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Review

Protein kinase B: signalling roles and therapeutic targeting

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Abstract. The serine/threonine kinase, protein kinase B (PKB, also known as Akt), is activated by a wide array of growth factors and insulin. PKB is a central player in the regulation of metabolism, apoptosis, transcription and the cell-cycle. PKB exists as three isoforms (alpha, beta and gamma) that may have unique as well as common functions within the cell. Deregulation of PKB is associated with several human diseases, including cancer, diabetes and schizophrenia. These findings underscore the medical relevance of the PKB pathway and make PKB an attractive drug target for the treatment of diseases that exhibit abnormal PKB signalling.

Keywords. Protein kinase B, Akt, insulin, apoptosis, cell survival, cell cycle, cancer, diabetes.

Introduction

Protein kinase B (PKB) is activated by insulin as well as by a variety of growth factors such as platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor and insulin-like growth factor I and plays a major role in signalling effects on metabolism, cell growth, cell cycle and apoptosis. Deregulated (activated) or overexpressed PKB has been found in numerous cancer types, with a $>50\,\%$ incidence rate in some cases. Conversely, activation of PKB is reduced in human patients suffering from type 2 diabetes. Type 2 diabetes can only be moderately controlled by diet regimes and current drugs that are weakly effective. Patients with type 2 diabetes tend to suffer long-term complications including loss of sight, kidney and heart disease and an average 5- to 10-year reduction in life expectancy. The medical significance of the PKB pathway underpins the need to fully understand the structure and regulation of PKB so that therapies targeted against PKB, and especially its individual isoforms, can be developed. This is reinforced by findings indicating that PKBβ is the predominant isoform that signals the actions of insulin, and so specific therapies that avoid affecting this isoform in cancer and others that activate it in diabetes may be particularly useful.

Structure of PKB

PKB, originally discovered through homology cloning using a protein kinase A (PKA)-specific probe and as the cellular homologue of the v-Akt oncogene, exists as three isoforms: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 [1] (Fig. 1). PKBα is ubiquitously expressed. PKBβ is predominantly expressed in insulin target tissues, such as fat cells, liver and skeletal muscle. PKBγ is less widely expressed, being high in brain but also expressed in at least one insulin-responsive cell type, namely 3T3-L1 adipocyte cells [2, 3]. The PKB family of kinases is conserved in higher eukaryotes, with PKBs from

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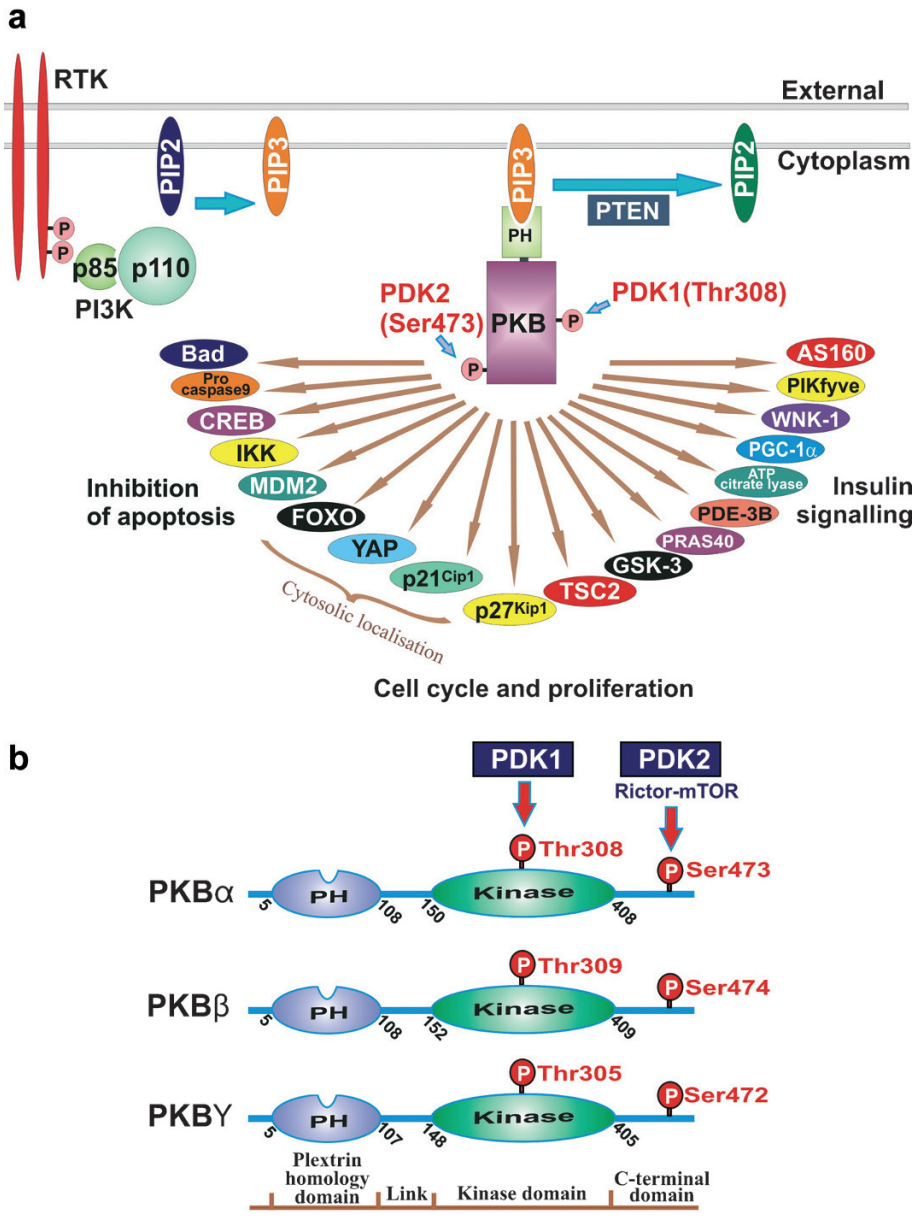

Figure 1. (*a*) Key substrates for PKB. (*b*) Structure of PKB isoforms.

mouse, rat and human having 95% amino acid sequence identity.

