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MINIREVIEW

Protein Kinases as Mediators of Phosphoinositide 3-Kinase Signaling

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Phosphoinositide 3-kinase (PI3K) is an enzyme that participates in a myriad of cellular processes and whose activity has been linked to cell growth and transformation, differentiation, motility, insulin action, and cell survival to name a few. Direct links between PI3K action and human diseases have also been made, most notably in cancer. Thus it is not surprising that considerable effort has gone into understanding the mechanisms by which PI3K mediates these responses. PI3K comprises a family of agonist-stimulated lipid signaling enzymes that initiate signaling cascades by generating three distinct membrane lipids, the polyphosphoinositides PtdIns-3-P, PtdIns-3,4-P_2, and PtdIns-3,4,5-P_3. Virtually all eukaryotic cells studied to date, including yeasts, have been found to contain one or more PI3K lipid products. In mammalian cells, three distinct classes of PI3Ks have been discovered, characterized, and cloned, and found to differ in their activation mechanisms by extracellular agonists, substrate specificity, and subcellular and tissue distribution. Type III PI3Ks are responsible for the synthesis of PtdIns-3-P in yeasts and higher eukaryotes. PtdIns-3-P is constitutively present in all cells, and its levels do not dramatically change following agonist stimulation. Conversely, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3, generated by type II and type I enzymes, are nominally absent in most cells and their levels rapidly accumulate on agonist stimulation (for a recent review on the PI3K family of enzymes, see Vanhaesebroeck and Waterfield, 1999). Thus, the accumulation of these two lipids at the plasma membrane, in particular PtdIns-3,4,5-P_3, has been extensively studied with respect to initiation of PI3K-dependent signaling cascades. Molecular genetic and biochemical studies in the last decade of the second millennium resulted in the identification of a multitude of PI3K effector molecules responsible for transducing the PI3K signal. Studies revealed that many enzymes (e.g., protein kinases, phospholipases, and G-proteins) are effector molecules of both PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3, and whose activities and/or cellular location is affected by the lipid-protein interaction.

In this review, we will focus exclusively on the regulation and function of protein kinases in the PI3K pathway. A wealth of information has come to light recently concerning the role of these enzymes in PI3K signaling, both with respect to their mechanism of regulation as well as their role in cell biology. In a number of cases, multiple downstream substrates for the protein kinases have been described that directly link PI3K, PtdIns-3,4-P_2/PtdIns-3,4,5-P_3, and the effector to cell function.

The Proto-Oncogene Akt/PKB

Although the serine/threonine protein kinase Akt/PKB was not the first PI3K effector discovered (this distinction belongs to the p70 ribosomal S6-kinase, p70S6K), intense interest in this field has led to what is arguably the best understood mechanism of activation and function of any PtdIns-3,4,5-P_3 target. Akt/PKB was originally discovered independently as the cellular homolog (c-Akt) of the transforming retrovirus AKT8 and as a novel kinase with similarities to both protein kinase C (PKC) and protein kinase A (PKA) (hence the name PKB). Interest in this kinase intensified when it was found to be activated by PI3K in platelet-derived growth factor (PDGF)-stimulated cells and that its pleckstrin homology (PH) domain was required for this activation (Franke et al., 1995). The lipid products of PI3K bind with high affinity and specificity to the Akt/PKB PH domain, with a preference of PtdIns-3,4-P_2 over PtdIns-3,4,5-P_3 both in vitro and in vivo (Franke et al., 1997). In addition to lipid binding, phosphorylation of a number of key residues in the

ABBREVIATIONS: PI3K, phosphoinositide 3-kinase; DAG, diacylglycerol; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PAK, p21-activated kinase; PDK-1, phosphoinositide-dependent kinase-1; PH, pleckstrin homology; PIF, PDK-1-interacting fragment; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PtdIns, phosphatidylinositol; PRK, PKC-related kinase; p70S6K, p70S6-kinase; RSK, ribosomal S6-kinase; TOR, target of rapamycin; PDGF, platelet-derived growth factor; SH2, Src homology 2; CREB, cAMP-responsive element-binding protein; SGK, serum and glucocorticoid-inducible kinase.

