PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle

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PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle. Am J Physiol Endocrinol Metab 289: E187–E196, 2005; doi:10.1152/ajpendo.00011.2005.—Activation of members of the protein kinase AGC (cAMP dependent, cGMP dependent, and protein kinase C) family is regulated primarily by phosphorylation at two sites: a conserved threonine residue in the activation loop and a serine/threonine residue in a hydrophobic motif (HM) near the COOH terminus. Although phosphorylation of these kinases in the activation loop has been found to be mediated by phosphoinositide-dependent protein kinase-1 (PDK1), the kinase(s) that catalyzes AGC kinase phosphorylation in the HM remains uncharacterized. So far, at least 10 kinases have been suggested to function as an HM kinase or the so-called “PDK2,” including mitogen-activated protein (MAP) kinase-activated protein kinase-2 (MK2), integrin-linked kinase (ILK), p38 MAP kinase, protein kinase Cα (PKCα), PKCβ, the NIMA-related kinase-6 (NEK6), the mammalian target of rapamycin (mTOR), the double-stranded DNA-dependent protein kinase (DNK-PK), and the ataxia telangiectasia mutated (ATM) gene product. However, whether any or all of these kinases act as a physiological HM kinase remains to be established. Nonetheless, available data suggest that multiple systems may be used in cells to regulate the activation of the AGC family kinases. It is possible that, unlike activation loop phosphorylation, phosphorylation of the HM site in the different AGC family kinases is mediated by distinct kinases. In addition, phosphorylation of the AGC family kinase at the HM site could be cell type, signaling pathway, and substrate specific. Identification and characterization of the bonafide HM kinase(s) will be essential to verify these hypotheses.

phosphoinositide-dependent protein kinase-2; phosphoinositide-dependent protein kinase-1; Akt; hydrophobic motif kinase

Members of the protein kinase AGC (for cAMP dependent, cGMP dependent, and protein kinase C) family play critical roles in receptor tyrosine kinase (RTK) signal transduction essential for regulating many important biological events, including cell cycle progression, growth, migration, differentiation, and apoptosis. Loss of regulation of these kinases may lead to serious diseases such as diabetes and cancer.

Activation of the AGC family kinases is controlled primarily through phosphorylation. In the case of protein kinase Bα (PKBα/Akt1), activation depends on phosphorylation at two sites, Thr308 in the activation loop and Ser473 in the hydrophobic motif (HM) at the carboxyl terminus. All members of the AGC family kinases possess phosphorylation sites equivalent to Thr308, which lies in a T*FCGTPXF/Y consensus motif where X is a variable residue and * indicates the phosphorylation site (Fig. 1). Phosphorylation of Akt and other AGC family kinases in the activation loop has been found to be mediated by phosphoinositide-dependent protein kinase-1 (PDK1) (70). Akt isoforms and many other AGC family members also possess a second phosphorylation site that lies in a hydrophobic region near their COOH terminus that contains the conserved HM sequence FXXF(S*/T*)(Y/F), where S*/T* is the phosphorylation site (Fig. 1). Some members of the AGC family kinases, such as the protein kinase C (PKC)-related kinase-1 (PRK1/PKN), possess a negatively charged acidic residue (Asp or Glu) instead of an S/T residue in the HM (Fig. 1). Phosphorylation of the conserved serine residue in the HM, as in Akt and p70 ribosomal S6 kinase (p70S6K), or substituting this residue with an acidic residue as in PRK1/PKN, seems to serve as a docking site to recruit PDK1 to these substrates and induce synergistic stimulation of the catalytic activity of PDK1 (43). Although available data have shown that phosphorylation in the HM, or the so-called “PDK2” site, plays a critical role in activating the AGC family kinases, the underlying mechanism(s) remains uncharacterized.

PDK1: THE KINASE THAT CATALYZES PHOSPHORYLATION OF AGC FAMILY KINASES IN THE ACTIVATION LOOP

PDK1 was originally identified as the kinase that catalyzes Akt1 phosphorylation at Thr308 in the activation loop (2, 3, 92). This pivotal serine/threonine kinase has since been found to phosphorylate the conserved threonine residue in the activation loop of other AGC family kinases, including p70S6K (4, 78), PKC isoforms (26, 34, 37, 63), PKA (23), serum- and glu-
corticoid-regulated kinase (SGK) (60, 61, 74), p90 ribosomal S6 kinase (RSK) (56, 80), PKC-related kinase-1 and -2 (PRK1/PKN and PRK2) (33, 41), and p21-activated kinase 1 (PAK1) (59). However, whether all these kinases are direct and physiological substrates of PDK1 and whether there are additional PDK1 substrates remain to be established.

