

PROTEIN KINASE B (PKB/AKT) –
A COMMON ELEMENT IN MULTIPLE SIGNALING
PATHWAYS INVOLVED IN INSULIN SIGNALING,
CELL SURVIVAL AND CANCER

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Introduction

All aspects of cell function depend on external signaling molecules in the form of growth factors, hormones or neurotransmitters or as proteins anchored to the surface of a neighbouring cell or the extracellular matrix. Signal transduction pathways function by coordinated activation or inactivation of proteins, within the cell membrane, cytoplasm or nucleus. Activation of a signal pathway usually involves binding of a ligand – hormones, mitogens, growth factors or neurotransmitters – to its cognate receptor followed by phosphorylation or dephosphorylation of a target protein on tyrosine, serine or threonine residues by the appropriate downstream protein kinases or phosphatases. The phosphorylation state of a target protein or enzyme depends upon the relative activities of protein kinases and phosphatases in the pathway. Modules of protein kinases control cellular processes. This discovery – perhaps the most important in signal transduction during the past 10 years – is typified by growth factor stimulation of the Ras-Raf-MAP kinase module (1-3). One of the many initial events occurring after growth factors bind their cognate growth factor receptor tyrosine kinases is the recruitment and activation of the phosphoinositide 3-kinases (PI 3-kinases). Inositol lipids phosphorylated at the D3 position by PI3-kinases act as second messengers somewhat analogous to cAMP and Ca^{2+} (4,5).

The receptors specific for insulin and insulin-like growth factor-1 (IGF-1) also signal by inducing the recruitment of PI3-kinase (reviewed in 6). However, this activation mechanism is unique in the use of an adapter molecule, termed insulin receptor substrate (IRS) that bridges the interaction of insulin receptors and PI3-kinase. Following ligand binding, the receptor undergoes autophosphorylation on tyrosine residues initiating recruitment of the IRS adapter molecules and

tyrosine phosphorylation, recruitment of the lipid kinase followed by lipid phosphorylation and generation of the inositol lipid second messengers. That insulin induces profound effects on tyrosine phosphorylation was established relatively early. One of the major challenges was to find the protein kinase functioning as an «integrator» that decodes the lipid signals produced by the activation of PI3-kinase, and subsequently promotes the serine/threonine phosphorylation of intracellular proteins. The ultimate read-out from this pathway is recruitment of glucose transport proteins to plasma membranes and the subsequent uptake of glucose from the blood stream. Thus, for many years, researchers searched for the protein kinases activated by insulin.

My work on insulin signaling actually started in 1980. At that time, I was a post-doctorate fellow in the laboratory of Philip Cohen (Dundee) charged with the purification of a protein kinase with the potential to activate a protein phosphatase. This was considered to be a vital step in insulin action (another obsession at the time was the identification of the insulin second messenger). The kinase in this instance became known later as glycogen synthase kinase-3 (GSK-3) and was found to be acutely regulated by insulin. The molecule that emerged to link these disparate events is the topic of this lecture. The discovery of this particular protein kinase was tortuous, as is reflected by its different names – RAC, Akt and PKB. My laboratory was the first to clone the gene, which we termed RAC, but the kinase is now known as PKB/Akt, depending on which side of the Atlantic you are.

Protein kinase B is a serine/threonine kinase of the second messenger or AGC subfamily (7, 8). It is of considerable medical and pharmaceutical interest due to its connection with diabetes, cell survival and cancer (reviewed in 9, 10). PKB is a major downstream target of PI 3-kinase (11, 12), the lipid kinase involved in insulin signaling. The first evidence for the involvement of PKB in insulin signaling came from our work in 1995 with the laboratory of Philip Cohen (13). We demonstrated that PKB is responsible for the inactivation of glycogen synthase kinase 3 (GSK3) and may thus regulate glycogen synthesis in insulin-sensitive tissues. Since then, PKB has been linked to a number of insulin- and insulin-like growth factor-1 (IGF-1)-induced responses, inclu-

ding glucose uptake, protein synthesis, preadipose cell differentiation, and the regulation of gene expression (reviewed in 9, 10). In parallel, new functions of this kinase have emerged from the fields of cancer biology and neurobiology (reviewed in 9, 14 – 16).

