Role of T-loop phosphorylation in PDK1 activation, stability and substrate binding.

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Abbreviations: PDK1, 3-phosphoinositide dependent protein kinase-1; AGC, cAMP-dependent, cGMP-dependent, Protein Kinase C; HM, hydrophobic motif; pHM, phosphorylated hydrophobic motif; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; S6K, p70 ribosomal S6-kinase; SGK, serum and glucocorticoid responsive kinase; RSK, p90 ribosomal S6 kinase; CDK, cyclin dependent kinase; PKA, cAMP dependent protein kinase; GST, glutathione-S-transferase; PRK2, PKC-related kinase-2; HM-PRK2, hydrophobic motif peptide derived from PRK2; HEK, human embryonic kidney; GST, Glutathione-S-transferase.

Key words: AGC kinase, T-loop phosphorylation, crystal structure, PKB/Akt.

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Summary

3-phosphoinositide dependent protein kinase-1 (PDK1) phosphorylates the T-loop of several AGC (cAMP-dependent, cGMP-dependent, Protein Kinase C) family protein kinases, resulting in their activation. Previous structural studies have revealed that the αC-helix, located in the small lobe of the kinase domain of PDK1, is a key regulatory element, as it links a substrate interacting site termed the hydrophobic motif (HM) pocket with the phosphorylated Ser241 in the T-loop. In this study we demonstrate by mutational analysis that interactions between the phosphorylated Ser241 and the αC-helix are not required for PDK1 activity or substrate binding through the HM-pocket, but are necessary for PDK1 to be activated or stabilised by a peptide that binds to this site. The structure of an inactive T-loop mutant of PDK1, in which Ser241 is changed to Ala, was also determined. This structure, together with surface plasmon resonance binding studies, demonstrates that the PDK1[S241A] inactive mutant possesses an intact HM-pocket as well as an ordered αC-helix. These findings reveal that, in contrast to the model of activation of other AGC kinases such as protein kinase B (PKB, also known as Akt), the integrity of the αC-helix and HM-pocket in PDK1 is not regulated by T-loop phosphorylation.
Introduction

3-phosphoinositide dependent protein kinase-1 (PDK1) is Ser/Thr protein kinase that activates at least 24 protein kinases belonging to the AGC (cAMP-dependent, cGMP-dependent, Protein Kinase C) family of protein kinases, by phosphorylating their T-loop (1). PDK1 possesses an N-terminal catalytic domain and a C-terminal Pleckstrin Homology (PH) domain (2,3). The latter binds with high affinity to the PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 second messengers that are the generated by PI 3-kinase following stimulation of cells with growth factors (3,4). The recent determination of the structure of the PDK1 PH domain has revealed the molecular basis for its interaction with phosphoinositides (5). The only PDK1 AGC kinase substrates to also possess a PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 binding PH domain are isoforms of PKB (also known as Akt) (1). Much evidence indicates that the mutual binding of PKB and PDK1 to PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 at the plasma membrane co-localises these enzymes and is necessary for PKB to be activated by PDK1 (6,7). In contrast, the other PDK1 AGC kinase substrates, including p70 ribosomal S6-kinase (S6K) (8), serum and glucocorticoid responsive kinase (SGK) (9) and p90 ribosomal S6 kinase (RSK) (10), do not bind PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 or possess PH domains. Instead, these kinases possess conserved PDK1 docking sites located in a C-terminal region known as the hydrophobic motif (HM). Growth factors and hormones lead to the phosphorylation of the hydrophobic motif of these AGC kinases, significantly enhancing their ability to interact with PDK1 (4,11,12). The region on PDK1 that interacts with the hydrophobic motif of its substrates is termed the HM-pocket (also known as the HM-pocket) and is located on the N-terminal smaller lobe of the catalytic domain (13,14). The structure of the PDK1 catalytic domain revealed that the HM-pocket consisted of a hydrophobic pocket adjacent to a positively charged phosphate-binding pocket (15).