How PDK1 is regulated in cells is a subject of intense investigation, but data suggest that growth factor-stimulated membrane translocation, conformational changes, and phosphorylation may play important roles in the regulation of PDK1 activity and function (15, 70, 99). PDK1 contains a catalytic domain near its amino terminus and a pleckstrin homology domain (PH) at its COOH terminus. The PH domain has been shown to be necessary for targeting PDK1 to the plasma membrane in order for PDK1 to interact with and phosphorylate its substrates such as Akt (5, 29). However, deletion of the PH domain stimulates PDK1 autokinase action in intact cells (39), suggesting that the PH domain of PDK1 may also act as a negative regulator of its enzymatic activity under basal conditions. Thus the binding of the phosphatidylinositol 3-kinase (PI3K) products to the PH domain not only functions to recruit PDK1 to the plasma membrane but also simultaneously induces a conformational change that eliminates the inhibitory effect of this domain, leading to activation of PDK1. In addition to a conformational change, recent studies suggest that PDK1 phosphorylation also plays an important role in regulating PDK1 activity. PDK1 contains several autophasorylation sites (18, 102) and is trans-phosphorylated in the activation loop (Ser241 for human PDK1 and Ser244 for mouse PDK1) (101), which is essential for activity. PDK1 phosphorylation in the activation loop in vivo is stimulated by insulin and is markedly downregulated in ob/ob mice (62), suggesting that phosphorylation at this site plays a role in insulin-stimulated PDK1 activation and function. Taken together, these results suggest that phosphorylation plays an important role in regulating the in vivo roles of PDK1.

MECHANISMS UNDERLYING PHOSPHORYLATION OF THE AGC FAMILY KINASES IN THE HM SITE (PDK2 SITE)

Although phosphorylation in the activation loop has been shown to be essential for the activity of Akt and other AGC family kinases, the role of phosphorylation at the HM site is less clear. However, two recent X-ray crystal studies of the active [PDB ID: 1O6K (104)] and inactive [PDB ID: 1MRY (54)] Akt kinase domains provide important insight into our understanding of the role of the HM site phosphorylation in Akt activation. In an inactive state, the activation loop of Akt adopts a disordered conformation that sterically hinders the binding of ATP and the peptide substrate (54). Phosphorylation of Ser473 in the HM promotes the interaction between the HM and the NH2-terminal lobe of the kinase domain, leading to a disorder-to-order transition of the activation loop and thus full activation of the kinase (104). Unlike PKA, which lacks the HM site and phosphorylation in the activation loop sufficient to stimulate its kinase activity, Ser473 phosphorylation appears to play a critical role in stabilizing an active configuration of Akt (54). Thus phosphorylation in the activation loop and at the HM site seems to provide a dual control for activation of Akt and other AGC family kinases.

Eight years have now elapsed since PDK1 was originally identified as the kinase responsible for phosphorylating Akt in the activation loop (3, 93). Surprisingly, the identity of the kinase responsible for phosphorylating Akt at Ser473 in the HM remains elusive (21). At least three models have been proposed for Akt phosphorylation at Ser473, including phosphorylation by PDK1, by autophosphorylation, or by a heterologous kinase (the so-called PDK2) distinct from PDK1, which is discussed below.

PHOSPHORYLATION OF THE HM SITE BY PDK1?

It was initially proposed that PDK1 may function as a kinase that phosphorylates Akt at both Thr308 and Ser473 in the HM. This hypothesis was based on the finding that incubation of a region of PDK1 at the COOH terminus of PRK2, termed the PDK1 interacting fragment, or PIF, with purified wild-type but not kinase-inactive PDK1, led to Akt phosphorylation at Ser473 in a phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3]- or phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P2]-dependent manner (8). The “PD2K” kinase activity was not due to a PIF-associated kinase, because a synthetic PIF peptide was found to be sufficient to induce purified PDK1 to phosphorylate Akt1 at Ser473 in a PtdIns(3,4)P2-dependent manner. PDK1 has also been reported to phosphorylate the HM site in p70S6K (Thr412 in the longer splice variant of the α-isofom), both in vitro and in cells (9). Surprisingly, binding of the PIF prevents PDK1-mediated p70S6K phosphorylation at Thr412 (9). These findings led the authors to hypothesize that p70S6K, but not Akt, needed to interact with PDK1 at a site that overlaps with the PIF binding site before the kinase can become phosphorylated by PDK1 (9).

Although the aforementioned studies suggest that the binding of PIF to the PIF pocket of PDK1 may induce a conformational change that regulates the PDK2 activity of PDK1, how the PIF fragment is generated in cells remains unclear. We found that coexpression of full-length PRK2, its isoform PRK1/PKN, or the carboxyl terminus of PRK1/PKN (which contains the PIF sequence) inhibited constitutively active PDK1 (PDK1A280V)-mediated Akt phosphorylation at Thr308 (100), suggesting that the full-length PRK isoforms play a different role in modulating PDK1 activity than that induced by
the PIF of the kinases. Thus, if the PIF fragment plays a role in converting PDK1 into PDK2, a major question concerning this mechanism is whether this event occurs in vivo under physiological conditions. Regardless of the origin of the PIF fragment in cells, Akt has been found to undergo IGF-I-stimulated phosphorylation at Ser$^{473}$ in PDK1$^{-/-}$ embryonic stem cells (103). This result provides strong evidence that PDK1 is dispensable for phosphorylating the HM of the AGC family kinases in cells under physiological conditions.