Protein kinase B (PKB/Akt) – structure and regulation

The identification of two isoforms of PKB isoform (β_1 and β_2) inaugurated a new sub-family of the second messenger-regulated kinases (7, 8, 17). Although both isoforms were found to be expressed ubiquitously, both their roles and their modes of regulation remained unclear. Work by Staal on the AKT8 retrovirus shed some light in this direction.

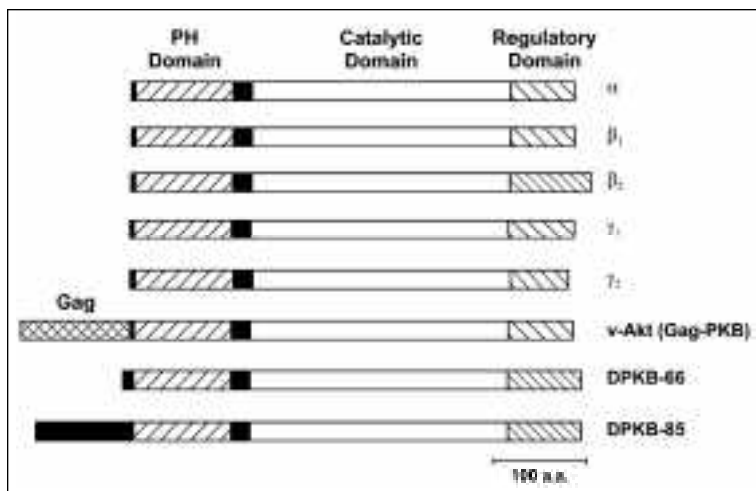


Figure 1. Domain structure of the mammalian and *Drosophila* PKB isoforms

AKT8 retrovirus induces T-cell leukemias and lymphomas in rodents and was isolated from a spontaneous lymphoma of an AKR mouse (18). The genome of the virus contains sequences of cellular origin designated v-Akt, and two homologues of the viral oncogene identified in the human genome were termed Akt1 (PKB β_1) and Akt2 (PKB β_2) (19). Cloning of v-Akt led to the exciting discovery that it corresponds

to mouse PKB or c-Akt (20). The third isoform PKB γ , cloned in 1995 from a rat cDNA library (21), has been connected recently to estrogen receptor-negative breast carcinomas in humans (22). Interestingly, the PKB γ human gene encodes two variants generated by alternative usage of 3' exons which results in different C-termini (23). All PKB isoforms have a common domain structure with a pleckstrin homology (PH) domain (24, 25) N-terminal to the central catalytic domain and a C-terminal regulatory region. The overall domain structure is preserved between the mammalian and *Drosophila* isoforms (Fig. 1).

Role of Phospholipids in PKB activation

PKB is activated by several growth and survival factors, including platelet-derived growth factor, epidermal growth factor, insulin, IGF-1, vascular endothelial growth, nerve growth factor, cytokines, chemokines, lysophosphatidic acid and serum (reviewed in 9, 10, 14 – 16). Several lines of evidence demonstrated that PKB activation is mediated through the action of PI 3-kinase. First, PKB activation is sensitive to the PI 3-kinase inhibitors (11, 12, 26) and LY 294002 (27). Second, PDGF receptor mutants which fail to activate PI 3-kinase are also unable to stimulate PKB (11, 12). Third, a dominant negative form of PI 3-kinase prevents PDGF- and insulin-induced activation of PKB (11, 28, 43). Fourth, constitutively active forms of PI 3-kinase stimulate PKB (29, 30, 31).