catalytic kinase core serves to potently activate the enzyme; this is a common feature of many AGC kinases [protein kinases A (PKA) G (PKG) and C (PKC)]. In the case of Akt/PKB, phosphorylation of Thr-308 in the activation loop and Ser-473 in the C-terminal hydrophobic motif is required for catalytic activity. Phosphorylation of both sites is mitogen- and PI3K-dependent, whereas an additional third site, Thr-450 appears to be constitutively phosphorylated in resting cells. The search for the Thr-308 kinase resulted in the discovery of the phosphoinositide-dependent kinase-1 (PDK-1), which specifically phosphorylates Thr-308 in vivo (Alessi et al., 1997, Stokoe et al., 1997). PDK-1 can only phosphorylate Akt/PKB in the presence of PtdIns-3,4,5-P_3, and thus a model has been proposed in which binding of PtdIns-3,4,5-P_3 to the PH domain of Akt/PKB causes a conformational change that relieves autoinhibition of the enzyme (by way of the PH domain masking the activation loop in the kinase domain), allowing PDK-1 access to Thr-308. Consistent with this model, deletion of the Akt/PKB PH domain renders this reaction PtdIns-3,4,5-P_3-independent (Stokoe et al., 1997). Thus, for activation of Akt/PKB by PDK-1, PtdIns-3,4,5-P_3 appears to be required at the level of the substrate (Akt/PKB), not the upstream kinase (PDK-1). However, as discussed below, the PtdIns-3,4,5-P_3 requirement for PDK-1 function is poorly understood. Phosphorylation of Ser-473 is also required for Akt/PKB activity, and a putative enzyme named PDK-2 was predicted to exist and be responsible for catalyzing this reaction (Alessi et al., 1997). Although a bonafide PDK-2 enzyme has yet to be described, two recent observations have provided contrasting models for the regulation of Ser-473 phosphorylation. A fragment from the PKC-related kinase-2 (PRK-2), termed PDK-1-interacting fragment (PIF) has been shown to interact with PDK-1 at its C terminus. This interaction was proposed to convert PDK-1 into a Ser-473 kinase in vitro thus causing an unprecedented switch in substrate specificity (Balendran et al., 1999). Our own studies have revealed that phosphorylation of Thr-308 triggers autophosphorylation of Ser-473 both in vitro and in

