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Cellular Signaling: Pivoting around PDK-1

Minireview

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The phosphoinositide 3-kinase (PI3K) signaling pathway mediates a multitude of cellular responses following extracellular stimulation by peptide growth factors and hormones. Deregulation of this pathway is associated with human diseases such as cancer and diabetes. The importance of this pathway in cell biology is underscored by the fact that PI3K signaling influences both cell survival and death, in addition to other fundamental cellular functions such as growth, motility, differentiation and insulin action. It does so by activating multiple distinct secondary signaling cascades, and considerable information exists about the precise biochemical mechanisms by which PI3K mediates these events. One group of enzymes that has emerged as a key mediator of the PI3K signal is the AGC superfamily of serine/threonine protein kinases (so named because it includes protein kinases A, G, and C), long known to be critical components of the signal transduction machinery. Most members of this family require an activating phosphorylation, setting off the search for a potential upstream kinase that was linked to the PI3K pathway.

The search for such a kinase culminated with the discovery in 1997 of a novel member of the AGC family, the phosphoinositide-dependent kinase-1 (PDK-1). PDK-1 has now been shown to stand at a pivotal point in signaling, initiating a flurry of studies into understanding how PDK-1 function is regulated. This review discusses how the primary regulators of PDK-1 function are substrate conformation and subcellular localization.

Akt/PKB, the Archetypal PDK-1 Substrate

The discovery that activation of the proto-oncogene Akt, also known as protein kinase B (PKB), is dependent on 3′ phosphoinositides spawned much of the interest in the role of PI3K in cell signaling. Both PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 bind with high affinity to the pleckstrin homology (PH) domain of Akt/PKB, thus recruiting the kinase to the plasma membrane. However, an additional event is required to fully activate Akt/PKB. A common regulatory mechanism of kinases is through phosphorylation of a segment near the entrance to the active site, the activation loop, and a second phosphorylation site at the carboxyl terminus, in the hydrophobic motif. In Akt, these sites correspond to Thr308 in the activation loop and Ser473 in the hydrophobic site. Phosphorylation of both is mitogen- and PI3K-dependent (Alessi et al., 1997; Stokoe et al., 1997). Extensive biochemical studies have clearly demonstrated that PDK-1 is the upstream kinase for Thr308. Following on the heels of the discovery that PDK-1 is the Akt/PKB upstream kinase came the observation that PDK-1 also phosphorylates a number of other kinases, including p70S6-kinase (p70S6-K) and protein kinase C (PKC) (Figure 1) (for a recent review, see Vanhaesebroeck and Alessi, 2000).

Substrate Conformation: A Key Regulator of PDK-1 Activity

The phosphorylation of Akt/PKB by PDK-1 is regulated by the conformation of Akt. Specifically, the engagement of the PH domain on the membrane by binding PtdIns-3,4,5-P_3/PtdIns-3,4-P_2 relieves autoinhibition of the active site, allowing PDK-1 to access Thr308 on the activation loop (Stokoe et al., 1997). Consistent with this, an Akt/PKB mutant lacking the PH domain no longer requires PtdIns-3,4,5-P_3 for PDK-1-mediated phosphorylation in vitro and is constitutively phosphorylated and active in cells (Filippa et al., 2000). Thus, the PH domain masks the activation loop site and its release is required for PDK-1 phosphorylation.

Similarly, access of PDK-1 to the activation loop of PKC is conformationally regulated. In this case, the autoinhibitory pseudosubstrate sequence of PKC must be removed from the substrate binding cavity in order for PDK-1 to phosphorylate PKC. The phosphorylation by PDK-1 of PRK/PKN is also conformationally regulated: these kinases require interaction with the small GTPase Rho to induce a conformational change which enables PDK-1 binding and phosphorylation at the activation loop. In addition, binding of sphingosine to PAK has recently been proposed to alter PAK in a manner that permits phosphorylation of this kinase by PDK-1. Lastly, p70S6-K requires prior phosphorylation on its autoinhibitory sequence by MAP kinase in order to expose its activation loop for phosphorylation by PDK-1. Thus, substrate conformation is a major determinant in allowing PDK-1 phosphorylation to occur.