PDK1 itself is also an AGC kinase member and like its substrates, requires to be phosphorylated at its T-loop (Ser241) in order to be activated (16). In vivo, PDK1 is capable of autophosphorylation at Ser241 by an intermolecular reaction and is thus constitutively phosphorylated at Ser241 (17). The structural analysis of the PDK1 kinase domain has revealed that, similar to what has been observed in other kinases, the phosphorylated Ser241 residue forms key interactions coordinating and aligning important catalytic motifs such as the αC-helix of the N-terminal lobe. The αC-helix plays a key role in all kinases as it contributes crucial residues to coordinating ATP. In PDK1 it positions the conserved Glu130 residue such that it coordinates the conserved Lys111, which interacts with the α-phosphate of ATP. This hydrogen bonding network is conserved in most protein kinases, and is required
for phosphoryl-transfer. Many kinases are regulated by controlling the position and/or formation of the αC-helix. For example, the binding of cyclins to cyclin dependent kinases (CDKs) induces a rotation of the αC-helix, leading to CDK activation (18-20). More recently the structure of PKBβ in the unphosphorylated, inactive state, was reported to possess a completely disordered αC-helix (21). PDK1-mediated phosphorylation of PKBβ in the T-loop led to ordering of this helix, resulting in PKB activation (22). This suggested a new regulatory mechanism for T-loop phosphorylation in PKB and posed the question of whether other AGC family members would be regulated in a similar manner. In PDK1, phosphorylated Ser241 interacts with 2 residues on the αC-helix (Tyr126 and Arg129, (15)) whereas PKBβ and PKA only possess a single interaction between the T-loop and the αC-helix (22,23). Interestingly, the αC-helix also contributes hydrophobic as well as phosphate-binding residues to the HM-pocket (Fig 1A) (15). Likewise, in active PKBβ the formation of the αC-helix leads to the creation of a pocket resembling the HM-pocket of PDK1. This pocket binds intramolecularly to the phosphorylated hydrophobic motif of PKBβ and this interaction is required for maximal activation. Similarly, occupancy of the HM-pocket of PDK1 with peptides encompassing the phosphorylated HM-docking residues of its substrates activates PDK1 4-6 fold and also stabilises the enzyme (13,24). These observations have been interpreted as implying that PDK1 is activated through interacting with its substrates. In this study we explore the role of T-loop phosphorylation of PDK1 in regulating enzyme structure, activity, stability and interaction with substrates.

Material & Methods
Protein expression, purification and crystallisation.
PDK1[S241A] (residues 51-359) was purified from baculovirus infected Sf-21 cells as described previously for the 51-359 wild type PDK1 catalytic domain (15). For crystallisation experiments, 50 µl of PDK1[S241A] at 6.8 mg/ml were mixed with 5 µl ATP and incubated on ice for 1 h. The protein crystallised at 20 °C, by mixing 1 µl protein solution with 1 µl mother liquor (2.2 M ammonium sulphate, 0.1 M Tris [pH7.5]). Prior to data collection, the crystals were soaked in mother liquor containing 15 % glycerol.

Data collection and structure determination.
Diffraction data on the PDK1[S241A]-ATP complex were collected at the ESRF beamline ID14-EH1 (Grenoble, France) to 1.95 Å. The protein crystallised in the same space group (P321) with similar unit cell dimensions compared to the wild type PDK1-ATP complex.
structure (pdb-id 1h1w, (15)) (Table I). The data were processed and scaled with the HKL suite (25), and the structure was solved by rigid body refinement using CNS (26) with the PDK1-ATP complex as a search model, followed by simulated annealing in CNS. Subsequent alternating rounds of refinement (in CNS and refmac5 (27)) and model building (in O (28)) were performed. Water and glycerol molecules as well as sulphate ions were included in final rounds of refinement, as was one molecule of ATP, respectively. ATP topologies were generated with PRODRG (29). Final statistics can be found in Table I.

