Activation of the essential kinase PDK1 by phosphoinositide-driven trans-autophosphorylation

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Abstract

3-phosphoinositide-dependent kinase 1 (PDK1) is an essential serine/threonine protein kinase, which plays a crucial role in cell growth and proliferation. It is often referred to as a ‘master’ kinase due to its ability to activate at least 23 downstream protein kinases implicated in various signaling pathways. In this study, we have elucidated the mechanism of phosphoinositide-driven PDK1 auto-activation. We show that PDK1 trans-autophosphorylation is mediated by a PIP₃-mediated face-to-face dimer. We report regulatory motifs in the kinase-PH interdomain linker that allosterically activate PDK1 autophosphorylation via a linker-swapped dimer mechanism. Finally, we show that PDK1 is autoinhibited by its PH domain and that positive cooperativity of PIP₃ binding drives switch-like activation of PDK1. Our work implies that the PDK1-mediated activation of effector kinases, including Akt, PKC, Sgk, S6K and RSK, many of whom are not directly regulated by phosphoinositides, is also likely to be dependent on PIP₃ or PI(3,4)P₂.

Keywords: autoinhibition, autophosphorylation, cooperativity, HDX-MS, kinase, membrane, PDK1, PIP₃, signaling.

Introduction

3-phosphoinositide-dependent kinase 1 (PDK1) is a serine/threonine protein kinase that plays a central role in regulating cell growth, survival, and proliferation. Knocking out PDK1 in mice leads to early embryonic lethality (1). PDK1 overexpression has been observed in breast and ovarian tumors (2, 3), and its activity is increased in prion-infected neurons (4). PDK1 belongs to the AGC kinase family (which derives its nomenclature from protein kinases A, G and C) that consists of 63 members of which 23 have been reported to be PDK1 substrates, including Akt/protein kinase B (PKB), protein kinase C (PKC), p70 S6 kinase (S6K), and ribosomal S6 kinase (RSK) (5). As such, it is often referred to as a 'master kinase'. The hyperactivation of Akt, downstream of PDK1, is a hallmark of many human cancers as well as a number of rare overgrowth disorders and immune diseases (6, 7).

PDK1 consists of an N-terminal kinase domain and a C-terminal phosphoinositide-binding PH domain. The PH domain binds to phosphatidylinositol-3,4-bisphosphate (PI(3,4)P₂) and phosphatidylinositol-3,4,5-trisphosphate (henceforth abbreviated to PIP₃) with low nanomolar affinity (8), allowing PDK1 to be translocated to the plasma membrane upon insulin- or growth factor-stimulated PIP₃ production by phosphoinositide 3-kinase (PI3K). Phosphorylation of a conserved serine (S241) in the activation loop leads to PDK1 activation. Mutation of Ser241 to alanine reduces its activity against Akt by 50-fold while rendering it inactive against Sgk (9). Activation loop phosphorylation of PDK1 has previously been observed to occur in *E. coli* (9) and with purified protein *in vitro* (10), suggesting that PDK1 can activate itself by *trans*-autophosphorylation. Consistent with these observations, PDK1 dimerization has been reported in cells (11, 12) and PH domain-mediated dimerization has been reconstituted on PIP₃-containing lipid bilayers (13). However, the

molecular mechanisms of lipid-mediated activation by dimerization and *trans*-autophosphorylation remain poorly understood.

Whether PDK1 activation is regulated at all remains controversial. Studies performed on overexpressed PDK1 in different cell lines have reported contradictory results. Some have reported no or minor changes in PDK1 localization in response to insulin (8, 14) whereas others have reported insulin-dependent membrane translocation (15) and growth-factor dependent PDK1 activation (12). High levels of PDK1 activation loop (S241) phosphorylation that are not affected by insulin stimulation are often observed in PDK1 overexpressed cells (16–18). However, S241 phosphorylation of endogenous PDK1 has been shown to be insulin-dependent in a mouse hypothalamic cell line (19). These contradictions highlight the difficulties of studying signaling events in cells under conditions of ectopic over-expression, cell population response heterogeneity, and the transient nature of the events themselves. Nevertheless, these observations have led many to conclude that PDK1 is constitutively active in cells and that signal propagation is regulated by a variety of mechanisms at the level of its substrates, including co-localization, allosteric activation, or post-translational modification. These mechanisms, however, beg the question of why PDK1 is regulated by activation loop phosphorylation at all. Moreover, the presence of a constitutively active kinase in cells is likely to lead to spurious off-target phosphorylation events, uncoupled from growth factor signaling, that are incompatible with the coordination of cellular events in a tightly regulated manner in both space and time.

These contradictions prompted us to address the question of how PDK1 activity is regulated at a molecular level. We show that PDK1 activation by *trans*-autophosphorylation is dependent on a specific face-to-face dimer mediated by its α G helix. *Trans*-

autophosphorylation is further enhanced by two previously uncharacterized motifs in the kinase-PH interdomain linker of PDK1. Finally, we show that PDK1 exists in an autoinhibited conformation in the absence of PIP₃ or PI(3,4)P₂, irrespective of its phosphorylation state. We propose a model of lipid-dependent PDK1 activation in which PH domain-mediated autoinhibition is relieved by PIP₃ or PI(3,4)P₂ binding, leading to kinase domain dimerization and trans-autophosphorylation at the membrane. Our findings suggest that PDK1 activation and activity, as well as the activation of 23 downstream kinases, themselves PDK1 substrates, are restricted to PIP₃ or PI(3,4)P₂-containing membranes.

Results

A transient but specific dimer controls PDK1 autophosphorylation

To investigate the mechanism of PDK1 autoactivation, we first sought to establish suitable construct boundaries of the PDK1 kinase domain that would permit a systematic and quantitative analysis of activation loop autophosphorylation. All constructs employed in this study are defined in Supplementary Table 1 and intact mass spectra for all recombinant proteins are provided in Supplementary Figures 1-2. Chordates encode a region of 50 amino acids immediately upstream of an alternative splice site that is not conserved or missing in older PDK1 orthologs. This region is predicted to be intrinsically disordered and its function has not been investigated. It is not present in isoform 2 of human PDK1. The reported PDK1 kinase domain comprising residues 51-359 (UniProt 015530-2) exhibited autophosphorylation of a second residue with low efficiency in an *in vitro* kinase assay (Supplementary Figure 1A). Since residues 51-71 are disordered in crystal structures of the PDK1 kinase domain, we truncated the N-terminus to create a construct comprising residues

73-359 (Figure 1A) that undergoes stoichiometric activation loop phosphorylation *in vitro* (Supplementary Figure 1B-D). Henceforth, we refer to this construct as PDK1^{SKD} (short kinase domain, where 'short' in this context refers to the C-terminal boundary of the construct).

Both the short kinase domain (PDK1^{SKD}) and near full-length PDK1 (PDK1^{FL}) are monomeric in solution as observed by size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) (Fig. 1B) at a concentration (2 μ M) three orders of magnitude higher than that reported in cells (20), consistent with expectations for a transient interaction. We therefore modeled the PDK1 kinase domain dimer using the ROSETTA symmetric modeling tool (21) using the crystal structure of the monomeric kinase domain (PDB code 2biy). Since the conformation of the activation loop in the context of the dimer is unknown, we deleted the corresponding residues 228-245 from the monomer structure for the purposes of modeling. The best scoring model exhibited a face-to-face arrangement of the two protomers (Fig. 1C) in which the surface of interaction is highly conserved and partially hydrophobic in nature (Supplementary Figure 3A). Strikingly, the dimer model is superimposable with crystal structures of MEK1 and MEK2 dimers (Supplementary Figure 3B), as well as the B-RAF:MEK1 complex (Supplementary Figure 3C), in which the α G helix is found in the dimerization interface. Independent modeling of the kinase domain dimer with AlphaFold2, which employs a deep learning neural network algorithm to combine evolutionary information with known physical and stereochemical constraints (22, 23), resulted in an essentially identical arrangement of the two protomers, with a root mean square deviation (r.m.s.d.) of 1.87-2.73 Å over all backbone C $_{\alpha}$ atoms of both chains for ten models generated with and without template matching (Supplementary Figure 3D).

