PKB/Akt: a key mediator of cell proliferation, survival and insulin responses?

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Summary

The serine/threonine protein kinase PKB (also known as Akt) is thought to be a key mediator of signal transduction processes. The identification of PKB substrates and the role PKB phosphorylation plays in regulating these molecules have been a major focus of research in recent years. A recently developed motif-profile scoring algorithm that can be used to scan the genome for potential PKB substrates is therefore a useful tool, although additional considerations, such as the evolutionary conservation of the phosphorylation site, must also be taken into account. Recent evidence indicates that PKB plays a key role in cancer progression by stimulating cell proliferation and inhibiting apoptosis and is also probably a key mediator of insulin signalling. These findings indicate that PKB is likely to be a hot drug target for the treatment of cancer, diabetes and stroke. There are, however, a number of pitfalls of methodologies currently employed to study PKB function, and therefore caution should be used in interpretation of such experiments.

Key words: Phosphoinositide 3-kinase, PDK1, AGC kinases, Docking sites, Phospho-specific antibodies, Insulin

Introduction

Growth factors, cytokines and insulin as well as the attachment of cells to the extracellular matrix stimulate the recruitment of a family of lipid kinases known as class 1 phosphoinositide 3-kinases (PI 3-kinases) to the plasma membrane. There, they phosphorylate the glycerophospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) at the D-3 position of the inositol ring, converting it to PtdIns(3,4,5)P_3 (reviewed by Cantrell, 2001). In many cancers, overexpression of growth factor receptors, as well as mutations that lead to the loss of the major PtdIns(3,4,5)P_3 3-phosphatase (PTEN), results in abnormally elevated levels of PtdIns(3,4,5)P_3 (Maehama et al., 2001). Studies using pharmacological inhibitors of PI 3-kinase and overexpression of dominant negative and constitutively active mutants of PI 3-kinase have provided compelling evidence that PtdIns(3,4,5)P_3 and perhaps its immediate breakdown product PtdIns(3,4,)P_2 play key roles in regulating cell cycle progression, apoptosis and cellular responses to insulin. Much recent work has therefore focused on dissecting the molecular mechanism by which PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 can trigger these processes. Recent evidence indicates that the serine/threonine protein kinase protein kinase B (PKB; also known as Akt) mediates many of the downstream events controlled by PI 3-kinase. The three widely expressed isoforms of PKB (PKBα, PKBβ and PKBγ; also known as Akt1, Akt2 and Akt3, respectively) are each composed of an N-terminal PtdIns(3,4,5)P_3- and PtdIns(3,4)P_2-binding PH domain and a C-terminal kinase catalytic domain. They belong to a subfamily of protein kinases that have been termed the AGC protein kinases and include protein kinase C (PKC) isoforms and the cyclic-AMP-dependent protein kinase (PKA). Following the activation of PI 3-kinase, PKB isoforms are recruited from the cytosol to the plasma membrane through their interaction with PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 where they are thought to undergo a conformational change and become activated by phosphorylation of two residues. One resides in the T-loop (also known as the activation loop) of the kinase domain of PKB, and the other is located ~160 residues C-terminal to this site in a non-catalytic region of PKB termed the hydrophobic motif. PKB isoforms are phosphorylated at the T-loop (Thr308 in PKBα) by 3-phosphoinositide dependent protein kinase 1 (PDK1) and this appears to be the major input required for the activation of PKB (reviewed by Alessi, 2001). Phosphorylation of PKB at its hydrophobic motif also contributes to the activation of PKB but has a lesser influence on this activation. Phosphorylation of the hydrophobic motif of PKB might conceivably also play roles in promoting its interaction with substrates and/or in stabilising PKB, given that the role of the equivalent site in conventional PKC isoforms is to stabilise these kinases. Whether PKB is phosphorylated at its hydrophobic motif by autophosphorylation or by a distinct upstream protein kinase remains controversial and no clear picture has emerged from recent studies aimed at addressing this question (Toker and Newton, 2000).

Identification of physiological substrates for PKB

Activation of PKB occurs rapidly and is usually maximal within a few minutes of a cell being stimulated with an agonist. After activation, PKB dissociates from the membrane and enters the nucleus. It is likely to phosphorylate numerous proteins in both the cytoplasm and the nucleus. One of the major challenges is to identify the substrates of PKB and characterise the role that phosphorylation plays in regulating the function of these proteins. It was originally established that the minimum motif in a peptide enabling PKB phosphorylation is Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa are preferably small residues other

