at the membrane and the cytosol of cells.

Materials and methods

Protein expression, purification, crystallisation and data collection

PDK1 PH domain (residues 409–556) was expressed in *Escherichia coli*, purified and crystallised as described previously (Komander *et al*, 2004). Further details on crystallisation conditions, data collection, as well as structure determination and analysis can be found in the Supplementary data. The refinement statistics can be found in Table I.

Analysis of PDK1 phosphoinositide and inositol phosphate interactions

For qualitative protein lipid overlay assays, wild type and mutants of full-length PDK1 were expressed as GST fusion proteins (pEBG vector) in human embryonic kidney HEK-293 cells, as described previously (Alessi *et al*, 1997). The phospholipid overlay assays were performed as described previously (Dowler *et al*, 2002), using di-palmitoyl-phosphoinositides from Cell Signals Inc. (Columbus, USA). The quantitative TR-FRET analysis of binding was performed as described by Gray *et al* (2003). To determine K_D values for biotinylated diC8-PtdIns(3,4,5)P_3, increasing amounts (0–500 nM) of biotinylated diC8-PtdIns(3,4,5)P_3 were incubated with GST-tagged wild type and mutant full-length PDK1 or wild-type PH domain of PDK1 (20 nM) in the presence of excess europium-labeled goat anti-GST antibody and streptavidin-conjugated allophycocyanin (APC). The binding of the biotinylated lipid to the protein allows FRET to occur between europium (donor) and APC (acceptor). Fluorescence is monitored at 665 and 505 nm; the ratio of these signals allows determination of relative amount of binding. Saturation-binding isotherms are presented in Figure 5A. Competition assays were performed with a fixed amount (80 nM) of biotinylated lipid and increasing amounts of competing agent (diC8 phosphoinositides or inositol phosphates). IC_{50} values were determined, from which K_i values were calculated, according to the equation below (Cheng and Prusoff, 1973). All curve fitting was performed using Prism (GraphPad).

$$K_i = \frac{IC_{50}}{1 + [L]/K_D}$$

Localisation studies

Cells were fixed in 4% (w/v) paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA and 2 mM $MgSO_4$, pH 7.0). The cells were washed three times with PBS and mounted on slides with Hydromount. The cells were imaged to monitor GFP fluorescence and confocal sections collected, using a Zeiss LSM 510 META confocal microscope.

A detailed description of all other methods employed in this study can be found in the supplementary data of this paper.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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