**Mutagenesis and activity assays.**

Mutants of PDK1 were generated using site-directed mutagenesis with the QuickChange mutagenesis kit (Stratagene) in the pEBG2T vector. PDK1 and the indicated mutants were expressed as glutathione-S-transferase (GST) fusion protein in human embryonic kidney (HEK)-293 cells and purified as described previously (13). PDK1 and mutants was assayed against the T308tide substrate peptide (KTFCGTPEYLAPEVRR, (13)) in the absence or presence of 1 µM or 10 µM HM-PRK2 (REPRILSEEEQEMFRDFDYIADWC (30)) for 10 min at 30°C in a 50 µl assay mixture in 50 mM Tris (pH 7.5), 0.1 mM EGTA, 0.1% 2-mercaptoethanol, containing 1 mM substrate peptide, 10 mM magnesium acetate, 100 µM [γ-32P] ATP (200 cpm/mol), as described previously (15). For the thermal stability experiments shown in Figure 2, prior to assay 150 ng of wild type GST-PDK1 or indicated mutant GST-PDK1 in the presence or absence of 100 µM HM-PRK2 in a volume of 25 µl, was placed in a 96 well plate PCR plate. Following incubation for 2 min on a 40-60 °C step temperature gradient in a PCR machine (MJ Research PTC-200 Gradient Cycler), PDK1 activity was assayed as described above employing T308tide.

**Surface Plasmon resonance spectroscopy.**

Binding was analysed in a BioCore 3000 system (BiaCore AB, Stevenage, UK) according to (13). The biotinylated 24-residue peptide HM-PRK2 (Biotin-C12-REPRILSEEEQEMFRDFDYIADWC) was bound to a streptavidin-coated sensor chip (SA) (25 response units, RU). 30 µl of the indicated concentration of wild type or mutant GST-PDK1 were injected at a flow rate of 30 µl/min, in buffer HBS-P (10 mM HEPES pH 7.4, 0.15 M NaCl, 0.005% (v/v) polysorbate-20) supplemented with 1 mM DTT. Specific interactions between HM-PRK2 and PDK1 proteins were obtained between the concentration range of 2.5-2000 nM PDK1. Steady state binding was determined at each concentration. Dissociation of PDK1 from the HM-PRK2 was monitored over a 1 min period. Regeneration
of the sensor chip surface was performed with 10 µl injections of 0.05% SDS. The $K_d$ of interaction was calculated as described previously (13) by fitting the data to a sigmoid curve using GraphPad Prism software.

**Results & Discussion**

**Mutation of residues on PDK1 that interact with phosphorylated Ser241.**

The crystal structure of PDK1 (15) revealed that the phosphorylated Ser241 makes hydrogen bond interactions to four residues, Arg204 and Lys228 from the C-terminal lobe and Tyr126 and Arg129 from the αC-helix in the N-terminal lobe. The highly conserved Arg204 immediately precedes the catalytic Asp205, and the presence of this RD-motif places PDK1 in the group of RD-kinases (31,32). Arg204 is directly connected to the catalytic machinery due to its position within the catalytic loop and controls the fold of the activation loop after interaction with the phosphorylated Ser241. Lys228 may also play a role in aligning catalytic site residues, such as the Mg$^{2+}$ interacting residue Asp223 in the DFG motif. This is similar to the suggested role of the corresponding residues Arg165 and Lys189 in PKA (33). Mutational analysis of the RD Arg in both Ser/Thr and Tyr protein kinases has confirmed its importance (34-36). Interestingly, the residue equivalent to PDK1 Arg204 in PKBβ (Arg274) was recently found to be mutated in a human family with severe diabetes and insulin resistance (37).

In order to assess the role of Arg204 and Lys228 in PDK1, these residues were mutated to Ala. The effect that this had on PDK1 catalytic activity was assessed with an assay employing a peptide substrate (T308tide). In addition, the ability of these mutants to be activated by a peptide encompassing the hydrophobic motif of the PKC-related kinase-2 (PRK2), termed HM-PRK2, was also measured. In these assays HM-PRK2 was utilised at 1 µM, which maximally activates wild type PDK1, and at 10 µM, so activation could be detected if the affinity of the PDK1 mutant for the peptide was reduced. The ability of wild type and PDK1 mutants to interact directly with biotinylated HM-PRK2 peptide was also assessed, using a quantitative surface plasmon resonance (SPR) assay. As expected, the PDK1[R204A] mutant possessed negligible catalytic activity in the presence or absence of HM-PRK2, similar to that of a previously characterised catalytically-inactive PDK1 mutant (PDK1[K111A]) (Fig 1B). However, the PDK1[R204A] mutant still interacted with HM-PRK2 ($K_d$ 478 ± 10 nM), albeit with reduced affinity compared to wild type PDK1 ($K_d$ 72 ± 5 nM), but with significantly greater affinity than two HM-pocket mutants of PDK1 (PDK1[Q150E] and PDK1[K115A]) which do not show detectable binding to HM-PRK2.
(Fig. 1C). In contrast, PDK1[K228A] was normally active, could be further activated by 1 μM HM-PRK2 and bound HM-PRK2 in the SPR assay with high affinity ($K_d$ 270 ± 7 nM). In PKA, mutation of the residue Lys189 equivalent to PDK1 Lys228, was similarly observed to have no effect on catalytic activity (38).