Table 1. Phosphorylation sites in PKB substrates*

		Sequence	(Algorithm score)
Reported PKB substrate			
High stringency		(0-0.2%)	
FKHR	(Thr21)	LPRPRSCTWPLPR	(0.033%)
6-PF2-K	(Ser466)	PVRMRRNSFTPLS	(0.056%)
GSK3-β	(Ser9)	SGRPRTTSFAESC	(0.067%)
GSK3-α	(Ser21)	SGRARTSSFAEPG	(0.146%)
mTOR	(Ser2448)	RSRTRTDSYSAGQ	(0.146%)
Medium stringency		(0.2-1%)	
BAD	(Ser99)	PFRGRSRSAPPNL	(0.460%)
FKHR	(Ser256)	SPRRRAASMDNNS	(0.717%)
6-PF2-K	(Ser483)	IRRPRNYSVGSRP	(0.717%)
Low stringency		(1-5%)	
Ask1	(Ser83)	ATRGRGSSVGGGS	(1.133%)
IKK-α	(Ser23)	EMRXRLGTGGFGN	(1.404%)
FKHR	(Ser318)	TFRPRTSSNASTI	(1.404%)
Raf-1	(Ser239)	SQRQRSTSTPNVH	(1.744%)
BRCA1	(Thr509)	LKRKRRPTSGLHP	(1.744%)
eNOS	(Ser1176)	TSRIRTQSFSLQE	(2.623%)
TRT-telomerase	(Ser227)	GARRRGGSASRSL	(3.803%)
$p21^{cip1/WAF1}$	(Thr145)	QGRKRRQTSMTDF	(3.808%)
Rac1/cdc42	(Ser71)	YDRLRPLSYPQTD	(4.542%)
No stringency		(>5%)	
Caspase-9	(Ser196)	KLRRRFSSLHFMV	
PDE-3B (human)	(Ser273)	VIRPRRRSSCVSL	
Putative PKB substrates			
High stringency		(0-0.2%)	
Mouse MDM2	(Ser185)	RKRKRSLSFDPSL	(0.0001%)
Protein kinase CLK2	(Thr127)	RRRRRSRTFSRSS	(0.0006%)
Pit1 transcr. fact.	(Ser220)	RKRKRRTTISIAA	(0.033%)
Huntingtin protein	(Ser421)	GGRSRSGSIVELI	(0.046%)
TSC2	(Ser939)	SFRARSTSLNERP	(0.067%)
NK tumour rec. prot.	(Ser1403)	HSRSRSYTYDSYY	(0.111%)
IRS1	(Ser307)	TRRSRTESITATS	(0.111%)
IRS1	(Ser527)	RFRKRTHSAGTSP	(0.186%)
Medium stringency		(0.2-1%)	
TSC2	(Thr1462)	GLRPRGYTISDSA	(0.111%)
PTP-D1 phosphatase	(Thr16)	VIRERRRTLPDQG	(0.229%)
TSC2	(Ser1130)	GARDRVRSMSGGH	(0.290%)
DAP kinase	(Ser726)	RRRPRLSSTNSSR	(0.290%)
TCS2	(Ser981)	AFRCRSISVSEHV	(0.717%)
IRS1	(Ser330)	SFRVRASSDGEGT	(0.717%)
Low stringency		(1-5%)	
IRS1	(Ser1101)	GCRRRHSSETFSS	(1.744%)
IRS1	(Ser270)	EFRPRSKSQSSSN	(2.140%)
Human MDM2	(Ser186)	RQRKRHKSDSISL	(3.808%)
Caspase-8	(Thr263)	SIRDRNGTHLDAG	(4.542%)

*Analysis of Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr motifs that have been reported to be phosphorylated by PKB and those that may be phosphorylated by PKB using the motif profile scoring algorithm (http://cansite.bidmc.harvard.edu/cantley85.phtml) (Yaffe et al., 2001). These motifs are found in >14,000 different sequences in ~9500 vertebrate protein sequences and have been ranked according to how well they fit the theoretical, optimal PKB-phosphorylation site. Their position from the top of this list is defined by the algorithm score. Unless otherwise stated, all sequences are human. The phosphorylated serine or threonine residue is indicated in blue, and the conserved arginine residues located three and five residues N-terminal to this site are in red. The residue on human that is equivalent to Ser136 on mouse BAD that has been shown to be phosphorylated by PKB is Ser99. Abbreviations: Ask1, apoptosis signal-regulating kinase 1; BRCA1, breast cancer susceptibility gene 1; DAP kinase, death-associated protein kinase; eNOS, endothelial nitric oxide synthase; FKHR, forkhead transcription factor; GSK3, glycogen synthase kinase 3; hTERT, human telomerase reverse transcriptase subunit; IKKα, IkB kinase-α; IRS1, insulin receptor substrate-1; MDM2, ubiquitin-protein ligase E3 that binds p53; mTOR, mammalian target of rapamycin; NK tumour rec. prot., NK-tumour recognition protein; PDE-3B, phosphodiesterase 3B; 6-PF2-K, cardiac isoform of heart 6-phosphofructo-2-kinase; Pit1 transcr. fact, pituitary-specific positive transcription factor; PTP-D1, protein tyrosine phosphatase D1; TSC2, tuberous sclerosis complex-2.

