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3'-PHOSPHOINOSITIDE-DEPENDENT KINASE-1 (PDK-1) IN PI 3-KINASE SIGNALING

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TABLE OF CONTENTS

1. ABSTRACT

The recently discovered 3'-phosphoinositide-dependent kinase-1 (PDK-1) is a serine/threonine protein kinase which phosphorylates several members of the conserved AGC kinase superfamily (comprising the prototypes protein kinases A (PKA), G (PKG) and C (PKC)). Phosphorylation of a threonine or serine residue in the activation loop (also known as the T-loop) of these kinases is a critical step in their activation, and is typically accompanied by additional phosphorylations elsewhere in the molecule. Phosphorylation of the activation loop is a common regulatory mechanism shared by most serine/threonine as well as tyrosine kinases as it facilitates alignment of amino acid residues in the active site which are involved in the phosphotransferase reaction. Therefore the discovery of PDK-1 as the enzyme which mediates this event in many protein kinases introduced a new and important step in signaling pathways which regulate numerous important cellular processes including cellular survival, glucose transport and metabolism, tumor progression as well as protein translation. Moreover, the finding that PDK-1 function is mediated in part by the phosphoinositide 3'-OH-kinase (PI 3-K) pathway also provided an explanation as to how the lipid products of PI 3-K, namely phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4-P_2) and phosphatidylinositol-3,4-5-trisphosphate (PtdIns-3,4,5-P_3) stimulate the activation of protein kinase-dependent signaling pathways. These initial landmark observations were followed by many important studies which provided additional mechanistic insight into both PDK-1 regulation as well as the role of this kinase in cellular function. This review will focus on the regulation of PDK-1 and the various mechanisms which it uses to contribute to the activation of target kinases.

2. INTRODUCTION

It has long been known that an important event in insulin and growth factor signaling is the activation of several members of the AGC kinase family, and that in some cases this occurs in a PI 3-K-dependent manner. Similarly, it is now well-established that signaling cascades initiated by the PI 3-K product PtdIns-3,4,5-P_3 mediate many of the metabolic actions of insulin and mitogenic actions of growth factors. In response to insulin and most all growth and survival factors, PI 3-K (class I phosphoinositide 3-OH-kinase) is recruited to the plasma membrane where it phosphorylates PtdIns-4,5-P_2 to produce PtdIns-3,4,5-P_3 (for recent reviews, see (1,2)).

	Activation Loop	Hydrophobic Motif
Akt/PKB	ATMKT^{308}FCGTPEYLAPE	HFPQFS^{473}YSASS
PKCalpha	VTTRT^{497}FCGTPDYIAPE	DFEGFS^{657}YVNPQ
PKCbetaII	VTTKT^{499}FCGTPDYIAPE	EFEGFS^{660}FVNSE
PKCdelta	NRAST^{509}FCGTPDYIAPE	AFAGFS^{664}FVNPK
PKCepsilon	VTTTT^{566}FCGTPDYIAPE	EFKGFS^{729}YFGED
PKClambda	DTTST^{402}FCGTPNYIAPE	EFEGFE^{573}YINPL
PKCzeta	DTTST^{410}FCGTPNYIAPE	EFEGFE^{578}YINPL
S6K-1	TVTHT^{229}FCGTIEYMAPE	VFLGFT^{389}YVAPS
RSK-1	KKAYS^{221}FCGTVEYMAPE	LFRGFS^{380}FVATG
SGK	STTST^{256}FCGTPEYLAPE	AFLGFS^{422}YAPPM
PAK-1	SKRST^{423}MVGTPYWMAPE	
PRK-1	DRTST^{774}FCGTPEFLAPE	AFLDFD^{936}FVAGG
PKA	GRTWT^{197}LCGTPEYLAPE	EFTEF
PDK-1	ARANS^{241}FVGTAQYVSPE	

Figure 1. Alignments of the activation loop motif and the hydrophobic motif of PDK-1 substrates. Members of the AGC kinase family are activated and stabilized by phosphorylation of T-loop and hydrophobic motifs. Shown is an alignment of known and putative targets of PDK-1 highlighting the homology surrounding the activation loop and hydrophobic threonine or serine residues (red). Shown in blue are acidic residues substituting the serine in the hydrophobic motif in PKCzeta, PKClambda and PRK.

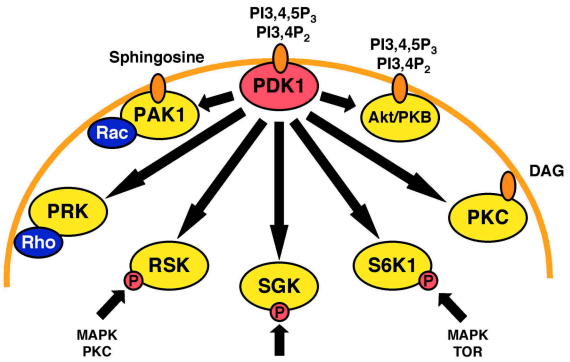

Figure 2. Scheme showing substrates of PDK-1. Lipids (orange), complexed proteins (blue) or phosphorylation events (red) represent activation mechanisms which induce a conformational switch in the substrate allowing subsequent interaction with PDK-1 and phosphorylation of the activation loop. Phosphorylation of RSK, SGK and S6K1 by MAPK (and possibly other pathways) is required prior to the PDK-1 step. Other distinct mechanisms include membrane binding of Akt/PKB or PKC *via* lipids, membrane targeting of PAK *via* the small GTPase Rac and sphingosine, and of PRK *via* the small GTPase Rho. Adapted from Ref. (73).

Conversely, PtdIns-3,4-P_2 is generated primarily by the action of the inositol phosphatases SHIP1 and SHIP2 which use PtdIns-3,4,5-P_3 as a substrate (3). The net effect is the rapid and local accumulation of these two second messengers at intracellular membranes (primarily the plasma membrane). Generation of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 allows the membrane localization and activation of downstream effector proteins. The first and best studied example of these is the Akt/PKB (protein kinase B) serine/threonine kinase, which was initially shown to be activated by growth factors in a PI 3-K-dependent manner (4). Importantly, it was also shown that activation required an intact Pleckstrin Homology (PH) domain, suggesting that lipid binding to this domain was a critical determinant in Akt/PKB activation (4). This was formally demonstrated for both PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 (5,6), and it is now well established that PH domains constitute a major mechanism by which PI 3-K lipids initiate signaling cascades (7). However, it was also known that in addition to membrane localization, a phosphorylation event at the activation loop Thr308 was required for full Akt/PKB activity (8). This was also coupled with phosphorylation at a second site, Ser473, at the very carboxyl-terminus of the protein in a sequence known as the 'hydrophobic motif', because the amino acid residues surrounding the phospho-acceptor are typically Tyr or Phe (Figure 1). The search was therefore underway to discover and characterize the upstream kinase which was responsible for Akt/PKB phosphorylation and activation. This search culminated with the discovery and subsequent cloning of the 3'-phosphoinositide-dependent kinase-1 (PDK-1) in 1997, which was shown to specifically phosphorylate Akt/PKB at the activation loop Thr308 reside, leading to activation (9-12). This was a landmark discovery in the field because of the critical role played by Akt/PKB in both cellular survival pathways as well as mediating the metabolic actions of insulin. This discovery also stimulated rapid progress in understanding the regulation of other related AGC kinases, as it was readily obvious that the homology in the activation loop suggested that PDK-1 might be the universal upstream kinase (13) (Figure 1). This was confirmed by the discovery that S6K1 (S6-kinase 1, also known as the p70 ribosomal S6-kinase) and members of the protein kinase C (PKC) family are also phosphorylated by PDK-1 at their respective activation loop motifs. Other AGC kinases are now known to be specific PDK-1 substrates *in vivo* (Figure 2), although it is worth noting that recent studies have shown that there are some exceptions, such that not all members of this family are physiological PDK-1 substrates (14).