In the PDK1 dimer, an invariant tyrosine or phenylalanine (human Y288, Supplementary Figure 4A) is contributed to the interface by the α G helix of each protomer (Fig. 1C). Reported substrate kinases of PDK1 that do not undergo autophosphorylation do not exhibit conservation at this position in their α G helices (Supplementary Figure 4B). Therefore, to test our *in silico* model *in vitro*, we mutated Y288 to either alanine (PDK1^{SKD} Y288A, Supplementary Figure 1E), thereby removing the side chain, or glutamate (PDK1^{SKD} Y288E, Supplementary Figure 1F), thereby creating a charge repulsion in the interface. The dimerization of wild-type and mutant kinase domains was evaluated with a radiometric autophosphorylation assay using labeled [γ -³²P] ATP. Mutation of Y288 to either alanine or glutamate resulted in complete abrogation of autophosphorylation under assay conditions in which the wild-type kinase domain is stoichiometrically phosphorylated on S241 (Fig. 1D, Supplementary Figure 1D). It is worth noting that expression of both PDK1^{SKD} Y288A and PDK1^{SKD} Y288E with an N-terminal dimeric GST-tag in insect cells resulted in stoichiometrically monophosphorylated proteins (Supplementary Figure 1G), indicating that mutation of Y288 does not completely abrogate autophosphorylation and can be overcome by artificial dimerization. Moreover, the crystal structure of the kinase domain of PDK1 bearing a Y288G mutation designed to disrupt lattice contacts (24) (Supplementary Figure 4C) illustrates that mutation of Y288 does not result in any conformational changes in the kinase domain. Together, these results indicate that PDK1 autophosphorylation is dependent on a specific kinase domain dimer centered on Y288.

PIF pocket occupancy promotes PDK1 trans-autophosphorylation

Typical AGC kinase domains contain a hydrophobic pocket in the N lobe and a long C-terminal tail containing multiple regulatory motifs, including a hydrophobic motif (HM) with the sequence $\phi XX\phi$, where ϕ = hydrophobic amino acid and X = any amino acid (25). Docking of the HM into the hydrophobic pocket promotes the active conformation of the kinase domain and its mutation is associated with reduced kinase activity (26–29). Some, but not all, AGC kinases also possess a regulatory phosphorylation site immediately C-terminal to the HM. AGC kinases with phosphorylatable HMs or with glutamate substitutions, including Akt, PKC, RSK, S6K, and SGK, are reported substrates of PDK1 (5). Phosphorylation of the HM of these substrate kinases is reported to drive association with the hydrophobic pocket of PDK1 and consequent activation loop phosphorylation of these substrates (10, 30–35).

In contrast to other members of the AGC kinase family, PDK1 does not contain a canonical C-terminal tail. Instead, the kinase domain is followed by a flexible linker and a C-terminal membrane-binding PH domain. Nevertheless, binding of a HM peptide to the hydrophobic pocket of PDK1 leads to conformational changes resulting in higher ATP binding affinity (36) and increased overall stability of the kinase domain (30, 37). The prevailing model of substrate phosphorylation by PDK1 therefore suggests that the phosphorylated HM of the substrate binds into the hydrophobic pocket of PDK1 and allosterically activates PDK1 against the docked substrate. However, binding of a HM peptide to the hydrophobic pocket has also been shown to promote PDK1 autophosphorylation (10). We therefore reasoned that PDK1 auto-activation likely depends on a functional HM.

To test whether a functional HM is necessary for kinase domain dimerization and autophosphorylation, we fused PIFTide, a peptide derived from the HM of PRK2 (QEMFRDFDYIADWC) that binds to PDK1 with low nanomolar affinity (38), to the N-terminus of the kinase domain (PDK1^{PIF-SKD}, Supplementary Figure 1H). The N-terminus (Q73) is just 8 Å away from the end of the PIFTide sequence (PDB 4RRV), which could be bridged with a simple poly-Gly (G₅) linker (Fig. 2A). This construct design ensured constitutive binding of the peptide to the hydrophobic pocket. To avoid autophosphorylation in the presence of ATP, which might disrupt dimerization, we introduced a S241A mutation (Supplementary Figure 1I). Whilst SEC-MALS revealed PDK1^{PIF-SKD} S241A to be monomeric, even in the presence of ATP (Fig. 2B), fusion of PIFTide to wild-type PDK1^{SKD} resulted in faster auto-phosphorylation kinetics (Fig. 2C), confirming that occupancy of its hydrophobic pocket promotes PDK1 autophosphorylation.

In order to obtain a stable kinase domain dimer amenable to structural and biophysical analysis, we next fused the PIFTide sequence to the C-terminus of the kinase domain (PDK1^{SKD-PIF}) (Fig. 2A). Our *in silico* model of the kinase domain dimer revealed that the C-terminus of the kinase domain and the first residue of a HM peptide (modeled using PDB 4RRV) docked in the hydrophobic pocket of the opposing protomer were just 29.4 Å apart. We therefore designed a minimal poly Gly-Ser (GS)₄ linker that would allow the C-terminal PIFTide to reach into the hydrophobic pocket of the other protomer. We hypothesized that, provided that the linker length is sufficient, the high affinity of the PIFTide towards the hydrophobic pocket should result in its constitutive binding and lead to stable dimer formation. SEC-MALS revealed that PDK1^{SKD-PIF} S241A (Supplementary Figure 1J) is a constitutive dimer in both the absence and presence of ATP (Fig. 2B). The presence of ATP

resulted in a noticeable reduction in the hydrodynamic radius of PDK1^{SKD-PIF}, consistent with the specific organization of the two activation loops in the center of the dimer and a consequent compaction of the particle.

The PDK1^{SKD-PIF} dimer was then used to analyze the conformational changes associated with dimer formation by crosslinking coupled to mass spectrometry (XL-MS) using the zero-length, heterobifunctional crosslinker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). We determined dimer- and monomer-specific crosslinks and mapped the crosslinks onto the dimer model (Fig. 2D). In the dimer, we observed 36 novel cross-links between residues in the C-terminal PIFtide sequence and residues in the N-terminus of PDK1 (Supplementary Table 2) consistent with a face-to-face dimer formed by exchange of the C-terminal PIFtide motifs. We also observed striking changes within the activation loop. 51 out of 53 crosslinks in the monomer that were formed between the activation loop and either the N lobe (glycine-rich loop and α B helix) or C lobe (α G and α H helices) as well as a specific cross-link in the catalytic loop of the kinase domain were not present in the dimer (Supplementary Table 3). This result is consistent with the activation loop being highly flexible when not phosphorylated and suggests that it adopts a different conformation in the dimer, which is presumably permissive for trans-autophosphorylation.

Further structural details were obtained by hydrogen-deuterium exchange-mass spectrometry (HDX-MS). HDX-MS reports on the solvent accessibility of backbone amide hydrogens, thereby permitting the mapping of conformational changes. We compared the dimeric PDK1^{SKD-PIF} to monomeric PDK1^{SKD} and mapped the differences in deuterium

incorporation onto the dimer model (Fig. 2E-H, Supplementary Figure 5A). In the N-lobe, the largest decrease in exchange is observed around the hydrophobic pocket (α C and α B helices), confirming binding of the PIFtide. Neighboring regions, as well as the ATP binding pocket, catalytic loop, and activation loop, are also protected in the dimer, reflecting conformational changes due to PIFtide-induced dimerization. Curiously, we did not observe any changes in the α G helix associated with dimerization, which most likely reflects the fact that there are no secondary structure changes in the α G helix upon dimerization. Finally, we observed deprotection of the α H- α I loop in PDK1^{SKD-PIF} suggesting that specific interactions present in the monomeric kinase domain are lost upon dimerization (Fig. 2H, yellow). Superimposition of the activation loop from the structure of PDK1 bearing a S241 to alanine mutation (Fig. 2H, red), revealed that the tip of the activation loop contacts the deprotected region, an observation that is consistent with our XL-MS data and confirms a conformational change of the activation loop during dimerization, an event which is a prerequisite for *trans*-autophosphorylation.

In conclusion, PDK1 autophosphorylation is promoted by occupancy of its hydrophobic pocket, and kinase domain dimerization leads to remodeling of the activation loop for *trans*-autophosphorylation.