than glycine, and Hyd is a bulky hydrophobic residue (phenylalanine or leucine; Alessi et al., 1996b). More recently, Cantley and colleagues have employed an oriented peptide library approach to determine both optimal and non-optimal amino acids at each of the seven residues located N-terminal and C-terminal to the site of phosphorylation. This work revealed that residues outside the originally proposed PKB phosphorylation motif strongly affect, either positively or negatively, the ability of PKB to phosphorylate peptides (Obata et al., 2000). This has permitted Yaffe and Cantley to use this information to generate a motif-profile scoring algorithm, which can compare all the serine/threonine residues in the vertebrate genome that lie in an Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr motif (of which there are ~14000 in ~9500 proteins) and rank each site according to how well it fits with the theoretical optimal PKB phosphorylation motif (Yaffe et al., 2001). This programme can be accessed freely on the Web (http://cansite.bidmc.harvard.edu/cantley85.phtml). A sequence is suggested to be a high-stringency hit if it falls within the top 0.2% of all the aligned potential PKB-phosphorylation sites. The sequences lying between 0.2% and 1% and between 1% and 5% of all aligned sequences are termed medium-stringency and low-stringency hits, respectively. In Table 1 we have aligned all the phosphorylation sites on substrates that to our knowledge have been proposed to be phosphorylated by PKB and indicated their positions in the Yaffe-Cantley list of optimal substrates. Only five out of the nineteen PKB phosphorylation sites identified to date lie within the high-stringency hit category; nine lie in the low-stringency group, and two do not lie in the top 5% of all aligned putative PKB-phosphorylation sequences (Table 1). Several interesting signalling molecules that have not yet been demonstrated experimentally to be PKB substrates produce high-stringency hits; a few of these are also shown in Table 1. It would certainly be interesting to test whether or not these molecules are phosphorylated by PKB in vivo. Before embarking on such projects one would have to verify whether the sites of phosphorylation are conserved in all mammalian species and how optimal these putative phosphorylation sites are in different species. For example, the protein in the entire database that best fits the theoretically optimal PKB-phosphorylation consensus sequence (Obata et al., 2000) is the mouse MDM2 ubiquitin-protein ligase E3 that binds to p53. This protein has not previously been shown to be a substrate of PKB. However, the human MDM2 protein instead of possessing a phenylalanine residue immediately C-terminal to the putative PKB-phosphorylation site possesses an aspartate residue, which is tolerated very poorly at this position in synthetic PKB-substrate peptides (Obata et al., 2000). Thus human MDM2, in contrast to mouse MDM2, would not be

predicted to be a substrate for PKB. Given that genuine PKB substrates would be expected to be phosphorylated by PKB in all mammalian species, MDM2 might not therefore be a physiological substrate of PKB.

Apart from the residues immediately surrounding the phosphorylation site on a protein, there is increasing evidence that more distant residues can also interact specifically with kinases, and these interactions are frequently referred to as docking interactions/sites. In the case of MAP kinase family members ERK1/ERK2, p38 and JNK, recent work shows that docking interactions play crucial roles in enabling these kinases to phosphorylate specific substrates (Holland and Cooper, 1999; Tanoue et al., 2001). Note that virtually all of the characterised PKB substrates listed in Table 1 physically interact with PKB, but, thus far, the residues that mediate these interactions have not been identified. PKB-docking sites, in addition to residues immediately surrounding the phosphorylated serine/threonine residue, might also play a key role in determining whether substrates are phosphorylated by PKB efficiently. Thus, a protein that has a non-optimal PKB-phosphorylation motif could still be a good physiological substrate if it can interact with PKB through a high-affinity docking site.

The role of PKB in promoting cancer progression

PKB was originally identified as a retroviral oncogene product, v-Akt, that can transform rodent cells (Bellacosa et al., 1991; Staal, 1987). It was subsequently observed that PKB isoforms are overexpressed in ovarian, breast and pancreatic cancers (Cheng et al., 1992; Cheng et al., 1996; Nakatani et al., 1999). Recent work has shown that overexpression of constitutively activated PKB mutants in many cell types promotes cellular transformation by two distinct mechanisms: it appears to promote proliferation under conditions in which cells should normally be growth arrested; and it inhibits apoptosis by virtually all cell-death-inducing agents, thus promoting the survival of tumour cells under conditions in which they should die.

Mechanisms by which PKB can promote proliferation

p21 and the related family members p27/Kip1 (p27) and p57/Kip2 block the cell cycle by reversibly inhibiting several cyclin-CDK (cyclin-dependent kinase) complexes. Interesting new work suggests that by phosphorylating p21, PKB can inhibit cell cycle arrest. The *HER2*/neu receptor is a member of the growth factor glycoprotein family that includes the epidermal growth factor receptor and is overexpressed in 30% of human breast and ovarian cancers. The *HER2*/neu receptor can activate PKB, which then phosphorylates p21 on Thr145 (Zhou et al., 2001). Interestingly, this does not prevent p21 from interacting with and inhibiting CDKs, but instead mislocalises p21 in the cytoplasm, preventing it from entering the nucleus, where it must reside to inhibit cell proliferation (Fig. 1; Zhou et al., 2001). This exclusion from the nucleus occurs because p21, once phosphorylated by PKB, interacts with 14-3-3 proteins that sequester it in the cytoplasm. Thr145 in p21 lies in the low-stringency hit category of PKB-phosphorylation consensus sites (Table 1). This is largely because a serine rather than a large hydrophobic residue precedes the Thr145 phosphorylation site. Since p21 also interacts with PKB (Zhou et al., 2001), it could possess a PKB-docking site and thus be