Next, Tyr126 and Arg129 were mutated either individually or together to Ala to disrupt the connection of phosphorylated Ser241 with the αC-helix. In the absence of HM-PRK2, these PDK1 mutants possessed similar activity as wild type PDK1. However, the PDK1[Y126A] and PDK1[R129A] mutants were activated only 2-fold, at either 1 and 10 μM HM-PRK2, compared to 5-fold activation for wild type PDK1 (Fig. 1B). As similar activation was observed at both concentrations of HM-PRK2, this indicates that the affinity of these mutants for HM-PRK2 is similar to that of wild type PDK1, but the transmission of binding to the catalytic residues is impaired. Consistent with this notion, the double PDK1[Y126A, R129A] mutant was barely activated by HM-PRK2 (Fig. 1B). Interestingly, the PDK1[Y126A] ($K_d$ 380 ± 3 nM), PDK1[R129A] ($K_d$ 365 ± 5 nM) and PDK1[Y126A, R129A] ($K_d$ 410 ± 8 nM) still interacted with significant affinity with HM-PRK2 (Fig. 1C). In PKA and PKBβ there is only a single interaction of the αC-helix with the phosphorylated T-loop, mediated by a His residue. Mutation of His87 in the αC-helix of PKA that interacts with the phosphorylated Thr197 at the T-loop impairs the catalytic activity of PKA (39), in contrast to what is observed for PDK1. To our knowledge the effect of mutating the equivalent residue on PKBβ (His196) has not been investigated.

It has previously been shown, using thermal denaturation studies, that binding of HM-PRK2 to PDK1 also significantly thermostabilised PDK1 (13). We investigated whether the interaction of the αC-helix of PDK1 with the phosphorylated T-loop was required for HM-PRK2 to stabilise PDK1. Wild type PDK1 and mutant PDK1[Y126A], PDK1[R129A] and PDK1[Y126A, R129A] were incubated for 2 min at temperatures ranging from 40-60 °C prior to assay at 30 °C. Consistent with our previous work (13), wild type PDK1 was inactivated in temperature dependent manner and was significantly stabilised in the presence of HM-PRK2 (Fig. 2). However, although the PDK1[Y126A], PDK1[R129A] and PDK1[Y126A, R129A] mutants showed similar stability to wild type PDK1 in the absence of HM-PRK2, they were not stabilised following the addition HM-PRK2. These results indicate that the ability of HM-PRK2 to stabilise PDK1 through binding to the HM-pocket requires the interaction of the αC-helix with the phosphorylated T-loop.
Structure of the inactive PDK1 [S241A] mutant kinase domain in complex with ATP