**PHOSPHORYLATION OF THE HM SITE BY AUTOPHOSPHORYLATION?**

During the studies investigating the mechanism underlying PKC$\beta$II phosphorylation, Behn-Krappa et al. (12) found that the purified kinase domain of PKC$\beta$II was autophosphorylated on the HM site in vitro. In addition, kinase-inactive mutants of PKC$\beta$II were not phosphorylated at the HM in intact cells. These findings led to the hypothesis that phosphorylation of PKC$\beta$II in the HM is mediated by autophosphorylation (12). A similar autophosphorylation mechanism was later proposed for Akt phosphorylation at the HM site, based on the findings that incubation of purified Akt with PDK1 led to phosphorylation at both Thr$^{308}$ and Ser$^{473}$ and that phosphorylation at Ser$^{473}$ requires the intrinsic Akt catalytic activity and prior phosphorylation at Thr$^{308}$ (96).

It is unclear whether phosphorylation at Thr$^{308}$ is sufficient to trigger Akt autophosphorylation at Ser$^{473}$ in intact cells. We found that expression of a constitutively active PDK1 is sufficient to induce Akt phosphorylation at Thr$^{308}$ to approximately the same extent as insulin stimulation, but only slightly increased Akt phosphorylation at Ser$^{473}$ in cells (100). In addition, it has been shown that PDK1-mediated phosphorylation of Akt at Thr$^{308}$ was primarily dependent on prior phosphorylation at Ser$^{473}$, rather than a prerequisite for Ser$^{473}$ phosphorylation (85), although other studies have shown that phosphorylation at Thr$^{308}$ and Ser$^{473}$ are completely independent events (1). The validity of the autophosphorylation mechanism was also put into question by several other observations. It has been shown that purified Akt phosphorylated at Thr$^{308}$ does not become phosphorylated at Ser$^{473}$ in vitro (1, 3). In addition, a kinase-inactive, membrane-targeted mutant Akt can be phosphorylated at both Thr$^{308}$ and Ser$^{473}$ in unstimulated cells (6). Furthermore, kinase-inactive Akt is still phosphorylated at Ser$^{473}$ in PDK1-deficient knockout cells (103). Therefore, although Akt may undergo autophosphorylation at the HM site under certain conditions, insulin and growth factor-stimulated phosphorylation of Akt at this site is most likely mediated by a heterologous kinase.

**PHOSPHORYLATION OF THE HM SITE BY A HETEROLOGOUS KINASE(S)?**

There is increasing evidence suggesting that phosphorylation of Akt on Ser$^{473}$ may be mediated by a heterologous kinase. However, the identity of the physiological PDK2 remains elusive. So far, at least 10 kinases have been shown to function as a PDK2 that catalyzes the phosphorylation of Akt and p70$^{56K}$ in the HM. Several of these kinases were identified using classical biochemical approaches such as protein purification, but most of the kinases were revealed on the basis of the finding that alterations of the expression levels of these kinases affect Akt or p70$^{56K}$ phosphorylation at the HM site.

**MAP kinase-activated protein kinase-2.** The first heterologous kinase reported to phosphorylate Akt at the HM site was mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP kinase-2 or MK2) (1). MK2 is a serine/threonine kinase directly phosphorylated and activated by the p38 MAPK/reactivating kinase as well as by sodium arsenite, heat shock, and osmotic stress (81). It has been found that incubation of MK2 with Akt led to stoichiometric phosphorylation of Ser$^{473}$ in vitro. In addition, an MK2 inhibitory peptide suppressed Akt phosphorylation at Ser$^{473}$ in freshly isolated neutrophils. These findings led to the conclusion that MK2 acts as the HM site kinase in intact cells (79).

The physiological role of MK2 in insulin- and mitogen-stimulated phosphorylation of Akt at the HM site has been questioned by the findings that activation of this kinase is independent of PI3K, whereas Akt phosphorylation at Ser$^{473}$ is completely inhibited by the PI3K inhibitors wortmannin or LY-294002 (1). In addition, Akt underwent IGF-I-stimulated activation in the absence of MK2 (1). In contrast to the findings in human neutrophils (79), it has been shown that suppressing MK2 activity in cells by inhibiting its upstream kinase p38 MAPK had no effect on Akt phosphorylation at Ser$^{473}$ in response to insulin, IGF-I, heat shock, or hydrogen peroxide stimulation in fibroblasts (88). These results suggest that MK2 may not act as the physiological PDK2.

**Integrin-linked kinase.** Shortly after the discovery of PDK1, Dedhar et al. (30) reported the identification of integrin-linked kinase (ILK) as the kinase responsible for Akt phosphorylation at the HM site. This conclusion was based on the following findings. First, ILK contains a potential PH domain, and its activity toward myelin basic protein is stimulated by PtdIns(3,4,5)P$_3$ in vitro. In addition, ILK activity in cells is suppressed by PI3K inhibitors and is activated by overexpression of a membrane-targeted and constitutively active p110 subunit of PI3K. Furthermore, recombinant ILK phosphorylates glutathione-S-transferase-Akt on Ser$^{473}$ in vitro, and kinase-deficient ILK inhibits endogenous Akt phosphorylation at Ser$^{473}$ in cells. The conclusion that ILK functions as the HM kinase is further supported by new findings from the same laboratory that showed that suppression of ILK expression by RNA interference (RNAi) as well as conditional knockout of ILK using the Cre/loxP system resulted in almost complete inhibition of phosphorylation of Akt on Ser$^{473}$ (97).