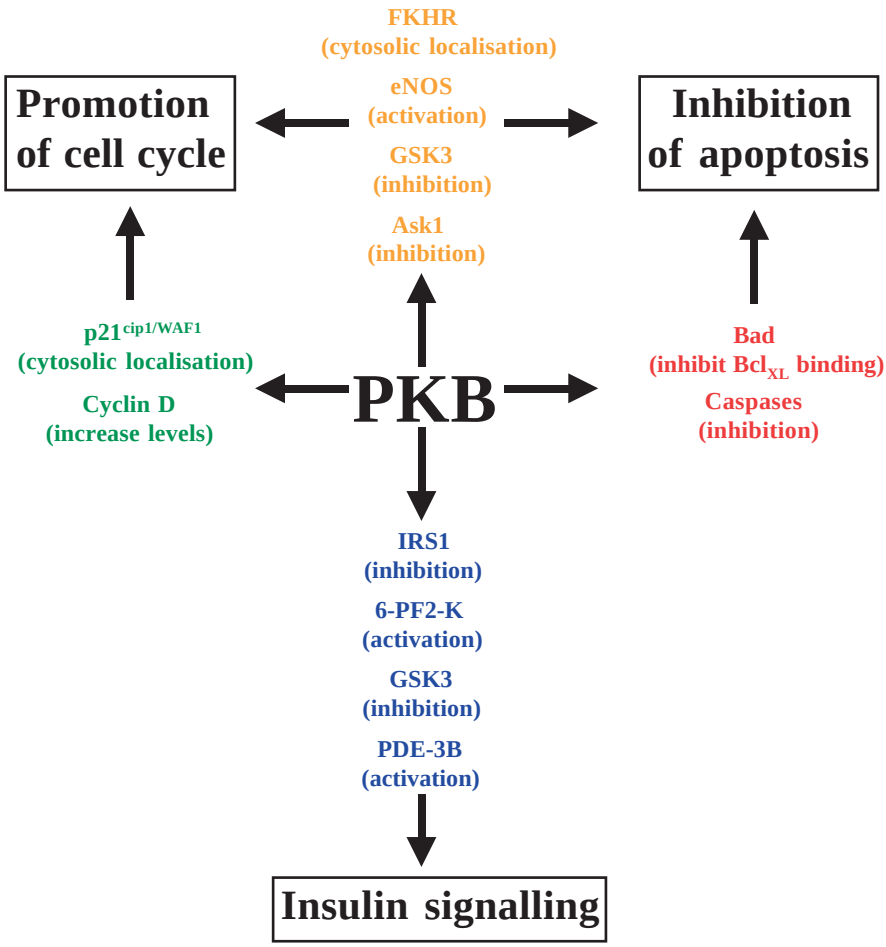

Fig. 1. The mechanisms by which PKB may regulate the cell cycle, apoptosis and insulin signalling. Only the substrates that have been proposed to be directly phosphorylated by PKB and discussed in the main text are shown. PKB may also promote cell survival by phosphorylating I-κB kinase-α, the breast cancer susceptibility gene 1, human telomerase reverse transcriptase, the GTPases Rac1/CDC42 and the serine/threonine protein kinase Raf1, but the roles that these phosphorylations play are controversial and require further investigation. Note that, because of the limitations in the experimental approaches that are being to dissect the PKB pathway, some of the proposed substrates for PKB may ultimately turn out not to be phosphorylated physiologically by PKB but by other protein kinases instead. Abreviations: FKHR, forkhead transcription factor; 6-PF2-K, cardiac isoform of heart 6-phosphofructo-2-kinase; GSK3, glycogen synthase kinase 3; eNOS, endothelial nitric oxide synthase; PDE-3B, phosphodiesterase 3B; Ask1, apoptosis signal-regulating kinase 1.

phosphorylated efficiently by PKB, despite possessing a relatively poor PKB-phosphorylation consensus motif. This will require further investigation.

PKB also regulates transcription of the p27 CDK inhibitor. Activation of PKB or overexpression of constitutively active forms of this enzyme decreases the cellular levels of p27, thereby promoting cell proliferation (Gesbert et al., 2000; Graff et al., 2000; Sun et al., 1999). More recent studies indicate PKB phosphorylates the forkhead transcription factor, which is required for transcription of p27. Again phosphorylation results in its binding to 14-3-3 proteins, which sequester it in the cytoplasm of cells, in which location it cannot induce p27 (Medema et al., 2000; Nakamura et al., 2000).