PDK-1 activates its substrate kinases by two mechanisms, direct or indirect. For Akt/PKB and the atypical PKCζ, phosphorylation at the activation loop serves as a direct "ON/OFF" switch for catalytic activity. Once phosphorylated by PDK-1, these kinases are directly activated. In contrast, phosphorylation at the activation loop of conventional PKC isozymes does not result in activation but rather "primes" PKC for subsequent activation (Dutil et al., 1998). Specifically, the PDK-1 phosphorylation triggers two C-terminal autophosphorylation reactions required to generate a catalytically competent, stable "mature" PKC. However, this species is maintained in an inactive conformation by its pseudosubstrate sequence. Relief of autoinhibition and subsequent phosphorylation of substrates results from binding its lipid second messenger, diacylglycerol, at the membrane.

The Elusive PDK-2

While the regulation of the activation loop by PDK-1 is widely accepted, that of the C-terminal hydrophobic site (Ser473 in Akt/PKB) is less clear. This site is also conserved in other AGC kinases, and a series of recent

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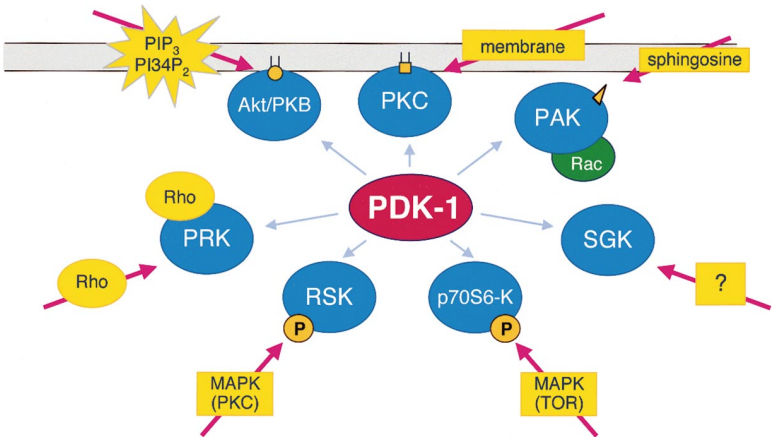

Figure 1. Schematic Showing Protein Kinase Substrates of PDK-1

Structures in yellow represent the activating mechanism which induce a conformational switch in the substrate allowing subsequent PDK-1 phosphorylation of the activation loop. Examples of this switch include: phosphorylation of p70S6-K and p90RSK by MAPK (and possibly other pathways comprising TOR [target of rapamycin] and PKC); binding of the small GTPase Rho to PRK; binding of sphingosine to Rac-bound PAK; PtdIns-3,4,5-P_3 binding to the PH domain of Akt/PKB; and membrane binding of newly synthesized PKC.

reports has provided contrasting views on how phosphorylation of this residue is controlled. Initial studies on Akt/PKB indicated that regulation of this site is both mitogen- and PI3K-dependent, leading to the proposal that an upstream kinase, also in the PI3K pathway but distinct from PDK-1, was responsible for Ser473 phosphorylation. Thus, the name PDK-2 was coined for the hydrophobic site kinase. Despite extensive biochemical analyses, such an enzyme has remained refractory to identification. Although an integrin-linked kinase (ILK) has been shown to increase Ser473 phosphorylation in transfected cells, it does so by an indirect mechanism suggesting that ILK is not the elusive PDK-2.

The mechanism of regulation of the hydrophobic site has been most clearly defined for the conventional PKCs. In the case of these isozymes, phosphorylation at the activation loop by PDK-1 triggers the intramolecular autophosphorylation at two C-terminal sites, the turn motif and the hydrophobic motif (Behn-Krappa and Newton, 1999). Autophosphorylation also appears to account for the mechanism by which the hydrophobic site is regulated in Akt/PKB (Toker and Newton, 2000). Similar to the conventional PKCs, the phosphorylation of the hydrophobic site of Akt/PKB depends on the intrinsic catalytic activity of Akt/PKB both in vitro and in vivo.