PKB is a 57-kDa soluble cytosolic protein, with three conserved domains in each isoform, namely an N-terminal pleckstrin homology (PH) domain, a central catalytic kinase domain and a C-terminal domain. Curiously, the PH domain is absent in the yeast orthologue, Sch9 [4]. Various crystal structures are now available for the protein kinase domain or PH domain yet there remains no complete crystal structure for PKB [5]. Overall PKBβ and PKBγ share 81% and 83% sequence homology respectively, with PKBα. The PH domain of just over 100 amino acids is 80% identical amongst PKB isoforms and binds phosphatidylinositol 3, 4, 5-trisphosphate [PtdIn(3,4,5)P_3] and phosphatidylinositol 3, 4, trisphosphate [PtdIn(3,4)P_2] with high affinity. The PH domain of PKB is unable to bind phosphoinositides lacking a D3 or D4 phosphate. Phosphoinositides bind to a shallow pocket in the PH domain with the phosphate side chains of the head group forming salt bridges with specific basic residues in the protein [5, 6].

The kinase domain of PKB, approximately 250 amino acids in length, is highly similar to PKC, PKA, serum- and glucocorticosid-regulated protein kinase (SGK) and S6 subfamilies of the PKA/protein kinase G/protein kinase C (PKC) (AGC) kinases and ~90% identical amongst the PKB isoforms [6]. It contains a conserved threonine whose phosphorylation is necessary for full activation. The threonine (T308 in PKBα,

T309 in PKBβ and T305 in PKBγ) is located between the DFG and Akt phosphorylation enhancer (APE) motifs in the activation loop [1, 4–6]. The C-terminal domain, approximately 40 amino acids in length, is ~70% identical amongst PKB isoforms and most closely related to the PKC family. It contains the hydrophobic motif characteristic of AGC kinases: F-X-X-F/Y-S/T-Y/F where X is any amino acid [1, 4–6]. A conserved serine in this region must also be phosphorylated for full activation of PKBα, PKBβ and PKBγ [1, 4–6].

Activation of PKB

Phosphatidylinositol-3 kinase (PI3K), a key regulator of fundamental cellular responses, promotes the phosphorylation of an array of protein kinases including PKB. Both PI3K and PKB are activated by numerous growth factors and insulin. PI3K generates the key second messenger PtdIn(3,4,5)P_3 in the plasma membrane that binds to the PH domain of PKB thus mediating the membrane translocation of PKB (Fig. 1). The interaction of PKB with PtdIn(3,4,5)P_3 does not directly activate PKB. Instead, binding of PKB to PtdIn(3,4,5)P_3 is believed to induce a conformational change in PKB which facilitates its phosphorylation on T308 and S473 by the upstream kinases phosphoinositide-dependent kinase 1 and 2 (PDK1 and PDK2), respectively [1, 4–6]. The inhibitors of PI3K, wortmannin and LY294002, as well as a dominant negative form of class1a PI3K reduce the level of PtdIn(3,4,5)P_3 and prevent the phosphorylation and activation of PKB [7]. Point mutants of PKB with Ala instead of T308 and S473 show little activity. The phosphorylation site double mutants T308D/S473D show constitutive kinase activation. Studies with mutants having just one site changed to Ala or Asp indicate that the singly phosphorylated forms are partially active (~20%). These observations indicate that these two sites are necessary and sufficient for full activation with phosphorylation at one site inducing partial activation [1]. Phosphorylation of S473 is believed to be the more crucial step in the activation of PKB because it stabilizes the kinase domain in an active conformation state almost identical to that found in the constitutively phosphorylated and activated catalytic subunit of PKA [8]. Once activated, PKB dissociates from the plasma membrane and phosphorylates many substrates in the cytoplasm and the nucleus. Most of the PKB substrates contain the minimal consensus sequence RXRXX(S/T) [4]. The number of substrates identified continues to grow and there is still much to learn about the signalling roles of PKB, especially in the brain.

PDK1, like PKB, has a PtdIn(3,4,5)P_3-binding PH domain [1, 4–6]. It is likely that colocalisation of PDK1 and PKB with PtdIn(3,4,5)P_3 facilitates the phosphorylation of PKB at T308 by PDK1. The identity of the kinase that phosphorylates S473, variously called PDK2, the S473 kinase and the hydrophobic motif (HM) kinase, has proved much more elusive. Given the importance of phosphorylation of PKB on S473 in its activation, much effort has been expended in trying to identify the kinase. One suggestion is that PDK2 activity is attributable to PDK1 [9, 10]. This has been proposed to occur through a small C-terminal fragment of PRK2 (protein kinase C-related kinase 2) which converts PDK1 into PDK2 [9, 10]. A second proposal is that a distinct PDK2 does not exist but that the serine phosphorylation occurs by virtue of PKB autophosphorylation [11]. Antisense knock-down studies and use of a PDK1 inhibitor showed that PKB autophosphorylation or phosphorylation by PDK1 were unlikely mechanisms and instead suggested the presence of a distinct PDK2 activity [3]. PKCζ has been proposed to function as an adaptor that delivers the PDK2 to PKB [3]. At least 10 kinases have been proposed to function as the actual PDK2. These include p38 MAP kinase, integrin-linked kinase, mitogen-activated protein kinase-activated protein kinase 2, PKCα, PKCβII, double-stranded DNA-dependent protein kinase and the ataxia telangiectasia mutated gene product [12]. It has been argued for a variety of reasons that these are unlikely candidiates, e.g. in many cases activation of the kinase is insensitive to PI3K inhibitors or the kinase has not been shown to directly phosphorylate S473 in vitro [12, 13]. Nonetheless, some of these kinases might play roles in specific cellular backgrounds or act indirectly by regulating the physiological PDK2 [12]. On the other hand, evidence is now mounting that mammalian target of rapamycin (mTOR) complex 2 (mTORC2) is the physiological PDK2 [13–18] (Fig. 2). mTORC2, also called rictor.mTOR, is a complex of the kinase mTOR, mLST8, mammalian stress-activated protein kinase (SAPK)-interacting protein (mSin1) and rictor. The S473 kinase activity of mTORC2 is insensitive to staurosporine and sensitive to PI3K inhibitors, known properties of the physiological kinase [3, 13–15]. Knock-down of rictor or mTOR expression using RNA interference (RNAi) decreased the phosphorylation of S473 in cancer cells [13]. Similarly, small interefering RNA (siRNA) knock-down of rictor from 3T3-L1 adipocytes suppressed insulin-activated S473 phosphorylation [14] and knock-out of mSin1, mLST8 or rictor in mice prevented S473 phosphorylation [15–18]. Moreover, mTORC2 directly phosphorylates PKB on S473 in vitro and this facilitates phosphor-