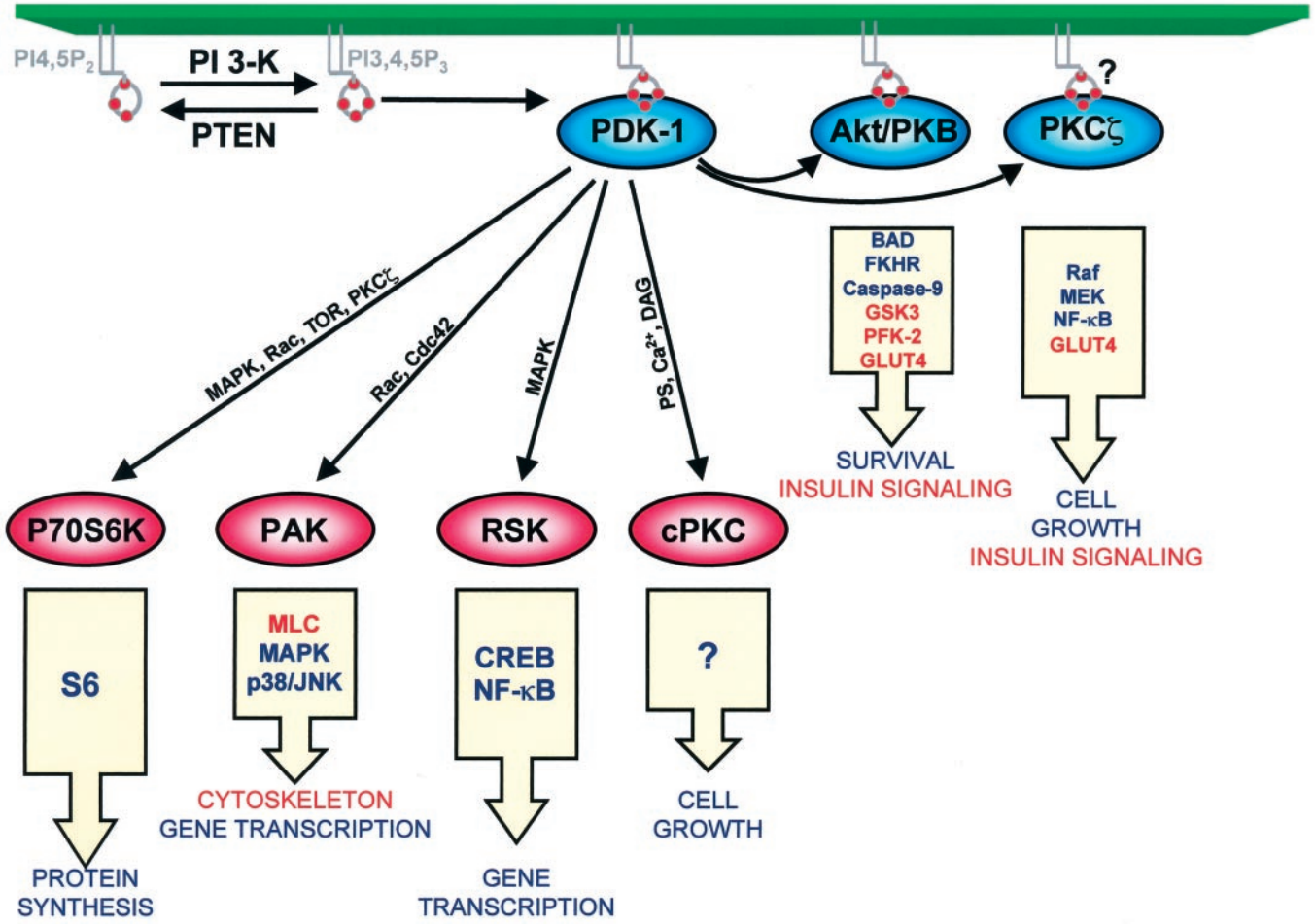

Fig. 1. Protein Ser/Thr kinases activated by the PI3K pathway. Activation of PI3K results in the local accumulation of PtdIns-3,4,5-P_3 at the plasma membrane. The action of the PTEN phosphatase inhibits PI3K signaling by converting PtdIns-3,4,5-P_3 back to PtdIns-4,5-P_2. Newly synthesized PtdIns-3,4,5-P_3 recruits both PDK-1, Akt/PKB, and possibly PKCζ to the plasma membrane where the combination of lipid binding and phosphorylation by PDK-1 serves to activate the enzymes. Both Akt/PKB and PKCζ are thus considered as true mediators of the PtdIns-3,4,5-P_3 signal. Additional substrates of PDK-1 are p70S6K, p90RSK, PAK, and conventional PKCs (cPKC). In addition to phosphorylation by PDK-1, these effectors require additional regulatory inputs for efficient kinase activation (shown along the black arrows). These PDK-1 targets do not require PtdIns-3,4,5-P_3 for PDK-1-mediated phosphorylation in vitro, so it is conceivable that they are phosphorylated in the cytosol rather than at the plasma membrane. Consequently, it is likely that one or more of these are capable of transducing PI3K-independent signaling. Once activated, these kinases can have profound effects on cell function by directly phosphorylating a number of identified substrate proteins, leading to gene transcription, cytoskeletal remodeling, cell survival, insulin-mediated metabolism, cell growth, and protein synthesis. This model accounts at least in part for the plethora of cellular responses, which have been attributed to the PI3K pathway.

cells, indicating that PDK-2 or a PDK-2-like activity is not required for Akt/PKB (Toker and Newton, 2000). One potential resolution to these contrasting results is that interaction of Akt/PKB with PDK-1 masks Ser-473 in the inactive conformation, and following activation, PIF may promote release of PDK-1 from Akt/PKB leading to autophosphorylation of this residue. Whether a true PDK-2 enzyme exists for other kinases remains to be established.

In addition to phosphorylation, Akt/PKB is regulated by other mechanisms. Dimerization has been shown to occur in cells, and this appears to require the PH domain (reviewed by Kandel and Hay, 1999). Negative regulation of Akt/PKB also appears to play an important role in signaling. Dephosphorylation of Thr-308 and Ser-473 by protein phosphatases is suggested by their sensitivity to vanadate and okadaic acid as well as osmotic shock (Meier et al., 1998). Phosphatase 2A may be the physiologically relevant phosphatase. Inactivation of Akt/PKB also occurs by removal of the PtdIns-3,4,5-P_3 signal, and this occurs by the action of the tumor suppressor PTEN, a PtdIns-3,4,5-P_3 phosphatase. Tumor cells expressing inactive PTEN alleles have elevated Akt/PKB activity (Myers et al., 1998). Finally, there are also recent reports of PI3K independent mechanisms of Akt/PKB regulation, including activation by cAMP/PKA and heat shock in a PI3K-insensitive manner (Filippa et al., 1999). The significance of these mechanisms over the PtdIns-3,4,5-P_3/PDK-1 pathway is presently unclear.

