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Minireview

Unravelling the activation mechanisms of protein kinase B/Akt

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Abstract Over the past decade, protein kinase B (PKB, also termed Akt) has emerged as an important signaling mediator between extracellular cues and modulation of gene expression, metabolism, and cell survival. The enzyme is tightly controlled and consequences of its deregulation include loss of growth control and oncogenesis. Recent work has better characterized the mechanism of PKB activation, including upstream regulators and secondary binding partners. This minireview refreshes some old concepts with new twists and highlights current outstanding questions.

Key words: Protein kinase; Signal transduction; Protein kinase B; Akt; 3-Phosphoinositide-dependent kinase-1; Phosphatidylinositol 3-kinase

1. Introduction

Protein kinase B (PKB, also termed Akt) is a serine/threonine kinase belonging to the 'AGC' superfamily of protein kinases (of which there are over 80 members). This group of kinases shares similarity within their catalytic domain structure and in their mechanism of activation, and many are frequently deregulated in human diseases including cancer. As with other AGC kinases, PKB is regulated by upstream, second messengers and secondary, activating enzymes. For PKB, this activating process involves multiple inputs that strictly control the location, duration and strength of response. The purpose of this article is to update current understanding of the complex PKB activation process. Interest in PKB has been piqued by its role in promoting cell survival signals through the phosphoinositide 3-kinase (PI3K) pathway, leading to inactivation of a series of pro-apoptotic proteins (see Fig. 1). The reader is directed to several recent reviews that describe the physiological substrates and cellular functions of this captivating enzyme [1–4].

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Abbreviations: PKB, protein kinase B; PI3K, phosphoinositide 3-kinase; PDK1, 3-phosphoinositide-dependent kinase-1; PH domain, pleckstrin homology domain; HM, hydrophobic motif; PIF, PRK2-interacting fragment

2. PKB – topography

In mammals, there are three isoforms of PKB (α, β, γ; Akt1, 2, 3). All three isoforms share a high degree of amino acid identity and are composed of three functionally distinct regions: an N-terminal pleckstrin homology (PH) domain, a central catalytic domain, and a C-terminal hydrophobic motif (HM). Together, these regions encompass a phosphoprotein of approximately 56 kDa. This general architecture is conserved across species including *Drosophila melanogaster* and *Caenorhabditis elegans*, suggesting that regulation of PKB is evolutionarily ancient. The Sch9 gene product of *Saccharomyces cerevisiae*, although lacking a lipid binding domain, is a likely a distant member due to similarities in its regulation.

The catalytic domain of PKB is structurally similar to other protein kinases of the AGC family including PKA [5]. To control the activity and specificity of the protein kinase domain, there are two important regulatory domains. The N-terminal PH domain is common to numerous signaling proteins and provides a lipid binding module to direct PKB to PI3K-generated phosphoinositides PI(3,4,5)P3 and PI(3,4)P2. Thus, growth factors that act to increase PI3K activity and lead to the subsequent generation of PI(3,4,5)P3 and PI(3,4)P2 provide a plasma membrane recruitment mechanism for PKB. Membrane recruitment of PKB is a hallmark of activation (discussed in detail below).

The crystal structure of the PH domain of PKB bound with the inositol head group of PI(3,4,5)P3 was recently solved [6]. Interestingly, this structure revealed differences in the binding between inositol(1,3,4,5)P4 and other PI(3,4,5)P3 binding PH domains, including BTK and DAPP1. The most significant of these differences is that the D5 phosphate of inositol(1,3,4,5)P4 does not physically interact with the PKB PH domain, agreeing with earlier studies demonstrating that PI(3,4,5)P3 and PI(3,4)P2 bind to PKB with equal affinities. This has important implications regarding the upstream phosphatase regulators of PI3K-generated lipids, PTEN [7,8]and SHIP [9]. Since PTEN catalyzes the dephosphorylation of PI(3,4,5)P3 and PI(3,4)P2 at the D3 position, the actions of this phosphatase would reduce the entire pool of lipids capable of binding with PKB. SHIP, however, catalyzes the dephosphorylation of PI(3,4,5)P3 to generate PI(3,4)P2. Thus, the actions of PTEN would likely have a greater negative impact on the activation of PKB than SHIP.