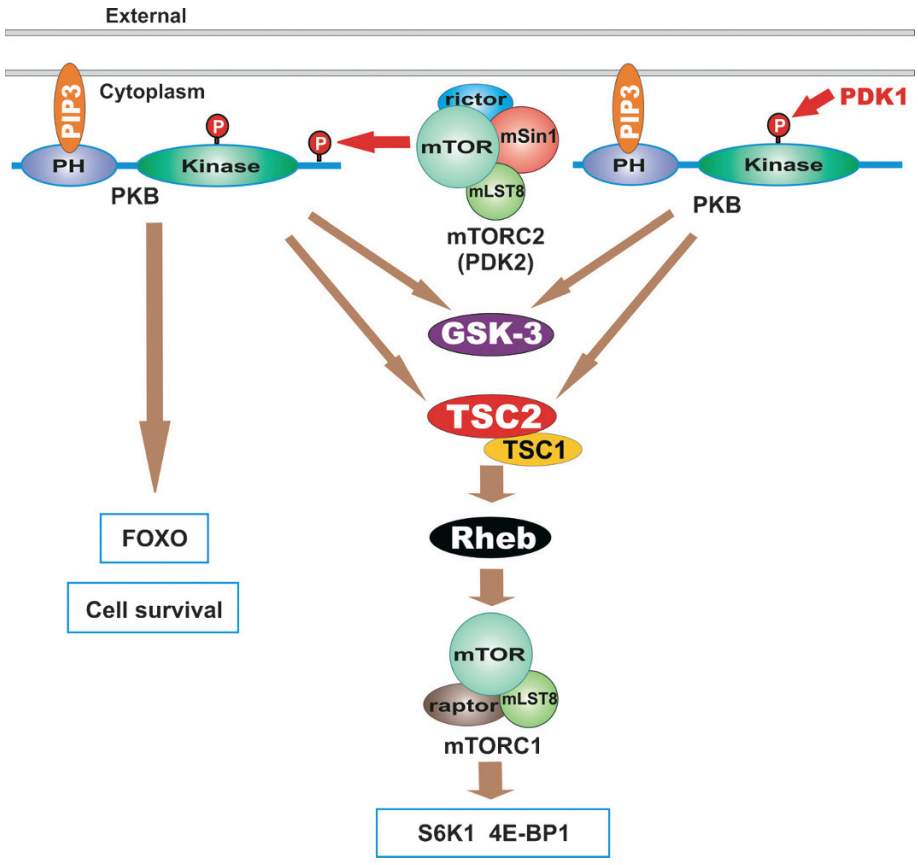

Figure 2. Phosphorylation of PKB on S473 acts as a regulatory switch that determines PKB substrate specificity. mTOR exists in two highly conserved complexes. mTORC2 acts upstream of PKB functioning as a PDK2 that phosphorylates S473. Activation of mTORC2 is inhibited by wortmannin and so is PI3K dependent. The mechanism by which growth factor and insulin-generated PtdIn(3,4,5)P_3 (PIP_3) activates mTORC2 is currently unknown. mTORC1 functions downstream of PKB and ultimately signals increases in translation. The kinase activity of mTORC1 is inhibited by rapamycin whereas that in mTORC2 is not. The presence of mSin1, mLST8 and rictor in the mTOR complex are all required for the capacity of mTORC2 to phosphorylate S473 [15]. Three isoforms of the mSin1 protein generate three distinct mTORC2 s. Even though all mTORC2 s can phosphorylate PKB in vitro, insulin regulates the activity of only two of them. Thus, cells may contain several mTORC2 subtypes that phosphorylate PKB in response to different signals [15]. Cells lacking functional mTORC2 are defective in PKB S473 phosphorylation and in the ability to phosphorylate FOXO and activate cell survival pathways. In such cells, phosphorylation of PKB on T308 and the phosphorylation of the PKB targets, GSK3 and TSC2, is unimpaired. In cells containing functional mTORC2, PKB is doubly phosphorylated and competent for pro-survival signalling and for phosphorylating GSK3 and TCS2. S473 phosphorylation may thus act as a molecular switch required for phosphorylation of anti-apoptotic targets. PKB can also activate mTORC1 through PRAS40 (not shown). It is not known which phospho PKB-species phosphorylates PRAS40.

ylation of T308 by PDK1 [13]. How growth factor- and insulin-generated PtdIn(3,4,5)P_3 activates mTORC2 is unknown.

The production of phosphoinositides by PI3K is reversed by phosphatase and tensin homologue (PTEN) which possesses 3'-phosphoinositide phosphatase activity [1]. PTEN is the second most frequently mutated tumour suppressor gene, again underscoring the medical relevance of the PKB pathway.

Tools for manipulating the activity and expression of PKB: the platform for therapeutic development

Strategies that eliminate or inhibit all three isoforms, PKBα, PKBβ and PKBγ, either individually or in combination are essential for a clear understanding of the roles of PKB isoforms in signal transduction and for developing effective therapies. In this regard, various approaches ranging from the use of molecular biological methods to pharmaceutical inhibitors have been employed.

Overexpression of constitutively active or dominant negative forms of PKB

Such mutants were the first available tools to study the role of PKB and are easily applied to a range of cell

types. The most effective dominant negative mutant has both phosphorylation sites and the ATP binding lysine changed to alanine (PKB-AAA) [19]. Constitutively active mutants have either both phosphorylation sites changed to aspartate or the gag membrane targeting v-Akt sequence added [19]. Much of the original fundamental information regarding the roles of PKB was deduced from using overexpressed constitutively active or dominant negative mutants. However, concerns have been raised about the specificity of these mutants [see e.g. ref. 20] and this helped fuel the drive for more unequivocal tools. Dominant negative mutants inhibit all PKB isoforms, not just the one from which they are constructed, and are thus not isoform specific.