During the last decade, PI 3-K-dependent signaling has emerged as a key regulatory mechanism which eukaryotic cells use to mediate numerous diverse physiological responses. Essential for most of these pathways is the contribution of AGC kinases, leading to the activation of secondary signaling cascades which are important for cell proliferation and cell survival (e.g., Akt/PKB) (15-17), glycogen synthesis (Akt/PKB) (18,19), glucose transport (Akt/PKB, PKCzeta) (20), and protein synthesis (S6K1) (21), again emphasizing the important role played by PDK-1 as a key regulator of PI 3-K signaling. However, the discovery of PDK-1 provided only a piece of the puzzle because as discussed below, phosphorylation of the activation loop is but one step in the activation of AGC kinases such as Akt/PKB, PKC and S6-kinases. Additional regulatory mechanisms exist to fully activate these enzymes, and many of these steps are still poorly understood or controversial. In addition, considering the importance of PDK-1 in signaling, one might expect a complex and tightly regulated mechanism of activation for PDK-1 itself. As discussed below, several mechanisms have emerged which fulfill this requirement.

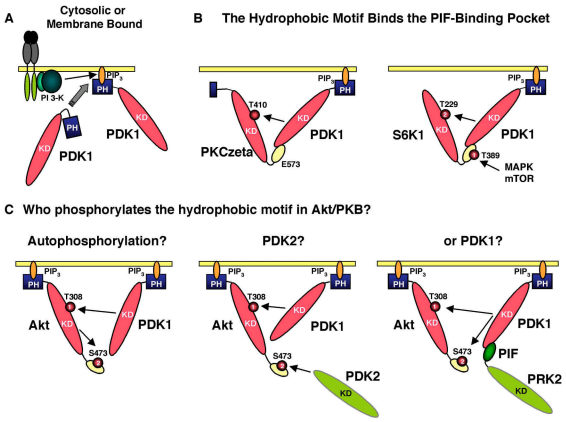

Figure 3. Potential mechanisms of PDK-1-mediated activation of PKC, S6K1 and Akt/PKB. [A] Membrane translocation of PDK-1 in response to growth factor signaling leads to PtdIns-3,4,5-P_3 (PIP_3) production by PI 3-K activation. PDK-1 shows high basal kinase activity and can phosphorylate substrates in the cytosol, but can also be recruited to the membrane by Pleckstrin Homology (PH) domain:PtdIns-3,4,5-P_3 interaction. [B] PKCzeta and S6K1 are phosphorylated and activated by PDK-1 after binding of the negatively-charged hydrophobic motif to the PIF-binding pocket in PDK-1. This is achieved either by the phosphorylation of the hydrophobic site (S6K1, Thr389) or by the presence of a negatively-charged Asp residue (E573 in PKCzeta). [C] Who phosphorylates the hydrophobic motif in Akt/PKB? Three models have been proposed to date. Left panel, Ser473 in the hydrophobic motif of Akt/PKB is regulated by autophosphorylation as a consequence of the PDK-1 step (i.e. Thr308 phosphorylation). Middle panel, a putative PDK2 functions as a heterologous kinase for Ser473 phosphorylation. Right panel, interaction of proteins (e.g., PRK) with PIF-containing sequences with the PIF-binding pocket in PDK-1 converts PDK-1 into a Ser473 kinase. KD, kinase domain.

3. PLECKSTRIN HOMOLOGY DOMAIN AND LOCALIZATION OF PDK-1

PDK-1 is a monomeric 63kDa serine/threonine kinase which was originally isolated from rabbit skeletal muscle (9) and brain cytosol (10) and is ubiquitously expressed in human tissues and cells (11). It contains an amino-terminal kinase domain, followed by a linker region and the Pleckstrin Homology domain at the carboxyl-terminus (9). The PH domain of PDK-1 has been shown to bind to the lipid products of PI 3-K, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 with low nanomolar affinity, causing a redistribution of the enzyme from the cytosol to the plasma membrane (12,22,23). In unstimulated cells, overexpressed PDK-1 is primarily cytosolic (22,24), although two reports have suggested a constitutive membrane localization in the absence of growth factor stimulation (24,25). It is possible that this constitutive membrane association is due to PtdIns-4,5-P_2 binding and this is supported by a report which showed that PDK-1 can interact with significant affinity with PtdIns-4,5-P_2, at least as judged by surface plasmon resonance (24). However, several other studies using distinct lipid binding assays have failed to demonstrate significant interaction of PDK-1 with PtdIns-4,5-P_2 (12,17) and thus an inducible membrane translocation of PDK-1 in response to growth factors remains controversial. On the one hand, PDGF (platelet-derived growth factor) stimulation of endothelial cells (22) and insulin stimulation of HeLa cells (23) and HEK293 cells (26) causes a redistribution of PDK-1 to the plasma membrane in a PI 3-K-dependent manner. On the other hand, IGF-1 (insulin-like growth factor-1) stimulation of human embryonic kidney cells (24) and insulin stimulation of adipocytes (27) did not elicit this translocation. These conflicting results must be rationalized when considering a model for the activation and localization of PDK-1 under normal physiological conditions. Considering the high affinity of PDK-1 for PtdIns-3,4,5-P_3, it is possible that unless this lipid can be quantitatively eliminated (by serum-starvation or with pharmacological agents) a sub-fraction of PDK-1 will be located at the membrane even under unstimulated conditions. It is equally likely that local generation of PtdIns-3,4,5-P_3 will cause redistribution of a pool of PDK-1 to the membrane, but it is also possible that there exist other pools of PDK-1 inside the cell which are prevented from doing so because of other constraints (Figure 3A). These may include scaffolding proteins or localization in discrete subcellular compartments.

Based on the above observations it is reasonable to speculate that the PH domains of PDK-1 and Akt/PKB (and possibly other PH domain-containing substrates) function to bring these two proteins into close spatial proximity. The importance of membrane location is underscored by the fact that artificial myristoylated (hence membrane-targeted) PDK-1 and Akt/PKB are constitutively active towards their substrates (28,29). However it is also possible that activation of Myr-Akt/PKB still requires PtdIns-3,4,5-P_3 to relieve the autoinhibition mediated by the PH domain. In support of this, the activity of a Myr-Akt/PKB allele can be reduced by long-term exposure of cells to PI 3-K inhibitors (30), also pointing to the contribution of membrane-bound PDK-1 in this reaction. However, it is also worth noting that there exist several PDK-1 substrates which do not possess a membrane-targeting module such as a PH or other domain, and which have not been reported to translocate to the membrane following cell stimulation. Examples of these are S6K1 and p90RSK (p90 ribosomal S6-kinase). Whether these kinases are phosphorylated by cytosolic PDK-1 in the absence of PtdIns-3,4,5-P_3 binding, or whether they bind to PDK-1 and are thus indirectly translocated to the plasma membrane for regulation is not clear. As discussed later, the latter possibility is supported by the fact that these kinases directly bind to PDK-1 *via* their respective carboxyl-termini. Finally, concerning PDK-1 intracellular localization, there is agreement with the fact that PDK-1 appears to be excluded from the nucleus (22,24).

4. REGULATION OF PDK-1

Initial studies suggested that despite its name, PDK-1 was a constitutively active kinase which was not dependent on either PI 3-K lipids or other mechanisms to

regulate its intrinsic protein kinase activity. Thus, PtdIns-3,4,5-P_3/PtdIns-3,4-P_2 binding would exclusively regulate its location. However more recent studies have provided good evidence for several mechanisms which regulate the ability of PDK-1 to phosphorylate its downstream substrates. These include lipids such as PtdIns-3,4,5-P_3 and sphingosine, as well as a number of phosphorylation events. Thus, although PDK-1 *per se* shows a high constitutive activity in cells, there exist mechanisms to upregulate its activity in response to growth factor stimulation.