The second important regulatory domain of PKB is a C-terminal HM present in many other AGC kinases [2,10]. Studies on PKB and other AGC kinases including protein kinase C, p70 ribosomal S6 kinase, serum and glucocorticoid-activated

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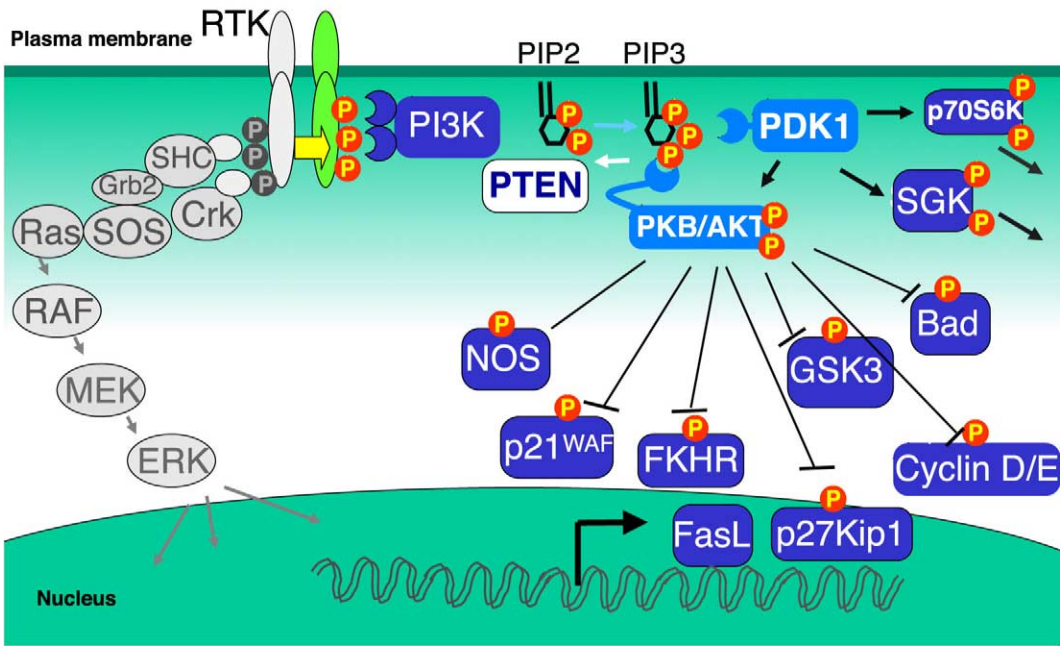

Fig. 1. Regulation and targets of PKB/Akt. Ligation of receptor tyrosine kinases (RTKs) with their ligands (or through other agonist pathways such as G protein-coupled receptors, not shown) leads to recruitment of members of the PI3K family of lipid kinases to the plasma membrane where they encounter the substrate lipid phosphatidylinositol 4,5 bisphosphate (PIP2), leading to formation of 3,4,5 PIP3. This molecule recruits PH domain containing proteins to the lipid bilayer including PKB and PDK1, where the former becomes activated (see also Fig. 2). Substrates of PKB include transcription factors such as FKHR which regulates expression of Fas ligand and other molecules that promote apoptosis and metabolism. PKB phosphorylation of Bad, FKHR, p27Kip1 and GSK-3 inactivates these proteins, helping to suppress apoptosis and promote glucose utilization.

kinase (SGK) and RSK indicate that the HM provides a dual role in regulating kinase activity. The HM provides a docking site for the upstream activating kinase, 3-phosphoinositide-dependent kinase-1 (PDK1) [11–13]. Interrupting this docking interaction severely attenuates phosphorylation of the activation loop (T-loop) of AGC kinases. More recent biochemical and structural studies have elegantly demonstrated that the HM also serves as an allosteric regulator of catalytic activity [5,12,14]. The HM provides stability to the catalytic core by association with hydrophobic and phosphate binding pockets created by a cleft formed at the junction of the αB-helix, αC-helix and β5-sheet in the N-lobe of the kinase domain. This pocket has been called the 'PRK2-interacting fragment (PIF)-pocket' because it was initially determined to be the site of binding for the HM fragment of a protein kinase termed PRK2, called PIF [15]. Stabilization of the N-lobe of the kinase domain by binding of the HM increases the phosphotransfer rate by 10-fold. Similar dynamics exist for PKA and the interactions are predicted in the crystal structure of this kinase [16]. For both PKB and PKA, mutations of key phenylalanine residues within the HM compromise catalytic activity [17].