PDK-1 and the Hydrophobic Motif

A series of studies from Alessi and co-workers provided the first evidence that PDK-1 interacts with high affinity with sequences corresponding to the C-terminal hydrophobic phosphorylation motif. Screens for PDK-1 interacting proteins identified the C terminus of the PKC-related kinase, PRK2, the C terminus of PKA, and the C terminus of PKCζ (Balendran et al., 1999; Biondi et al., 2000; Ziegler et al., 1999). These sequences all include part, or all, of the hydrophobic phosphorylation motif except that the phospho-acceptor position contains an acidic residue in place of a Ser. In addition, the phosphorylation of p90RSK at the equivalent hydrophobic motif provides a binding site for PDK-1 (Frodin et al., 2000). Mutagenesis using the *P*DK-1 *I*nteracting *F*ragment (PIF) of PRK2 revealed a preference for negative charge at the phospho-acceptor position and the importance of flanking hydrophobic residues.

What is the significance of PDK-1 binding the hydrophobic motif? The first clue came from a report showing that inclusion of PIF in in vitro phosphorylation assays of Akt/PKB by PDK-1 promoted the phosphorylation of Ser473 (in addition to the activation loop site, Thr308). This PIF-stimulated phosphorylation of Ser473 depended on the intrinsic catalytic activity of Akt/PKB (Biondi et al., 2000), making it unlikely that PIF converts PDK-1 into a PDK-2 kinase, as originally suggested. An alternative explanation is that PIF renders Ser473 more accessible to autophosphorylation. This could occur by displacing PDK-1 from the hydrophobic site of its target kinases, thus unblocking the autophosphorylation sites. Proteins containing PIF sequences could effectively compete for binding to PDK-1, releasing it from the C terminus and unmasking the hydrophobic site. Such a mechanism could explain why PKCζ was identified in a screen for a hydrophobic site kinase for PKCδ; PKCζ is similar to PIF in that it has a negatively charged residue at the phospho-acceptor position of its hydrophobic motif, and could thus compete for PDK-1 binding to PKCδ and allow autophosphorylation (Ziegler et al., 1999). Such a model appears to describe the regulation of PKC by PDK-1 (Figure 2). It is worth noting that the precise identity of the physiological "PIFs" remains to be clarified, but could include kinases that contain acidic residues at their hydrophobic motifs (e.g., PRK2 and PKCζ).

Such a mechanism leads to the predication that cells lacking PDK-1 should have higher basal autophosphorylation of the hydrophobic motif of substrate kinases because PDK-1 is no longer masking the hydrophobic site and preventing autophosphorylation. (Even without activation loop phosphorylation, most kinases have some basal activity and, in fact, the T308A mutant of Akt/PKB has residual activity [Stokoe et al., 1997].) This is, in fact, the case with Akt/PKB: PDK-1 null cells have elevated Ser473 phosphorylation (Williams et al., 2000). Note that in these cells, Ser473 phosphorylation is marginally increased with mitogen stimulation, again as would be expected because engagement of the PH domain on the membrane likely renders the active site more accessible.

Regulation of PDK-1: Lipids, Location, Phosphorylation, and "PIF"

In contrast to its substrates, no significant switches for the intrinsic kinase activity of PDK-1 have yet to be defined. Rather, recent studies converge on the idea that PDK-1 function is regulated primarily by substrate conformation (as discussed above) and by cellular re-