4.1. Regulation by lipids

Although PtdIns-3,4,5-P_3 regulates the cellular localization of PDK-1, this binding does not significantly contribute to its intrinsic protein kinase activity. Initial experiments demonstrated that *in vitro*, PDK-1 activity towards Akt/PKB is strongly stimulated in the presence of lipid vesicles containing PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 (9,10). However, the PtdIns-3,4,5-P_3 dependence for Akt/PKB phosphorylation was shown to be mediated by the Akt/PKB PH domain, because full-length Akt/PKB is not phosphorylated by PDK-1 in the absence of PtdIns-3,4,5-P_3, whereas an Akt/PKB mutant deleted in the PH domain is (11). Thus, a model was proposed in which the Akt/PKB PH domain acts as an autoinhibitory module such that in the inactive conformation, the PH domain blocks access to the catalytic kinase domain by steric hindrance, and PtdIns-3,4,5-P_3 binding at the membrane relieves this inhibition. PDK-1 is now able to access and phosphorylate Thr308 in the activation loop (11,31) (Figure 1). Thus, the PtdIns-3,4,5-P_3 requirement for this reaction is substrate-dependent. It is also worth noting that PtdIns-3,4-P_2 can also support Akt/PKB activation. For example, PtdIns-3,4-P_2 also binds with high affinity to the Akt/PKB and PDK-1 PH domains (5,12). Further evidence for a role for PtdIns-3,4-P_2-mediated Akt/PKB regulation comes from studies on SHIP (SH2-containing inositol 5'-phosphatase) which dephosphorylates PtdIns-3,4,5-P_3 and PtdIns-4,5-P_2 to produce PtdIns-4,5-P_2 and PtdIns-4-P respectively. Studies with SHIP null cells in combination with PI 3-K inhibitors have demonstrated a requirement for PtdIns-3,4-P_2 in the phosphorylation of Akt/PKB, thus contributing to its activation (32). The notion that phosphorylation by PDK-1 is substrate-dependent is further supported by the finding that certain PDK-1 substrates which do not have PtdIns-3,4,5-P_3-binding domains, such as S6K1, are stoichiometrically phosphorylated by PDK-1 *in vitro* in the absence of PtdIns-3,4,5-P_3 (33). However, recent experiments have suggested a more complex role for PtdIns-3,4,5-P_3 in controlling PDK-1 activity towards substrates. Firstly, according to one study, PtdIns-3,4,5-P_3 is still able to increase the activity of PDK-1 towards a PH domain-deleted Akt/PKB *in vitro* by three-fold (10). Secondly, PtdIns-3,4,5-P_3 is able to stimulate, albeit weakly, the activity of PDK-1 towards certain PKCs, such as PKCzeta, which does not have a PH domain (34,35). In addition, an Akt/PKB delta.PH mutant is a better substrate for PDK-1 in complex with the 'PIF' peptide (see below) in the presence of PtdIns-3,4,5-P_3 (36). More recent experiments have shed some light on these seemingly contradictory results. The PDK-1 PH domain, in addition to its role in mediating membrane association, also acts as an autoinhibitory module, and PtdIns-3,4,5-P_3 binding relieves this inhibition (23). Whether the net effect of this release is also to directly increase the catalytic activity of PDK-1 towards substrates is still the subject of some debate. Clearly additional studies are required to resolve this issue, but it is clear that for both Akt/PKB and PDK-1, the PH domain is important for the regulation of the kinases, and PtdIns-3,4,5-P_3 is absolutely required to relieve autoinhibition.

Recently it was shown that the presence of sphingosine in *in vitro* kinase assays increased the autophosphorylation of PDK-1 dramatically, and the elevation of intracellular sphingosine levels induces Akt/PKB phosphorylation in a PDK-1-dependent manner (37). A serine-rich region between the kinase domain and the PH-domain of PDK-1 was identified as the region of sphingosine-stimulated autophosphorylation (37). This region contains Ser393 and Ser396 which have previously been shown to be constitutively phosphorylated *in vivo* (38). Since sphingosine is also prerequisite for the activation of PAK-1 (p21-activated kinase-1) by PDK-1, it has been suggested that sphingosine is an important activator of PDK-1 (37,39) (Figure 2). Whether sphingosine-mediated activation of PDK-1 acts in concert with PtdIns-3,4,5-P_3 binding, or whether it is a separate event, remains to be determined, although it is worth noting that certain growth factors (e.g., PDGF) which stimulate PtdIns-3,4,5-P_3 synthesis also cause elevation of sphingosine levels (40,41). Similarly, a *Saccharomyces cerevisiae* PDK-1 homologue (Pkh1) which does not possess a PH domain has been shown to use sphingosine as the lipid mediator to activate downstream targets (42,43). However, it is unclear whether the sphingosine requirement in eukaryotic cells is substrate-dependent and is thus restricted to only a subset of PDK-1 targets, such as PAK-1.

4.2. Regulation by phosphorylation

Initial studies addressing the regulation of PDK-1 by phosphorylation suggested that it is a constitutively active kinase whose activity is not increased following mitogenic stimulation of cells (38). However, more recent experiments have suggested otherwise. Although PDK-1 is an AGC kinase with a conserved activation loop (Figure 1), regulation of this motif, specifically at Ser241, is mediated by autophosphorylation, and this explains why this kinase, unlike most other members of this family, is active when expressed in bacteria (38). Alessi and co-workers mapped Ser241 and four other residues as *in vivo* phosphorylation sites, Ser25, Ser393, Ser396 and Ser410. Only Ser241 phosphorylation is required for catalytic activity (38). Ser241 is highly conserved between different species, whereas the other four residues are not (11,44-47). In contrast to these results, two more recent independent studies have demonstrated an agonist-dependent activation of PDK-1 in response to insulin and pervanadate stimulation of cells, as judged by immune-complex protein kinase assays (26,48). This activation was accompanied by a modest increase in phosphorylation of Ser241 and Ser25. More striking is the increase in tyrosine phosphorylation of PDK-1 in response to pervanadate stimulation of cells.

Tyrosine phosphorylation and activation of PDK-1 in response to treatment with H_2O_2, pervanadate (which globally inhibits tyrosine phosphatases and activates tyrosine kinases) or NGF (nerve growth factor) was recently demonstrated (27,49). Both H_2O_2 (50,51) and pervanadate (52,53) activate PI 3-K and Akt/PKB (51,54). H_2O_2, pervanadate or overexpression of the tyrosine kinase c-Abl increases the activity of PDK-1 towards the substrates SGK (serum and glucocorticoid-induced protein kinase) and Akt/PKB, but interestingly the tyrosine phosphorylation pattern mediated by these agonists differ in their sensitivity to wortmannin, a PI 3-K inhibitor. For example, c-Src and H_2O_2-mediated tyrosine phosphorylation of PDK-1 is wortmannin insensitive, whereas pervanadate-induced tyrosine phosphorylation is dependent on PI 3-K activity (27,49). This suggests that pervanadate may act on a tyrosine phosphatase upstream of PI 3-K, whereas H_2O_2 likely acts on a tyrosine phosphatase independent or downstream of PI 3-K. In addition, the tyrosine kinases c-Src and c-Abl are both capable of directly phosphorylating PDK-1 *in vitro*. The relevant phosphorylation sites were mapped as Tyr9 and Tyr373, and mutation of Tyr373 resulted in reduced phosphorylation in response to pervanadate (26,27). Moreover, mutagenesis studies revealed that phosphorylation of Tyr9 is dispensable for PDK-1 kinase activity, whereas phosphorylation of Tyr373/376 in response to pervanadate and insulin is required for kinase activity *in vivo*. Finally, PDK-1 tyrosine phosphorylation is concomitant with membrane translocation in response to these same agonists (26). Thus, phosphorylation of PDK-1 at both Ser and Tyr residues has emerged as an important mechanism of regulation, though there is some debate as to the relative contribution of these events in controlling PDK-1 activation. Whether additional upstream kinases exist in cells remains to be determined, but considering that PDK-1 has optimal phosphorylation motifs for PKC, PKA, casein kinase I/II and S6K1, it is tempting to speculate that additional complexity exists in PDK-1 regulation at this level.