Other key regulators of cell cycle progression are the cyclin D proteins (D1, D2 and D3), which accumulate during G_1 phase and are required for the activity CDK4 and CDK6. The levels of cyclin D are controlled at the levels of transcription, translation and protein stability. Many growth factors promote transcription of this cell cycle regulator, and multiple signal transduction pathways, including the ERK1/ERK2 pathway (Cheng et al., 1998; Lavoie et al., 1996; Schwartz and Assoian, 2001), are involved. Initially, it was shown that a PI-3-kinase-dependent pathway also plays an important role in this process (Klippel et al., 1998; Takuwa et al., 1999), and subsequent studies have suggested PKB is involved (Crowder and Freeman, 1998; Gille and Downward, 1999). There is also evidence that overexpression of PI 3-kinase and PKB in cells stimulates the rate of translation of cyclin D (Muise-Helmericks et al., 1998). The transcriptional and translational components that PKB phosphorylates to induce cyclin D expression remain to be identified.

Another important mechanism by which cyclin D1 levels are regulated is by translocation of cyclin D1 from the nucleus to the cytoplasm, where it is degraded by proteosomes following its ubiquitination (Diehl et al., 1998). A key step in promoting this pathway is the phosphorylation of cyclin D1 at Thr286 by glycogen synthase kinase 3 (GSK3), which promotes the interaction of cyclin D1 with the nuclear exportin CRM1 (Alt et al., 2000). GSK3 was the first substrate of PKB to be identified, and its phosphorylation by PKB at an N-terminal non-catalytic residue is inhibitory (Cross et al., 1995). In principle, therefore, activation of PKB should result in GSK3 inactivation and decreased phosphorylation of cyclin D1 by GSK3 and therefore maintain high cyclin D1 levels in the nucleus. Indeed, overexpression of a constitutively active PKB promotes stabilisation of cyclin D1 protein (Diehl et al., 1998). However, we believe that this situation could be more complicated. Many of the substrates that GSK3 phosphorylates, such as glycogen synthase and eIF2B, require a priming phosphorylation at residue n+4 (where n is the site of phosphorylation) in order to be phosphorylated by GSK3 efficiently. The molecular basis of this effect has recently been elucidated by the finding that the kinase domain of GSK3 possesses a phosphate-docking site that interacts with the primed phosphorylation site on the substrate (Dajani et al., 2001; Frame et al., 2001). Phosphorylation of GSK3 by PKB results in binding of the phosphorylated N-terminal residues of GSK3 to this docking motif and inhibits phosphorylation of these substrates by GSK3. A group of GSK3 substrates, however, do not require a priming phosphorylation site. These include components of the Wnt signalling pathway, such as axin and β-catenin. Cyclin D1 is likely to fall into the same category, given that it lacks phosphorylatable residues at the n+4 position. If this is the case, then phosphorylation of GSK3 by PKB might not necessarily affect the rate at which GSK3 phosphorylates cyclin D, and thus PKB might promote stabilisation of cyclin D through a non-GSK3-dependent mechanism. Further studies are required to clarify this situation.

Mechanisms by which PKB can inhibit apoptosis

PKB can promote cell survival by inhibiting proteins that mediate apoptosis. The first of these substrates to be identified was the Bcl2 family member BAD, which promotes apoptosis by interacting with Bcl-X_L on the mitochondrial membrane. Phosphorylation of BAD by PKB (and other AGC kinases) enables it to interact with 14-3-3 proteins, which prevents it from binding to Bcl-X_L and thereby suppresses apoptosis (Downward, 1999). The forkhead transcription factor may be required for the expression of pro-apoptotic molecules such as the Fas ligand, and its sequestration in the cytoplasm of cells following phosphorylation by PKB could result in reduced expression of these proteins and thereby promote cell survival (Brunet et al., 1999). However, the recent finding that the dominant negative forkhead mutant used in these studies potently inhibits PKB activity in vivo (Rena et al., 2001) indicates that some of the reported effects of overexpression of forkhead mutants may be indirect and result from the suppression of the phosphorylation of other PKB substrates.

PKB could directly phosphorylate and inhibit the caspase proteases – key executioners of apoptosis. Indeed several caspases possess putative PKB-phosphorylation sites (Table 1). Significantly, the site in human caspase-9 (Ser196) that is phosphorylated by PKB (Cardone et al., 1998) is not conserved in monkey and rodent homologues, making it unlikely that phosphorylation of caspase-9 is a key mechanism regulating apoptosis. A more recently identified substrate is the apoptosis signal-regulating kinase 1 (Ask1), which stimulates MAP kinase kinases that activate the JNK and p38 MAP kinases. Overexpression of activated PKB phosphorylates ASK1 at Ser83, resulting in inhibition of Ask-1 activity and reduced JNK activity (Kim et al., 2001). In some situations JNK can promote apoptosis; in this context, inactivation of ASK1 by PKB could promote cell survival. PKB activation might inhibit apoptosis by promoting the increased expression of survival molecules or the degradation of pro-apoptotic molecules. There are no examples, thus far, of the latter but overexpression of a constitutively active PKB in cells does induce transcription of Flip, an inhibitor of caspase-8 (Panka et al., 2001) and the p21 CDK inhibitor (Lawlor and Rotwein, 2000; Mitsuuchi et al., 2000). The molecular mechanisms by which this occurs remain uncharacterised.