However, other studies have cast doubt on whether ILK is a direct upstream kinase responsible for Akt phosphorylation at the HM site. In marked contrast to the results reported by Delcommenne et al. (30), studies from several other laboratories showed that ILK could not phosphorylate Akt at Ser$^{473}$ in vitro (8, 51, 66). In addition, it has been found that Akt underwent normal phosphorylation at Ser$^{473}$ in ILK-deficient fibroblasts (82) and chondrocytes (47), suggesting that ILK is not essential for Akt phosphorylation at the HM site in cells. Indeed, there is some uncertainty as to whether ILK has catalytic activity, because it lacks sequences thought essential for kinase function (21). Interestingly, overexpression of both wild-type and a mutant ILK with a mutation in the activation loop, which is not expected to have kinase activity, still promotes insulin-induced phosphorylation of Akt at Ser$^{473}$ (66). These findings suggest that ILK may function as an
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adaptor in cells and its effect on Akt phosphorylation may be indirect. In this regard, it is worth mentioning that mutant studies in Drosophila melanogaster (106) and Caenorhabditis elegans (67) both suggest that ILK functions primarily as an adaptor protein and that the kinase activity of ILK is not critical to its function. Thus whether ILK acts as a HM kinase remains questionable.

p38 MAP kinase. Studies from several groups have suggested that p38 MAPK may function as a kinase capable of mediating Akt phosphorylation at Ser473. Evidence supporting this notion first came from the study that showed that inhibition of p38 MAPK activity with SB-203580 inhibited formyl-methionyl-leucyl-phenylalanine-, FcγR cross-linking-, or PtdIns(3,4,5)P3-stimulated Akt phosphorylation at Ser473 in human neutrophils (79). In addition, overexpression of constitutively active MKK3 or M KK6, two upstream kinases of p38 MAPK, activated both p38 MAPK and Akt in HEK-293 cells (79). Inhibition of p38 MAPK also inhibited lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S-1-P)-stimulated Akt phosphorylation at Ser473 (11). The involvement of p38 MAPK in Akt phosphorylation and activation seems to be pathway specific, since suppression of p38 MAPK activity inhibited platelet-derived growth factor (PDGF), epidermal growth factor (EGF), thrombin, and endothelin-1-stimulated Akt phosphorylation, whereas insulin-induced Akt phosphorylation at this site was not affected (11). p38 MAPK-mediated Akt phosphorylation and activation are also found during myoblast differentiation (46). It has been shown that, during myogenesis, activation of p38 MAPK precedes Akt phosphorylation at Ser473 (11). Moreover, inhibition of p38 MAPK with SB-203580 or by overexpression of dominant negative p38 MAPK reduced Akt phosphorylation and activity during myogenesis. On the other hand, overexpression of the constitutively active upstream p38 MAPK activator M KK6 stimulated Akt phosphorylation at Ser473. Taken together, these results suggest p38 MAPK has a role in Akt phosphorylation at Ser473. However, it remains to be established whether p38 MAPK directly phosphorylates Akt at Ser473. In addition, it has been shown that insulin (11), IGF-I, heat shock, or hydrogen peroxide (88) activates Akt in a p38 MAPK-independent manner. These findings suggest that, although p38 MAPK may play a role in the phosphorylation and activation of Akt under certain conditions, other kinases exist that assume the PDK2 function.

PKC isoforms. PKC isoforms are classified into three categories: conventional or classical (PKCα, PKCβ1, PKCβII, and PKCγ), novel (PKCδ, PKCe, PKCθ, and PKCν), and atypical isoforms (PKCζ and PKCa/δ). Conventional PKC isoforms are activated by diacylglycerol (DAG) and Ca2++. Activation of novel PKC isoforms, on the other hand, depends only on DAG. Neither DAG nor Ca2++, however, is required for activating atypical PKC isoforms.

Two conventional PKC isoforms, PKCα (76) and PKCβII (57), have been found to be capable of mediating Akt phosphorylation at the HM site. PKCα underwent IGF-I-stimulated membrane translocation in rat fat pad endothelial cells (RFPEC), and overexpression of this kinase increased Akt phosphorylation at Ser473 (76) and Akt activity. Inhibition of PKCα activity by dominant negative constructs or suppressing PKCα expression by siRNA decreased IGF-I-stimulated Akt phosphorylation at Ser473. PKCα directly phosphorylated Ser473 in vitro in the presence of phospholipids and calcium, leading to partial activation of Akt (76). PKCβII has also been found to directly phosphorylate Akt at Ser473 (57). Akt phosphorylation on the HM site is substantially reduced in IgE/antigen-stimulated mast cells derived from PKCβ-deficient, but not from PKCe- or PKCθ-deficient, bone marrow cells. Reconstitution with PKCβII, but not PKCβI, restored IgE/antigen-stimulated Akt phosphorylation at Ser473, suggesting that this isoform plays a major role in IgE/antigen-stimulated Akt phosphorylation on the HM site. In addition to the two conventional PKC isoforms, the atypical PKCζ isoform has also been suggested to play a role in Akt phosphorylation at the HM site. PKCζ interacts with Akt3 in intact cells, and overexpression of this PKC isoform stimulates Akt3 phosphorylation on the HM serine residue (Ser472 of Akt3) (52). However, purified PKCζ was unable to phosphorylate Akt3 in vitro, suggesting that PKCζ functions as an adaptor to recruit PDK2 that catalyzes the phosphorylation of Akt (52).