How does PI 3-kinase promote PKB activation? The crucial role in this process is performed by 3-phosphoinositides. This class of phospholipid second messenger is produced by the action of PI 3-kinase, which synthesises phosphatidylinositol 3,4,5-bisphosphate [PtdIns(3,4,5)P₃] (reviewed in 4). The latter phospholipid is a substrate of inositol polyphosphate 5-phosphatase, which modifies it to phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P₂], thus changing the ratio between the two classes of 3-phosphorylated phosphoinositides (32). Both PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ act as second messengers able to recruit a number of target proteins to the membrane (4). The lipid signal is «switched off» by the tumor suppressor MMAC/PTEN, a phosphatidy-

linositol phosphatase which removes the phosphate from the 3' position of the inositol ring in PtdIns(3,4,5)P₃. (33 – 35).

The major targets of 3-phosphoinositides are PH domain-containing proteins (36, 37). Such signaling modules of about 100 amino acids have been identified in over 150 signaling or cytoskeletal proteins, most of them able to associate with the cell membrane (25, 38). Conservation between different PH domains at the primary structure level is low (reviewed in 38). However, the three-dimensional structures of PH domains from β -spectrin, pleckstrin, dynamin and phospholipase C 1 revealed remarkable conservation. It was anticipated from the structure that this signaling module would bind lipophilic molecules and this was first demonstrated for PtdIns(4,5)P₂ and isolated PH domains of pleckstrin, RasGAP, and protein kinases Tsk and β -Ark (39). As PKB was found to be a target of PI 3-kinase, it was not difficult to foresee the binding of 3-phosphoinositides to its PH domain. Indeed, the PH domain of PKB binds PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ *in vitro* with similar submicromolar affinities (40, 41). PKB molecules carrying mutations in the PH domain do not respond to PDGF stimulation and are not activated by PtdIns(3,4)P₂ (12, 42). The high-affinity interaction between the PH domain and 3-phosphoinositides is sufficient for recruitment of PKB to the site of their production *in vivo*. Thus, growth factor binding to their cognate receptors results in PI 3-kinase activation and production of the lipid second messenger at the plasma membrane, which represents the «switch-on» mechanism for PKB activation. Translocation of the kinase to the plasma membrane has been demonstrated following IGF-1 stimulation and is sensitive to the PI 3-kinase inhibitor wortmannin, which requires the PH domain (43). This step is needed for priming the kinase for activation by phosphorylation (43). Targeting of PKB to the plasma membrane results in a constitutively active kinase (43) and reduces the requirement for PI 3-kinase in PKB activation. This also offers an explanation for the oncogenic potential of v-Akt, a chimera of viral gag protein and PKB, in which the N-terminal myristic acid group alters the subcellular localisation and makes the kinase constitutively active (43, 44).

Role of phosphorylation in PKB activation

The results of stimulating PKB activity by treating cells with phosphatase inhibitors provided the clue that phosphorylation is a mechanism for regulating activity (28). Furthermore, the kinase isolated from stimulated cells could be inactivated *in vitro* by treatment with protein phosphatase 2A(PP2A). PKB was found to be phosphorylated in unstimulated cells and phosphorylation levels increased following stimulation by PDGF or pervanadate (11, 28). While PDGF augmented Ser phosphorylation (11), the potent PKB activator pervanadate (an insulin mimetic) induced phosphorylation on both serine and threonine residues (28). Mapping the phosphorylation sites of PKB from human