Mouse knock-out models

All three PKB isoforms have been targeted for deletion in mice via homologous recombination. Ablation of PKBα affects growth, neonatal mortality and adipogenesis but does not alter glucose metabolism [21–24]. Removal of PKBβ causes insulin resistance and severe diabetes, suggesting that PKBβ has a key role in glucose metabolism [24, 25]. Knock-out of PKBγ reduces brain size but has no effects on growth or glucose metabolism [26, 27]. Removal of both PKBβ and PKBγ does not increase the intensity of diabetes compared with removal of PKBβ alone [27]. On a cautionary note, phenotypes of knock-out mice could be influenced by unrelated changes in gene expression and developmental regulation and thus effects are not necessarily direct.

siRNA knock-down

siRNA knock-down tools that target each PKB isoform have been developed and have proved useful in a range of studies [28–30]. A problem that can occur is failure of the siRNA probe to completely remove the targeted isoform, e.g. 30% remaining. Thus if no effect is seen upon depletion there is the dilemma of not knowing whether the isoform is unimportant for the response or that the residual isoform is sufficient to transduce the response. Recent studies have also demonstrated that siRNA may cross-react with targets of limited sequence similarity, leading to some further concerns regarding this strategy [31].

Antisense knock-down

Antisense oligonucleotide probes (typically 18mers) of general applicability have been developed that enable the specific and potent knock-down of endogenous PKBα, β or γ isoforms, individually or in various combinations, including concurrent removal of all three isoforms [32]. Knock-down of the targeted isoform is routinely >95%, making these extremely powerful tools. Assessment by a range of criteria has shown that they act specifically.

Small-molecule inhibitors of PKB

In recent years, a number of pharmacological inhibitors against PKB have been described. Inhibitors targeting the ATP-binding site include H-89 and analogues, NL-71–01 and indazole-pyridine-based compounds [33–35]. These compounds show moderate to high selectively for PKB over PKA, which has 50% identity in the kinase domain and 70% identity in the 25-residue ATP-binding site [5]. Given that the ATP-binding site shows 96–100% identity amongst PKB isoforms it will be difficult to develop isoform-specific inhibitors that act at the ATP binding site [5]. Allosteric inhibitors that are PKBα selective, PKBβ selective and dual PKBα/PKBβ isoform selective have recently been described [36, 37]. These inhibitors did not inhibit a range of other kinases and are believed to interact with multiple domains on PKB. They are PH domain dependent and are also postulated to bind to the linker region (link) that connects the PH domain to the kinase domain, consistent with the sequence diversity of this region. These compounds have poor pharmokinetic properties and solubility that have prevented their evaluation in animal models [5]. Chemically modified peptide substrate mimetic inhibitors have recently been described [38]. By targeting the protein substrate binding site these avoid the potential specificity problems of targeting the kinase active site but they are not PKB isoform specific. Phosphatidylinositol ether lipid analogue (PIA) inhibitors that target the PH domain have also been described [39, 40]. These inhibitors did not affect upstream kinases such as PI3K and PDK1 or those in other signalling pathways such as ERK2. These compounds show poor solubility, only moderate potency, aggregation and weak pharmokinetics which may limit their usefulness. As PH domains are present in many proteins there is also concern about the specificity of PIAs. Although there are issues of specificity and selectivity, the inhibitors so far developed provide a foundation for developing future pharmacological inhibitors of PKB that are useful clinically and as research tools.

A number of other compounds have been reported to inhibit the activation of PKB, like curcumin [41], deguelin [42], indo-3-carbinol [43] and CMEP [44]. Whether these compounds directly inhibit PKB and their underlying molecular mechanisms of action remain unknown. Perifosine interferes with recruitment of PKB to the plasma membrane and inhibits PKB phosphorylation and activation but does not directly affect PKB activity [45]. The action of perifosine is not specific to the PKB pathway since

this drug also causes inhibition of p42 and p44 mitogen-activated protein (MAP) kinases and the activation of c-*Jun* NH_2-terminal kinase and $p21^{cip1}$. Lastly, because mTORC1 and ribosomal S6 kinase (S6K) negatively regulate insulin receptor substrate (IRS) 1/2 function, it may be advantageous to combine PKB inhibition with mTOR inhibitors under certain circumstances to increase blockage of the pathway [46].

Role of PKB in insulin signalling

PKB is highly activated within the first minute of exposure of responsive cells to insulin and appears to be common to signalling pathways that mediate the metabolic effects of insulin in several physiologically important target tissues.