Once activated, Akt/PKB leaves the plasma membrane to phosphorylate intracellular substrates. Consistent with this, translocation of Akt/PKB to the nucleus has been reported (Andjelkovic et al., 1997), and this undoubtedly links Akt/PKB to phosphorylation of transcription factors such as cAMP-responsive element-binding protein (CREB), forkhead transcription factors (see below), E2F and NF-κB (Kandel and Hay, 1999). The majority of Akt/PKB substrates described are implicated either in insulin signaling or in cell survival pathways. The glycogen synthase kinase 3 (GSK3) is phosphorylated and inactivated by Akt/PKB leading to an increase in glycogen synthesis (Cross et al., 1995). 6-Phosphofructo-2-kinase was also shown to be phosphorylated by Akt/PKB leading to an increase in glycolysis (Deprez et al., 1997). The phosphodiesterase PDE3B is also an Akt/PKB target in insulin-stimulated cells, and the phosphorylation of the repressor of transcription, the 4E-BP protein (eucaryotic initiation factor-4E-binding protein) is also Akt/PKB-dependent, leading to mRNA translation (Gingras et al., 1998; Kitamura et al., 1999). Other components of the translational machinery, including p70S6K and the target of rapamycin (TOR) are Akt/PKB targets, although the precise role of Akt/PKB in these pathways is presently unclear.

One of the major functions of Akt/PKB is as a cell survival factor, and a number of proteins have been shown to mediate its anti-apoptotic function. The pro-apoptotic Bcl-2 family member BAD is phosphorylated and inactivated by Akt/PKB leading to protection from apoptosis (Datta et al., 1997). However, it is unlikely that this represents the major mechanism by which Akt/PKB acts as an inhibitor of apoptosis, as BAD is not ubiquitously expressed. Both the pro-apoptotic cysteine protease, caspase-9, as well as forkhead transcription factors such as FKHRL1 are potent at inducing apoptosis, an event that can be inhibited by Akt/PKB-mediated phosphorylation of both proteins (Cardone et al., 1998; Brunet et al., 1999). With such a wide array of substrates that have been shown to mediate the anti-apoptotic effect of Akt/PKB, it is still unclear whether one or all of these pathways are necessary for full protection from cell death, or whether there are additional substrates that fulfil this function. Finally, the discovery of Akt/PKB as a proto-oncogene suggests that it is also likely to play a fundamental role in cellular transformation and cancer, phenotypes that represent an imbalance of both cell growth and death. Akt/PKB substrates relevant for cell growth are the Raf-1 kinase, which is phosphorylated by Akt/PKB leading to inhibition of mitogen-activated protein kinase (MAPK) signaling (Zimmermann and Moelling, 1999), and induction of cyclin D1 levels during the G_1 phase of the cell cycle (Muise-Helmericks et al., 1998). Despite these exciting observations, clearly more work is required to fully understand the role of this important kinase in normal and aberrant cell growth.

Protein Kinase C Isozymes as Mediators of PI3K-Dependent and -Independent Signaling

The PKC superfamily comprises 12 distinct mammalian protein kinases, which are subdivided into three subfamilies according to their activation profiles: conventional PKCs (cPKC, α, βI, βII, γ), novel PKCs (δ, ϵ, η, θ), atypical PKCs (aPKC, ζ, ι/λ), and the more distantly related PKCμ/PKD and PKCν. The role of diacylglycerol (DAG)/phorbol ester, phosphatidylserine, and calcium (Ca^{2+}) in activation of PKC has been extensively studied and is well understood (reviewed by Newton, 1997). More recently, the role of phosphorylation in PKC activation has been examined and has provided a clear link between PI3K/PtdIns-3,4,5-P_3 signaling and PKC function. The first indication of such a link was when several laboratories reported activation of various PKCs by either PtdIns-3,4-P_2 or PtdIns-3,4,5-P_3 in in vitro assays. The discovery of the PDK-1 enzyme provided an explanation for these findings; PKCs also require phosphorylation at the activation loop and hydrophobic motif for catalytic activity, and the amino acid sequence surrounding these sites is highly conserved not only between PKC and Akt/PKB, but also many other AGC kinases. Consistent with this, PDK-1 phosphorylates and activates PKCζ, a non DAG-responsive PKC whose activity is mitogen-dependent (Chou et al., 1998, Le Good et al., 1998). In vitro, maximal phosphorylation and activation of PKCζ by PDK-1 requires PtdIns-3,4,5-P_3. Because a PtdIns-3,4,5-P_3-binding site on PKCζ has not been described, the PtdIns-3,4,5-P_3 requirement for this reaction remains a mystery. Curiously, cPKCs such as PKCβII are also phosphorylated by PDK-1, but in a PtdIns-3,4,5-P_3-independent manner. This phosphorylation is required to process catalytically competent PKC but does not in itself activate the enzyme (Dutil et al., 1998). The activating step for cPKCs is binding of DAG at the plasma membrane, leading to release of the autoinhibitory pseudosubstrate domain from the active site. Thus, different PKCs have the ability to mediate both PI3K-independent (PKCβII) and -dependent (PKCζ) responses. The PtdIns-3,4,5-P_3 requirement for nPKCs such as PKCϵ has yet to be determined, but there is evidence that PI3K activation in cells leads to an increase in PKCϵ activity (Moriya et al., 1996). PDK-1 is thus likely to represent the upstream kinase for all PKCs. As with Akt/PKB, phosphorylation of the PKC hydrophobic site is a more