Questions remain to be answered as to the physiological role of the conventional PKC isoforms in phosphorylating AGC family kinases at the HM site. It is well established that activation of PI3K is essential for insulin- and growth factor-stimulated Akt phosphorylation at the PDK2 site. However, PKC activation involves mainly membrane translocation of the PKC isoform and its interaction with DAG, which is generated by mitogen-stimulated membrane translocation and activation of PtdIns(4,5)P2-specific phospholipases Cγ1/2 rather than by activation of PI3K (72, 75, 95). In fact, it has been shown that processing of conventional PKC isoforms is not regulated by PI3K in cells (90). In addition, in contrast to the idea that PKCα may function as PDK2, knocking out this PKC isoform led to enhanced Akt phosphorylation at the HM site in mouse skeletal muscle and adipose tissues (64). Furthermore, a large number of studies have shown that PKC isoforms typically phosphorylates a basic consensus motif RXX(S/T*)XR (72, 95), which is distinct from the HM sequence FXXF(S/T*)(Y/F). Thus it remains to be determined whether or not conventional PKCs phosphorylate the HM site in vivo. Nonetheless, the purified kinase domain of PKCβII has been found to autophosphorylate at the HM site by an intramolecular mechanism (12), suggesting that the HM site could be a substrate of PKC. It is also worth pointing out that, although knocking out PKCβ greatly reduces IgE/antigen-stimulated Akt phosphorylation at Ser473, no difference in stem cell factor- and IL-3-induced Ser473 phosphorylation was found between wild-type and PKCβ-deficient cells (57). It is unclear why in the same cell distinct kinases are brought into play to catalyze Akt phosphorylation at the HM site, but one possibility is that regulation of Akt phosphorylation by different kinases may provide a mechanism for signaling specificity. Nevertheless, these results do suggest that a kinase(s) distinct from PKCβ serves a PDK2 function.

The NIMA-related kinase-6. Like Akt, the p70 (p70S6K) and p85 (p85S6K) S6 kinases, which are generated through alternative translation initiation start sites of the same transcript and differ by a 23-amino acid extension at the amino terminus, also undergo insulin- and growth factor-stimulated activation in cells through sequential multisteps phosphorylation (7). Two critical phosphorylation sites have been shown to be essential for full activation of the p70S6K isoforms, including Thr239/242 in the activation loop and Thr389/413 at the COOH-terminal HM. Phosphorylation of p70S6K at Thr239/242 is
mediated by PDK1 (4, 78, 103). The identity of the kinase responsible for the insulin/growth factor-stimulated phosphorylation of p70S6K at the COOH-terminal HM site, however, remains elusive.

To identify the kinase that phosphorylates p85S6K at the HM site, Belham et al. (13) undertook a biochemical approach. They purified a kinase activity from rat liver that phosphorylated p85S6K at Thr412. This purification led to the identification of the NIMA (never in mitosis, gene A)-related kinases NEK6 and the closely related NEK7. NEK6 phosphorylates p85S6K at Thr412 and synergized with PDK1 to activate p85S6K in vitro and in cells. Overexpression of kinase-inactive NEK6 interferes with insulin phosphorylation of p85S6K at Thr412 and activation of this kinase in CHO-IR cells.

Whether NEK6 functions as a physiological PDK2 in vivo remains unclear. NIMA, the founding member of NEKs, was first identified in Aspergillus and was found to be normally expressed in G2 phase and plays a role in chromosome condensation and M phase entry (73). At least seven NIMA-related kinases have been found in mammalian cells, but their functional roles are poorly understood. The best characterized is NEK2, which localizes to the centrosome and plays a role in cell cycle regulation (44). Several recent studies suggest that, like other members of the NEK family, NEK6 also plays a role in regulating mitotic progression. NEK6 protein levels are increased three- to fourfold in H4 cells arrested in mitosis compared with the amount in the nonmitotic cells, and its kinase activity increases in parallel to its abundance, suggesting that endogenous NEK6 is activated in mitosis (14). In addition, NEK6 is phosphorylated and activated during M phase and inhibition of the kinase arrests cells in M phase and triggers apoptosis (105). Furthermore, NEK6 functions as a mitotic histone kinase that regulates chromatin condensation in mammalian cells (50). Thus the finding that NEK6 functions as a PDK2 is somewhat surprising, as phosphorylation of the AGC family kinases at the HM site has not been shown to be cell cycle dependent. The finding that NEK6 activity is only modestly inhibited by PI3K inhibitors such as LY-294002 and wortmannin (13) also raises some concern as to whether NEK6 is a physiological relevant kinase responsible for phosphorylation of the PDK2 site in cells, considering the fact that phosphorylation of the HM of AGC kinases is completely dependent on PI3K activation. A possible explanation may be that NEK6 is constitutively active in cells and activation of PI3K only regulates its interaction with its targets. However, the findings that NEK6 activity is regulated by phosphorylation (13) and is cell cycle dependent (14, 105) make this scenario unlikely. Consistent with the notion that NEK6 is not a physiological PDK2, it has been shown that NEK6 does not phosphorylate the HM of p70S6K and SGK in vivo (65).