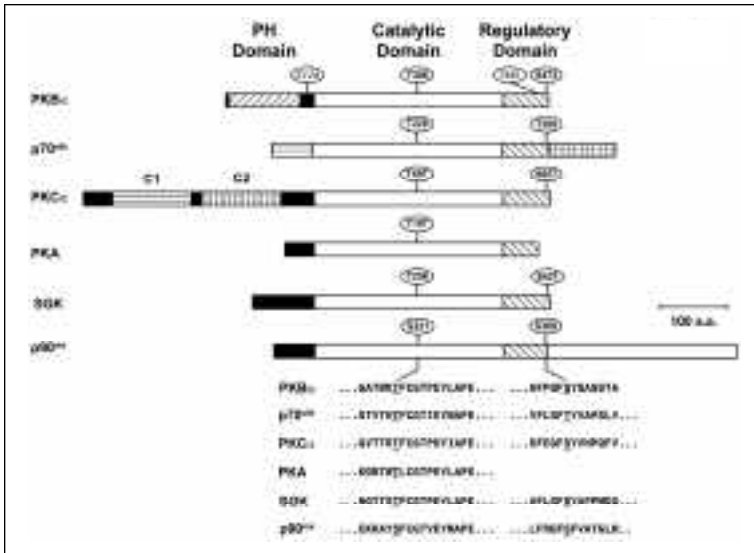


Figure 2. Constitutive and regulatory phosphorylation sites of protein kinase B, and other related members of the AGC family of kinases.

embryo kidney (HEK) 293 cells revealed that the kinase is constitutively phosphorylated on Ser124, in the hinge region between the PH and catalytic domains, and on Thr450, in the C-terminal region in unstimulated cells (45). Concomitant with insulin- or IGF-1-induced

PKB activation, two additional sites appeared: Thr308/Thr309 in the activation loop of the catalytic domain and Ser473/Ser474 in the C-terminus of the α and β isoforms, respectively, (45, 46). Like PKB activation, phosphorylation of the two induced sites was found to be sensitive to PI 3-kinase inhibitors (45, 46). Mutation of either site to Ala abolished insulin-induced PKB activation. Conversion of both sites to Asp (PKB-T308D/S473D) to mimic phosphorylation yielded a constitutively active form of the kinase resistant to wortmannin, which could not be activated further (45). Constitutive and regulatory phosphorylation sites on PKB are depicted in Figure 2.

The mechanism of PKB phosphorylation at Thr308 and Ser473 was quickly elucidated. Several lines of evidence excluded PKB itself as the enzyme responsible for this phosphorylation. Firstly, only a restricted amount of overexpressed wild-type PKB is activated following stimulation of the cells, implying the existence of an upstream limiting factor (28). Secondly, the use of kinase-defective PKB excluded autophosphorylation as a regulatory mechanism (26, 28). Thirdly, the sequences flanking these regulatory residues are not compatible with the consensus phosphorylation sequence for PKB. The different con-

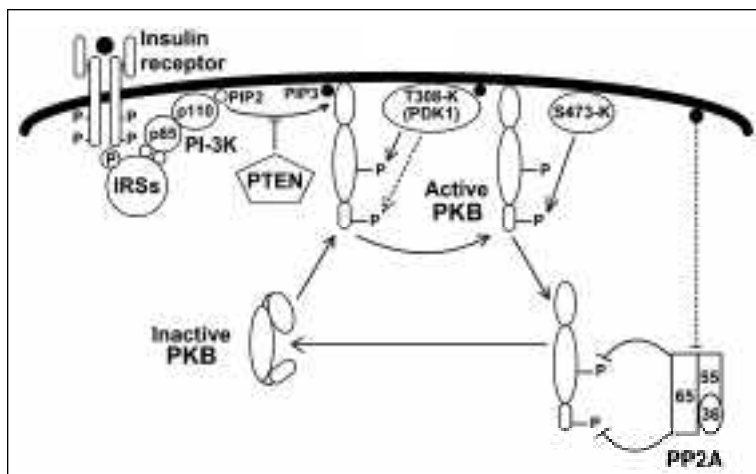


Figure 3. Model for regulation of PKB activity by subcellular localization and phosphorylation by upstream kinases.

texts of the Thr308 and Ser473 phosphorylation sites pointed towards the existence of two upstream kinases. The fact that PKB becomes phosphorylated on these residues simply by membrane targeting without cell stimulation implies that the upstream kinases are already membrane-associated in unstimulated cells and may themselves be regulated by PI 3-kinase (43, 52). A current model depicting some of the major steps in PKB activation is shown in Figure 3.