contentious issue, and the same putative PDK-2 enzyme has been assumed to regulate C-terminal site phosphorylation. One group has reported that PKCζ can act as a PDK-2 for nPKCδ, and that phosphorylation of both PKCδ and PKCϵ at the hydrophobic Ser occurs via the TOR pathway because of rapamycin sensitivity (Ziegler et al., 1999). Conversely, autophosphorylation is responsible for the regulation of the equivalent site in PKCβII (Behn-Krappa and Newton, 1999), and other studies have shown a similar mechanism for PKCϵ (V. Cenni and A.T., unpublished observations). As with Akt/PKB, this brings into question the existence of a PDK-2 for PKCs.

Based on the above discussion, PKCζ is good candidate for mediating PI3K-dependent responses, but little is known about specific substrates for this enzyme in the PI3K signaling cascade. PKCζ has been shown to activate an NF-κB-like activity in vivo and to phosphorylate a novel Iκ-B kinase (IKKb) leading to gene transcription (LAllemain et al., 1989). PKCζ has also been implicated as a Ras effector in certain cell types. One mechanism by which PKCζ may mediate transcriptional activation and cell growth is by activation of the MAPK pathway. Lysophosphatidic-stimulated activation of MAPK requires both PI3K and PKCζ, but not Ras (Takeda et al., 1999). Both Raf-1 and MAPK kinase (MEK) have been reported to be activated by PKCζ in vivo (Cai et al., 1997; Schonwasser et al., 1998) and thus the PI3K/PKCζ pathway may have multiple mechanisms for regulating MAPK activation. PI3K is a critical mediator of many of insulin's physiological functions. PI3K-dependent PKCζ and PKCλ activation leads to insulin-stimulated glucose uptake mediated by the glucose transporter-4 vesicle (Standaert et al., 1997; Kotani et al., 1998). Insulin-stimulated MAPK activation and protein synthesis are also dependent on PI3K/PKCζ activity (Mendez et al., 1997; Sajan et al., 1999). The latter is consistent with the observation that dominant negative PKCζ antagonizes activation of p70S6K (Romanelli et al., 1999). Thus, evidence is mounting that activation of PKCζ by PI3K is required for many cellular responses, in particular mitogenesis, insulin signaling, and protein synthesis. The PKCζ substrates that mediate these responses remain elusive.

Ribosomal S6-Kinase

p70S6K is a mitogen-regulated protein kinase of the AGC superfamily and was the first identified downstream target of PI3K in vivo. However, as discussed here, p70S6K appears to be a more distal effector compared to either PKCs or Akt/PKB. Initial studies showed that mutants of the βPDGF-receptor defective in PI3K binding and activation were also impaired in p70S6K activation. Similarly, inhibitor studies using the PI3K antagonists wortmannin and LY294002, as well as the immunosuppressant rapamycin indicated that several distinct pathways were responsible for phosphorylation and activation of the enzyme. Understanding the regulation of p70S6K has in part been confounded by the fact that up to eight distinct phosphorylation sites exist in the mammalian enzyme, and that mutation of one or more of these to nonphosphorylatable residues impairs kinase activation. The initial step in p70S6K activation appears to involve a phosphorylation-induced conformational change in the C terminus of the kinase domain, revealing additional phosphorylation sites. MAPK is thought to be the relevant kinase for mediating this initial step (reviewed by Dufner and Thomas, 1999). Subsequently, phosphorylation of the newly exposed sites (Thr-229, Thr-389, and Ser-371) occurs and is dependent on both the PI3K and TOR pathways, based on wortmannin and rapamycin sensitivities. Although the role of PI3K in mediating p70S6K activation remained a mystery for several years, the discovery of PDK-1 as an activation loop kinase once again came to the rescue. Thr-229 is in the activation loop of p70S6K, and PDK-1 was shown to be the relevant upstream kinase (Alessi et al., 1998; Pullen et al., 1998). Interestingly, PtdIns-3,4,5-P_3 is not required for the activation of p70S6K by PDK-1 in vitro, and this may be an indication that this step does not require membrane association of either PDK-1 or p70S6K. How the two remaining sites (Thr-371 and Ser-389) are phosphorylated is less clear. Phosphorylation of Ser-371 is wortmannin-sensitive, once again implicating the PI3K pathway. Recent work (Romanelli et al., 1999) has shown that PKCζ is necessary for p70S6K activation, suggesting a linear pathway: PI3K →174→ PtdIns-3,4,5-P_3 →174→ PDK-1 →174→ PKCζ →174→ p70S6K. However, it is not known whether PKCζ can directly phosphorylate p70S6K or which residue is involved. Finally, Ser-389 (equivalent to the C-terminal hydrophobic motif in PKCs and Akt/PKB) has been shown to be phosphorylated by PDK-1 although curiously, interaction of PDK-1 with PIF in vivo abrogates the ability of PDK-1 to act as a Ser-389 kinase (Balendran et al., 1999b). There are also reports that TOR is capable of directly phosphorylating Thr-389 (Pearson et al., 1995). Thus, the precise mechanism of Ser-371 and Ser-389 phosphorylation remains elusive. This elaborate sequence of events in p70S6K activation by at least three pathways (MAPK, PI3K, and TOR) is further complicated by other mechanisms of regulation, which include the small GTPases Rac and Cdc42, Akt/PKB, and amino acids, all of which have been shown to activate p70S6K in vivo (Dufner and Thomas, 1999).