PKB also phosphorylates and activates endothelial nitric oxide synthase, thereby promoting angiogenesis (formation of new blood vessels). In tumours this increases the supply of nutrients to cancer cells, thereby promoting their survival (Snyder and Jaffrey, 1999). PKB activity is also stimulated following hypoxia. This has been shown to result in the activation of the hypoxia-inducible factor 1, a transcription factor that induces the expression of several genes that promote angiogenesis (Zhong et al., 2000).

Inhibition of GSK3 following its phosphorylation by PKB has also been suggested to play a role in inhibiting apoptosis in neuronal cells. This is based on the finding that overexpression of dominant negative GSK3 or overexpression of a GSK3 inhibitor protein promotes neuronal cell survival in response to inhibitors of PI 3-kinase (Ding et al., 2000). Furthermore, selective and potent small-molecule inhibitors of GSK3 have recently been developed, termed SB-415286 and SB-216763 (Coghlan et al., 2000), and these have also been shown, together with lithium, a less specific GSK3 inhibitor, to protect both central and peripheral nervous system neurons in culture from death induced by reduced PI 3-kinase pathway activity (Cross et al., 2000). The proteins that GSK3 phosphorylates to induce apoptosis remain to be defined.

Mechanisms by which PKB promotes insulin-signalling responses

Inhibitors of PI 3-kinase and overexpression of dominant negative PI 3-kinase mutants block many, if not all, of the physiological responses of a cell to insulin, indicating that PI 3-kinase lies upstream of these events (Bevan, 2001). Work carried out over the past six years indicates that PKB could be a key downstream target of PI 3-kinase in insulin-regulated processes. PKBβ is highly expressed in insulin-responsive tissues such as adipose tissue (Walker et al., 1998). Exciting new data from Birnbaum and colleagues establishes PKBβ as an essential gene for the maintenance of normal glucose homeostasis (Cho et al., 2001). Mice deficient in PKBβ display many of the typical features of Type II diabetes mellitus in humans, namely hyperglycemia, elevated blood insulin levels, and insulin resistance in the liver and to a minor extent muscle. Consistent with the elevated insulin levels, the size and number of pancreatic islets is significantly increased in the PKBβ knockouts. The authors also show that PKBβ is not required for expression of Glut 4, the main glucose transporter in muscle. These findings suggest that PKBβ is likely to represent a critical intermediate in the insulin signal transduction pathway and indicate perhaps surprisingly that the PKBα and PKBγ isoforms are not able to compensate for the lack of PKBβ expression. This is indeed the first physiological evidence that PKB isoforms may phosphorylate different substrates.

Other data suggest that PKB phosphorylation and inactivation of GSK3 is likely to stimulate the conversion of nutrients such as glucose and amino acids to storage macromolecules (glycogen and protein) in skeletal muscle, adipose tissue and liver (reviewed by Alessi, 2001). Kasuga and colleagues have shown that PKB phosphorylates and activates the cAMP-phosphodiesterase 3B in adipocytes (Kitamura et al., 1999). This reduces the levels of cAMP and hence the activity of PKA, which antagonises some insulin signalling events. Note that the Yaffe-Cantley algorithm indicates that the site in PDE3B phosphorylated by PKB lies outside the top 5% of optimal PKB-phosphorylation motifs in the database. This is probably due to the presence of a serine residue, rather than a hydrophobic residue, following the residue phosphorylated by PKB (Table 1). The cardiac-specific isoform of 6-phosphofructo 2-kinase is also activated following its phosphorylation on two residues by PKB, and this is thought to underlie the mechanism by which insulin stimulates glycolysis in the heart. Another potential substrate for PKB is the insulin receptor substrate 1 (IRS1) adaptor molecule. Its phosphorylation is proposed to inhibit recruitment of PI 3-kinase to the membrane and may play a role in a negative feedback loop shutting off PI 3-kinase activity following prolonged insulin stimulation (Li et al., 1999). The sites on IRS1 phosphorylated by PKB have not been mapped; however, IRS1 contains five potential PKB-phosphorylation sites: two high-stringency hits (Ser307 and Ser 527, Table 1) and three low-stringency hits (Ser270, Ser 330 and Ser1101). Overexpression of PKB in several insulin-responsive cell lines also stimulates the uptake of nutrients such as glucose and amino acids, and induces gene expression normally mediated by insulin (reviewed by Hajduch et al., 2001). The mechanisms by which PKB mediates these effects remain unknown.