Glucose transport

It is now widely believed that PKB plays a key role in signalling the translocation of glucose transporter GLUT4 to the plasma membrane and the uptake of glucose. This role for PKB was initially suggested by observations that overexpression of constitutively active mutants of PKB mimicked the action of insulin in increasing glucose transport and GLUT4 translocation whereas overexpression of dominant negative mutants was inhibitory [47, 48]. Consistent with this, PKBβ binds to GLUT4-containing vesicles in response to insulin [47, 48]. Targeted disruption of PKBβ, but not PKBα or PKBγ, in mice caused insulin resistance and profound effects on glucose metabolism [21–27]. At physiological insulin concentrations, reduced glucose transport in response to insulin was observed in cells isolated from extensor digitorum longus, but not soleus, muscle of the $PKB\beta^{-/-}$ mice when compared with controls [25]. Adipocytes derived from PKBβ-deficient mice showed significant impairment in glucose uptake and GLUT4 translocation whereas PKBα was dispensable for this effect [49]. siRNA knock-down experiments in 3T3-L1 adipocytes show that PKBβ, and PKBα to a lesser extent, has an essential role in insulin-stimulated GLUT4 translocation and glucose uptake [28, 29]. Moreover, pharmacological inhibition of PKB with the dual allosteric PKBα/PKBβ isoform inhibitor impairs insulin-induced GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes [50]. Taken together, all these data point towards a key role of PKBβ in the insulin signalling of glucose transport. There is also a wealth of evidence showing that the atypical PKCs, ζ/λ, play crucial roles in signalling glucose uptake [51, 52]. This may account for the partial effects of PKB blockage on glucose uptake, indicating that both kinase systems are required to achieve full insulin stimulation of glucose transport. It is not known how PKB interfaces with and mobilizes GLUT4-containing vesicles. Phospho-PKB substrate antibodies have been used to identify potential PKB substrates. One candidate substrate identified is AS160 (Akt substrate of 160 kDa) [53]. AS160 contains six PKB consensus phosphorylation sites, five of which are phosphorylated in response to insulin, in addition to a rab GTPase activating protein (GAP) domain [54]. Rab proteins are known to regulate a number of steps in membrane transport and vesicle movement. Use of a dominant negative form of AS160 as well as siRNA knock-down of endogenous AS160 in 3T3-L1 adipocytes indicate that AS160 is necessary, but not sufficient, for maintaining the intracellular location of GLUT4 under basal conditions [55, 56]. AS160 is proposed to function by maintaining a rab protein in the GDP-bound, inactive state. This rab protein has recently been proposed to be rab10 [57]. PKB phosphorylation of AS160 turns off the GAP activity, allowing the rab protein to switch to the GTP-bound, active state. However, in AS160 knock-down 3T3-L1 adipocytes, the dual allosteric PKBα/PKBβ isoform inhibitor still inhibited insulin-stimulated exocytosis of GLUT4 by approximately twofold, indicating that AS160 is not the sole PKB substrate required for PKB-dependent effects on GLUT4 translocation [50]. Moreover studies dynamically tracking GLUT4 storage vesicles (GSVs) indicate that the preparation of GSVs for fusion competence after docking at the surface is a key step regulated by insulin, whereas the docking step is regulated by AS160 [58]. This suggests that PKB-dependent phosphorylation of AS160 is not the major regulated step in GLUT4 trafficking, implicating alternative PKB substrates or alternative signalling pathways downstream of GSV docking at the cell surface as the major regulatory node.

Using antibodies directed against the phospho-Akt/PKB substrate motif, RXRXXS/T(P), the phosphoinositide 5-kinase PIKfyve (phosphoinositide kinase for five position containing a Fyve finger) was identified as a further potential PKB substrate [59]. Overexpression of a mutant PIKfyve, in which the insulin-regulated phosphorylation site S318 was changed to alanine, enhanced insulin-stimulated translocation of GLUT4 vesicles. Further work is required to determine the precise steps that might require PIKfyve activity. Insulin has also been reported to stimulate the phosphorylation of synip on S99, a site that appears to be a specific substrate for PKBβ but not PKBα or PKBγ [60]. A working model is that synip binds to syntaxin4 in the basal state and blocks the ability of the GLUT4 vesicle to dock, by

preventing the interaction of (vesicle-associated membrane protein 2(VAMP2) in the GLUT4 vesicle with syntaxin in the plasma membrane [61]. Insulin has been observed to induce the dissociation of synip from syntaxin4 thus enabling docking. Mutation of S99 has resulted in controversial results, with one group reporting that the overexpressed mutant inhibits GLUT4 translocation (mutated to phenylalanine) while another group found no effect (mutated to alanine) [60, 62]. Munc18c also binds to syntaxin4, and as this association is stronger than that between VAMP2 and syntaxin4 [63], it has been proposed that munc18c acts as a clamp preventing the GLUT4 vesicle binding to the plasma membrane in the basal state. There is controversy over whether or not this actually involves dissociation of munc18c from syntaxin4 [64]. Insulin stimulation reduces the binding of munc18c to syntaxin4 thereby enabling the GLUT4 vesicle to dock with the plasma membrane. Recent experiments indicate that PKCζ acts to signal GLUT4 translocation through a protein called 80K-H [65, 66]. 80K-H is closely related to vacuolar system-associated protein-60 (VASAP-60) a protein known to regulate vesicle trafficking [67]. Overexpression of constitutively active 80K-H constructs mimicked the action of insulin in stimulating both glucose uptake and translocation of GLUT4 [65]. As 80K-H was also shown to associate with munc18c, this raises the prospect that PKCζ delivers a signal through 80K-H, and that 80K-H may function by releasing the clamp action of munc18c on syntaxin-4 thereby allowing VAMP2 to bind [65, 66]. Clearly, it is of importance to determine whether PKB regulates 80K-H as well. Myosin 5a has recently been shown to be phosphorylated in an insulin-stimulated manner by PKBβ and, upon insulin stimulation, phosphorylated myosin 5a has been proposed to facilitate anterograde movement of GLUT4 vesicles along actin to the cell surface [68]. It is therefore likely that a complex spectrum of signalling events is required to induce GLUT4 vesicle translocation.

Glycogen synthesis

Another key action of insulin is the stimulation of glycogen synthesis. Insulin activates glycogen synthase mainly by inducing the dephosphorylation of serines phosphorylated by glycogen synthase kinase 3 (GSK-3). Insulin causes the phosphorylation and inactivation of both isoforms of GSK-3 (α and β) and both are phosphorylated by PKB *in vitro* [1]. Many studies now demonstrate that GSK-3 plays a more diverse role in the cell other than just phosphorylating glycogen synthase and that it acts as a critical downstream regulatory switch for a divergent array of responses from multiple stimuli [69]. These pathways, when dysregulated, have been implicated in diseases such as diabetes, cancer, Alzheimer's and bipolar disorder [70–72] thereby underscoring the importance of a clear understanding of the mechanism by which GSK-3 is regulated. GSK3 was originally implicated as a substrate for PKB *in vivo*, based on overexpression experiments with constitutively active and dominant negative mutants of PKB [28, 29, 32]. siRNA and antisense knock-down experiments have now established that both GSK-3α and GSK-3β are genuine physiological targets of endogenous PKB [28, 29, 32]. Each PKB isoform can contribute to GSK-3α and GSK-3β phosphorylation, with PKBβ having the predominant role [28, 29, 32]. However, knock-down of total PKB incompletely blocked insulin-stimulated phosphorylation of GSK-3α and GSK-3β, and a pathway involving atypical PKCs, ζ/λ, also contributes to the signal [32]. In support of a dual mechanism for GSK-3 regulation, PKB and PKCζ have been shown to come together to form a complex containing PDK2 [3]. Formation of such an active signalling complex may be crucial in ensuring that GSK-3 becomes fully phosphorylated [3].