PDK1 encodes a hydrophobic motif that drives *trans*-autophosphorylation

Since PDK1 autophosphorylation is promoted by binding of PIFtide to its hydrophobic pocket, we next questioned the source of the HM in the autophosphorylation reaction. Sequence alignment of PDK1 orthologs revealed strong conservation in the flexible linker

between the kinase and the PH domains (residues 359-389), which was especially pronounced within chordates (Fig. 3A, Supplementary Figure 6A). We therefore tested whether this sequence influences autophosphorylation by comparing the kinetics of PDK1^{SKD} (residues 73-359, encompassing just the kinase domain) with a construct of PDK1 comprising an additional 30 amino acids containing the kinase-PH interdomain linker sequence, which we henceforth refer to as PDK1 long kinase domain (PDK1^{LKD}, residues 73-389; Supplementary Figure 1K-L). PDK1^{LKD} autophosphorylation kinetics were dramatically faster than PDK1^{SKD} (Fig. 3B) indicating that the 30 amino acids C-terminal to the kinase domain promote autophosphorylation. However, the linker did not affect the oligomeric state of PDK1^{LKD} S241A (Supplementary Figure 1M), which remained monomeric at 5 μ M even in the presence of 1 mM ATP (Fig. 3C).

Since PDK1^{PIF-SKD} and PDK1^{LKD} both resulted in accelerated PDK1 autophosphorylation kinetics, we hypothesized that the kinase-PH linker might contain a previously unidentified HM that could bind into the hydrophobic pocket of the opposing protomer in the dimer. We reasoned, that, if this was the case, fusion of PIFtide to the N-terminus of PDK1^{LKD} (PDK1^{PIF-LKD}) would compete with the linker for binding to the hydrophobic pocket, thereby inhibiting stimulation of PDK1 autophosphorylation by the linker. To test this hypothesis, we compared the autophosphorylation kinetics of PDK1^{PIF-LKD} (Supplementary Figure 1N) to PDK1^{LKD}. For this and subsequent kinase assays, it was necessary to adjust the reaction conditions in order to slow the rate of PDK1^{LKD} autophosphorylation sufficiently to obtain high quality kinetic data. This was achieved by reducing the ATP concentration from 200 μ M to 50 μ M and PDK1 concentration from 5 μ M to 1 μ M. PDK1^{PIF-LKD} exhibited reduced autophosphorylation kinetics (Fig. 3D), suggesting

that the PIFtide sequence competes with the kinase-PH linker for binding to the hydrophobic pocket and indicating that PDK1 encodes a *bona fide* HM in its linker.

We next sought to identify a putative HM sequence in the linker. The linker sequence contains several patches of hydrophobic residues, but only one corresponds to a typical HM motif (³⁸³Phe-Gly-Cys-Met³⁸⁶) and is capable of reaching into the hydrophobic pocket of the opposing protomer in our *in silico* model. The sequence of this motif aligns favorably with the structurally validated HMs of 57 out of 62 other AGC kinases (Supplementary Figure 6B). Mutation of both of these hydrophobic residues to alanines (PDK1^{LKD} F383A/M386A, Supplementary Figure 10) completely abrogated the effect of the linker (Fig. 3E), confirming that the linker contains a HM that binds to the hydrophobic pocket and acts as an allosteric modulator.

However, the increased autophosphorylation kinetics of PDK1^{LKD} compared to PDK1^{PIF-SKD} (Fig. 3D) suggested that the HM is not the only part of the linker that promotes autophosphorylation. A conserved Asn-Phe-Asp (NFD) motif in the C-terminal tail of AGC kinases PKC iota and PKA has been shown to be important for the binding of ATP and consequently, for kinase activity (39, 40). Within the PDK1 linker sequence, we observed an Asn-Tyr-Asp (NYD) motif five residues N-terminal to the HM. Mutation of Y376 to alanine (PDK1^{LKD} Y376A, Supplementary Figure 2A) resulted in slower autophosphorylation kinetics than the wild-type long kinase domain (PDK1^{LKD}) (Fig. 3F) indicating that the NYD motif also promotes PDK1 autophosphorylation.

Taken together, these results suggest that the linker between the kinase and PH domains encodes previously unidentified, but equivalent, NFD and HM motifs found in the

C-terminal tail of other AGC kinases. We conclude that PDK1 autophosphorylation is allosterically regulated by its kinase-PH interdomain linker in a mechanism analogous to a domain-swap.

PDK1 is autoinhibited by its PH domain

A C-terminal membrane binding PH domain that binds to PI(3,4)P₂ and PIP₃ with high affinity allows PDK1 to be targeted to membranes in response to growth factor signaling (8). PI3K-dependent plasma membrane translocation and a consequent increase in PDK1 dimerization have previously been observed in cells (11). Based on this, we hypothesized that PDK1 auto-activation might be restricted to PIP₃ and PI(3,4)P₂-containing membranes. Such a mechanism of membrane-restricted kinase activity has previously been observed in the related AGC kinases Akt and Sgk3, which contain a PIP₃- and PI(3,4)P₂-binding PH domain or PI3P-binding PX domain respectively (41–43) (Truebestein et al., article in press, *PNAS* 2021). In its inactive state, the catalytic cleft of Akt is occluded by its PH domain, an autoinhibitory interaction which is relieved by PIP₃ or PI(3,4)P₂ binding (42, 44) (Truebestein et al., article in press, *PNAS* 2021). We therefore hypothesized that PDK1 activation might be regulated in a similar way.

To test whether the PH domain inhibits PDK1 autophosphorylation, we compared the autophosphorylation kinetics of near full-length PDK1 containing its PH domain (residues 73-560; PDK1^{FL}, Supplementary Figure 2B-C) to PDK1^{LKD} and PDK1^{SKD} (Fig. 4A). The autophosphorylation kinetics of PDK1^{FL} were significantly slower than PDK1^{LKD}, suggesting that the PH domain exerts an inhibitory effect on the kinase domain. It is worth noting that the inhibitory effect of the PH domain could only be observed by comparing PDK1^{FL} with

PDK1^{LKD}, since PDK1^{SKD} does not contain the regulatory NYD or HM motifs provided by the kinase-PH linker. Comparison of PDK1^{SKD} with PDK1^{FL} would lead to the erroneous conclusion that the PH domain activates PDK1.

We next sought to investigate the mechanism and structural basis of PH domain-mediated autoinhibition by HDX-MS. We compared the deuterium incorporation kinetics for the kinase (PDK1^{SKD}) and PH (PDK1^{PH}, Supplementary Figure 2D) domains of PDK1 with full-length PDK1 (PDK1^{FL}) in pairwise HDX-MS experiments. Comparison of PDK1^{FL} with PDK1^{SKD} revealed a large area of deprotection on one side of the kinase domain in the absence of the PH domain (Fig. 4B, Supplementary Figure 7A). The putative PH domain binding interface covers the whole of the catalytic cleft, including the substrate-binding helix α D, as well as the α G-containing dimerization surface, suggesting that the PH domain mediates PDK1 autoinhibition by impairing dimerization and *trans*-autophosphorylation as well as downstream substrate engagement. To further confirm the interaction between the two domains, we compared PDK1^{FL} to the isolated PH domain (PDK1^{PH}). In the absence of the kinase domain, a large part of the PH domain, including the PIP₃ binding pocket, shows increased H/D exchange (Fig. 4C, Supplementary Figure 7B), suggesting that the PH domain engages in an intramolecular interaction with the kinase domain in full-length PDK1. This surface is highly conserved (Supplementary Figure 7C) and covers the positively charged phosphoinositide binding pocket (Supplementary Figure 7D), but does not include the highly divergent N-bud extension that is peculiar to the PH domain of PDK1 (Supplementary Figure 7E).

We observed in the HDX-MS data that the surface of the PH domain including the phosphoinositide binding pocket exhibited lower rates of hydrogen-deuterium exchange in full-length PDK1 compared to the isolated PH domain (Supplementary Figure 8A-F). This implied that the binding pocket may be sequestered in the autoinhibitory interface between the kinase and PH domains. This property of lipid-regulated kinases has previously been observed for Akt (41), Sgk3 (43), PKC (45, 46) and PKD (47). To test this hypothesis, we performed a liposome pelleting assay in which we compared the binding affinities of full-length PDK1 with its isolated PH domain. We observed that the PH domain bound to PIP₃-containing liposomes with approximately 2-fold higher affinity than full-length PDK1 (Figure 4D). The binding of the isolated PH domain could not be fit with a one-site binding model, but exhibited positive cooperativity (Hill coefficient = 1.91), consistent with dimerization of the PH domain on the membrane, as has previously been reported (13). PDK1^{FL} exhibited sigmoidal binding with a Hill coefficient of 6.01. This strong positive cooperativity likely reflects additional interactions that stabilize the PIP₃-bound conformation, including kinase domain dimerization and HM exchange (Figure 4E).