3. Phosphorylation in PKB regulation

PKB is activated by, and dependent upon, multisite phosphorylation. As introduced above, a main site of phosphorylation is within the activation T-loop at Thr308 (for PKBα). Phosphorylation of PKB on Thr308, similar to other AGC kinases, causes a charge-induced change in conformation allowing substrate binding and greatly elevated rate of catalysis. Some of the AGC kinases are constitutively phosphorylated on the equivalent activation loop residue and regulation of activity is provided through other means, such as allosteric effectors or release from pseudosubstrate inhibitory domains. However, PKB is one example of the AGC kinases for which there is very low activation loop phosphorylation in resting cells and rapid increase in phosphorylation upon agonist stimulation. The phosphorylation of Thr308 strictly governs the activation of PKB, and its mutation to non-phosphorylatable alanine greatly reduces activity [18].

There is convincing evidence that Thr308 is phosphorylated by the PDK1 [19,20]. PDK1 is a 'master kinase' responsible for activation loop phosphorylation of numerous AGC kinases [2,21]. PDK1 phosphorylates PKB in vitro, and overexpression of PDK1 in cells also leads to elevated Thr308 phosphorylation in the absence of natural agonists. PDK1 contains a C-terminal PH domain, and the rate of PKB phosphorylation by PDK1 is greatly enhanced in vitro by the inclusion of PI(3,4,5)P3 or PI(3,4)P2, which concentrates both upstream kinase and kinase substrate onto a lipid-micelle surface [19]. In cells in which PDK1 has been genetically disrupted, PKB is unresponsive to mitogenic stimulation as a consequence of a loss of Thr308 phosphorylation [22]. Similar results are observed with the disruption of PDK1 mRNA [23].

A key difference between the HM of PKB (and other AGC kinases) compared with PKA is an additional layer of regulation provided by HM phosphorylation. For PKBα, this target residue is Ser473. Phosphorylation of Ser473 increases the affinity of the HM for the PIF-pocket by several orders of magnitude [12–14]. The mechanism of Ser473 phosphoryla-

tion is not completely understood, and there is evidence suggesting both autophosphorylation [24] and phosphorylation by distinct serine kinases, including the integrin-linked kinase (ILK; [25,26]). Furthermore, Hill and coworkers recently purified a Ser473 kinase activity isolated from buoyant lipid rafts, distinct from both PDK1 and ILK [27].

4. Determinants of Thr308 and Ser473 phosphorylation

Several distinct criteria and inputs are required to trigger Ser473 phosphorylation. Recent work with conditionally active PKB models has assessed the requirements for Ser473 phosphorylation [28,29]. These studies revealed that plasma membrane localization is a necessary criteria for Ser473 phosphorylation [29]. In cooperation with membrane location, some catalytic activity of PKB is necessary for Ser473 phosphorylation, since kinase-dead forms (Lys179 to alanine mutant) and Thr308 to alanine mutants are refractory. Inhibition of PDK1 activity with staurosporine reduces the extent of Thr308 phosphorylation, without affecting Ser473 phosphorylation, indicating that plasma membrane localization cooperates with basal PKB activity to activate Ser473 phosphorylation [29,30]. On the other hand, cytosolic PKB containing an aspartic acid substitution at the Thr308 position is not significantly phosphorylated at Ser473 [29]. It is difficult to interpret these observations to completely accommodate either autophosphorylation or transphosphorylation by a distinct Ser473 kinase.