Many key discoveries in the insulin/PI 3-kinase/PKB signaling pathway have been made in model organisms. For example, the finding that PKB regulates the forkhead transcription factor was first established in *Caenorhabditis elegans* (Paradis and Ruvkun, 1998). Inactivation of components of the PI 3-kinase signaling pathway in *Drosophila* has been shown to markedly reduce cell size. Recent data have identified a new downstream component of the PI 3-kinase pathway in *Drosophila*, namely the Tuberous Sclerosis Complex genes *Tsc1* and *Tsc2*. Loss of *Tsc1* and *Tsc2* expression results in increased cell size, and conversely overexpression of these genes decreases cell size considerably. Genetic evidence from these studies suggests that *Tsc1* and *Tsc2* function to inhibit the insulin signaling pathway downstream of PKB (Gao and Pan, 2001; Potter et al., 2001; Tapon et al., 2001). Interestingly, there are number of potential PKB-phosphorylation sites in human and *Drosophila* TSC2 (but not TSC1), of which one is a high-stringency site (Table 1).

PKB as a therapeutic target for the treatment of cancer and diabetes

PKB is inappropriately activated in a significant number of different types of cancer. This is most commonly a consequence of overexpression of PKB isoforms or growth factor receptors such as Her/Neu or mutations that result in inactivation or loss of PTEN (PtdIns(3,4,5)P_3 5-phosphatase). Therefore PKB is, in principle, an attractive drug target for the treatment of cancer. Ideally, a drug that inhibits PKB should cause both cell cycle arrest and promote apoptosis. However, such drugs may be toxic to non-cancer cells and result in serious side effects such as insulin resistance and diabetes. Drugs that directly inhibit the catalytic activity are one option; however, one can envisage that they could also work by binding to the PH domain of PKB and preventing it from interacting with PtdIns(3,4,5)P_3. The latter type of drug might be more specific given that there are far fewer PtdIns(3,4,5)P_3-binding PH-domains than protein kinases. Since different PH domains normally share <20% sequence identity, this may also facilitate the development of drugs that bind specifically to the PH domain of PKB.

There is also interest in generating drugs that can activate PKB, which could potentially be used to trigger insulin-dependent processes for the treatment of diabetes. These

compounds could also be used to promote survival and inhibit apoptosis of neuronal cells following a stroke. Although it is intrinsically much harder to develop an activator of a kinase than an inhibitor, in the case of PKB a drug that binds to the PH domain of PKB, instead of inhibiting PtdIns(3,4,5)P_3 binding, might mimic PtdIns(3,4,5)P_3 and enable PDK1 to phosphorylate and activate PKB in unstimulated cells. If PKB activators could be developed, an obvious potential side effect is cancer. Although this might be a problem for the treatment of diabetes, where it would be necessary to administer the drug over a long period of time, it is less of a problem for the treatment of strokes, for which these drugs might only be required for a relatively short time.

Potential pitfalls in approaches used to study PKB function

Although all the PKB substrates listed in Table 1 are phosphorylated by PKB in vitro, the evidence that the endogenous forms of these proteins are phosphorylated in vivo in response to agonists that activate PI 3-kinase is in most cases lacking. Furthermore, many studies investigating the role of PKB are dependent on the overexpression of constitutively active PKB mutants. PKB is only one member of the AGC family of protein kinases; the other members include isoforms of p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (p90RSK), serum- and glucorticoid-induced protein kinase (SGK) and PKC. Because these protein kinases possess substrate specificities similar to those of PKB, and they are also activated by PDK1, overexpression of PKB in cells is likely to result in the phosphorylation of substrates that are normally phosphorylated by other AGC kinase members and perhaps other kinases as well. Thus, some substrates listed in Table 1 may not be physiological substrates for PKB but only become phosphorylated artefactually following the overexpression of PKB in cells.

Insulin and growth factors frequently induce a transient activation of PKB, and it is doubtful that the overexpression of high levels of activated PKB mutants in cells for hours or even days is physiological. To overcome this problem, Richard Roth and colleagues have generated a conditionally active version of PKB fused to the estrogen receptor, which is inactive when expressed in cells but is activated within minutes of stimulation by 4-hydroxytamoxifen (Kohn et al., 1998). Another concern is that most of the constitutively active PKB mutants that have been employed are forcibly attached to the plasma membrane by the addition of a membrane-targeting motif. In contrast, endogenous PKB is likely to phosphorylate most of its substrates either in the nucleus or cytoplasm of cells and not at cell membranes. Thomas and colleagues have demonstrated that the overexpression of a membrane-targeted PKB induces activation of S6K1, whereas overexpression of a non-membrane-targeted PKB mutant that has identical activity does not have this effect (Dufner et al., 1999). This demonstrates that a membrane-targeted PKB can trigger non-physiological processes. There are two forms of non-membrane-targeted constitutively active PKB that can be employed: one is a PKB mutant in which both the Thr308 and Ser473 activating phosphorylation sites are changed to aspartate residues (Alessi et al., 1996a), and the other is mutant of PKB in which the hydrophobic motif of PKB is replaced with that found in PKC-related kinase 2 (PRK2), which possesses a very high intrinsic affinity for PDK1 (Biondi et al., 2001).