Such an interaction between the PH and the kinase domain should be reflected in the overall dimensions of PDK1. We therefore analyzed the conformation of PDK1^{FL} in solution by small-angle X-ray scattering (SAXS) (Fig. 4F-K). We compared PDK1^{FL} to the previously determined dimensions of Akt, since both proteins have almost identical molecular weights (57 and 56 kDa respectively) and domain compositions. The calculated radius of gyration ($R_g=29.3 \text{ \AA}$) and maximum dimension ($D_{\max}=82.9 \text{ \AA}$) of PDK1^{FL} are closer to the ones obtained for Akt in the autoinhibited state ($R_g = 27 \text{ \AA}$ and $D_{\max} = 97 \text{ \AA}$) than for an interface mutant of Akt that adopts an open, PIP₃-independent, conformation ($R_g = 32 \text{ \AA}$; $D_{\max} = 122$

Å) (42). Furthermore, the overall dimensions of PDK1^{FL} were not affected by activation loop (S241) phosphorylation, demonstrating that PDK1 is autoinhibited even when stoichiometrically phosphorylated. These observations imply that PH domain-mediated autoinhibition could restrict both PDK1 *trans*-autophosphorylation and activity against downstream substrates to membranes enriched in either PIP₃ or PI(3,4)P₂.

Discussion

Our work describes hereto unknown molecular mechanisms of PDK1 activation. In contrast to previous assumptions that PDK1 is constitutively active in cells, we propose a model in which PDK1 auto-activation and subsequent substrate phosphorylation are restricted to PI(3,4)P₂ and PIP₃-containing membranes (Fig. 5). Prior to pathway activation by insulin or growth factors, PDK1 is present in the cytoplasm in an autoinhibited conformation in which the substrate binding cleft and the dimerization surface of the kinase domain, as well as the lipid binding site of the PH domain, are sequestered in an intramolecular interaction. Upon PI(3,4)P₃ or PI(3,4)P₂ production in the membrane, PDK1 is recruited via its PH domain, which binds specifically to either lipid, thereby breaking the autoinhibitory interaction. Endogenous PDK1 concentrations have been reported to be approximately 30 nM (20), a concentration at which we do not observe any dimer formation *in vitro*. Lipid-mediated recruitment to the membrane results in local concentration of PDK1, which presumably drives kinase domain dimerization. The linker-swap ensures the specificity of PDK1 dimerization and allosterically promotes *trans*-autophosphorylation via a previously uncharacterized HM. Upon autophosphorylation, the kinase domains of PDK1 dissociate to

permit downstream substrate phosphorylation. Whether autophosphorylation drives the dissociation of the kinase domains or dimerization is sufficiently transient that a dedicated mechanism of dissociation is not required, however, remains an open question.

PDK1 dimerization and trans-autophosphorylation have been previously reported (10, 11, 18). Masters et al, however, have proposed a model of the PDK1 kinase domain dimer (11) that is quite different to the model proposed here. Our biochemical and structural modeling show that the HM we have identified in the kinase-PH inter-domain linker can reach into the hydrophobic pocket of the other protomer in the dimer. In the model proposed by Masters et al, the α G helix of one protomer interacts with the hydrophobic pocket of the other protomer. The hydrophobic pockets in this dimer are therefore oriented in a way that is incompatible with the binding of the HM from the opposing protomer. Although our structural model is derived from *in silico* docking of an existing crystal structure, evidence for this mode of kinase domain dimerization is supported by crystal structures of MEK1 and MEK2 homodimers as well as the BRAF:MEK1 heterodimer, all of which are arranged in a face-to-face arrangement that is superimposable with our model of PDK1. Furthermore, the relevance of α G-mediated heterodimerization in B-Raf:MEK signaling is well established (48, 49). α G helix-mediated homodimerization of PDK1 is, however, dependent on PIP₃ or PI(3,4)P₂, since the PH domain obscures the dimerization surface of the kinase domain in the absence of lipids. The same surface of other kinases has been observed to mediate myriad protein-protein interactions that control (auto)inhibition (42, 44, 50, 51), substrate docking (52), recruitment of phosphatases (53), and homo- and heterodimerization-driven trans-autophosphorylation (48).

The PH domain has previously been proposed to regulate PDK1 kinase domain dimerization (11, 13, 18). These studies propose a model in which PDK1 forms an autoinhibitory dimer in the cytoplasm that is dissociated upon trans-autophosphorylation of T513 in the PH domain following the binding of PDK1 to PIP₃ or PI(3,4)P₂ in the membrane. It is unclear, however, what would drive PDK1 dimerization in the cytoplasm considering low nanomolar PDK1 expression levels and the fact that dimerization cannot be observed up to 2 μ M *in vitro*. Moreover, we did not observe any T513 phosphorylation in full-length PDK1 following stoichiometric activation loop phosphorylation (Supplementary Figure 2C). Our HDX-MS analysis, however, revealed that T513 is located within the intramolecular interface between the PH and kinase domains. Comparison of the rates of deuterium incorporation for the peptide comprising residues 511-515 of PDK1 shows that the rate of deuterium incorporation is significantly higher in the absence of the kinase domain (Supplementary Figure 8F). This was, in fact, the largest difference in deuterium incorporation observed over the whole protein. We therefore hypothesize that the insulin- and growth factor-independent activity of PDK1 T513E previously observed in cells (11, 54) may be due to disruption of the autoinhibitory interaction between the kinase and PH domains. In this respect, it is also worth noting that T513 is invariant in chordate PDK1 orthologs. However, though we doubt the physiological role of T513 phosphorylation, PH domain-mediated dimerization of PDK1 on membranes (13) is very much substantiated by the cooperative binding of the PH domain to PIP₃-containing liposomes. This mechanism enhances the specificity of PDK1 dimerization and, together with intramolecular sequestration of both the PIP₃ binding site and the dimerization surfaces of the PH and kinase domains, ensures high positive cooperativity in PIP₃ sensing, leading to switch-like

behavior in PDK1 activation. Although the affinities of PDK1 for PIP₃ and PI(3,4)P₂ were previously reported to be 1.6 and 5.2 nM respectively (8), it should be noted that these experiments were performed with GST-tagged PDK1. As such, the discrepancy between the reported binding affinities and the values of 3.5-6.5 μM that we have measured is likely attributable to an avidity effect that arises from the use of a dimeric fusion tag. Interestingly, the binding affinity of PDK1^{FL} for PIP₃-containing liposomes is only marginally higher than the 15-20 μM determined for Akt1 binding to liposomes of identical composition (41).

The PH domain of PDK1 is essential for PDK1 activity. Abrogation of PIP₃ binding by mutagenesis of arginines 472, 473 and 474 to leucine leads to embryonic lethality in mice as a consequence of the inability of PDK1 to bind PIP₃ or PI(3,4)P₂ (55). However, introduction of a single point mutation, K465E, that disrupts PIP₃ binding did not completely abrogate PDK1 substrate phosphorylation, despite leading to impaired mouse development (56). Our analysis of PIP₃ binding in full-length PDK1 indicates that the PIP₃ binding site is obscured, an observation corroborated by HDX-MS analysis of the intramolecular interface. In fact, peptides containing K465 exhibit significantly increased rates of deuterium incorporation in the absence of the kinase domain (Supplementary Figures 7B, 8A-B). These observations suggest that this mutation might also destabilize the autoinhibitory interaction between the PH and the kinase domains, potentially explaining the residual PDK1 activity and viability observed.

We have identified uncharacterized NFD and HMs in the interdomain linker of PDK1 that are equivalent to those found in the C-terminal tail of all other AGC kinases and that allosterically regulate PDK1 activity. Previously, PDK1 was thought to lack the canonical C-

terminal extension to its kinase domain. While the fact that the HM sequence in PDK1 is only conserved in chordates raises questions about the mechanism of PDK1 activation in evolutionarily older species, we cannot discount that sequence alignments are not powerful enough to identify an analogous sequence in these species. We show that the HM within the interdomain linker binds to the hydrophobic pocket of PDK1 during trans-autophosphorylation. This interaction, while dramatically stimulating autophosphorylation, is necessarily of low affinity compared to the high affinity interaction of PIFtide, since the kinase domain dimer of PDK1 must be capable of dissociation post-activation loop autophosphorylation in order to engage its substrates. This explains why PKD1^{LKD} is monomeric, while our C-terminal PIFtide fusion protein, PDK1^{SKD-PIF}, is constitutively dimeric. Mutation of the central residue in the PDK1 hydrophobic pocket (L155E) was previously shown to result in early embryonic lethality in mice (55). To date, the hydrophobic pocket in PDK1 has been reported to facilitate the interaction with its substrates. However, our findings indicate that the hydrophobic pocket plays an important role in PDK1 auto-activation, which may explain the strong phenotype observed.