The double-stranded DNA-dependent protein kinase. The finding that membrane localization is sufficient to activate Akt suggests that the Akt upstream kinase may be constitutively active at the membrane. To identify the kinase responsible for Akt phosphorylation at Ser473, Hill et al. (51) undertook a protein purification approach. They identified a constitutively active Akt Ser473 kinase activity enriched in plasma membrane rafts of HEK-293 cells. This activity, which is distinct from ILK and PDK1, was subsequently shown to contain the 465-kDa catalytic subunit of the double-stranded DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (38). DNA-PKcs directly phosphorylated Akt on Ser473 in vitro and its activity in cells is potently inhibited by LY-294002 and wortmannin, two PI3K-specific inhibitors. Akt phosphorylation at Ser473 was greatly diminished in DNA-PKcs short interfering (si)RNA-treated cells, and DNA-PK-deficient cells. However, low levels of Akt phosphorylation at Ser473 were persistently observed in DNA-PKcs-deficient cells (38), suggesting the existence of an additional kinase(s) that can phosphorylate Akt at the HM site.

The identification of DNA-PKcs as an HM kinase is somewhat unexpected, as this kinase has been found to be localized predominantly in the nucleus and to play a major role in the repair of DNA strand breaks, DNA replication, and gene transcription (28, 89). Electron microscopic and crystallographic studies showed that DNA-PKcs has a structure similar to other double-stranded DNA-binding proteins with a symmetrical crown-shaped top separated from a round bottom, suggesting that the mechanistic function of DNA-PKcs involves internalization of double-stranded DNA (28). DNA-PKcs has been shown to possess a preference for S/TQ motifs (20), which are different from the HM consensus phosphorylation sequence FXXF(S/T)(Y/F). The interaction between DNA-PK and its substrates has been found to be primarily determined by substrate colocalization through DNA tethering and protein-protein interactions with Ku rather than by a specific protein sequence (20, 45, 87, 91). DNA-PK is activated by binding double-stranded DNA and is negatively regulated by autophosphorylation after cellular ionizing radiation exposures (19, 32, 35). However, little is known on whether this kinase is activated by insulin or mitogen, factors known to stimulate Akt and p70S6K phosphorylation at the PDK2 site. Unlike Akt1 (22, 25) or Akt2 (24) null mice, DNA-PKcs null mice (94) show no significant growth retardation or insulin-signaling defects. These results suggest that DNA-PK is unlikely to be a physiological upstream kinase mediating Akt phosphorylation at the HM site.

Ataxia telangiectasia. DNA-PK belongs to the PI3K-related kinase (PIKK) family, which also includes the ataxia telangiectasia mutated (ATM), ATM- and RAD-3-related kinase (ATR), and mTOR (89). The findings that DNA-PK and mTOR (see below) are capable of stimulating Akt phosphorylation at Ser473 raise an interesting question as to whether other members in this family are involved in phosphorylating Akt at the HM site. There are some data suggesting that this could be the case (97a). Overexpression of ATM greatly enhanced phosphorylation of Akt at Ser473 but had little effect on the phosphorylation at Thr308. The ATM-induced Akt phosphorylation at Ser473 was blocked by the PI3K inhibitors LY-294002 and wortmannin and by siRNA for ATM. However, ATM did not directly phosphorylate Akt at Ser473 in vitro, suggesting that the ATM-induced phosphorylation of Akt at the HM site is mediated through an indirect event, such as inactivation of an HM site phosphatase.

mTOR. mTOR is a kinase that has been shown to directly phosphorylate p70S6K at the HM site. mTOR is a Ser/Thr kinase that regulates cell growth and proliferation in response to nutrient (e.g., amino acids and glucose) and growth factor stimulation (40). Growth factor-stimulated activation of mTOR is mediated by the PI3K-PDK1-Akt signaling pathway (40). The mechanism by which nutrients activate mTOR remains...
unclear, but activation of the small G protein Rheb has been implicated in the activation process (Ref. 40 and Fig. 2).