Translation

Insulin, as well as growth factors, promote protein synthesis by stimulating the initiation and elongation steps in protein translation. This is achieved by regulating the function of multiple translation components. These actions appears to involve PKB since constitutively active PKB increases protein synthesis and dominant negative PKB blocks insulin-stimulated protein synthesis [73]. PKB functions by activating mTOR in the raptor.mTOR complex (also called mTOR complex 1 or mTORC1) which consists of mTOR, raptor and mLST8. Activation of mTORC1 by PKB occurs through inactivation of the tuberous sclerosis complex (TSC). Unphosphorylated TSC2 is bound to TSC1 in a complex that prevents mTOR activation. PKB-mediated phosphorylation of TSC2 at Ser939 and Ser981 disrupts this complex, which relieves the Rheb GAP activity of TSC2 [74]. Conversely, AMPK-mediated phosphorylation of Ser1345 and subsequent GSK3-dependent phosphorylation of Ser1337 and Ser1341 positively regulate the ability of TSC2 to function as a GAP towards Ras enriched in brain (Rheb) [75]. All these events allow Rheb to bind ATP. In the presence of ATP, Rheb switches from the inactive GDP state to the active GTP form and subsequently activates mTOR [76].

The mTOR-raptor binding partner proline-rich Akt/PKB substrate 40 kDa (PRAS40) has also been proposed to mediate PKB signals to mTOR in response to insulin. Unphosphorylated PRAS40 interacts with raptor and inhibits mTOR kinase activity.

Insulin stimulates PKB-mediated phosphorylation of PRAS40 on Thr 246, which prevents its inhibition of mTORC1 [77]. Activated mTOR phosphorylates and activates S6 ribosomal protein kinase 1 (S6K1) and the 4E-BP1 initiation factor binding protein that inhibit eukaryotic polypeptide chain initiation factor 4E (eIF4E). S6K1 phosphorylates eIF4B. The enhanced phosphorylation of the translational targets increases both cap-dependent and cap-independent translation, with a subsequent collective increase in the translation of existing mRNA transcripts [78]. In addition, insulin induces the dephosphorylation of the guanine nucleotide exchange factor, eIF2B, at the site phosphorylated by GSK-3 [79]. eIF2B controls the initiation phase of protein synthesis. mTOR can thus be both downstream (mTORC1) and upstream (mTORC2) of PKB depending on whether its associated interacting partner is raptor or rictor (Fig. 2).

Other pathways

PKB has also been implicated in the control of lipolysis and lipogenesis. Insulin has a potent antilipolytic effect by decreasing the cyclic AMP concentration and inhibiting hormone-sensitive lipase. This regulation by insulin is dependent on phosphorylation and activation of phosphodiesterase 3b (PDE-3B), which results in a decrease in levels of cAMP and in PKA-induced phosphorylation and activation of hormone-sensitive lipase. PDE-3B is phosphorylated by PKB *in vitro* and the insulin-induced phosphorylation of PDE-3B in 3T3-L1 adipocytes was inhibited by a dominant negative PKB. However, the broad specificity kinase inhibitor ML9, which inhibits PKB and many other protein kinases, failed to inhibit either the antilipolytic affect of insulin or PDE-3B activation by insulin [80]. PKBβ is also able to inhibit fatty acid oxidation, as well as gluconeogenesis, through phosphorylating S570 on the transcriptional coactivator peroxisome proliferator-activated receptor-coactivator 1α (PGC-1α) [81]. PGC-1α and forkhead box O1 (FOXO1) work synergistically to curb the transcription of glucose 6 phosphatase and phosphoenol pyruvate carboxykinase. PGC-1α works alone to suppress an entirely separate set of genes related to lipid catabolism such as medium-chain acetyl-CoA dehydrogenase. Insulin also promotes the conversion of glucose into fatty acids. Endogenous ATP citrate lyase is one enzyme in the pathway shown to be a substrate for PKB [32]. Additionally, GSK-3 phosphorylates sterol regulatory element-binding proteins (SREBPs), thus targeting them for proteasomal degradation. SREBPs are transcription factors that switch on genes involved in fatty acid and cholesterol biosynthesis [1]. PKBα, but not PKBβ, has been reported to be required for adipocyte differentiation. This action may involve the induction of peroxisome-proliferator-activated receptor γ (PPARγ) transcription through regulation of transcription factors such as FOXO1 and perhaps Krüppel-like transcription factor 15 [27]. Antisense knock-down studies of PKB and the classical p42 and p44 MAP kinases have shown that insulin-triggered DNA synthesis is mediated by the MAP kinase pathway and not the PKB pathway [32, 82].

PKP and apoptosis

PKB was originally implicated as a controller of apoptosis based on observations that overexpression of constitutively active forms of PKB protected a range of cell types from induction of apoptosis to a variety of triggers and that dominant negative forms of PKB induced apoptosis. Such studies led to the idea that PKB acts as a prosurvival kinase. This view was validated by the proposal that PKB phosphorylates an array of downstream proteins that are known to regulate apoptosis. Given the number of substrates identified the mechanisms by which PKB protects cells from apoptosis are likely to be complex. The substrates identified include Bad, GSK-3, pro-caspase9, IκB kinase (IKK), forkhead transcription factors and Yes-associated protein (YAP) (Fig. 1).

Bad is capable of forming a non-functional heterodimer with the anti-apoptotic protein Bcl-xL and antagonizes its anti-apoptotic activity. Bad can be phosphorylated at S136 by PKB. Phosphorylation of Bad by PKB prevents this interaction, restoring the anti-apoptotic function of Bcl-xL [1]. PKB has been proposed to directly block the capsase cascade by phosphorylating and inhibiting pro-caspase9 on S196. An S196A mutation on pro-caspase9 rendered pro-caspase9 resistant to phosphorylation and PKB was consequently unable to rescue cells from apoptosis [83]. However, the PKB phosphorylation site found in human pro-caspase9 is absent from lower species such as mouse and rat. Therefore, it is probable that the inhibition of pro-caspase9 by PKB is not conserved across various species and may be restricted to primates. PKB also appears to regulate the SAPK survival pathway by phosphorylating MAP kinase kinase kinases, including apoptosis signal-regulating kinase 1 (ASK1) [84–86].