Complicating matters further, PI3K activity is required to achieve Ser473 phosphorylation, in a manner distinct from the actual membrane localization of PKB [28,29]. This could indicate that PI3K-derived lipids activate a distinct Ser473 kinase, inhibit a Ser473 phosphatase, or regulate the binding of a Ser473-regulatory protein (such as CTMP; [31]). An interesting possibility is that the phosphorylation of Thr308 promotes a conformation of the PIF-pocket more suitable for HM binding. The HM of PKB binds with much lower affinity to the PIF-pocket than the HM of other AGC kinases, such as S6K and SGK, do to their own PIF-pockets [13]. Therefore, small changes in the ternary structure of the kinase domain may influence the packing of the HM into the PIF-pocket. Without Thr308 phosphorylation, the HM may be more susceptible to phosphatases, leading to a net reduction in phosphorylation. This possibility could have an impact on the duration of PKB activity as it moves throughout the cell: the phosphorylated and fully active form of PKB is stable, allowing migration to the nucleus and other sites.

We recently showed in a study utilizing mast cells derived from SHIP-knockout mice that the Ser473 activity was sensitive to PI3K inhibition, even though under the conditions tested phosphatidylinositol 3,4,5 trisphosphate (PIP3) levels were high, PKB membrane localization was strong, and Thr308 phosphorylation was normal [32]. In cells engineered to lack SHIP, generation of mitogen-induced PIP3 is so robust that reduction of PI3K activity by 90% still leads to a significant pool of PIP3, while completely abolishing PI(3,4)P2. Adding back this lipid partially restores Ser473 phosphorylation and PKB activation. Thus, SHIP-derived PI(3,4)P2 may serve a role in promoting and sustaining Ser473 phosphorylation. This would be consistent with earlier characterization of PKB showing that PI(3,4)P2 added to cells could activate PKB efficiently [33].

5. The HM and Ser473 bring PDK1 into the fold

A major goal for a number of research groups is understanding of the spatial and temporal constraints that limit non-physiological phosphorylation of PKB by PDK1 and the Ser473 kinase. This is particularly complex since PDK1 itself appears to be constitutively active, and the primary means of its control is likely through PH-domain subcellular targeting. For some substrates, PDK1-directed phosphorylation is constitutive, while for others, such as PKB, phosphorylation is coupled to the activation of PI3K.

Biochemical and structure studies of PDK1 [12,34] and PKB [14,5] provide important insight into this complex relationship. PDK1 harbors a hydrophobic and phosphate binding pocket similar to other AGC kinases, but lacks a complementary HM. In the absence of its own HM, PDK1 compensates by utilizing the HM of its substrates. Thus, the HM of AGC kinases provides PDK1 with a docking and activation mechanism. For PKB, PDK1 interacts with the Ser473-phosphorylated HM, bringing PDK1 into proximity to Thr308 and by causing a stabilizing effect on the N-lobe, increases its specific activity.

The scenario that arises from these criteria suggests the following order of events in PKB activation (Fig. 2). First, PI3K-generated lipids recruit PKB and PDK1 to the plasma membrane. Autophosphorylation (perhaps increased by conformational changes induced by lipid binding of the PH domain), or a membrane-localized kinase, promotes PKB phosphorylation on Ser473. The now phosphorylated HM promotes interaction with PDK1, via its PIF-pocket. PDK1, now activated and in stable contact with PKB, phosphorylates Thr308. The Ser473-phosphorylated HM now prefers intramolecular association with its own PIF-pocket, releasing PDK1 and adopting a fully active state for PKB.

Arguing against this hypothetical mechanism, recent work suggests that the HM of PKB is not critical for docking PDK1 in vivo [13,35]. This is argued because addition of PIF-tide, which inhibits the PDK1-dependent phosphorylation of other AGC kinases, including p70 S6 kinase and SGK, does not prevent PKB phosphorylation by PDK1 in vitro. This finding suggests that competition for the PIF-pocket of PDK1 does not restrict PDK1–PKB interaction, which may instead be facilitated by the PH domain of PKB. However, PKB and PDK1 are colocalized to PIP3-containing vesicles, while ΔPH-PKB, p70 S6 kinase and SGK are not. It is unclear what effect concentrating both substrate and kinase on the lipid surface would have on the IC_{50} of the PIF-tide. Also, while it is notable that ΔPH-PKB is sensitive to PIF-tide, behaving similarly to p70 S6 kinase and SGK, it is very poorly phosphorylated by PDK1 [13]. This is consistent with the finding that the HM of PKB binds with lower affinity to PDK1 than to the HM of SGK or p70 S6 kinase [12]. Overall, we interpret these data as indicating that phosphorylation of Ser473, in the cytosol, is insufficient to dock PDK1 and achieve Thr308 phosphorylation. Rather, both kinases must be stably colocalized on lipid membranes to drive PDK1-dependent phosphorylation. In support of this view, we have shown that conditionally active PKB proteins, with and without PH domains, require both Ser473 phosphorylation and membrane anchoring to achieve full Thr308 phosphorylation [29]. This is evidenced by the observation that cytoplasmic ΔPH-PKB S473D mutants of PKB are much