4.3. Regulation by PIF

An important mechanism controlling PDK-1 activity is the binding of PDK-1 to the hydrophobic motif present in several of its targets. An initial screen for PDK-1-interacting proteins by Alessi and co-workers yielded the very carboxyl-terminal sequence of the PKC-related kinase-2 (PRK-2) (36). This sequence comprised the so-called hydrophobic motif found in many AGC kinases including Akt/PKB, PKC and S6K1 (Figure 1). A fragment of this sequence from PRK-2 which bound with high affinity to PDK-1 was termed 'PIF' (PDK-1-interacting fragment). A similar sequence is also found in the carboxyl-terminus of PKCzeta, and a unique feature of this sequence is that the phospho-acceptor position in the hydrophobic motif is replaced by an acidic Asp residue, in place of the Ser residue which is present in Akt/PKB, conventional and novel PKCs and S6K1 (55,56) (Figure 1). Similarly, phosphorylation of the equivalent site in the hydrophobic motif of p90RSK provides a docking site for PDK-1 (57). In the case of PKA, the hydrophobic motif lacks a phosphoacceptor or acidic residue (Figure 1). Initial attempts to delineate the significance of PIF binding to PDK-1 suggested that it converted PDK-1 into an enzyme which could phosphorylate Akt/PKB at the hydrophobic Ser473 residue (thus converting it to the putative 'PDK-2' kinase) (36). However, later studies using a catalytically inactive variant of Akt/PKB demonstrated that an alternative explanation for the effect of PIF on PDK-1 is to promote accessibility of Akt/PKB Ser473 to autophosphorylation (56). Regulation of Akt/PKB Ser473 phosphorylation is discussed in more detail below.

What then is the function of PIF binding to PDK-1? As shown for PKCbetaII, binding of PDK-1 to the hydrophobic motif of its target kinases renders the phospho-acceptor residue in that sequence inaccessible for phosphorylation (58). PIF-containing proteins have a higher affinity for PDK-1 than does the hydrophobic motif, and would thus compete for binding effectively displacing PDK-1 from the carboxyl-terminus, allowing (auto)phosphorylation to occur (Figure 3A). This would also account for the observation that a screen for the kinase which phosphorylates the novel PKCdelta isoform at the hydrophobic motif yielded atypical PKCzeta (59). PKCzeta has an acidic Asp residue in the hydrophobic motif, and would thus be predicted to compete with PDK-1 for binding to PKCdelta, leading to autophosphorylation. This model presumes direct interaction of PDK-1 with its target kinases though the hydrophobic motif, and this has indeed been demonstrated for PKCs (58), S6K1 (60), p90RSK and SGK-1 (55), although curiously not Akt/PKB (36,55). This is further supported by the discovery of a PIF-binding pocket in PDK-1 (56), a hydrophobic pocket which directly interacts with the hydrophobic motif of these kinases and which is similar to a hydrophobic pocket in PKA which binds to its own carboxyl-terminus. Alessi and co-workers hypothesized that binding of PIF to this pocket could stabilize the active conformation of PDK-1 (56), and this is consistent with the fact the rate at which PDK-1 can phosphorylate an artificial peptide substrate *in vitro* is accelerated in the presence of PIF (56). Therefore the binding of the PDK-1 PIF-binding pocket to the hydrophobic motif might represent a mechanism by which PDK-1 can phosphorylate cytosolic substrates (Figure 3B). However, for membrane-bound PDK-1 and Akt/PKB, PtdIns-3,4,5-P_3:PH domain interactions might be sufficient for substrate phosphorylation. Clearly, more *in vivo* studies are required to further clarify these models, particularly because the precise nature of physiologically-relevant 'PIFs' remains to be determined.

4.4. Inhibition of PDK-1 signaling

The PtdIns-3,4,5-P_3 phosphatase and tumor suppressor gene PTEN can induce growth arrest, an effect which can be effectively rescued by expression of active PI 3-K (61). PTEN acts by dephosphorylating PtdIns-3,4,5-P_3 at the 3'OH position, effectively converting PtdIns-3,4,5-P_3 back to PtdIns-4,5-P_2 (62). Therefore the activity of PTEN indirectly regulates PDK-1 activity towards Akt/PKB and other substrates. This is evidenced by the finding that deletion of PTEN results in increased cellular levels of PtdIns-3,4,5-P_3 and a concomitant increase in Akt/PKB activity (63-67). Moreover, PTEN-induced cell death can

be inhibited by co-expression of active Akt/PKB (66). This therefore points to an important negative regulatory role for PTEN in the regulation of PtdIns-3,4,5-P_3-dependent PDK-1 activation. It is however necessary to note that there are isolated reports claiming PTEN-dependent signaling with no impact on Akt/PKB activation (68). Termination of PtdIns-3,4,5-P_3 signaling is also effectively achieved by activation of the SHIP phosphatase, which converts PtdIns-3,4,5-P_3 to PtdIns-3,4-P_2, and PtdIns-4,5-P_2 to PtdIns-4-P (reviewed in (69)). As discussed above, cells deleted in both SHIP alleles have been used to further implicate PtdIns-3,4-P_2 in regulation of Akt/PKB.

It has also been shown that the universal second messenger cyclic AMP (cAMP) has inhibitory effects on the PI 3-K/PDK-1/Akt/PKB signaling pathway. PDK-1 membrane translocation (but not kinase activity) can be negatively regulated by elevation of cAMP and as a consequence, Akt/PKB activation loop phosphorylation is compromised (70). Similar effects of cAMP on S6K1 activation have also been reported (71). The inhibitory effect of cAMP on Akt/PKB can be rescued by overexpressing myristoylated, active PDK-1 (29). Since cAMP also inhibits PI 3-K activity, the likely effect is a decrease in PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 generation, which is translated to PDK-1 inhibition (29).

N-alpha-tosyl-L-phenylalanylchloromethylketone (TPCK) exerts anti-tumorigenic and anti-proliferative effects. Interestingly, TPCK inhibits phosphorylation of the PDK-1 phosphorylation sites in S6K1 and Akt/PKB, but has no direct effect of total cellular PDK-1 activity (72). It is possible that TPCK interferes with PDK-1-substrate interactions e.g., the hydrophobic docking motif found in PDK-1 substrates (57), such that it does not alter PDK-1 activity *per se*, but rather its accessibility to a given substrate (72). Thus far, no other small molecule inhibitors of PDK-1 have been described, but development of specific inhibitors for this kinase would clearly be a valuable tool for researchers in the field.

5. TARGETS OF PDK-1

The concerted efforts of many laboratories working on PDK-1 signaling have provided detailed mechanistic insight as to how this kinase phosphorylates and regulates members of the AGC kinase family. As mentioned above, the discovery that PDK-1 is the Akt/PKB activation loop upstream kinase prompted other researchers to investigate the regulation of PKCs, S6K1 and p90RSK, SGK and other kinases as potential PDK-1 substrates (Figure 2), which has been the subject of many recent reviews (17,73,74). Although initial *in vitro* experiments suggested that this was indeed the case, more recent *in vivo* studies as well as the use of cells deleted in both PDK-1 alleles have shown beyond doubt that some of these molecules are true targets in physiological conditions. However, it has also been appreciated that for these kinases there exist additional phosphorylation events which are a pre-requisite for activation, and which in some cases act in concert with the PDK-1-dependent step. Below we review these mechanisms on a case-by-case basis, focusing on the best understood PDK-1 targets, Akt/PKB, PKCs and S6-kinases. We also briefly discuss the regulation of other less well understood examples, such as PRKs, SGK, PAK and PKA.