The regulation site Thr308 is located between subdomains VII and VIII of the catalytic domain of PKB, between the conserved DFG-APE motifs (47). The crystal structures of PKA, cyclin-dependent kinase 2, MAPK and Ca²⁺-calmodulin-dependent protein kinase (CaMK-I) identified this region as the activation- or T-loop (48). The first PKB upstream kinase was isolated independently by two research groups using its unique property to phosphorylate PKB on Thr308 in the presence of 3-phosphoinositides (49, 50). Because of the phospholipid requirement for phosphorylating PKB on Thr308, the kinase was named 3-phosphoinositide-dependent protein kinase 1 (PDK1) (51). Cloning of PDK1 revealed that it contains a PH domain at the C-terminal region, which binds 3-phosphoinositides (51), similar to PKB. Interestingly, removal of the PH domain of PKB ablated the phospholipid demand for phosphorylation by PDK1 (49-51). This may be explained by the fact that the PH domain in the inactive PKB sterically hinders the phosphorylation site in the catalytic domain. Phospholipid binding to the PH domain opens up the kinase conformation, allowing the upstream kinase access to its substrate. The inhibitory effect of the PH domain has been confirmed by *in vivo* studies. Its removal has a stimulatory effect on PKB activity and it facilitates activation by PDK1 *in vivo* (52).

The isolation and cloning of PDK1 marked a new era in the regulation of second messenger-regulated kinases. Striking similarities between the regulatory phosphorylation sites in the activation loop of PKB and those identified in other second messenger-regulated kinases opened up the possibility that PDK1 can also phosphorylate the homologous site in the catalytic domain of other kinases (Figure 2). This has been confirmed in several instances. For example, PDK1 readily phosphorylates a site in the catalytic domain of p70 S6K both *in vivo* and *in vitro*,

when the main requirement – free access to the substrate – is fulfilled (53). In contrast to PKB, where this is achieved by acting on the N-terminal PH domain, p70 S6K requires prior hierarchical phosphorylation C-terminal to the catalytic domain. PKA and PKC are also substrates for PDK1. Two PKC isoforms have been reported to be phosphorylated both *in vitro* and *in vivo* by PDK1 (54). Likewise PDK1 can phosphorylate Thr197 or PKA, resulting in activation of the catalytic subunit *in vitro* (55). Therefore, PDK1, or a related kinase, is likely to be the PKA kinase *in vivo*.

Serum and glucocorticoid-inducible kinase (SGK) appears to be another target of PDK1. This transcriptionally-regulated kinase is activated by insulin, IGF-1, peroxide and pervanadate in a PI 3-kinase-dependent manner, which makes it similar to PKB (56, 57). Coexpression of PDK1 activates SGK *in vivo* and PDK1 was found to interact with the catalytic domain of SGK (57). Despite a high degree of similarity to PKB (57% identity in the catalytic domain) and conservation of the phosphorylation sites, SGK does not possess a PH domain; it is still not clear how the signal from PI 3-kinase is translated into phosphorylation. Based on these properties, SGK represents a new kinase on the horizon of insulin signaling. p90RSK is a kinase with two catalytic domains. The N-terminal kinase domain has all the properties of second messenger-regulated kinases and is involved in substrate phosphorylation. Its activation by phosphorylation on Ser227 in the activation loop is carried out by PDK1 both *in vitro* and *in vivo* (58).

The second PKB regulatory site Ser473, which is surrounded by aromatic, hydrophobic residues, is homologous to the major rapamycin-sensitive site of p70 S6K, the Thr389 located C-terminal to the catalytic domain (59). A similar consensus sequence is present in conventional and novel PKC isoforms, p90 RSK, SGK and Ndr (Figure 3) (57, 58, 60). While phosphorylation of Ser473 has been firmly demonstrated in PKB regulation, the identity of the kinase has not been clearly established. Several candidates listed below have been implicated directly or indirectly in phosphorylation of this site but in my opinion, none of them appears to fulfil the criteria for the authentic *in vivo* kinase.