The kinase-PH interdomain linker has previously been reported to mediate PDK1 translocation to the nucleus following insulin stimulation (17, 57). The authors of this study identified a putative nuclear export signal (NES) in mouse PDK1 that overlaps with the HM we have identified in this study. However, data from this study also indicates that the nuclear-cytoplasmic distribution of PDK1 is unaltered in cells lacking PTEN, where PI3K signaling is dramatically up-regulated. Whilst we cannot discount the presence of a NES in the interdomain linker, the activation of a nuclear pool of PDK1 would require actively signaling pools of PIP₃ or PI(3,4)P₂. Though some evidence has hinted at their existence (58),

further work is undoubtedly required to probe whether the PI3K pathway is physiologically relevant in the nucleus.

A striking difference between the PDK1 interdomain linker and the C-terminal tail of PDK1 substrates is the absence of a phosphorylated turn motif. The turn motif, reported to be co-translationally phosphorylated by mTORC2 (59), binds to a conserved basic patch on the surface of the N-lobe ($\beta 1$ and $\beta 2$) of the kinase domain, and is important for regulating the stability of both Akt and PKC (60). In our model of the trans-autophosphorylating PDK1 kinase domain dimer, the N-lobe surface to which the phosphorylated turn motif binds in other AGC kinases forms a part of the dimerization surface. As such, transient heterodimerization of PDK1 with any of its substrates to accomplish their activation loop phosphorylation might be expected to be incompatible with docking of the turn motif to the basic patch. Intriguingly, evidence that turn motif phosphorylation results in substrate dissociation from PDK1 has previously been proposed for PRK2 (38). However, little is known about the structural details of PDK1 interaction with its substrates. One obvious hypothesis that arises from this study is that the HM of PDK1 may play an important role in substrate phosphorylation by binding to the vacant hydrophobic pocket of its substrate. Such a mechanism, in which the HMs of both the substrate and PDK1 are exchanged, would undoubtedly enhance the specificity of the interaction, with obvious consequences for signal amplification and fidelity. Further work, however, will be required to test this hypothesis.

Regardless of whether PDK1 forms a specific heterodimer with its substrate or not, our data suggests that PDK1 autoactivation and subsequent substrate phosphorylation is dependent on the presence of PI(3,4)P₂ or PIP₃ in the membrane. Our finding that even S241

mono-phosphorylated PDK1 adopts an autoinhibited conformation in the absence of PIP₃ suggests that PDK1 may be unable to phosphorylate its substrates in the cytosol. Presumably, therefore, all PDK1 substrates must be targeted to PIP₃- or PI(3,4)P₂- containing membranes in order to interact with active PDK1. Akt co-localization with PDK1 is ensured by its own PH domain that binds to PI(3,4)P₂ and PIP₃. Sgk3 phosphorylation by PDK1 is likely restricted to endosomes that present both PI3P, required for Sgk3 activation, and PI(3,4)P₂ for PDK1 activation (43). However, the mechanism of phosphorylation of PDK1 substrates that do not contain a membrane-binding domain, such as RSK, S6K or PRK2, remains to be elucidated. Whether they require a scaffold protein that would target them to the membrane or they rely on a high-affinity interaction between their HM and the hydrophobic pocket of PDK1 for co-localization is not yet known. However, published data suggests that this may be the case, at least for PRK2 and S6K, which respectively encode either a constitutively high-affinity HM or a HM which exhibits a dramatic increase in affinity for PDK1 upon phosphorylation (32).

Several PDK1 substrates belong to the PI3K (Akt, SGK) or MAPK pathways (RSK), both of which are frequently dysregulated in human cancers. PDK1 is, therefore, an attractive target for therapeutic intervention. However, the development of PDK1 inhibitors is challenging, since as little as 10% of normal PDK1 expression levels are enough to maintain viability, while the absence of PDK1 is embryonically lethal (1). So far, no PDK1 inhibitors have progressed into clinical trials. Several inhibitors targeting the hydrophobic pocket of PDK1 have been developed to target substrate-specific inhibition (24, 61). However, our data showing that the hydrophobic pocket of PDK1 is important for PDK1 autoactivation suggests that inhibitor binding to the pocket would impair activation of all PDK1 substrates.

Moreover, the hydrophobic pocket is common to all AGC kinases, with obvious potential for pleiotropic, off-target effects. Our finding that PDK1 is autoinhibited by its PH domain opens up new possibilities for the development of drugs with greater specificity. Allosteric inhibitors targeting the autoinhibited conformation of Akt have been shown to be highly specific (62) with anti-tumor activity in patient-derived xenograft models of pancreatic, colon, and endometrial cancer (63, 64) and tumor regression and Akt signaling blockade in advanced solid tumors (65). Given the essential role of PDK1 in signal transduction downstream of growth factors and upstream of Akt, the development of allosteric inhibitors that target PDK1 may have enormous implications in the treatment of cancer.

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Author Contributions

A.L. purified all proteins and performed all biochemical experiments, with the exception of HDX-MS experiments. T.L. performed *in silico* modeling. A.L. and T.L. analyzed SAXS data. K.F. carried out all HDX-MS experiments and K.F. and J.B. analyzed the corresponding data. A.L. and T.L. wrote and edited the manuscript. T.L. conceived the project.

Declaration of Interests

The authors declare no competing interests.

Materials and Methods

Protein expression and purification

PDK1^{SKD} (73-359), PDK1^{LKD} (73-389), N-terminal PIFtide SKD (PDK1^{PIF-SKD}) or LKD (PDK1^{PIF-LKD}) fusion and C-terminal PIFtide SKD (PDK1^{SKD-PIF}) fusion were cloned into the pFastBac Dual expression vector with an N-terminal GST-tag for expression in baculovirus-infected Sf9 insect cells. PDK1^{SKD} S241A, PDK1^{SKD} Y288A, PDK1^{SKD} Y288E, PDK1^{LKD} F383A/M386A, PDK1^{LKD} Y376A, and PDK1^{SKD-PIF} S241A were generated by site-directed mutagenesis. Near-full length PDK1 (residues 73-556; PDK1^{FL}) was cloned into a pFastBac Dual expression vector with an N-terminal GST tag followed by a 3C cleavage site and a C-terminal His₁₀-Strep II tag preceded by a TEV cleavage site.

All dephosphorylated proteins were purified according to the following protocol. The pellet from 0.5 L of baculovirus-infected Sf9 cells was lysed in 50 ml of lysis buffer: 50 mM Tris pH 7.5, 300 mM NaCl, 2% glycerol, 1 mM TCEP, 0.25% CHAPS, 1 mM PMSF, 1 mM MgCl₂, 0.5 µl benzonase (25 U/µl), 1x protease inhibitor cocktail (made in-house: 100 µM bestatin, 14 µM E-64, 10 µM pepstatin A, 1 µM phosphoramidon). The lysate was cleared by centrifugation at 39 000 g for 30 min and incubated with 3 ml of glutathione sepharose beads (Cytiva) for 2h at 4°C. The beads were washed with Buffer A (50 mM Tris pH 7.5, 300 mM NaCl, 2% glycerol, 1 mM TCEP) and Buffer B (50 mM Tris pH 7.5, 500 mM NaCl, 2% glycerol, 1 mM TCEP) and resuspended in Buffer A containing 1 mM MnCl₂ and 40 µM of lambda phosphatase (purified in-house). The protein was dephosphorylated on beads overnight at 4°C and the next day the beads were extensively washed with Buffer A and Buffer B to remove the lambda phosphatase. The protein was cleaved off the beads in Buffer A with 0.7

μM of 3C protease (generated in-house) for 2h at RT. FL constructs were cleaved with 3C and TEV to remove both tags. The cleaved protein was separated from the beads using a gravity column (Bio-Rad), salt concentration was diluted down to 250 mM using Q_A buffer (50 mM Tris pH 7.5, 1 mM TCEP) and the protein was loaded on a HiTrap Q FF column (Cytiva). Protein was collected in the flow-through, concentrated, and loaded on a HiLoad S200 Increase 10/300 gel filtration column (Cytiva) equilibrated in gel filtration buffer (50 mM Tris pH 7.5, 100 mM NaCl, 2% glycerol, 1 mM TCEP). S241A mutants were purified in the same way with the omission of the lambda phosphatase step.