5.1. Akt/PKB

The serine/threonine kinase Akt/PKB is an important mediator of the physiological actions of insulin and several growth factors including EGF (epidermal growth factor), PDGF and IGF-1 (31,75). It is regulated by the PI 3-K products PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 (4,5) and plays an important role in protecting cells from apoptosis (31). Three steps are required to activate Akt/PKB. In the first step, binding of PtdIns-3,4,5-P_3 and/or PtdIns-3,4-P_2 to the PH domain effectively recruits Akt/PKB to the plasma membrane, and this has been visualized in living cells (22,23). This binding leads to a small increase in Akt/PKB activity (5,6,76), but is also necessary to relieve autoinhibition of the kinase, the second step. This is because the PH domain is proposed to occlude access to the activation loop Thr308, and phosphorylation of this residue by PDK-1 is the rate-limiting step in Akt/PKB activation. This model is supported by the finding that *in vitro* Akt/PKB can only be phosphorylated at Thr308 in the presence of PtdIns-3,4,5-P_3 or PtdIns-3,4-P_2-containing vesicles (10-12). It is important to note that structural evidence for this model is not yet available. Thus, the net effect of membrane localization is to facilitate Thr308 phosphorylation, but for full activity phosphorylation of a serine residue (Ser473) in the hydrophobic motif is also necessary, representing the third step (8). Because regulation of this site was also shown to be mediated in a mitogen- and PI 3-K-dependent manner, and because *in vitro* purified PDK-1 alone could not phosphorylate Ser473, it was postulated that this site was regulated by a distinct PtdIns-3,4,5-P_3-dependent kinase, and thus the name 'PDK-2' was coined for the Ser473 kinase (8) (Figure 3C). As discussed below, a kinase which fulfils this requirement has not yet been identified. It was also appreciated that whereas phosphorylation of both sites is necessary for kinase activity, the contribution of each site to Akt/PKB activity is not equivalent. For example, phosphorylation of Thr308 alone is able to increase Akt activity, but phosphorylation of Ser473 alone does not significantly stimulate the kinase (8,77). There remains no doubt that PDK-1 is the Thr308 kinase, the best evidence coming from embryonic stem cells deleted in both PDK-1 alleles, which show no appreciable Thr308 phosphorylation upon mitogenic stimulation (14). Similarly, PDK-1 has been shown to phosphorylate all three isoforms of Akt/PKB (Akt1 (PKBalpha), Akt2 (PKBbeta) and Akt3 (PKBgamma) in their activation loops (78). What has remained much more contentious is the mechanism of regulation of Ser473, with a number of competing theories currently available. As already mentioned, although binding of PIF to PDK-1 was suggested to convert it to the putative PDK-2 (36), this was later shown to be an unlikely regulatory mechanism in vivo (56). The regulation of Ser473 phosphorylation is reviewed in more detail below (see section 6).

5.2. Protein kinase C

Protein kinase C enzymes are a family of conserved serine/threonine kinases which play key roles in

cell physiology by responding to two equally important regulatory mechanisms, lipid interactions and phosphorylation. Three separate PKC subfamilies exist, conventional (cPKC), novel (nPKC) and atypical (aPKC) isotypes, which are differ with respect to co-factor requirements (79,80). cPKCs and nPKCs respond to extracellular signals which elicit the hydrolysis of PtdIns-4,5-P_2 leading to the generation of Ins-1,4,5-P_3, Ca^{2+} and diacylglycerol (DAG). Binding of DAG to the PKC C1 domain in the regulatory region contributes to the activation of the kinase because it mediates membrane binding and importantly, causes release the autoinhibitory pseudosubstrate domain from the substrate-binding pocket. For a review on how lipid signals regulate PKC activity and function, see (81). However, before PKC is competent to bind DAG, it must first be phosphorylated at three positions in the catalytic kinase core.

Because the activation loop sequence in PKCs shows high homology to that in Akt/PKB (Figure 1), it was perhaps not that surprising to find that PDK-1 also phosphorylates the T-loop residue in conventional (82), novel (34) and atypical PKCs (35). The conventional PKCs (alpha, betaI, betaII and gamma) are all phosphorylated at the activation loop *in vivo* and mutation of the site abolishes the ability of PKCs to gain catalytic competence. In the case of PKCbetaII, phosphorylation of the activation loop (Thr500) by PDK-1 triggers autophosphorylation of two carboxyl-terminal sites (Thr641 in the 'turn motif' and Thr660 in the hydrophobic site). This results in what is considered to be the fully mature species of PKCbetaII (82,83). It is this fully phosphorylated species which is now activatable by DAG. This distinction in the regulation of cPKC also explains why unlike Akt/PKB, cPKCs cannot be immunoprecipitated in the active conformation to measure an increase in protein kinase activity following stimulation, because the activating step is not phosphorylation, but DAG binding. This interaction is presumably lost upon detergent lysis of cells, allowing PKC to fold back into the inactive conformation. However, PDK-1 phosphorylation of cPKCs is the rate-limiting step, because co-expression of kinase-inactive PDK-1 results in a non-phosphorylated, inactive PKCbetaII (82). Once PKCbetaII is phosphorylated at the activation loop triggering the subsequent autophosphorylations, phosphate at Thr500 is dispensable and dephosphorylation at this site occurs without any appreciable loss of activity (84). Although the phosphorylation of PKCbetaII by PDK-1 appears to be constitutive and is not dependent on PI 3-K signaling or PtdIns-3,4,5-P_3 (85), the case with novel and atypical PKCs is somewhat different. Both novel (PKCdelta and PKCepsilon) and atypical (PKCzeta) are phosphorylated and activated by PDK-1 both *in vitro* and in co-transfection experiments (34). Because phosphorylation of PKCzeta and PKCdelta at their activation loop is stimulated *in vitro* by PDK-1 in the presence of PtdIns-3,4,5-P_3 and inhibited *in vivo* by wortmannin (34,35), this suggests a PI 3-K-dependence for the rate-limiting step in the activation of these kinases. This is also consistent with reports that the increase in the activity of these PKCs induced by mitogens can be detected in immune-complex kinase assays, because phosphorylation provides a direct ON/OFF switch. The notion that PDK-1 is the universal PKC upstream kinase is reinforced by the finding that in PDK-1 knockout cells, PKCzeta phosphorylation at Thr410 is abolished, and degradation of cPKCs and nPKCs is greatly accelerated (86). Because PDK-1-triggers phosphorylation of PKCs is necessary for protein stability, the inference is that phosphorylation at the activation loop cannot occur in the absence of PDK-1.

All PKCs also require phosphorylation at the turn motif and hydrophobic motif for full activity, and as with Akt/PKB, there is some debate as to the mechanism by which this is achieved. As mentioned above, in PKCbetaII this is mediated by an autophosphorylation mechanism. However in the case of novel PKCdelta, studies indicate that phosphorylation at the hydrophobic motif is mediated by a heterologous kinase (59,87), one possibly regulated by the mTOR (mammalian target of rapamycin) pathway, because phosphorylation of this site is compromised by rapamycin which inhibits TOR activity, although it is possible that this actually reflects the action of this drug on a phosphatase, rather than a kinase. In the case of atypical PKCs, the hydrophobic phosphoacceptor position is replaced by a negatively-charged Asp residue (Figure 1), an effective mimic of phosphorylation, and it is possible that this alteration is responsible for the high basal kinase activity of PKCzeta. In summary, phosphorylation of the activation loop site of PKCs by PDK-1 is an essential step in the activation cycle of PKC, and is followed by subsequent phosphorylations which lock the enzyme into a conformation which is necessary for the interaction with DAG.