PKB also regulates apoptosis through an array of transcription factors. PKB inactivates by phosphorylation the forkhead transcription factors FOXO1 (FKHR), FOXO2, FOXO3a (FKHRL1) and FOXO4 (AFX) [87–90]. Three phosphorylation sites are highly conserved among the FOXO isoforms and across species (T21, S256 and S318 in human

FOXO1). The phosphorylation results in their exclusion from the nucleus and decreased transcriptional activity that is required to promote apoptosis. The targets of the forkhead transcription factors are the forkhead response elements found in the regulatory region of genes that control the expression of proteins crucial for inducing apoptosis. These are thought to include the Fas ligand [tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and tumour necrosis factor receptor-associated death domain (TRADD)], Bim and Bcl-6 [91]. PKB may activate by phosphorylation the transcription factor cyclic AMP response element-binding protein (CREB) [92] and the IκB kinase (IKK, a positive regulator of NF-κB) which together regulate the expression of genes with anti-apoptotic activity such as Bcl-2, Mcl-1, Bfl-1, Bcl-xL, survivin and inhibitor of apoptosis proteins (IAPs) [93]. YAP triggers activation of pro-apoptotic genes like p53. YAP appears to be phosphorylated on S127 by PKB, which localises YAP to the cytosol, thereby preventing its action [94].

Despite its anti-apoptotic role, PKB activation renders cancer cells absolutely dependent on the availability of glucose for their survival [95]. High glucose utilisation results in phosphorylation and inactivation of GSK3 and the anti-apoptotic GSK3 substrate myeloid cell leukaemia-1 (Mcl-1), a Bcl-2 family member, being dephosphorylated and not targeted for degradation [96]. Activation of PKC isoforms through glucose-induced alterations in diacylglycerol levels or distribution and the subsequent phosphorylation of GSK3α/β by PKC [3] may also be important in this effect [96]. The anti-apoptotic kinase, casein kinase 2 (CK2), is also invariably activated in tumour cells compared with normal cells. CK2 has been reported to induce hyperactivation of PKB through phosphorylation of Ser129 in the link region [97].

Scaffolding interactions may also contribute to the regulation of apoptosis by PKB [98]. For example, association with Jun N-terminal kinase (JNK)-interacting protein 1 (JIP1) may inhibit the signalling of apoptosis by JNK in primary neurons. Additionally, the scaffold protein 'plenty of SH3 domains' (POSH) has recently been identified as a substrate for PKB. This phosphorylation decreases binding of POSH to Rac, again leading to lowered JNK activity [99]. Both carboxyl-terminal regulator protein (CTMP) and APE bind to the C terminus of PKB and have been reported to enhance PKB phosphorylation and activation [100, 101]. However, the role of CTMP is controversial because an earlier study suggested that CTMP is a negative regulator of PKB [102].

In summary much is now known about how PKB controls apoptosis. However, there is still a dearth of information about the contribution of the various PKB isoforms to regulating apoptosis and to the phosphorylation of the substrates described above.

Cell cycle regulation

The regulation of the cell cycle by PKB is well established. PKB promotes cell cycle progression at the G1/S transition in the following ways. PKB directly phosphorylates the cell cycle inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ which causes them to adopt a cytosolic location through binding of 14–3-3 proteins, in an analogous way to the FOXO transcription factors [103–107]. In addition, phosphorylation of the FOXO transcription factors by PKB results in the lowering of the level of $p27^{Kip1}$. The PKB phosphorylation site in $p27^{Kip1}$ (T157) is not conserved in species like mouse, suggesting that the transcriptional regulation is more important in such species. In addition, phosphorylation by PKB may stabilize mouse double minute 2 (MDM2) and cause its translocation to the nucleus, where it promotes degradation of p53, leading to a reduction in transcription of $p21^{Cip1}$ mRNA. However, the identity of the phosphorylation sites and the effect of the phosphorylation on the subcellular localization of MDM2 are still contended [108–111]. Activation of mTOR in mTORC1, as described above, stimulates the translation of proteins required for the cell cycle progression from the G1 to the S phase. PKB also regulates the G2/M transition. This may involve phosphorylation of Myt1 and FOXO3a [112, 113]. The latter is believed to enable activated PKB to bypass the DNA repair checkpoint in cells.

PKB and cancer

PKB is crucial for the survival of cancer cells and is elevated and/or activated in many major cancers including breast, ovarian, lung, pancreatic, prostate, stomach and melanomas [114–117]. PKB has also been implicated as a key player in the regulation of tumorigenesis, cell invasiveness and, in some cancers, resistance to hormone therapy [114–117]. The incidence rate for increased PKB levels or activation in cancers can exceed 50%. For example, in >60% of prostate tumour samples, PKB expression is elevated [118] and in ~50% of prostate cancers, immunohistochemical studies show that the level of phosphorylated (active) PKB is increased [118–120]. Similarly, immunohistochemical studies showed that the levels of phosphorylated PKB in breast tumour samples correlate with prognosis [121]. All isoforms of PKB have been found to be overexpressed or amplified in cancers, with the incidence for a particular isoform

dependent on the type of cancer. For example, increases in the level of PKBα have been reported in gastric cancers, PKBβ in ovarian, pancreatic and breast cancers (rare) and PKBγ in hormone-resistant prostate and breast cancers [122]. Activation of PKB is primarily the result of aberrant upstream signalling molecules of PKB, in particular receptor tyrosine kinases and PTEN [122]. Mutations of PKB have not been identified in human tumours. The levels of phosphorylated PKB and PKB expression have been shown to correlate with prognosis in a range of cancers [121–124]. Much evidence supports the crucial role of aberrantly expressed PKB or activated PKB in signalling cancer. For example, introducing PTEN into cancer cells that have lost PTEN results in the inhibition of PKB activation leading to apoptosis or cell cycle arrest [125–127]. However, as PTEN negatively regulates other pathways, this does not prove the involvement of PKB. Direct evidence for the role of PKB is provided by PKB knock-down experiments using antisense or siRNA treatments, which induce apoptosis and cell growth arrest and inhibit tumour growth and invasiveness but only in tumour cells overexpressing PKB [128–132]. Additionally, expression of dominant negative PKB induced apoptosis selectively in tumour cells containing a high level of active PKB but not in normal cells or cells containing only a low level of active PKB [133]. Lastly, a number of small-molecule inhibitors of PKB have been shown to induce apoptosis in a range of cancer cell types [33–40]. In summary, these data show that overexpressed or activated PKB isoforms enable cancer cells to evade apoptosis which can be reversed by blockade at PKB thereby establishing PKB as a therapeutic target for the treatment of cancer.