The PH domain of PDK1 (408-556) was cloned into pGST parallel with an N-terminal GST-tag and transformed into BL21 STAR electrocompetent *E. coli* cells. Cells were grown in 4 L of LB medium containing 100 $\mu\text{g}/\text{ml}$ ampicillin at 37°C, 200 rpm to an $\text{OD}=0.5$. They were then induced with 250 μM isopropylthiogalactopyranoside (IPTG) and grown for 20h at 20°C. Cells were centrifuged at 4000g for 30 min and pellets were lysed in 200 ml of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glucose, 1 mM EDTA, 1 mM EGTA, 1 mM TCEP, 1x protease inhibitor cocktail, 0.5 μl benzonase (25 U/ μl), 2 mM MgCl_2 , 1 mM PMSF, 0.25% CHAPS). The lysate was sonicated and centrifuged at 39 000 g for 30 min and the supernatant was incubated with 7 ml glutathione sepharose beads (Cytiva) for 2h at 4°C. The beads were then washed with 500 ml of buffer A (50 mM Tris pH 7.5, 500 mM NaCl, 2.5 mM DTT) and 200 ml of Buffer B (50 mM Tris pH 7.5, 300 mM NaCl, 2.5 mM DTT). The protein was cleaved off the beads with 500 μM of 3C protease overnight. The protein was concentrated to 5 ml and injected onto a 16/600 Superdex 200 pg column (Cytiva) equilibrated in buffer B.

Intact mass spectrometry

Intact protein samples were diluted in H₂O and up to 100 ng protein were loaded on an XBridge Protein BEH C4 column (2.5 µm particle size, dimensions 2.1 mm X 150 mm; Waters) using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific) with a working temperature of 50 °C, 0.1% formic acid (FA) as solvent A, 80% acetonitrile, 0.08% FA as solvent B. Proteins were separated with a 6 min step gradient from 10 to 80% solvent B at a flow rate of 300 µL/min and analysed on a Synapt G2-Si coupled via a ZSpray ESI source (Waters). Data were recorded with MassLynx V 4.1 (Waters) and analyzed using the MaxEnt 1 process to reconstruct the uncharged average protein mass.

Mass spectrometry of cross-linked samples

Crosslinking of PDK1^{SKD-PIF} S241A with the zero-length crosslinker EDC was performed in 40 mM MES pH 6.5, 100 mM NaCl. 10 µM protein was mixed with 2 mM EDC, 5 mM Sulfo-NHS, 1 mM ATP and 2 mM MgCl₂ and incubated for 1h at RT in the dark. The reaction was quenched with 50 mM Tris pH 7.5 and 20 mM beta-mercaptoethanol, 15 min at RT. Cross-linked monomer and dimer species were separated by polyacrylamide gel electrophoresis. Gel bands were excised, cut up, and destained with acetonitrile (ACN) and 50 mM ammonium bicarbonate (ABC). Before each of the following reaction steps, gel pieces were washed with 50 mM ABC and dehydrated in 100% ACN in order to facilitate the uptake of the solutions. Disulfide bridges were reduced in 10 mM dithiothreitol in 50 mM ABC for 30 min at 56 °C. Subsequently the free thiols were alkylated with 50 mM iodoacetamide in 50 mM ABC in the dark (30 min at RT). Proteins were digested with trypsin (Promega) in 50 mM ABC overnight at 37 °C. The reaction was stopped by adding 10 µl of 10% (v/v) formic

acid (FA) and peptides were extracted by sonication with 5% FA, 50% ACN. The samples were dried in the vacuum centrifuge and taken up in 0.1% trifluoro acetic acid, 2% ACN. Peptide samples were injected on an Ultimate 3000 RSLC nano-flow chromatography system, set up with a pre-column for sample loading (PepMapAcclaim C18, 2 cm × 0.1 mm, 5 µm). Peptides were separated on the C18 analytical column (PepMapAcclaim C18, 50 cm × 0.75 mm, 2 µm; all HPLC parts Thermo Fisher Scientific) applying a linear gradient from 2 to 40% of solvent B (80% ACN, 0.08% FA; solvent A 0.1% FA) at a flow rate of 230 nl/min over 120 minutes. Eluting peptides were analyzed on an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). For the data-dependent mode, survey scans were acquired in the m/z range of 350–1550 at a resolution of 120,000 at 200 m/z, with lock mass on. The AGC target value was set to 4E5 with a maximal injection time of 50 ms. The 15 most intense ions were selected within an isolation width of 1.2 Thomson, fragmented in the HCD cell with a normalized collision energy of 30%. Spectra were recorded at a target value of 1E5 with a maximal injection time of 200 ms and a resolution of 30,000. Only peptides with an assigned charge state between +3 and +6 were selected for fragmentation, the peptide match and exclude isotope features were enabled and selected precursors were dynamically excluded from repeated sampling for 30 s. The MaxQuant software package, version 1.6.0.16 (66) was used to identify the most abundant protein hits searching against the sequence of the PDK1 chimera protein, the proteome of *Bombyx mori* and *Spodoptera* from uniprot (2019.01 UP000005204_7091_Bombyx_mori_all.fasta, 2019.09_Spodoptera.fasta) and common contaminants. Search settings were set to default values. To identify cross-linked peptides, the spectra were searched against the sequences of the top 10 proteins from the MQ search sorted by iBAQ using pLink software v2.3.9 (67).

Carbamidomethylation of cysteine was set as fixed, and oxidation of methionine as variable modification. The enzyme specificity was set to tryptic allowing 4 missed cleavage sites, and EDC/sulfo-NHS was specified according to the cross-linking chemistry. Search results were filtered for 1% FDR (false discovery rate) on the PSM level (peptide-spectrum matches) and a maximum precursor mass deviation of 5 ppm. To remove low quality PSMs, additionally an e-Value cutoff of < 0.001 was applied. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (68) partner repository with the dataset identifier PXD026933.

In silico modeling

The structure of monomeric PDK1 S241A (PDB: 2biy) was modified to remove activation loop residues 223-245, the conformation of which is unknown for a putative dimeric assembly undergoing trans-autophosphorylation. The remainder of the model was not modified. Two independent approaches to modeling a homodimer were employed. In the first, the ROSETTA Symmetric Docking protocol (21) was used to model the dimer by imposing C2 symmetry and searching for dyad-symmetric assemblies with the lowest free energy of docking. The best hit from the modeling was further refined using the ROSETTA protocol for local refinement. The final model was then used to search the Protein Data Bank for homologous assemblies and to design interface mutations to test the model. In the second approach, AlphaFold2 (22, 23) was used to predict the homodimer for residues Q73-T359. Modeling was performed with and without experimental structure input (templates) and the

output models compared to ROSETTA by calculating the r.m.s.d. over all C $_{\alpha}$ atoms for both chains.

Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS)

The oligomeric state and polydispersity of purified recombinant proteins were assessed by SEC-MALS. 50 μ l of PDK1^{SKD} S241A (3 mg/ml), PDK1^{LKD} S241A (5 mg/ml), PDK1^{FL} S241A (1.5 mg/ml), PDK1^{PIF-SKD} S241A (5.6 mg/ml) or PDK1^{SKD-PIF} S241A (4 mg/ml) was injected onto a Superdex 200 10/300 column (Cytiva) operated by a 1260 Infinity HPLC (Agilent Technologies). Light scattering of a 690 nm laser was detected by a MiniDawn Treos (Wyatt) and the refractive index was measured by a Shodex RI-101 (Shodex) detector. The runs were done in 50 mM Tris pH 7.5, 100 mM NaCl, 2% glycerol, 1 mM TCEP, 2 mM MgCl₂, +/- 1 mM ATP.