The finding that the double PDK1[Y126A, R129A] mutant still interacted with HM-PRK2 ($K_d$ 67 ± 4 nM), suggested that formation of the αC-helix of PDK1 was independent of Ser241 phosphorylation. This notion was confirmed by the finding that the inactive PDK1[S241A] still interacted with HM-PRK2 with high affinity (Fig. 1C). These observations were arguably surprising as they contrast with the role of T-loop phosphorylation inducing αC-helix formation in PKBβ. In order to investigate this further, the PDK1[S241A] mutant (residues 51-359), was crystallised in the presence of ATP without Mg$^{2+}$ ions, employing similar conditions described for the wild-type PDK1 kinase domain complexes, and the structure was solved by molecular replacement using the previously determined PDK1 kinase domain structure (pdb-id 1h1w, (15)). The overall structure of the PDK1[S241A] mutant is similar to the wild-type PDK1-ATP complex (RMSD 0.19 Å), with most of the larger deviations observed in the T-loop region, introduced by the mutation (Fig. 3A, D). One molecule of ATP is present at the nucleotide-binding site, which shows no structural deviations from the ATP molecule in the wild type PDK1-ATP complex ((15), maximum atomic shift 0.4 Å). In contrast to the wild type PDK1 kinase domain structure, in which several T-loop residues (residues 233-236) were disordered, electron density for these residues is clearly defined in the PDK1[S241A] structure (Fig. 3C). The region of the T-loop surrounding Ser241 (residues 230-245, yellow in Fig. 3C,D) in the PDK1[S241A] structure adopts a markedly different conformation compared to wild type PDK1 (Fig. 3D). The Cβ carbon of Ala241 is shifted by 10.1 Å compared to the Cβ carbon of Ser241 in the wild-type structure, and is buried in a hydrophobic pocket lined by Leu254, Val249, Phe291 and Leu253 of the C-lobe. Moreover, the movement of Ser241 in the PDK1[S241A] structure displaces Asn240, Phe242 and Val243 by several Å compared to the wild-type structure, resulting in a shift of the whole T-loop by a distance equivalent to two residues, so that the carbonyl function of Arg238 in the PDK1[S241A] structure takes the position of the carbonyl of Asn240 in the wild type structure (located within 1.3 Å, Fig. 3D).

Consistent with the biochemical data, we find that the PDK1[S241A] structure displays fully ordered density for the αC-helix as well as the adjacent αB-helix (Fig. 3B, maximum atomic shift 0.4 Å). The PDK1[S241A] also displays a well ordered HM-pocket, indistinguishable from that of wild type PDK1. This shows that T-loop phosphorylation of PDK1, in contrast to PKBβ, only has a role for enzyme activity and stability, but not for formation of the αC-helix and hence docking to substrates via the HM-pocket. Furthermore, this finding suggests that the mechanism of T-loop phosphorylation leading to stabilisation of
the αC-helix in PKBβ may not be applicable to all AGC kinase members. To date no dephosphorylated T-loop structure of PKA has been solved and the only other inactive AGC kinase crystallised apart from PKBβ, is the N-terminal catalytic domain of MSK1 (40). This structure revealed an auto-inhibited conformation, in which a novel, three-stranded β-sheet, occupies the position of the αC-helix in the N-terminal lobe, rendering the protein inactive (40). Recently, the phosphorylated, active structure of the catalytic domain of PKCθ AGC kinase was reported, which possesses similar features to that of PKA, PDK1 and activated PKBβ (41). In PKCθ, similar to PKBβ, the αC-helix creates a HM-pocket, which forms an intra-molecular interaction with its own phosphorylated hydrophobic motif (41). A major difference, however, is that the phosphorylated T-loop in PKCθ does not interact with the αC-helix. The only charged interaction, conserved in all PKC isoforms, between the PKCθ αC-helix and the C-terminal lobe of the kinase domain, is between Glu528 (in the C-lobe), which interacts with Arg430 (in αC-helix). As the phosphorylated T-loop in PKCθ does not interact with the αC-helix, PKCθ may be similar to PDK1 in that formation of the αC-helix is independent of T-loop phosphorylation.