It has been found that wild-type, but not a kinase-inactive mutant, of mTOR catalyzes p70S6K phosphorylation at the HM site in vitro and that this phosphorylation was inhibited in the presence of a rapamycin-FK506/12-binding protein (FKBP12) complex or wortmannin (55). mTOR-catalyzed phosphorylation of p70S6K at the HM site appears to be required for efficient phosphorylation of p70S6K in the activation loop by PDK1 (17, 55). Several recent studies have shown that the mTOR-mediated phosphorylation of p70S6K at the HM site is enhanced in vitro and in cells in the presence of the regulatory associated protein of mTOR (raptor), a newly identified scaffold protein that binds both mTOR and p70S6K to properly configure p70S6K for phosphorylation (49, 58) (Fig. 2).

The mechanism by which mTOR regulates p70S6K phosphorylation at the PDK2 site in vivo deserves some discussion. It is well established that binding of the rapamycin-FKBP12 complex to mTOR leads to dephosphorylation of p70S6K at Thr389. However, there is some evidence suggesting that this dephosphorylation may be due to an activation of the Thr389 phosphatase rather than inhibition of the Thr389 kinase. Phosphorylation of p70S6K at the HM site has been found to be inhibited by both wortmannin and rapamycin. However, rapamycin blocks mitogen- or 12-O-tetradecanoylphorbol 13-acetate-induced p70S6K (27), but not Akt (16, 42), activation. In addition, it has been shown that phosphorylation at the HM site of a double truncated mutant form of p70S6K, which lacked the first 54 amino acids and last 104 amino acids, was not inhibited by rapamycin but was still wortmannin sensitive (31, 98). These results suggest that different mechanisms may be involved in the phosphorylation of Akt and p70S6K on their respective HM sites (see OPEN QUESTIONS). There is also some evidence suggesting that mTOR may control p70S6K phosphorylation indirectly by restraining protein phosphatase 2A (PP2A) (77). PP2A associates with wild-type, but not a mutated, p70S6K that is resistant to rapamycin- and amino acid deprivation-mediated dephosphorylation. In addition, mTOR phosphorylates PP2A in vitro, suggesting a direct regulation of the activity of this phosphatase. Consistent with the notion that mTOR regulates p70S6K phosphorylation through an indirect mechanism, it has been reported that mTOR was unable to directly phosphorylate p70S6K at the HM (8). A possible explanation for these conflicting results may be that phosphorylation of the HM site in p70S6K is mediated by a P3K stimulated kinase, whereas the principle role of mTOR may be to induce dephosphorylation at this site (Fig. 3).

Earlier studies suggested that the mTOR-mediated mechanism may not to be operative in mitogen-stimulated phosphorylation of Akt at the HM site. In agreement with this notion, mTOR was found to be unable to phosphorylate Akt at Ser473 (8). In addition, rapamycin, which inhibits mTOR activity, has no effect on insulin-stimulated phosphorylation of Akt at the HM site in vivo (16, 42). However, a very recent study shows that mTOR, when bound to rictor [rapamycin-insensitive companion of mTOR (83)], is able to directly phosphorylate Akt at the HM site (84). Knocking down mTOR or rictor expression by siRNA decreased Akt phosphorylation at both Thr308 and Ser473 in mammalian cells. On the other hand, suppression of p70S6K or its associated protein raptor increased Akt phosphorylation at the HM. Because the mTOR-rictor complex is insensitive to rapamycin (83), this finding explains why mTOR was not previously recognized as an HM kinase for Akt. Thus, by interaction with either rictor or raptor, mTOR switches its specificity toward Akt or p70S6K, respectively (Fig. 2).

Fig. 3. Potential mechanisms underlying p70S6K phosphorylation of the HM site. mTOR may directly or indirectly phosphorylate p70S6K in the COOH-terminal (C-tail) HM. Alternatively, phosphorylation of the HM site may be mediated by a distinct PDK2, and the major role of mTOR is to inhibit a PDK2-specific phosphatase, such as protein phosphatase 2A (PP2A), leading to an increase in p70S6K phosphorylation at the PDK2 site.
OPEN QUESTIONS

Despite extensive efforts by many investigators over the past several years, a clear consensus on the nature of PDK2 remains to be achieved. Nevertheless, available evidence overwhelmingly supports the notion that phosphorylation of Akt at the HM site is mediated by a heterologous kinase rather than by autophosphorylation or by PDK1. The question remaining to be answered is, therefore, what is the identity of this kinase(s)?

Some basic properties of the kinase responsible for Akt phosphorylation at the HM site. Insulin and mitogen-stimulated Akt phosphorylation at Ser473 are greatly inhibited in cells by wortmannin or LY-39402 (86), suggesting that the kinase(s) mediating the phosphorylation at the HM site is PI3K dependent. In this regard, a particularly intriguing question concerns the physiological roles of the 10 kinases discussed above in the phosphorylation of AGC family kinases at the HM site. Another important feature of PDK2 is its constitutive plasma membrane (PM) association, which has been demonstrated by two recent studies using the classic biochemical purification approach (51, 53). Unlike PDK1, which is predominantly cytosolic, PDK2 activity has been found to be principally associated with the PM raft (51). Further purification of a partially purified PDK2 activity from HEK-293 cells by ion exchange chromatography led to a total loss of activity, suggesting that the kinase may be a multisubunit enzyme or require accessory factors for activation (51). A similar result was obtained by Mueckler’s group [Hresko et al. (53)], who showed that the PDK2 activity is associated with the PM and the low-density microsomes. In addition, they found that the HM kinase activity could be dissociated from the PM by salt extraction in functional form, which was co-sedimentary with proteins present in focal adhesions (53). It is unclear whether the same PM-associated kinase is also responsible for p70S6K phosphorylation at the HM. However, since membrane translocation is not essential for p70S6K activation, it is therefore conceivable that distinct mechanisms may be involved for phosphorylation of Akt and p70S6K at their respective HM sites (see below).