Angiogenesis

PKB plays key roles in angiogenesis. In endothelial cells, vascular endothelial growth factor (VEGF) triggers PKB to activate endothelial nitric oxide synthase (eNOS) through phosphorylation of S1177 [1]. The released nitric oxide stimulates vasodilation, vascular remodelling and angiogenesis. These mechanisms may be important in pathological angiogenesis in cancer cells where PKB is aberrantly activated.

Dephosphorylation of PKB

Direct dephosphorylation of PKB is mediated by PP2A-type phosphatases and the PH domain leucine-rich repeat protein phosphatases, PHLPP1 and PHLPP2, which directly dephosphorylate the hydrophobic motif serine phosphorylation site in PKB [134]. Knock-down studies reveal that PHLPP1 and PHLPP2 differentially terminate PKB signalling by regulating distinct PKB isoforms [135]. This provides a mechanism to selectively terminate PKB signalling pathways through the differential inactivation of specific PKB isoforms by specific PHLPP isoforms and the control of different downstream substrates of PKB. For example, PHLPP1 has been proposed to dephosphorylate PKBβ and PKBγ which regulates the phosphorylation status of p53 ubiquitin ligase HDM2 and GSK3α, whereas PHLPP2 acts on PKBα and PKBγ and controls $p27^{Kip1}$ through its action on PKBγ. It will be important to determine whether these specificities for both PHLPP and PKB isoforms hold true in all cell types with all stimuli, as most of the results were obtained in one cell line.

Concluding remarks

The availability of several tools that target specific PKB isoforms should now facilitate understanding the functions of the individual PKB isoforms more fully. In particular, since well over 50 putative substrates have been reported, this should enable the isoform specificity to be determined and how that specificity is controlled. There is also much to be learnt about the function of PKBγ in the brain and its substrates involved in post-natal brain development. Very recent studies have suggested a new level for the control of PKB substrate selection [16–18]. Thus, genetic ablation studies of the mTORC2 components mSin1, mLST8 or rictor indicate that mTORC2 controls the substrate specificity of PKB. In the absence of any one of these components, mTORC2 function is abrogated with S473 phosphorylation abolished and T308 phosphorylation maintained. The defective S473 phosphorylation affected only a subset of PKB targets in vivo, including FOXO1/3a, while other PKB targets, TSC2 and GSK3, and the mTORC1 effectors, S6K1 and 4E-BP1, were unaffected. These results suggest that PKB-S473 phosphorylation is required for mTORC2 function in cell survival but is dispensable for mTORC1 function. Moreover, as different mSin1 isoforms are present in functionally distinct mTORC2 s [15], the results raise the possibility that mSin1 may be involved in controlling the specificity of the different PKB isoforms. To validate the role of S473 phosphorylation as a molecular switch that regulates PKB substrate specificity, it will be important to show in the cells containing non-functional mTORC2 that PKB-T308 rather than a substitute kinase is catalysing the phosphorylation of GSK3 and

TCS2. In this regard, both GSK3 and TSC2 are known to be phosphorylated by other kinases [32]. Additionally, it will be important to show that the effects are not merely due to different levels of active PKB in the presence and absence of S473 phosphorylation and the differing targets requiring different levels of PKB activity for phosphorylation.

A better understanding of the PKB isoforms elevated in various types of cancer is also crucial for delivering targeted and specific therapies aimed at PKB. This is important since inhibition of all PKB isoforms in vivo has been reported to cause abnormalities in glucose metabolism, possibly arising through inhibition of PKBβ [35]. Thus specific blockade of PKBα or PKBγ would be beneficial where those isoforms are aberrant. Cancers are heterogeneous diseases so that the development of suitable biomarkers is required to categorise cancers and tailor the required therapies. Moreover, differential roles of PKB isoforms in signalling tumorigenesis, cell migration, cell proliferation and invasiveness of cancer cells have been reported [27]. Importantly, such differential roles could be exploited therapeutically, according to cancer phenotype. Small-molecule inhibitors so far developed provide a platform for developing future inhibitors that may be isoform specific and useful both clinically and as research tools. siRNAs targeted against specific PKB isoforms are also potentially useful therapeutically. However, many hurdles need to be surmounted before siRNAs become generally useful in a clinical context, including addressing the issues of delivery, pharmokinetics and off-target effects [136]. Notwithstanding, a few siRNAs against targets other than PKB are in clinical trials but only for diseases where specific local delivery can be achieved [137]. Antisense agents against PKB also hold great promise as anticancer agents. Unlike siRNA, antisense technology is a proven therapy. Additionally, antisense molecules generally enter cells more easily than naked siRNA. Approximately 100 antisense oligonucleotides against various targets are currently undergoing clinical trials for a number of different diseases with one already on the market, thereby giving proof-of-principle to the technology [138, 139]. Although clinical trials of some antisense agents have given mixed results, this seems likely due to the nature of the target selected or the use of first-generation phosphorothioate oligonucleotides. Much more potent second- and third-generation oligonucleotides that have new backbone structures are now available [138, 139]. These are being used in clinical trials and show much promise. For example, the antisense market has recently received a boost with the cholesterol-lowering antisense drug, ISIS-301012, successfully completing phase II human trials as a drug with low toxicity. Antisense agents that specifically target each PKB isoform are available [32], with one against PKBα entering phase II clinical trials.

In conclusion specific blockade of each PKB isoform will be crucial for the treatment of diseases that exhibit abnormal PKB signalling. With the availability already of specific antisense molecules, the prospect of improved small-molecule inhibitors and the resolution of issues concerning the therapeutic use of siRNAs, PKB isoform-targeted treatments are likely to become a reality.

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