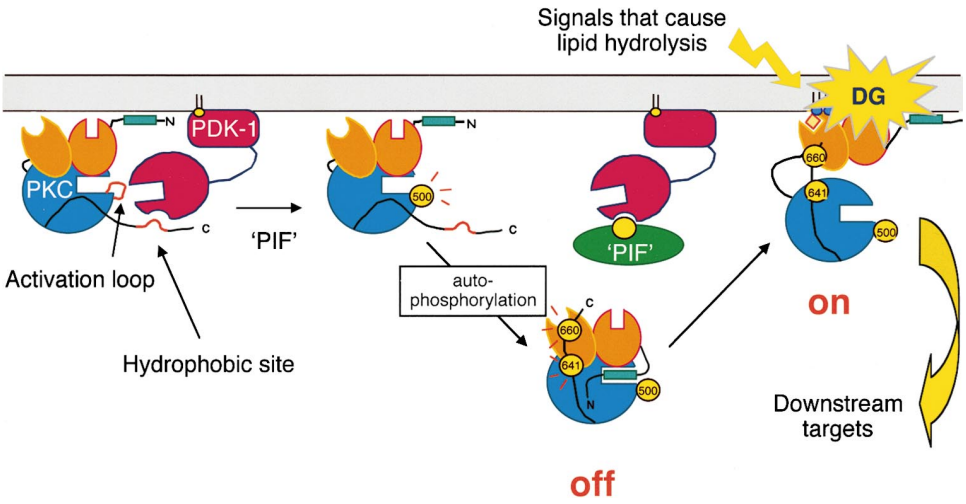

Figure 2. Model Showing the Role of PDK-1 in Controlling the Phosphorylation of the Activation Loop and the Hydrophobic Motif of Conventional PKC

Unphosphorylated PKC binds the membrane where it adopts a conformation in which the pseudosubstrate sequence (green rectangle) is removed from the kinase core (blue circle), thus exposing the activation loop (red loop). Binding of PDK-1 to PKC masks the autophosphorylation site in the hydrophobic motif (red bump). PDK-1 phosphorylates the activation loop Thr and is released from PKC. PDK-1 has a much higher affinity for hydrophobic motifs with negative charge (e.g., "PIF" sequence). Release of PDK-1 is promoted by proteins containing "PIF"-like sequences which effectively compete for binding to PDK-1. The unmasked C terminus now becomes rapidly autophosphorylated. Fully phosphorylated PKC localizes to the cytosol, where it is maintained in an inactive conformation by binding of the pseudosubstrate in the substrate binding cavity. Autoinhibition is relieved following interaction with its lipid second messenger, diacylglycerol (DG) at the membrane.

localization. Nonetheless, there are several potential points of regulation. One of these is the PH domain of PDK-1: it selectively binds PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 and with higher affinity than the PH domain of Akt/PKB (Stephens et al., 1998). This explains the observations that stimulated cells show a PH domain–dependent relocalization of PDK-1 from the cytosol to the plasma membrane (Anderson et al., 1998; Filippa et al., 2000). These studies have also shown that PDK-1 effectively recruits Akt/PKB to the plasma membrane in stimulated cells: mutants of PDK-1 deleted in its PH domain prevent translocation of Akt/PKB, whereas an Akt/PKB PH domain mutant efficiently translocates in the presence of intact PDK-1 (Filippa et al., 2000). Thus, the weight of evidence is in favor of PDK-1 translocating to the membrane in a PH domain– and agonist-dependent manner. In this regard, it is worth noting that one report did show a constitutive association of PDK-1 with the membrane in a wortmannin- and growth factor–independent manner, though this may be due to high overexpression and mislocalization of PDK-1, or the degree of serum starvation used in different cell types (Currie et al., 1999; Filippa et al., 2000).

Although PI3K lipids appear to trigger the translocation of PDK-1 to the membrane, how this membrane interaction affects kinase activity is poorly defined. Initial attempts to detect activation of PDK-1 by PtdIns-3,4,5-P_3 yielded negative results, but more recent observations suggest otherwise. Mutations of the PDK-1 PH domain impair phosphorylation of Akt/PKB in a PtdIns-3,4,5-P_3-dependent manner (Stokoe et al., 1997; Currie et al., 1999), and PtdIns-3,4,5-P_3 added to a complex of PDK-1:PIF increases the rate at which it can phosphorylate an Akt/PKB mutant lacking the PH domain (Balendran et al., 1999). Similarly, phosphorylation of PKCζ by PDK-1 requires a PI3K-signal operating through the PDK-1 regulatory domain rather than that of PKCζ (Le Good et al., 1998). These data suggest that PtdIns-3,4,5-P_3 could modulate the activity of PDK-1 in cells. Consistent with this notion, the PDK-1 PH domain acts as a negative regulator of its activity, and PtdIns-3,4,5-P_3 binding relieves this autoinhibition (Filippa et al., 2000). The precise mechanism by which PtdIns-3,4,5-P_3 modulates the intrinsic catalytic activity of PDK-1 remains to be elucidated, and is in part complicated by the fact that certain PDK-1 substrates themselves have a PtdIns-3,4,5-P_3 requirement (e.g., Akt/PKB).