Unfortunately, no specific PKB inhibitors or cell lines that lack expression of all PKB isoforms have been developed. Without such tools we cannot rule out the possibility that the phosphorylation of even a potential PKB substrate protein, such as GSK3 or forkhead, is in fact not mediated by PKB but rather by another PI-3-kinase-activated protein kinase, such as SGK. Although dominant negative forms of PKB have been used extensively to dissect the signalling networks that are regulated by PKB, great caution must also be employed when one uses these reagents, because the mechanism by which a dominant negative PKB mutant is functioning in cells is not known. For example, dominant negative PKB might work by interacting with and inhibiting PDK1, which could affect the phosphorylation of other AGC kinases that it activates, such as SGK. Additionally, dominant negative PKB could also interact non-physiologically with a substrate of another PI-3-kinase-activated protein kinase when overexpressed in cells, and this could prevent this substrate from becoming phosphorylated by its natural upstream kinase.

Finally, analysis of phosphorylation sites of proteins in cells is increasingly being performed using phospho-specific antibodies, many of which are generated by commercial companies and, unfortunately, in most cases these have not been adequately characterised. In our opinion an essential control to be carried out with every immuno-blot shown in a paper using a phospho-specific antibody is that the phosphopeptide antigen used to raise the antibody, but not the dephosphopeptide antigen must be shown to prevent the recognition of the phosphorylated protein by the antibody. This is also important when using commercial polyclonal antibodies, because different batches of antibodies are likely to be derived from different animals and different bleeds. Thus the specificity of the antibody may vary. Unless the companies selling phosphopeptide antibodies are willing to provide the phospho-specific and dephospho-peptide immunogens as controls with each batch of antibody, we recommend that these antibodies not be employed. Given that a phospho-specific antibody can frequently crossreact with other phosphorylation sites on a protein, it is also essential to demonstrate that mutation of the phosphorylatable serine/threonine to alanine or valine abolishes recognition of the protein by the antibody. Unfortunately, these simple but key controls are not being performed routinely, and this can lead to unreliable results. For example, a study by our colleagues in Dundee found that a commercially produced phospho-specific antibody raised against one phosphorylation site on Bad also crossreacts with another phosphorylation site on this protein. This could potentially lead to inaccurate conclusions being obtained by other groups who used this antibody (Lizcano et al., 2000). It is also important to bear in mind that one cannot establish stoichiometries of phosphorylation by using phospho-specific antibodies. These reagents can readily detect trace levels of phosphorylation of a substrate in vitro. Especially for in vitro phosphorylation experiments, one must demonstrate that the substrate is phosphorylated to a significant stoichiometry by using standard peptide mapping procedures and not rely exclusively on the use of phospho-specific antibodies to infer that a given substrate is becoming phosphorylated significantly.

Concluding remarks

Although many exciting roles for PKB have emerged over recent years, because of the limitations in the approaches used to investigate the function of PKB, there must still be significant doubt as to whether all of the processes attributed to PKB are truly mediated by this enzyme in vivo. It is essential that future work in this area is aimed at developing pharmacological reagents and genetic and biochemical approaches that not only identify novel roles for PKB but also verify whether the physiological functions ascribed to PKB that have been discussed here are indeed correct. The generation of a potent and specific PKB inhibitor, or even an activator, would certainly revolutionise the study of the processes mediated by PKB in the same way as the inhibitors of MAP kinase kinase 1 activation, (e.g. PD98059, PD184352, U0126) have done for our understanding of processes regulated by the classical MAP kinase pathway. Furthermore, such drugs would also enable the validation of PKB as a target for the treatment of cancer, diabetes and stroke. There is still much to be learned about how PKB activity and its localisation in cells is regulated. The mechanism by which PKB is phosphorylated at Ser473 is still obscure, and the forces that drive the dissociation of activated PKB from PtdIns(3,4,5)P_3 at the membrane and into the cytoplasm, and then the nucleus, remain uncharacterised. PKB activity and cellular localisation may also be regulated through its interaction with regulatory proteins. One such protein could be TCL1, a protein that, when overexpressed in cells, interacts with the PH domain of PKB, which apparently promotes its phosphorylation and activation (Laine et al., 2000). PKB has also recently been shown to interact with heat shock protein 90 (HSP90) and this has been proposed to play a role in maintaining PKB in a phosphorylated and active form by preventing its dephosphorylation and inactivation by protein phosphatase 2A (PP2A; Sato et al., 2000), a phosphatase previously implicated in inactivating PKB in vivo (Millward et al., 1999). Another important question that has not so far been addressed is how the interaction of PKB with PtdIns(3,4,5)P_3 at the plasma membrane of cells affects the rate at which PKB is dephosphorylated at its T-loop and hydrophobic motif by PP2A.

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