p70S6K is directly responsible for the phosphorylation of the 40 S ribosomal protein S6. Translation of several hundred mRNAs with 5′ oligopyrimidine tracts is controlled by p70S6K. These mRNAs largely encode for proteins necessary for the assembly of the translational machinery, including ribosomes and elongation factors. Because these proteins constitute up to 30% of total cellular protein, it is not surprising that the mammalian p70S6K1 knockout results in a small mouse phenotype, consistent with the notion that this enzyme controls cell size, growth, and proliferation (Shima et al., 1998). It is also worth noting that the p70S6K1 knockout led to the identification of a second distinct S6-kinase, p70S6K2, also capable of phosphorylating the S6 ribosomal subunit.

Regulation of MAPK Signaling by PI3K

There is ample evidence that activation of the MAPK cascade is mediated, at least in part, by PI3K depending on the cell type and stimulus used. The variability observed in MAPK activation by PI3K seems to stem from the strength of the stimulus used in different cell types, such as that subsaturating doses of PDGF demonstrate a PI3K requirement, whereas at saturating doses PI3K becomes redundant (Duckworth and Cantley, 1997). There are a number of potential mechanisms by which PI3K could mediate activation of

MAPK in cells. As discussed above, PKCζ has been shown to activate and phosphorylate both Raf-1 and MEK. Similarly, Akt/PKB can directly phosphorylate Raf-1, although this leads to inactivation of Raf-1 and inhibition of MAPK signaling. The p21-activated kinase (PAK), a PI3K effector (see below) is an important regulator of MEK, because it phosphorylates Raf-1 at a site necessary for the Raf-1/MEK interaction (Frost et al., 1996). Perhaps the most intriguing mechanism that has come to light recently is the protein kinase activity of PI3K and its role in MAPK activation. In addition to being a lipid kinase, PI3K possesses an intrinsic Ser/Thr kinase activity (PI3K-protein kinase or PI3K-PK), and autophosphorylation inactivates the lipid kinase. Few physiologically relevant exogenous substrates for PI3K-PK have been described, although one may be a component of the MAPK cascade. Through some elegant protein engineering studies, Bondeva et al. (1998) constructed a PI3K mutant deficient of lipid kinase activity, but which retained protein kinase activity, and in cotransfection studies, this mutant activated MAPK but not Akt/PKB. This suggests that PtdIns-3,4-P_2/PtdIns-3,4,5-P_3 are dispensable for MAPK activation. The precise nature of the PI3K-PK target is still a mystery, although MEK might be a relevant substrate. Regardless of the mechanism, the implication from these various studies is that PI3K is capable of activating MAPK at various points in the cascade, involving both protein kinases activated by PtdIns-3,4,5-P_3 as well as PI3K-PK. This is not entirely surprising considering the complexity of MAPK regulation by multiple pathways.