Phosphorylation of PDK-1 may also regulate its activity. The activation loop phospho-acceptor of PDK-1, Ser241, is regulated by autophosphorylation, and mutation of this site essentially abolishes kinase activity (Casamayor et al., 1999). Tyrosine phosphorylation of PDK-1 in response to oxidative stress and vanadate has recently been reported, and both Src and Abl tyrosine kinases can phosphorylate and activate PDK-1 in vitro (Prasad et al., 2000). These data suggest that signaling through these tyrosine kinases can positively regulate PDK-1 signaling. Whether this pathway can wholly or partially bypass the PI3K requirement for PDK-1 activity toward substrates remains to be determined.

Modulation of the intrinsic kinase activity of PDK-1 could also be modulated by protein:protein interactions. In this regard, in vitro studies have shown that the activity of PDK-1 toward a synthetic peptide substrate is increased up to 4-fold in the presence of PIF (Biondi et al., 2000). Thus, binding of PIF to PDK-1 could stabilize the active conformation of PDK-1. A possible mechanism suggested by Alessi and coworkers is that PIF binds a hydrophobic pocket in the kinase core of PDK-1: this pocket corresponds to one in the structure of PKA

which binds the C terminus of PKA (Biondi et al., 2000). In the case of PKA and PKC, phosphorylation of the C terminus stabilizes the kinase core and it is likely that this stabilization results from nestling of the C terminus in this hydrophobic pocket. Because PDK-1 does not have a C terminus like that of PKA and PKC, it may depend on protein:protein interactions to stabilize the catalytically competent conformation of the enzyme. This stabilization could occur by its docking to the C terminus of its substrates (e.g., Figure 2) or by binding PIF-like sequences that are not coupled to substrates.

Perspectives

PDK-1 is a key enzyme in transducing signals to multiple effector pathways, and thus represents a pivotal point in PI3K-dependent and -independent signaling. Thus, understanding how the enzyme is regulated is critical to understanding how multiple signaling pathways are regulated. Initial findings that PDK-1 has a high basal activity even in unstimulated cells led to the notion that it is constitutively active, and that its activity is not critically regulated. However, recent studies have clearly demonstrated that the function of PDK-1 is under tight control, with phosphorylation depending on substrate conformation and subcellular location. The regulation by substrate conformation provides an attractive mechanism to allow PDK-1 to discriminate between one subset of targets over another, leading to a specific cellular response. Additional control could occur by interaction with adaptor of scaffold proteins, as suggested by findings that PDK-1 is found in a complex with many of its substrates. What is less clear is the PI3K-dependency of PDK-1 function. It now appears that the PtdIns-3,4,5-P_3 requirement for phosphorylation of Akt/PKB by PDK-1 results from effects on substrate conformation and localization. In fact, the phosphorylation of conventional PKCs is independent of PI3K, suggesting that 3′-phosphoinositides may be entirely dispensable for PDK-1 signaling in some cases. An additional unresolved issue is whether isoforms of PDK-1 exist. On the one hand, this may seem unlikely considering that PDK-1 null cells are devoid of Akt/PKB and p70S6-K phosphorylation. On the other, it is possible that PDK-1 isoforms might have a substrate specificity which is distinct from one another. The emergence of PDK-1 as a critical regulator of several AGC kinases has in part provided an explanation as to how PI3K can regulate so many distinct cellular processes. The challenge remains to attribute true PI3K-dependent signaling to specific PDK-1 targets.

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