5.3. p70 S6-kinases

p70 ribosomal S6-kinases (S6K1 and S6K2) execute important roles in the regulation of cell growth by controlling the synthesis of ribosomal proteins and other components of the translational apparatus (88). The prototype p70 S6-kinase, S6K1, is regulated by several phosphorylation steps in a complex hierarchical signaling cascade, with some steps not yet fully elucidated. Eight distinct phosphorylation sites contributing to kinase activation have been identified to date. Upon mitogen stimulation, activation is initiated by phosphorylation of a cluster of four Ser/Thr residues in the carboxyl-terminal auto-inhibitory domain (88). These sites are regulated by the Ras/MAPK (mitogen-activated protein kinase) pathway, and phosphorylation induces release of the carboxyl-terminal tail of the kinase which acts as an autoinhibitory pseudosubstrate domain. This release provides accessibility to additional upstream kinases which are also rate limiting for S6K1 activation. The first of these to be described was PDK-1, which phosphorylates the activation loop residue Thr229 (33,89) (Figure 1). PDK-1 also phosphorylates the activation loop of the recently discovered S6K2 homologue (90). Thus, phosphorylation of this site is PI 3-K-dependent and wortmannin sensitive (91). Curiously however, *in vitro* PDK-1 can stoichiometrically phosphorylate S6K1 in the absence of PtdIns-3,4,5-P_3, suggesting that this reaction can occur in the absence of membrane binding *in vivo* (33). One possible reaction mechanism is that the PIF-binding pocket

of PDK-1 can interact with the phosphorylated hydrophobic motif of S6K1, at Thr389 (Figure 3B). Phosphorylation of this site is also wortmannin-sensitive but is also reportedly inhibited by rapamycin (91), suggesting an mTOR input, although a mutant S6K1 which retains mitogen and PI 3-K sensitivity and is phosphorylated at Thr389, is rapamycin insensitive (92). As with Akt/PKB and PKCs, the precise mechanism of regulation of the S6K1 at the hydrophobic site is still under debate. One report indicated that PDK-1 can directly phosphorylate Thr389 *in vitro* in a reaction inhibited by the inclusion of PIF (60). The physiological relevance of this is not entirely clear because PDK-1-mediated phosphorylation of this site is <5% than that of the activation loop Thr229. In addition to mTOR and PDK-1, competing theories concerning regulation of Thr389 include Akt/PKB (93), the NEK6/7 family of NIMA-related kinases (94), as well as the atypical PKCzeta isotype (95). The latter example is noteworthy because PKCzeta appears to cooperate with PDK-1 to mediate Thr389 phosphorylation in transfected cells, although it is not yet known whether PKCzeta directly phosphorylates this site or whether it does so by an indirect mechanism. Considering the reports that for PKCs and Akt/PKB, phosphorylation of this site can be mediated by autophosphorylation it will be interesting to see if such a mechanism can also exist for S6K1. Finally, it is important to note that in the PDK-1 knockout cells, S6K1 activity (and Thr229/Thr389 phosphorylation) is compromised and cannot be increased by IGF-1 stimulation (14). It therefore appears that unlike PKCs and Akt/PKB, phosphorylation of S6K1 by PDK-1 at the activation loop is the last step in the activation of the kinase, and a pre-requisite is phosphorylation of the hydrophobic motif. To complicate matters even further, S6K1 is subject to yet additional phosphorylation events which also contribute to activation, and these are poorly understood (88).

5.4. Other targets

The PKC-related kinases (PRK1 and PRK2), also known as protein kinase N, can be activated *in vitro* by fatty acids and phospholipids (96). *In vitro* and *in vivo*, activation of PRK requires activation loop phosphorylation by PDK-1 (Figure 2). Importantly, activation of PRKs requires the small GTPase Rho, and activation loop phosphorylation can be increased by overexpression of Rho (96,97). As already discussed, the PIF peptide was derived from the carboxyl-terminal hydrophobic motif of PRK1 which harbors an acidic Asp residue (36). More recent studies have shown that the interaction between PRK and PDK-1 is dependent on Rho (96) and an amino-terminal domain in PRK termed HR1 mediates interaction with Rho (97-100). Rho binding disrupts the autoinhibitory effect mediated by a pseudosubstrate domain in PRKs (101). Therefore, Rho binding increases the activity of these kinases slightly (102,103), but is also likely responsible for intracellular localization (104).

The 90kDa ribosomal S6 kinase (RSK1, RSK2, RSK3) consists of two kinase domains connected by a linker region. The carboxyl-terminal kinase domain and linker regions play important roles in the regulation and activation of the enzyme, whereas the amino-terminal kinase domain exerts substrate specificity (105,106). Full activation of RSK by mitogens requires the cooperation of MAPK and PDK-1 resulting in the coordinated phosphorylation of multiple serine residues (107-109). MAPK mediates phosphorylation of Ser369 in the linker region and of Ser577 in the activation loop of the carboxyl-terminal kinase domain (105,106), and this is followed by autophosphorylation of Ser386 in the linker region. This now serves as a docking site for PDK-1, increasing its activity (57). PDK-1 is now able to phosphorylate the amino-terminal kinase domain, specifically at Ser227 in the activation loop, and this has been demonstrated both *in vitro* and in transfected cells (107,109). The importance of PDK-1 phosphorylation of RSK is underlined by the finding that mutation of Ser227 to an Ala abolishes the ability of all three RSK isotypes to phosphorylate exogenous substrates *in vitro* (105,110,111). Moreover, the Ser227Ala mutation in RSK2 can cause Coffin-Lowry Syndrome, which is characterized by severe mental retardation and progressive skeletal deformations (110).

The serum and glucocorticoid-induced protein kinase (SGK) is subject to complex regulatory mechanisms. SGK is transcriptionally regulated by serum, glucocorticoids and mineralcorticoids. Furthermore, SGK kinase activity can be regulated by multiple protein kinases including PKA and PDK-1 in response to stimulation of cells with serum, insulin, IGF-1 and oxidative stress (50,112,113). The structure of SGK is most similar to that of Akt/PKB, although SGK lacks a PH domain. However, the activation of the PI 3-K pathway plays a crucial role in SGK activation. SGKs (SGK1, SGK2, SGK3) are phosphorylated and activated by PDK-1 *in vitro* and *in vivo* (114) (Figure 2) and as predicted, PDK-1 phosphorylates SGK at Thr256 in the activation loop. This phosphorylation can be enhanced by mutation of Ser422 in the hydrophobic motif to an acidic Asp residue (50,113). However, this mutant is not active *per se*, and because the activation of SGK by PDK-1 is abolished by a Ser422Ala mutation, this suggests that Ser422 phosphorylation is the rate-limiting step in the RSK activation. Since the activation of SGK by PDK-1 is unaffected by PtdIns-3,4,5-P_3, but Ser422 phosphorylation is dependent on PI 3-K activity, a putative PtdIns-3,4,5-P_3-dependent Ser422 kinase is proposed to exist (113).

Although PAK-1 (p21-activated kinase-1) is not a member of the AGC kinase family, its kinase domain contains a conserved threonine (Thr423) equivalent to the consensus phosphorylation motif of PDK-1 (Figure 1). This site was initially reported to be an autophosphorylation site, but recent studies have also shown that it can serve as a PDK-1 substrate *in vitro* and in transfected cells (39). PAK-1 is regulated by the GTP-bound form of the Rho-family GTPases Cdc42 and Rac, and by the lipid sphingosine (37,39,115). PDK-1-mediated activation of PAK-1 is dependent on sphingosine but independent of PI 3-K activity (39). Sphingosine was shown to increase the rate at which PDK-1 phosphorylates the activation loop of PAK-1 (37,39). Because the phosphorylation of PAK-1 in PDK-1 null cells has not yet been evaluated, it is not entirely clear

whether autophosphorylation or PDK-1 phosphorylation is the dominant event in controlling activation loop phosphorylation.

5.5. Is PKA a PDK-1 substrate?

PDK-1 was shown to phosphorylate Thr197 in the activation loop of the catalytic subunit of the cAMP-dependent protein kinase (PKA) *in vitro* (116). The physiological relevance of this event remains unclear for a number of reasons. Firstly, cAMP is reportedly a negative regulator of Akt/PKB activity and PDK-1 translocation (29). Similarly, the PDK-1 antagonist TPCK has no effect on PKA activity at concentrations which inhibit PDK-1 in cells (72). However, it is important to note that PKA activation may not require membrane-bound PDK-1, and PKA does not possess a complete hydrophobic motif where TPCK is predicted to bind (72). More striking are the observations that constitutive phosphorylation of PKA at Thr197 can be catalyzed by autophosphorylation in bacteria (13), and that in PDK-1 null cells PKA activity and activation loop phosphorylation is not compromised. It is still possible however that under certain physiological conditions in eukaryotic cells, PDK-1 can mediate phosphorylation at Thr197.