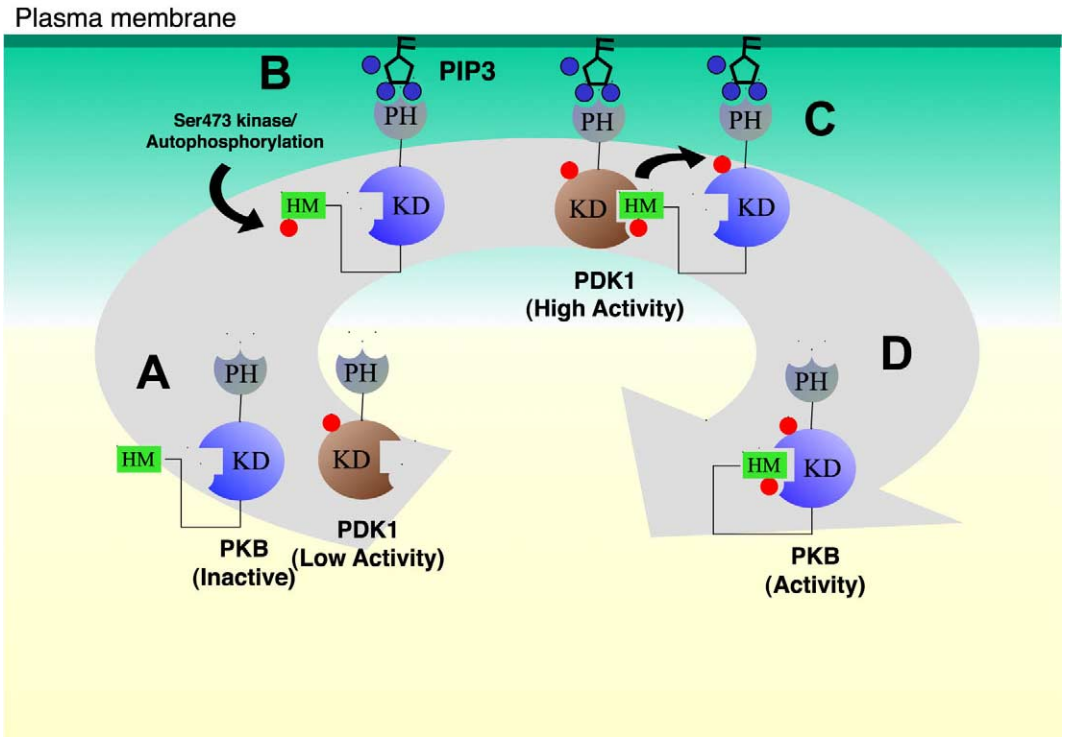

Fig. 2. Activation Cycle of PKB/Akt. A: PKB is cytosolic and inactive. PDK1 has low activity, and Thr308 phosphorylation is not favorable. B: PI3K-generated PIP3 And PI(3,4)P2 recruit PKB and PDK1 to the plasma membrane. PKB undergoes HM phosphorylation by the Ser473 kinase or by autophosphorylation. C: The HM of PKB stabilizes and activates PDK1, which then phosphorylates PKB on Thr308. D: The HM of PKB associates with and stabilizes its kinase domain. The fully active kinase phosphorylates cytosolic and nuclear targets (Fig. 1). HM, hydrophobic motif; PH, pleckstrin homology domain; KD, kinase domain. Red circles indicate phosphorylation events.

less sensitive to PDK1 phosphorylation than their equivalent membrane-localized form.