Are there multiple PDK2s? As discussed above, at least 10 kinases have been found to be able to phosphorylate AGC family kinases in the HM. These results raise the interesting question whether there are multiple PDK2s. Although a definitive answer to this question remains to be obtained, the recent finding that the mTOR/rictor complex catalyzes phosphorylation of Akt at the HM site suggests that mTOR is most likely a physiologically relevant HM kinase, at least for p70S6K and Akt. However, a number of issues remain to be resolved. First, although the HM kinase for Akt appears to be constitutively PM associated (51, 53), endogenous and recombinant mTOR proteins have been found to localize predominantly in the endoplasmic reticulum (ER), the Golgi apparatus, and the nucleus, but not to the PM (36). In addition, targeted deletion of PDK1 has been shown to cause an abolishment of p70S6K phosphorylation at the HM site (Thr389), but had no effect on Akt phosphorylation at Ser473 (103). Furthermore, mTOR is activated by a number of stimuli in addition to insulin and IGF-I, including phorbol ester (69); but phorbol ester has been shown to inhibit, rather than stimulate, insulin-stimulated Akt phosphorylation and activation (10, 71). Thus it will be important to determine whether there is a novel mTOR-independent and PM-associated HM kinase for Akt. In addition to the question of whether mTOR is the sole HM kinase for Akt, it will also be interesting to determine whether mTOR catalyzes HM site phosphorylation in other AGC family kinases in addition to p70S6K and Akt. Nevertheless, we reason that regulating phosphorylation of different AGC family kinases at their respective HM sites by distinct kinases or kinase-complexes may be advantageous for regulating specific signaling pathways that mediate diverse upstream stimuli in response to distinct functional requirements. For example, the mTOR-p70S6K pathway senses levels of environmental nutrients and energy efficiency, and excess activation of this pathway has been shown to attenuate insulin signaling (68). Although activation of Akt and p70S6K requires phosphorylation of these kinases in the activation loop by the same upstream kinase PDK1, full activation of these kinases via phosphorylation in the HM site by distinct kinases would provide a mechanism to prevent a futile cycle in these signaling processes.

Are there multiple mechanisms for AGC family kinase phosphorylation at the HM site? In addition to the identity of PDK2, another interesting question that needs to be answered is whether there is a unified mechanism regulating all AGC kinase phosphorylation at the HM site. Taking into consideration all of the data obtained so far, we think that the answer is most likely to be no. In the case of PKCβII, for example, phosphorylation at the HM site has been found to be mediated by autophosphorylation (12), although it remains to be determined whether a similar mechanism is used by the other PKC isoforms. In addition, although mTOR has been shown to be the kinase catalyzing the phosphorylation of both p70S6K and Akt in the HM, the specificity of mTOR is entirely dependent on its association with either raptor (49, 58) or rictor (84). If mTOR is the sole HM kinase, then regulation of the mTOR/riotor/mTOR/rictor complex formation may be key to understanding of the specificity of mTOR to recognize various targets. It is worth also mentioning that, for some AGC family kinases such as PKCζ and the PKC-related kinase-1 (PRK1/ PKN), the residue corresponding to the PDK2 site in Akt is either glutamate or aspartate (Fig. 1). These negatively charged residues may mimic phosphorylation at the HM site, and thus interaction of the kinases with PDK2 appears not to be necessary. Thus multiple mechanisms appear to be used in cells to regulate the activation of the AGC family kinases necessary for regulating specific downstream biological events.

CONCLUDING REMARKS

Despite the conflicting data on the mechanisms and the identity of the HM kinase, a new picture is steadily emerging that ascribes the regulation of Akt and other AGC family kinases in response to RTK signaling. An attractive model that takes all the available data into account is that phosphorylation of the HM site in different AGC family kinases may be mediated by distinct mechanisms. In addition, phosphorylation of the AGC family kinase at the HM could be cell type, signaling pathway, and substrate specific. The picture, however, is far from complete, and a major challenge to solve the signaling puzzle is to determine whether there is a unified kinase that mediates the phosphorylation of different AGC kinases.
family kinases at the HM. Biochemical and genetic studies have provided valuable information on some of the characteristics of the kinases responsible for phosphorylating Akt and p70S6K on the HM site. It can be anticipated that progress along the above-mentioned directions will lead to the unambiguous identification of PDK2(s) and allow a better understanding of RTK signaling and regulation, which is essential for developing novel therapeutic treatment for diseases such as diabetes and cancer.

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