Radiometric kinase assay

Autophosphorylation reactions were done using radiolabeled [γ -³²P] ATP (Hartman Analytic). All reactions were performed in the following buffer: 25 mM Tris pH 7.5, 100 mM NaCl, 2% glycerol, 5 mM DTT. In the reactions comparing autophosphorylation kinetics between PDK1^{SKD}, PDK1^{SKD} Y288A and PDK1^{SKD} Y288E; PDK1^{SKD} and PDK1^{PIF-SKD}; PDK1^{SKD} and PDK1^{LKD}, 5 μ M of dephosphorylated protein, 200 μ M of ATP (Promega) spiked 1:10 with [γ -³²P] ATP and 400 μ M MgCl₂ were added to the buffer. In the reactions comparing autophosphorylation kinetics between PDK1^{LKD}, PDK1^{PIF-LKD} and PDK1^{PIF-SKD}; PDK1^{LKD}, PDK1^{FL} and PDK1^{SKD}; PDK1^{LKD} and PDK1^{LKD} F383A/M386A; PDK1^{LKD} and PDK1^{LKD} Y376A, 1 μ M of dephosphorylated protein, 50 μ M ATP (1:10 [γ -³²P] ATP) and 100 μ M MgCl₂ were

added to the buffer. At every time point (5, 10, 15, 30, 45 min, 1 and 2 hour), 20 µl aliquots were taken from the reaction and mixed with 5 µl of 500 mM EDTA to terminate the reaction. The quenched reactions were spotted on 0.45 µm nitrocellulose membrane (Cytiva). The membrane was washed 4x5 min with 50 ml of 75 mM H₃PO₄ to remove non-incorporated ATP. The washed membranes were placed in scintillation tubes containing 5 ml of dH₂O. Cerenkov radiation was measured with a Tri-Carb 4910 TR liquid scintillator (Perkin Elmer). Phosphate incorporation in the assay comparing PDK1^{LKD} to PDK1^{LKD} Y376A was quantified by phosphorimaging. The washed membrane was wrapped in Saran film and exposed to a phosphor screen in a cassette overnight. The screen was imaged with an Amersham Typhoon phosphorimager and the radioactive signal was quantified in ImageJ.

Hydrogen-Deuterium Exchange Mass Spectrometry

Sample preparation

HDX reactions for PDK1^{SKD} S241A (monomer) and PDK1^{SKD-PIF} S241A (dimer) were conducted in a final reaction volume of 10 µL with a final concentration of 6.7 µM and 26.8 µM for PDK1^{SKD} S241A and PDK1^{SKD-PIF} S241A, respectively. The reaction was initiated by the addition of 7.0 µL of D2O buffer (20mM pH7.5 HEPES, 100mM NaCl, 94% D2O (V/V)) to 3.0 µL of protein solution (final D2O concentration of 66%). The reaction proceeded for 3, 30, 300, or 3000s at 18°C, before being quenched with ice cold acidic quench buffer, resulting in a final concentration of 0.6M guanidine-HCl and 0.9% formic acid post quench. All conditions were stored at -80°C, and timepoints were created and run in triplicate.

HDX reactions comparing PDK1^{FL} and PDK1^{SKD} were conducted in a final reaction volume of 20 μ L with a final protein concentration of 26 μ M. The reaction was initiated by the addition of 45 μ L of D2O buffer (20mM pH7.5 HEPES, 100mM NaCl, 94% D2O (V/V)) to 5.0 μ L of protein solution (final D2O concentration of 89.6%). The reaction proceeded for 3s at 4°C and 3, 30, 300, or 3000s at 18°C, before being quenched with ice cold acidic quench buffer, resulting in a final concentration of 0.6M guanidine-HCl and 0.9% formic acid post quench. All conditions were stored at -80°C, and timepoints were created and run in triplicate.

HDX reactions comparing PDK1^{FL} and PDK1^{PH} were conducted in a final reaction volume of 50 μ L with a final protein concentration of 5 μ M. The reaction was initiated by the addition of 19 μ L of D2O buffer (50mM pH7.5 HEPES, 100mM NaCl, 94% D2O (V/V)) to 1.0 μ L of protein solution (final D2O concentration of 81.2%). The reaction proceeded for 3s at 4°C and 3, 30, 300, or 3000s at 18°C, before being quenched with ice cold acidic quench buffer, resulting in a final concentration of 0.6M guanidine-HCl and 0.9% formic acid post quench. All conditions and timepoints were created and run in triplicate. Samples were flash frozen immediately after quenching and stored at -80°C until injected onto the ultra-performance liquid chromatography (UPLC) system for proteolytic cleavage, peptide separation, and injection onto a QTOF for mass analysis, described below.

Protein digestion and MS/MS data collection

Protein samples were rapidly thawed and injected onto an integrated fluidics system containing a HDx-3 PAL liquid handling robot and climate-controlled (2°C) chromatography system (LEAP Technologies), a Dionex Ultimate 3000 UHPLC system, as well as an Impact

HD QTOF Mass spectrometer (Bruker). The protein was run over either one (at 10°C) or two (at 10°C and 2°C) immobilized pepsin columns (Trajan; ProDx protease column, 2.1 mm x 30 mm PDX.PP01-F32) at 200 µL/min for 3 minutes. The resulting peptides were collected and desalted on a C18 trap column (Acquity UPLC BEH C18 1.7mm column (2.1 x 5 mm); Waters 186003975). The trap was subsequently eluted in line with an ACQUITY 1.7 µm particle, 100 × 1 mm² C18 UPLC column (Waters), using a gradient of 3-35% B (Buffer A 0.1% formic acid; Buffer B 100% acetonitrile) over 11 minutes immediately followed by a gradient of 35-80% over 5 minutes. Full details of all LC methods can be found at (69). Mass spectrometry experiments acquired over a mass range from 150 to 2200 m/z using an electrospray ionization source operated at a temperature of 200°C and a spray voltage of 4.5 kV.

Peptide identification

Peptides were identified from the non-deuterated samples of PDK1 using data-dependent acquisition following tandem MS/MS experiments (0.5 s precursor scan from 150-2000 m/z; twelve 0.25 s fragment scans from 150-2000 m/z). MS/MS datasets were analysed using PEAKS7 (PEAKS), and peptide identification was carried out by using a false discovery based approach, with a threshold set to 1% using a database of known contaminants found in *Sf9* and *E. coli* cells (70). The search parameters were set with a precursor tolerance of 20 ppm, fragment mass error 0.02 Da, charge states from 1-8, leading to a selection criterion of peptides that had -10logP scores of 31.8, 30.7, 19.7, and 19.1.

Mass analysis of peptide centroids and measurement of deuterium incorporation

HD-Examiner Software (Sierra Analytics) was used to automatically calculate the level of deuterium incorporation into each peptide. All peptides were manually inspected for correct

charge state, correct retention time, appropriate selection of isotopic distribution, etc. Deuteration levels were calculated using the centroid of the experimental isotope clusters. Results are presented as relative levels of deuterium incorporation, no correction for back exchange, and with the only correction being applied correcting for the deuterium oxide percentage of the buffer utilized in the exchange (66%, 75.5%, 89.6%, and 81.2%). Differences in exchange in a peptide were considered significant if they met all three of the following criteria: $\geq 5\%$ change in exchange, ≥ 0.4 Da difference in exchange, and a two tailed t-test value of $p < 0.01$. The raw HDX data are shown in two different formats. Samples were only compared within a single experiment and were never compared to experiments completed at a different time with a different final D₂O level. The data analysis statistics for all HDX-MS experiments are in Supplementary Table 4 according to the guidelines of (71). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (68) with the dataset identifier PXD027401.

Small-angle X-ray scattering (SAXS)

SAXS data for unphosphorylated and S241-monophosphorylated PDK1^{FL} were collected on BM29 at the ESRF, Grenoble, France using an in-line SEC-SAXS setup. Proteins were applied to a Agilent Bio SEC 300 column equilibrated in 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mM EDTA and 1 % (v/v) glycerol and images were acquired every second for the duration of the size exclusion run. Buffer subtraction was performed by averaging 50 frames either side of the peak. All subsequent data processing steps were performed using

the ATSAS data analysis software 3.9.1. The program DATGNOM (72) was used to generate the pair distribution function $[P(r)]$ for each isoform and to determine $D_{\max}$ and R_g from the scattering curves $[I(q) \text{ vs. } q]$ in an automatic, unbiased manner.

Preparation of sucrose loaded vesicles (SLVs) and pelleting assay

Cholesterol, DOPC, DOPS and DOPE were dissolved in chloroform, and PIP_3 was dissolved in a chloroform:methanol:water (1:2:0.8) mixture. To generate SLVs, lipids were mixed in the following molar ratio: 20% cholesterol, 30% DOPC, 15% DOPS, 35% DOPE and 0-2% PIP_3 was added at the expense of DOPC. The lipid mixture was first dried under a nitrogen stream and then rehydrated in 20 mM HEPES pH 7.4, 0.3M sucrose buffer. Lipid mixtures were then frozen in liquid nitrogen and sonicated at RT. The freeze-thawing steps were repeated four times after which the vesicles were pelleted in a Beckman Coulter Optima MAX-XP Ultracentrifuge using a TLA 100 rotor operated at 50 000 rpm/20°C for 30 min. Liposome pellets were then resuspended in the reaction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM TCEP) to a final lipid concentration of 1 mM. 2 μ M of $PDK1^{FL}$ and 2 μ M $PDK1^{PH}$ were mixed 1:1 with liposomes in 50 μ l final volume and the mixture was incubated for 30 min. After that, the reaction was spun down at 9800g for 30 min at 20°C. The supernatant was collected and the pellet was resuspended in the reaction buffer. Equal volumes of supernatant and pellet were loaded on the SDS-PAGE and the fraction of protein bound to the liposomes was determined by Coomassie densitometry.