6. PHOSPHORYLATION OF THE HYDROPHOBIC MOTIF

As readily evidenced by the discussions above, while the phosphorylation of the activation loop of several AGC kinases is firmly established, the regulatory mechanisms leading to hydrophobic site phosphorylation remain controversial and the subject of considerable discussion. This is perhaps best illustrated for Akt/PKB, as several mechanisms leading to Ser473 phosphorylation have been proposed (Figure 3C). The original identification that Ser473 phosphorylation was mitogen- and PI 3-K-dependent, but not mediated by PDK-1 suggested the existence of a PtdIns-3,4,5-P_3-regulated kinase, the putative 'PDK-2' (8). At this point it is necessary to note that no single heterologous kinase which is able to directly phosphorylate this site has yet been described. However, it is equally important to describe attempts to delineate such a kinase. As already discussed, the notion that PDK-1 acquires PDK-2 (i.e., Ser473) activity in the presence of the PIF peptide is not likely to represent a viable mechanism because a catalytically inactive variant of Akt/PKB is not phosphorylated under these conditions(36,56). In addition, Akt/PKB in PDK-1 null cells is not phosphorylated at Thr308, but shows a high basal Ser473 phosphorylation, indicating that PDK-1 is not the Ser473 kinase (14). Coupled with the observation that a catalytically inactive variant of Akt/PKB is phosphorylated at Thr308, but not at Ser473 in transfected cells suggested that Akt/PKB autophosphorylates at this site (117), as reported for PKCbetaII. An attractive model emerging from these studies is that since PDK-1 binds to the hydrophobic motif in PKC and other AGC kinases effectively masking this site, PIF and other proteins containing this motif might displace PDK-1 binding thus allowing autophosphorylation of Ser473 (73) (Figure 3). This model would also take into account the fact that in the absence of PDK-1, Akt/PKB would have a higher basal kinase activity and Ser473 phosphorylation, because PDK-1 is no longer masking the hydrophobic motif. Mitogenic stimulation of such cells would be predicted to further increase activity because of the additional accessibility of the active site induced by release of the autoinhibitory PH domain, newly engaged with PtdIns-3,4,5-P_3. This is in fact what has been observed in PDK-1 null cells (14). However, recent studies have challenged this model of Akt/PKB autophosphorylation. For example, staurosporine, a broad-based kinase inhibitor can inhibit Akt/PKB activation exclusively by attenuating Thr308, but not Ser473 phosphorylation (118). Therefore it was suggested that Ser473 phosphorylation is not a consequence of Thr308 phosphorylation and not due to autophosphorylation but might be executed by a staurosporine-insensitive Ser473-kinase (118). However, an equally plausible explanation is that the low specificity of staurosporine as an inhibitor is actually positively affecting a Ser473 phosphatase, rather than acting on a Ser473 kinase, such that the net effect would be uncoupling of the two phosphorylation events. In addition, previous demonstrations of a catalytically-inactive Akt/PKB allele becoming phosphorylated at Ser473 in response to insulin may be due to trans-phosphorylation of the hydrophobic motif by the endogenous Akt/PKB (8).

In human neutrophils the PI 3-K-dependent and p38-mediated activation of MK2 (MAP kinase-activated protein kinase-2, MAPKAPK-2) was proposed to mediate Akt/PKB Ser473 phosphorylation (119) and was demonstrated *in vitro* (8). In contrast to neutrophils however, where the p38 inhibitor SB203580 is able to inhibit Akt/PKB activation (119), in other cells this is not the case (8,51) and MK2 is not generally activated by stimuli that activate Akt/PKB. For example, in HEK293 cells IGF-1 induces Akt/PKB activation and this is not mediated *via* MK2 (8). Therefore it is unlikely that MK2 is the common Ser473 kinase.

The integrin-linked kinase (ILK) was reported to phosphorylate Akt/PKB directly at Ser473 *in vitro* (120) and overexpression of a kinase-inactive ILK allele compromised Ser473 phosphorylation in cells (121). However, it has been argued that since ILK lacks certain required motifs found in all serine/threonine kinase domains, it might in fact not be an active kinase (36,118,122). Although additional studies demonstrated that ILK does have kinase activity *in vitro* (123) an argument against this model was based on the findings that catalytically-inactive ILK mutants are still able to mediate Ser473 phosphorylation in transfected cells (122). Interestingly, ILK possesses a hydrophobic motif similar to that of AGC kinases and mutation of the relevant phosphorylation site into an acidic residue induces phosphorylation of Akt/PKB at Ser473 (122). One can therefore postulate that ILK may have a PIF-like function by competing with PDK-1 from the hydrophobic region of Akt/PKB, allowing Ser473 phosphorylation. Whether this can be achieved by autophosphorylation has not been formally addressed. Finally, one report suggested that activation of conventional PKC isoforms might play a role

in PI 3-K-independent phosphorylation of Akt/PKB at Ser473 in platelets (124). However this may be platelet-specific since Ser473 phosphorylation in all other cell types is PI 3-K-dependent.

In summary, in addition to autophosphorylation a number of competing models are available to explain phosphorylation of Ser473 in Akt/PKB, and indeed in other AGC kinases such as S6K1 and PKCs (see Figure 3C). Although it is possible that more than one mechanism exists to regulate the hydrophobic motif in cells, there is yet no clear evidence that a *bona-fide* heterologous kinase for this site exists. One might ask the question, why is this important? The answer is two-fold: phosphorylation of the hydrophobic motif in many cases is necessary for the full activation of the kinase, and secondly, if a true PDK-2 did exist for some AGC kinases, it might represent a novel target for rational drug design for diseases in which these enzymes have been implicated.

7. CELLULAR FUNCTIONS MEDIATED BY PDK-1

PDK-1 in Insulin and Growth Factor Signaling. A complete understanding of insulin signaling mechanisms is of obvious importance for the treatment of several insulin-linked diseases, such as type II diabetes. The disease is caused by the reduced action of insulin on its major targets, such as the liver, fat and muscle. It is well-established that the PI 3-K pathway plays a key role in insulin signaling, and it can thus be surmised that this translates into an essential role for PDK-1. Insulin signals *via* its receptor, a tyrosine kinase which after ligand binding phosphorylates the substrate IRS (insulin receptor substrate). Phosphorylated IRS then serves as a docking site for several SH2 domain-containing proteins including PI 3-K. The PI 3-K/PDK-1/Akt/PKB pathway plays an essential role in mediating nearly all of the metabolic actions of insulin (125). Akt/PKB mediates insulin's metabolic functions such as glycolysis *via* direct phosphorylation of 6 phosphofructo-2-kinase (PFK-2) (126), glycogen synthesis *via* direct phosphorylation and inhibition of GSK-3 (18,127) and glucose uptake *via* the translocation of the insulin-regulated glucose transporter GLUT-4 (28). Furthermore, activation of Akt/PKB in a PDK-1-dependent manner leads to the induction of adipocyte differentiation (128). Furthermore, the importance of Akt/PKB in insulin signaling is underscored by the finding that mice lacking the Akt2/PKBbeta isoform display insulin resistance and diabetes-like syndromes (129). However, some of the functions of Akt/PKB in insulin signaling overlap with other PDK-1-activated kinases, most notably PKCzeta and the recently identified WISK (wortmannin-sensitive and insulin-stimulated protein kinase). WISK may represent a new element in insulin signaling pathways as it phosphorylates and activates cardiac 6-phospho-fructo-2-kinase (PFK-2) and may itself be activated by PDK-1 (126,130). PDK-1 has also been implicated in GLUT-4 translocation in adipocytes (131) and PDK-1-induced activation of PKCzeta has been shown to play an important role in the translocation of the inducible GLUT-4 (132). Similarly, PDK-1-activated PKCzeta also plays a role in mediating insulin-stimulated MAPK activation (133). Finally, in response to insulin PDK-1 stimulates protein synthesis in an S6K1-dependent manner (89,134,135).