Other Protein Kinases of the AGC Superfamily

Examination of the sequence surrounding the activation loop Thr-308 of Akt/PKB indicated that many members of the AGC kinase superfamily might also be substrates for PDK-1. Accordingly, a number of other AGC kinase have recently been shown to be direct PDK-1 targets. The prototype of the AGC kinase family, PKA, is also phosphorylated in vitro by PDK-1, although it is not clear if this is a predominant mechanism for regulating PKA activity in cells (Cheng et al., 1998). The p90 ribosomal S6-kinase (p90RSK) is also phosphorylated and activated by PDK-1, and thus in a manner analogous to p70S6K, receives signals from both MAPK and PDK-1 for kinase activation (Richards et al., 1999). However, unlike p70S6K, p90RSK is not a physiologically relevant ribosomal S6 kinase, instead it is responsible for phosphorylation of CREB and may also regulate transcription through NF-κB. Of particular importance to p90RSK regulation and function is the finding that inactivating mutations in its gene are responsible for human Coffin-Lowry syndrome, which is characterized by mental retardation and skeletal deformities.

The serum and glucocorticoid-inducible kinase (SGK) is also phosphorylated by PDK-1 and this activates the enzyme in vivo (Park et al., 1999). Although the activation of SGK is sensitive to PI3K inhibitors, the role of PtdIns-3,4,5-P_3 in activation of SGK is unknown. Finally, the Rac effector kinase, PAK, also shares an activation loop sequence similar to that of Akt/PKB and PKCs. In vitro studies indicate that PDK-1 may phosphorylate and activate PAK in the presence of the lipid sphingosine (King et al., 2000). It has been known for some time that PAK is downstream of PI3K in cells, by virtue of the fact that the small GTPase Rac is regulated by PI3K. The finding that PDK-1 may also lie upstream of PAK adds another level of complexity to this enzyme, which has been implicated in both gene transcription as well as cytoskeletal remodeling. Considering the long list of protein kinases with similar activation loop sequences, it is tempting to speculate that PDK-1 may be the universal upstream kinase for AGC family members. Although undoubtedly the list of physiologically relevant PDK-1 targets is by no means complete, at least one observation suggests that the above assumption is not correct: the Ca^{2+}-calmodulin kinase IV isoform, which has an activation loop similar to that of Akt/PKB, is not a PDK-1 substrate (Pullen et al., 1998).

PDK-1—A Pivotal Enzyme in PI3K Signaling

Considering the importance and diversity of cellular processes regulated by PDK-1 substrates, it is clear that this enzyme is a critical element in PI3K signaling. It is also reasonable to assume that PDK-1 must be very tightly regulated by multiple mechanisms, and recent studies have begun to address this issue. The regulation of PDK-1 activity and cellular location by PtdIns-3,4,5-P_3 has been extensively studied. Initial studies indicated that none of the PI3K lipid products could activate purified PDK-1 in vitro (Alessi et al., 1997), despite the fact that the PDK-1 PH domain has a remarkably high affinity for PtdIns-3,4,5-P_3 (K_D 1.6 nM) (Stephens et al., 1998). However, these studies made use of Akt/PKB as the PDK-1 substrate, and Akt/PKB itself has a PtdIns-3,4,5-P_3 requirement complicating the issue. More recent studies have in fact shown that partially purified PDK-1 is activated 3-fold by PtdIns-3,4,5-P_3 (Stephens et al., 1998), and also that binding of PIF to PDK-1 converts it into a PtdIns-3,4,5-P_3-activated enzyme (Balendran et al., 1999a). Similarly, the PtdIns-3,4,5-P_3 requirement for PDK-1 to phosphorylate PKCζ in vitro is lost when the PDK-1 PH domain is deleted (Le Good et al., 1998). These studies suggest that PtdIns-3,4,5-P_3 may in fact be required PDK-1 activity, either by directly increasing its intrinsic kinase activity, or by promoting access to the substrate. PtdIns-3,4,5-P_3 is also required to relocalize PDK-1 from the cytosol to the plasma membrane where it can gain access to its membrane-associated substrates such as Akt/PKB. This has been shown for PDGF stimulation of endothelial cells (Anderson et al., 1998), although one group has reported that in human embryonic kidney cells, PDK-1 is constitutively membrane-associated and that localization is agonist-independent (Currie et al., 1999). One important feature of PDK-1 function is the fact that it is a constitutively active enzyme even in unstimulated cells. This lends support to the idea that PDK-1 may function in a PI3K-independent manner, and phosphorylate substrates in the absence of a PtdIns-3,4,5-P_3 signal. Although some PDK-1 targets, such as p70S6K do not require PtdIns-3,4,5-P_3 for PDK-1-mediated phosphorylation, wortmannin is still a potent inhibitor of these kinases and thus it is not clear what the PtdIns-3,4,5-P_3 requirement is here. At any rate, PDK-1 has the potential for regulating multiple cellular responses in both a PI3K-dependent and -independent manner. Distinguishing which of its targets belongs in which category is clearly a priority.