Strikingly, in the PDK1[S241A] structure, at the site where the phosphate group is located in wild type PDK1, strong electron density is observed (Fig. 3E). The large, bulky electron density does not resemble any obvious component from the crystallisation condition, and was therefore not interpreted in the refinement procedure. The electron density is surrounded by residues Tyr126, Arg129 and Arg204. Tyr126 and Arg204 adopt the same conformation as in wild type PDK1, whereas Arg129 adopts a different conformation (Fig. 3D). The fourth residue interacting with the phosphorylated Ser241 in the wild type structure, namely Lys228, is disordered in the PDK1[S241A] structure. These observations suggest that a potentially negatively charged ligand occupied the unassigned density possibly in the shape of a tetrahedral oxy-anion. A range of common oxy-anions (such as phosphate, sulphate, pyrophosphate) was included in the refinement procedure in an attempt to identify the ligand, but without convincing results (data not shown). Interestingly, similar observations with negatively charged ligands in a positively charged pocket in a similar location have been made in a number of other crystal structures of protein kinases such as glycogen synthase kinase 3β (42,43), Chk1 (44), casein kinase 2 (CK2) (45) and Phosphorylase kinase (PhK) (36). A common feature of these kinases with PDK1 is that they all belong to the RD class of protein kinases, and the site of oxy-anion interaction has recently been named the RD-pocket, as the RD Arg (Arg204 in PDK1) is a main contributor to the positively charged nature of the pocket (32). PhK is not activated by phosphorylation in the T-loop, but instead contains a
negatively charged Glu residue which serves to align the catalytic machinery (36,46). Interestingly, in the structure of a T-loop mutant of PhK, in which the T-loop Glu residue was mutated to a Ser, a phosphate molecule occupies the site of the former negative charge contributed by the Glu residue, and no overall conformational changes are observed (36). This situation is similar to that observed in PDK1. From the current knowledge of how PDK1 is regulated it is not clear whether the ability of this site in unphosphorylated PDK1 to bind oxy-anions is physiologically important.
Conclusions.
We have investigated how PDK1 T-loop phosphorylation affects activation and peptide binding in the HM-pocket. The biochemical and structural data show that T-loop phosphorylation of PDK1 is not required for the structural integrity of the αC-helix and the HM-pocket. Furthermore, the basal activity of PDK1 in the absence of the HM-PRK2 is not influenced by the interaction of the αC-helix with the phosphorylated T-loop residue. However, activation of PDK1 by HM-PRK2 is abolished in the PDK1[Y126A,R129A] mutant which lacks contacts between the phosphorylated T-loop and the HM-pocket through the central αC-helix. Moreover, this mutant can no longer be stabilised to thermal denaturation in the presence of HM-PRK2. Thus it appears that these interactions enable the binding of PDK1 to the HM-motif of its substrates to be communicated to the catalytic residues. Interestingly, the three inactive structures of AGC kinases crystallised to date, namely PKBβ (21), MSK1 (40) and PDK1[S241A] (Fig. 3), display markedly different features indicating that a variety of mechanisms exist to account for the activation of these enzymes by T-loop phosphorylation. In contrast, the active forms of the four AGC kinases crystallised to date namely PDK1 (15), PKA (47), PKBβ (22) and PKC0 (41), display a very similar overall conformation. As there is much interest to develop specific AGC kinase inhibitors for the treatment of disease, the greater diversity shown between inactive kinase structures indicates that more selective protein kinase inhibitors would be obtained if the inactive forms of kinases were targeted.
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Table I

**Data collection and refinement statistics.** Values between brackets are for the highest resolution shell. All measured data were included in structure refinement.

|                          | PDK1[S241A] (51-359) ATP complex |
|--------------------------|----------------------------------|
| Beamline                 | ID14-EH1                         |
| Wavelength (Å)           | 0.933                            |
| Space Group              | P3_{21}                          |
| Unit Cell (Å)            | \(a, b = 121.83\) \(c = 47.75\) |
| Resolution (Å)           | 25.0-1.95 (2.02-1.95)            |
| Observed reflections     | 123183                           |
| Unique reflections       | 28782                            |
| Redundancy               | 4.3 (3.0)                        |
| Completeness (%)         | 90.5 (99.2)                      |
| \(R_{merge}\)            | 0.083 (0.416)                    |
| \(I / \sigma I\)         | 14.2 (2.3)                       |
| Reflections in test set  | 588                              |
| \(R_{cryst}\)            | 0.185                            |
| \(R_{free}\)             | 0.230                            |
| Protein residues         | 287                              |
| Water                    | 166                              |
| Ligand atoms             | 31                               |
| SO_{4}^{2-} atoms        | 35                               |
| Glycerol atoms           | 42                               |
| Wilson B (Å\(^2\))      | 24.2                             |
| \(<B>\) protein (Å\(^2\)) | 28.3                             |
| \(<B>\) water (Å\(^2\)) | 39.5                             |
| \(<B>\) ligand (Å\(^2\)) | 33.7                             |
| Bond length (Å)          | 0.014                            |
| Bond angles (°)          | 1.5                              |
| Main chain B (Å\(^2\))  | 0.9                              |
**Figure legends**