Regulation of Cell Survival via PDK-1/Akt/PKB. The PI 3-K/PDK-1 pathway is a key regulator of cellular survival (16). For example, PDK-1 antisense oligonucleotides cause a reduction in cellular proliferation and survival by inhibiting S6K1 and Akt/PKB activation (136). Akt/PKB mediates cellular survival signaling and it does so by a variety of mechanisms (137-139). It can either directly phosphorylate and inhibit the activities of the pro-apoptotic proteins BAD (137,138,140), caspase 9 (141) and forkhead transcription factors (142), or it induces anti-apoptotic signals *via* NF-κB activation (142-144). The beta isoform of Akt/PKB is overexpressed in several human cancers such as ovarian carcinoma (12%), pancreatic carcinoma (10%) and breast carcinoma (3%) (145-147). The inhibition of apoptotic effects by PDK-1/Akt/PKB may therefore contribute to the generation of malignancies by making cells independent of extracellular survival signals.

PDK-1 in development. PDK-1 is broadly expressed in mouse tissue and also at all embryonic stages (148). Thus PDK-1 is likely to play important roles in both adult individuals as well as in development. A high level of a testis-specific mPDK-1 mRNA has been shown and the implication is a role for PDK-1 in sex differentiation (148). This is consistent with the finding that the *D. melanogaster* homologue to PDK-1, DSTPK61 (DPDK1) has been shown to regulate sex differentiation, oogenesis and spermatogenesis (11). Transgenic studies in *D. melanogaster* further revealed a new role for the PI 3-K/PDK-1 signaling pathway, namely an essential role in the control of cell size. Down-modulation of many of the components of the PI 3-K pathway, such as or Dakt1 (*Drosophila* Akt) and *Drosophila* p70 S6-kinase resulted in a dramatically reduced cell and body size (149-151). As expected, DPDK-1 is the key player. Impairment of PDK-1 signaling causes ectopic apoptosis and developmental arrest of the mutant fly at the embryonic stage (152). Similarly, PDK-1 loss-of-function mutants in *D. melanogaster* failed to grow into larvae and do not form proper cuticles (152). This is consistent with studies in budding and fission yeast, where disruption of PDK-1 homologues resulted in lethal phenotypes (45,46,153). Finally, loss-of-function mutations of PDK-1 show a developmental arrest at the dauer larvae stage in *C. elegans* (47). All of these results support the hypothesis that PDK-1 activity is important for the normal development of eukaryotic organisms. The phenotype of PDK-1 null mice has yet to be reported.

8. PDK-1 HOMOLOGUES IN EUKARYOTIC CELLS

Since PDK-1 and its target kinases regulate numerous cellular responses, these proteins are expected to be evolutionarily highly conserved in structure and function. PDK-1-like proteins have been described not only in *D. melanogaster* and *C. elegans* (11,47), but also in plants (*Arabidopsis*) (44) and in yeasts (45,46).

Functional counterparts for PDK-1 (Pkh1, Pkh2 and Pkh3) have been described in the budding yeast (*Saccharomyces cerevisiae*). Pkh1 and Pkh2 are essential for cell growth and viability of the cells. This was demonstrated in cells lacking Pkh1 and Pkh2 (pkh1delta/pkh2delta), which are not viable, an effect which can be rescued with the human PDK-1 allele (45). Human PDK-1 or PDK-1-deltaPH can restore viability, consistent with the fact that Pkh1/2 do not have a PH domain. Conversely, Pkh1 can phosphorylate human Akt/PKB at Thr308 indicating functional similarity. Interestingly, single mutants such as pkh1delta or pkh2delta are viable, indicating that both PDK-1 homologues share a role essential to cell growth (153). Pkh3 has been described to phosphorylate the same targets as Pkh1 and Pkh2, but plays a minor role in cell growth under physiological conditions (153). In yeast, PDK-1 homologues have similar targets as the mammalian counterpart, including Pkc1 (PKC homologue) and Ypk1 and Ypk2 (SGK homologues) (45,153). The lack of a PH domain in the yeast alleles points to independence from PtdIns-3,4,5-P_3, and there is some evidence that sphingolipids such as sphingosine might be important for activity, instead of phosphoinositides (42,43). In fission yeast (*Schizosaccharomyces pombe*), which requires inositol for growth, mating and sporulation, the PDK-1 homologue ksg1 is essential for these responses. Ksg1 shows 56% structural homology to human PDK-1 in its ATP-binding site, active site and the substrate recognition site (46). There is somewhat lower homology in the PH domain, however it is well known that divergence in the amino acid sequence of many PH domains is not translated to function, such as lipid binding (154). Compared to the human, *D. melanogaster*, or *C. elegans* PDK-1, the PH domains of plant and *S. pombe* PDK-1 lack important conserved amino acid residues which are a prerequisite for the specific binding of PtdIns-3,4,5-P_3 (44,155). Importantly, as these organisms do not possess class I PI 3-Ks (44,156), it is possible that phospholipids or phosphoinositides other than PtdIns-3,4-P_2 or PtdIns-3,4,5-P_3 may interact with these PH domains (44).

A plant homologue of mammalian PDK-1 containing a kinase and PH domain has been identified in rice and *Arabidopsis* (44). *Arabidopsis* PDK-1 (AtPDK-1) can bind to PtdIns-3,4,5-P_3 and is able to activate human Akt/PKB in the presence of this lipid, albeit at a much lower rate. Furthermore, the *Arabidopsis* PDK-1 was able to rescue the above described yeast mutant (pkh1delta and pkh2delta), and thus may activate similar targets such as PKC and SGK homologues (44).

In *C. elegans* two PDK-1 homologues have been described (47,157). One cePDK-1 contains a PH domain and is thus dependent on lipids, whereas PIAK (phospholipid-independent Akt/PKB kinase) lacks the PH domain and is independent of PtdIns-3,4,5-P_3 (47,157). However, both act on Akt/PKB by phosphorylating the Thr308 residue (157).

9. PERSPECTIVE

In the last few years the importance of PDK-1 in the regulation of several protein kinases which mediate essential cellular functions has been clearly defined. Numerous studies have addressed the precise mechanism by which PDK-1 regulates activation loop phosphorylation of these target kinases, and these are now largely understood. However, several questions still remain, one of the most important being the mechanism of hydrophobic site phosphorylation of Akt/PKB, S6K1 and SGK, and in particular the contribution of PDK-1 to this event. The current literature suggests that in some cases phosphorylation of the activation loop by PDK-1 is a prerequisite for hydrophobic site phosphorylation, whereas in others it may occur as a consequence of this step. The data so far also suggest that a common unifying mechanism for hydrophobic motif phosphorylation is substrate conformation, an event which can be mediated by a variety of regulatory elements, such as PIF binding and release, phosphorylation of Ser/Thr residues causing release of autoinhibitory domains, or binding of adapter proteins. Thus, a number of mechanisms have emerged to regulate the accessibility of PDK-1 to its substrates, and perhaps surprisingly this is not restricted to PtdIns-3,4,5-P_3 binding at the membrane alone. This helps to explain why some substrates of PDK-1 do not need to relocalize to the plasma membrane for phosphorylation.

Future functional studies may also shed more light on additional mechanisms which control PDK-1 activity, whether by lipids, phosphorylation or others. Considerable progress has been made recently on the role of PI 3-K lipids in controlling PDK-1 localization, though this is still the subject of some debate. The advent of fusion proteins comprising the green fluorescent protein (GFP) coupled with live real-time imaging of cells has greatly accelerated progress on these issues, but what remains unclear is the precise role of PI 3-K in controlling PDK-1 activity and location under normal physiological conditions. Similarly, it is likely that other important mechanisms controlling PDK-1 function exist in cells but which have yet to be appreciated. For example, many enzymes in the PI 3-K pathway are subject to control by binding to scaffolding proteins such as AKAPs (A-kinase anchoring proteins), as well as dephosphorylation by protein phosphatases. So far, no such mechanisms have been reported for PDK-1. Thus, a greater understanding of the regulatory mechanisms which PDK-1 uses to activate its targets is likely to occupy researchers in the field in the near future. More distant goals will likely focus on the development of inhibitors as well as activators of this important protein kinase with a view to manipulate its action in aggressive human diseases in which the PI 3-K pathway is often deregulated.

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