6. PKB and tyrosine phosphorylation

Several recent reports have investigated the possible role of tyrosine phosphorylation in PKB regulation [36–38]. One report indicated that two tyrosine residues located within the catalytic domain of PKB, Tyr315 and Tyr326, are phosphorylated following receptor activation and are required for activity, since mutation to phenylalanine abolished kinase activity [36]. However in the absence of either mass spectrometry analysis or phosphopeptide mapping it is not clear that these are true sites of tyrosine phosphorylation (see below). Also, these residues are highly conserved in protein kinases, which might suggest that their mutation to phenylalanine could impact the structure and activity of the kinase domain independently of phosphorylation.

A second report identified Tyr474, based on phosphopeptide mapping, as a possible site of phosphorylation in response to insulin and pervanadate [38]. Mutation of this residue to phenyalanine reduced Thr308 phosphorylation and reduced PKB activation by about 55%. The crystal structure of PKB indicates that Tyr474 participates in the binding of the HM with the PIF-pocket: the aromatic ring of Tyr474 interacts with Leu214, while the hydroxyl group forms a hydrogen bond with Arg207 [5]. Thus, it is not necessarily surprising that the mutation of Phe474 would result in reduced activity, due to a loss of pHM-PIF-pocket affinity. This might also extend to a reduced ability of PDK1 to dock with and phosphorylate PKB. A very interesting point is that the authors show that Ser473 and Tyr474 phosphorylation are mutually exclusive: phosphoamino acid analysis and N-terminal sequencing of the tryptic peptide containing Ser473 and Tyr474 show that neither are phosphorylated together. This could indicate that phosphoTyr474 blocks the action of a Ser473 kinase, or, by preventing the HM from packing into the PIF-pocket might increase the susceptibility of Ser473 to the actions of phosphatases. Based on these observations, it might be considered that phosphorylation of Tyr474 represents an inhibitory input designed to limit the extent of PKB activation. This tyrosine is conserved widely in the AGC family, suggesting that a possible mechanism of regulation could be a general feature. Further experiments are needed to evaluate the role of Tyr474 in PKB regulation. Indeed, since phospho-Ser473 antibodies are widely used to monitor the activation state of PKB, it will be important to assess whether the epitope detected by these antibodies is affected by phosphorylation of Tyr474.

7. Conclusions

Clearly, PKB activation is complex, exhibiting a series of spatial restrictions that limit phosphorylation by upstream activators (Fig. 2). A principle component of PKB activation is the generation of PIP3 and PI(3,4)P2 by PI3K. This is necessary to localize PKB to membrane surfaces, where Ser473 phosphorylation then occurs. The sustained phosphorylation of Ser473 is also dependent on PI3K, through a process that is distinct from membrane localization. This might indicate that a Ser473 kinase, phosphatase or binding partner is also influenced by PI3K. PDK1 associates with the Ser473-

phosphorylated HM of PKB, which likely activates PDK1 sufficiently to catalyze phosphorylation of Thr308 of PKB. The affinity for the phosphorylated HM of PKB for PDK1 is less than the HM of other AGC kinases, including p70 S6 kinase and SGK. Thus, strict dependencies for membrane localization to both fully promote Ser473 phosphorylation, and to increase the relative concentration of kinase to substrate are likely in place to prevent inappropriate PDK1 activation of PKB. Finally, the Ser473-phosphorylated HM then plays a second major role, in which it binds with the PIF-pocket of PKB generating a fully active, multiphosphorylated PKB enzyme. In this stable form, PKB dissociates from the plasma membrane and target substrates located in the cytoplasm and nucleus.

Remaining questions include the rationale for the complexity of the activation process. Is it a reflection of specificity, allowing selective members of the AGC family to adapt to activation by distinct agonist pathways? Is there a temporal constraint to the process, such that the activation events are ordered? Is the processing and activation of PKB a trigger for other AGC kinase activations? Are there points of intervention which act as gateways for regulation by other pathways? There are likely many more surprises and confounding elements to this molecular machine. For example, PKB is trimerized by binding to Tcl proteins [39,40], introducing yet more possibilities for intramolecular control. The function of CTMP in the process is also a conundrum [31]. Given the importance of this protein kinase in a variety of human diseases including cancer, diabetes and inflammatory disease, understanding of the precise molecular switches that must be flipped to achieve activation should lead to design of antagonists that are selective for this process.

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