**Figure 1**

**Analysis of residues coordinated by T-loop phosphorylation**

A) Structure of the native PDK1 kinase domain (pdb-id 1h1w, (15)) in cartoon representation. Residues discussed in the text are drawn as a stick representation with blue nitrogen atoms, red oxygen atoms and purple phosphorus atoms. Residues with orange carbon atoms have been mutated in this study, whereas residues with grey carbon atoms have been mutated in a previous analysis (15). The ATP molecule and the sulphate molecule present in the phosphate pocket are drawn as coloured spheres, and hydrogen bonds are indicated as black dotted lines.

B) Activity measurements of wild type (WT) PDK1, kinase dead (KI) PDK1 and indicated mutants of PDK1. Activity was measured in the absence of HM-PRK2 (black), or in the presence of 1 µM (yellow) or 10 µM HM-PRK2 peptide. Equivalent amounts of wild type and mutant PDK1 were used as shown by a Coomassie stained gel.

C) Binding of wild type or indicated mutants of GST-PDK1 to the HM-PRK2 peptide was analysed by surface plasmon resonance BiaCore analysis as described in Materials and Methods. Binding was analysed over a range of PDK1 proteins concentrations (2.5-2000 nM) and the response level at the steady-state binding was plotted versus the log of the PDK1 concentration. For the mutants for which significant binding could be detected, estimated $K_d$’s were obtained by fitting the response curves to the 1:1 Langmuir binding model using GraphPad Prism. The apparent $K_d$’s were: wild type PDK1 = 72 ± 5 nM, PDK1[S241A] = 67 ± 4 nM, PDK1[K228A] = 270 ± 7 nM, PDK1[R129A] = 365 ± 5 nM, PDK1[Y126A] = 380 ± 3 nM, PDK1[Y126A/R129A] = 410 ± 8 nM, PDK1[R204A] = 478 ± 10 nM. For the PDK1[Q150E] and PDK1[K115A] mutants the binding was too weak to be able to quantitatively assess the $K_d$.

**Figure 2**

**Stability analysis of PDK1 αC-helix mutants.**

The wild type GST-PDK1 (circles) or the indicated mutants of GST-PDK1 (triangles) were incubated in the presence (closed symbols) or absence (open symbols) of 100 µM HM-PRK2. The samples were then incubated for 2 min at the indicated temperature prior to assay at 30ºC as described in the Materials and Methods. The activity of PDK1 obtained by incubation at 40ºC was taken as 100%. The data is presented as the average value of two separate experiments in which each condition was assayed in triplicate.
Figure 3
Structure of the PDK1[S241A] mutant

A) Overall structure of the PDK1[S241A]-ATP complex (orange), superimposed onto the PDK1-ATP complex (black). The ATP molecule is drawn in sphere representation. The only marked difference observed concerns the mutated T-loop (yellow and grey, respectively). B) In the PDK1[S241A] mutant structure, residues of the αC-helix are drawn in stick representation, with blue nitrogen atoms, red oxygen atoms and yellow carbon atoms. A 2|F_o|-|F_c| electron density map, contoured at 1 σ, is drawn in blue, and displays well ordered density for all residues of the αB- and αC-helices, and therefore an intact PIF- and phosphate-pocket. C) A 2|F_o|-|F_c| electron density map (blue), contoured at 1 σ, is drawn for the residues of the T-loop (colouring as in B). D) The T-loop position of PDK1[S241A] (yellow) deviates significantly from wild-type PDK1 (grey). E) Strong |F_o|-|F_c| electron density, in red and contoured at 2.5 σ, is present at the site where the phosphate group was located in the wild type PDK1 structure, indicating a presumably negatively charged unidentified ligand. Residues Tyr126, Arg129 and Arg204 are ordered in the structure, whereas Lys228 lacks electron density.
Figure 1
Figure 2
Figure 3
Role of T-loop phosphorylation in PDK1 activation, stability and substrate binding
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