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Figure Legends

Figure 1. A transient, but specific dimer controls PDK1 autophosphorylation.

- (A) Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) of PDK1^{FL} S241A (green) and PDK1^{SKD} S241A (black). The table summarizes protein polydispersity, theoretical ($M_{r_{theor}}$) and experimentally determined ($M_{r_{exp}}$) molecular weights, as well as the calculated oligomeric state ($M_{r_{exp}}/M_{r_{theor}}$).
- (B) Model of the PDK1 kinase domain dimer obtained using the ROSETTA symmetric modeling tool. Kinase domains (PDB: 2biy) are shown in dark and light grey. Activation loop residues 228-245 that were removed from the structure for the purpose of modeling are shown in dashed blue lines. The hydrophobic motif of PRK2 (PIFtide) bound to the hydrophobic pocket (PDB: 4rrv) is depicted with a black line. Y288 in the dimer interface is highlighted in red.
- (C) Radiometric PDK1 autophosphorylation assay. PDK1^{SKD} (closed black squares), PDK1^{SKD} Y288A (closed red diamonds) and PDK1^{SKD} Y288E (closed blue circles). Error bars are the standard deviation of three independent experiments.

Figure 2. PIF pocket occupancy promotes PDK1 trans-autophosphorylation.

- (A) Graphical schematics of PDK1^{SKD-PIF} and PDK1^{PIF-SKD} fusion proteins. PIFtide is depicted with a black line, (GS)₄ in PDK1^{SKD-PIF} is shown in green dashed line and G₅ linker in PDK1^{PIF-SKD} is shown in red dashed line. Activation loops that were removed from the structure are shown in dark blue dashed lines.

- (B) Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) of PDK1^{SKD-PIF} S241A run in the presence (light green) or absence (dark green) of 1 mM ATP and PDK1^{PIF-SKD} S241A run in the presence (light red) or absence (dark red) of 1 mM ATP. The table summarizes protein polydispersity, theoretical ($M_{r_{theor}}$) and experimentally determined ($M_{r_{exp}}$) molecular weights, as well as the calculated oligomeric state ($M_{r_{exp}}/M_{r_{theor}}$).
- (C) Radiometric autophosphorylation assay. PDK1^{SKD} (closed black squares) and PDK1^{PIF-SKD} (open red diamond). Error bars are the standard deviation of three independent experiments.
- (D) Cross-linking mass spectrometry analysis of PDK1^{SKD-PIF} S241A in the presence of ATP. EDC crosslinked peptides are mapped on the model of the kinase domain. Monomer-specific crosslinks are shown in purple, dimer-specific crosslinks are shown in cyan. The activation loop is highlighted in blue.
- (E) – (H) Comparison of deuterium incorporation in the PDK1 monomer (PDK1^{SKD}) and dimer (PDK1^{SKD-PIF}). PIFtide is depicted with a solid black line, the (GS)₄ linker in dashed line. The activation loop is shown in red mesh representation. The sequence below the model (in cyan) is the sequence of the activation loop that was removed for modeling. Peptides showing significant deuterium exchange differences were mapped on the PDK1 kinase domain dimer model. Color code for the magnitude of changes in deuterium incorporation is shown to the bottom right of panel H.

Figure 3. A hydrophobic motif in PDK1 drives trans-autophosphorylation.

- (A) Alignment of the kinase-PH interdomain linker of chordate PDK1 orthologs. Below: schematic of the PDK1^{SKD} and PDK1^{LKD} constructs with relative positions of the 'NYD' and hydrophobic (Φ xx Φ) motifs.
- (B) Radiometric autophosphorylation assay. PDK1^{SKD} (closed black squares) and PDK1^{LKD} (closed blue triangles). Error bars are the standard deviation of three independent experiments.
- (C) Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) of PDK1^{LKD} S241A in the presence of 1 mM ATP. The table summarizes protein polydispersity, theoretical ($M_{r_{theor}}$) and experimentally determined ($M_{r_{exp}}$) molecular weights, as well as the calculated oligomeric state ($M_{r_{exp}}/M_{r_{theor}}$).
- (D) Radiometric autophosphorylation assay. PDK1^{LKD} (closed blue triangles), PDK1^{PIF-LKD} (open black circles) and PDK1^{PIF-SKD} (open red diamonds). Error bars are the standard deviation of three independent experiments.
- (E) Radiometric autophosphorylation assay. PDK1^{LKD} (closed blue triangles), PDK1^{LKD} F383A/M386A (closed red circles) and PDK1^{SKD} (closed black squares). Error bars are the standard deviation of three independent experiments (red asterisk indicates removal of one outlier).
- (F) Radiometric autophosphorylation assay. PDK1^{LKD} (closed blue triangles) and PDK1^{LKD} Y376A (closed red squares). Error bars are the standard deviation of three independent experiments.

Figure 4. PDK1 is autoinhibited by its PH domain.

- (A) Radiometric autophosphorylation assay. PDK1^{SKD} (closed black squares), PDK1^{LKD} (closed blue triangles) and PDK1^{FL} (closed green circles). Error bars are the standard deviation of three independent experiments.
- (B) Comparison of deuterium incorporation between PDK1^{FL} and PDK1^{SKD} in solution. Peptides showing significant deuterium exchange differences were mapped on the PDK1 kinase domain structure (pdb code: 2biy). Color code for the magnitude of changes in deuterium incorporation is shown on the right.
- (C) Comparison of deuterium incorporation between PDK1^{FL} and PDK1^{PH}. Peptides showing significant deuterium exchange differences were mapped on the PDK1 PH domain structure (PDB: 1w1d). Color coding is the same as in Figure 4B.
- (D) Binding of PDK1^{FL} and PDK1^{PH} to 0-2 mol % PIP₃ liposomes. Error bars are the standard deviation of three independent experiments. Data were fit with a Hill-Langmuir isotherm in which no parameters were constrained. The equilibrium dissociation constants derived from curve fitting, together with the respective Hill coefficients, are reported in the table (inset).
- (E) Schematic of the conformational changes associated with PIP₃ binding that give rise to the positive cooperativity in binding.
- (F) Small-angle X-ray scattering (SAXS) curves for S241-monophosphorylated (black) and dephosphorylated (red) PDK1^{FL} in solution.
- (G) Normalized residuals plot comparing the scattering curves of S241-monophosphorylated and dephosphorylated PDK1^{FL}.

- (H) Guinier plot of the solution scattering data for S241 mono-phosphorylated (black) and dephosphorylated (red) PDK1^{FL}. The interval of $0.32 < qR_g < 1.29$ was used to determine the radius of gyration (R_g) by linear regression of $\ln I(q)$ versus q^2 .
- (I) Residuals plots for the Guinier fits of S241-monophosphorylated (black) and dephosphorylated (red) PDK1^{FL}.
- (J) Pair distribution functions for S241-monophosphorylated (black) and dephosphorylated PDK1^{FL} (red) with R_g and maximum particle dimension ($D_{\max}$) derived from it.
- (K) *Ab initio* calculation of the molecular envelope of PDK1^{FL}.

Figure 5. Model of phosphoinositide-driven PDK1 activation.

Cytosolic PDK1 is inhibited by its PH domain. PIP₃ binding relieves autoinhibition by displacing the PH domain from the dimerization surface and catalytic cleft of the kinase domain. Dimerization of the PH domain upon PIP₃ binding leads to PDK1 kinase domain dimerization, inter-domain linker exchange (which includes the specific binding of NFD and hydrophobic motifs), and trans-autophosphorylation of the activation loop on S241. Upon trans-autophosphorylation, the phosphorylated kinase domain dimer dissociates, rendering it competent to bind and phosphorylate downstream substrate kinases. The substrate kinase Akt is used to illustrate the final step in PDK1-mediated downstream kinase activation (far right).

Figure 1. A transient, but specific dimer controls PDK1 autophosphorylation.

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Figure 2. PIF pocket occupancy promotes PDK1 *trans*-autophosphorylation.

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Figure 3. A hydrophobic motif in PDK1 drives *trans*-autophosphorylation.

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Figure 4. PDK1 is autoinhibited by its PH domain.

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Figure 5. Model of phosphoinositide-driven PDK1 activation.

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