Phosphorylation of PDK-1 is also equally critical for its function. PDK-1 is phosphorylated at its activation loop Ser-241 residue by autophosphorylation, rather than by an upstream kinase (Casamayor et al., 1999). A number of phosphorylation sites in the enzyme have been mapped (Ser-25, Ser-393, Ser-396, and Ser-410), although the mechanisms by which these sites are phosphorylated is not known. Recent studies have suggested two novel mechanisms of PDK-1 regulation. First, PDK-1 is phosphorylated at Thr-37, Ser-231, and Ser-250 by the c-Jun N-terminal (JNK) and p38 kinases and this leads to inhibition of PDK-1 activity in vivo (W. Hou and B. Schaffhausen, unpublished observations). Considering the role of c-Jun N-terminal kinase in apoptosis, this provides an alternate route for programmed cell death. Conversely, pervanadate and mitogen stimulation of cells leads to tyrosine phosphorylation of PDK-1, and this activates the enzyme. Both p60Src and Abl are implicated in tyrosine phosphorylation of PDK-1 (W. Hou and B. Schaffhausen, unpublished observations). Thus evidence is mounting that not only is PDK-1 a critical player in many signal transduction pathways, but also that it is regulated by multiple upstream pathways, involving both lipid and protein kinases.

Tyrosine Kinases

Although the majority of protein kinases activated by PI3K are Ser/Thr kinases, there is good evidence that some tyrosine kinases are also activated by this pathway. Tec kinases are nonreceptor tyrosine kinases implicated in B and T cell signaling and development (Scharenberg and Kinet, 1996). Bruton's tyrosine kinase (Btk) and inducible T-cell kinase (Itk) are PH domain-containing Tec kinases that are activated by PI3K. In the case of Btk, PtdIns-3,4,5-P_3 binds with high affinity to the PH domain, leading to activation and tyrosine autophosphorylation (Scharenberg et al., 1998). Interestingly, activation of Btk by PI3K requires the synergistic activity of p60Src, and this is somewhat reminiscent of the regulation of Akt/PKB by both PtdIns-3,4,5-P_3 and the upstream kinase PDK-1. In this case, p60Src acts as the activation loop kinase for Btk. Activation of Btk by p60Src and PtdIns-3,4,5-P_3 leads to tyrosine phosphorylation and activation of phospholipase Cγ and increased inositol-1,4,5-trisphosphate production and Ca^{2+} release in B cells (Scharenberg et al., 1998). These exciting results link PI3K activation with PtdIns-4,5-P_2 hydrolysis and inositol-1,4,5-trisphosphate/DAG generation in cells, although it is not clear if this is a general phenomenon or whether it is restricted to B cell function. Etk is another Tec family member not found in the hematopoietic system but expressed in cells of epithelial origin, such as prostate carcinoma (Xue et al., 1999). Activation of Etk is wortmannin sensitive, and the fact that it has a PH domain suggests a similar PI3K dependence. Regulation of p60Src itself may also be under the influence of PI3K, as PtdIns-3,4,5-P_3 has been shown to bind to the Src homology 2 (SH2) domain of the enzyme (Rameh et al., 1995). Whether regulation of tyrosine kinases by PtdIns-3,4,5-P_3 is as widespread as that of Ser/Thr kinases remains to be seen.

Summary

Although a large number of effectors of PI3K and PtdIns-3,4,5-P_3 are known to exist in cells, protein kinases have emerged as enzymes whose regulation by this pathway controls many important cellular processes. A major feature of protein kinase activation by PI3K is that multiple mechanisms act in synergy to efficiently activate the enzyme, including cellular localization, PtdIns-3,4,5-P_3 binding and phosphorylation by heterologous upstream kinases. Although much has been learned in the past few years about the specific mechanisms that lead to activation of these enzymes, a number of critical questions remain unanswered. What is the precise role of PtdIns-3,4,5-P_3 in regulating these effectors? In some cases, additional pathways converge on the effector and these are also required for kinase activity (e.g., p70S6K), and both the identity and nature of these pathways is poorly understood. How many additional PDK-1 targets exist in the cell? What is the precise role for PtdIns-3,4,5-P_3 in PDK-1 function? Perhaps most critical to the field is understanding how PDK-1, through its various effectors, mediates PI3K-dependent and -independent signaling and cell physiology. Considering the role of PI3K in human diseases, these are pressing questions that may ultimately provide novel therapeutic targets directed at the enzymes described here.

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