The «classical» biochemical approach, where the C-terminal peptide of PKB including the phosphorylation site was used as a substrate to purify kinase activity, resulted in the isolation of MAPkinase-activated protein kinase 2 (27). This enzyme is a component of the stress- and cytokine-activated signaling pathway. Although MAPKAP-K2 can phosphorylate PKB on Ser473 and activate it *in vitro*, it does not appear to be the *in vivo* upstream kinase. First, it is activated by cellular stresses which do not activate PKB and, second, inhibition of the signaling pathway on which MAPKAP-K2 lies does not block PKB activation *in vivo*.

The integrin linked kinase (ILK) is activated by insulin and fibronectin *in vivo* in a PI 3-kinase-dependent fashion and can also be stimulated by 3-phosphoinositides *in vitro* (61). ILK does not contain a canonical PH domain but a putative phospholipid-binding motif has been identified, with a sequence corresponding to subdomains I and II of PH domains from several signaling molecules. ILK phosphorylates PKB on Ser473 *in vitro* and coexpression of a kinase-inactive form suppresses PKB activation and Ser473 phosphorylation *in vivo*. ILK overexpression in cells promotes adhesion-independent growth and survival and cell cycle progression (62) and induces tumors in nude mice (63). Its anti-apoptotic role and oncogenic potential makes it similar to PKB and it is thus a likely candidate for Ser473 kinase. However, we were not able to detect changes in Ser473 phosphorylation in PKB upon coexpressing ILK in either unstimulated or insulin-stimulated HEK 293 cells (52). Thus, the exact role of ILK in PKB regulation remains open.

PKC β has been shown to phosphorylate the site homologous to Ser473 in PKC α and PKC γ in the C-terminal variable 5 (V5) domain (64). Serum-induced phosphorylation at this site is sensitive to PI 3-kinase inhibition and to the immunosuppressant rapamycin, similar to p70 S6K. PKC β itself possesses the hydrophobic motif in the V5 domain found in conventional and novel PKC isotypes, but with an acidic residue instead of phosphorylatable Ser (64). Coexpression of constitutively active PKC β overcomes LY294002 and rapamycin sensitivity of PKC α phosphorylation on Ser661, whereas a dominant-negative mutant pre-

vents phosphorylation at the same site. PKC is itself a downstream target of PI 3-kinase and has been implicated in insulin signaling (65, 66), which opens up the possibility that this kinase also acts on PKB. A link between the two kinases was made by Konishi et al., (21), who identified PKC as a protein interacting with the PH domain of PKB. However, the activation of PKB by insulin, PDGF, or serum is not sensitive to the inhibitory effects of rapamycin (11, 13, 28), as well as serum-induced phosphorylation on Ser473 (Andjelkovic and Hemmings, unpublished observation). Therefore, the role of PKC in PKB regulation still remains to be clarified.

Downstream signaling and the biological function of PKB

In order to understand the physiological function of PKB, it was important to identify authentic substrates. Since 1995 the range of PKB-regulated functions has dramatically increased. This is in part due to the re-

PKB substrate proteins			
Proteins	PKB phosphorylation sites	Species	Effects of phosphorylation
Bcl-1	100 R S R H S S Y 101 102 G I S R S A M 103	human	Blocks Bcl-1-induced apoptosis
C-Raf	284 Q K E S T S T 285	human	inhibits C-Raf activity
B-Raf	199 R D E S S S A 200 201 R F E R S S S 202	human	inhibits B-Raf activity
BBCA-1	60 K K E R P T S 61	human	interferes with the nuclear localization of BBCA-1/7
Caspase-9	111 R R E F S S L 112	human	inactivates Caspase-9 activity
CREB	133 L S G R P S Y 134	human	increases its association with CBP and p/38
eNOS	171 R R L H S S Y 172	human	activates eNOS activity and leads NO production
PKHRL1	100 R R R A A S M M 101	human	inhibits transcription activity of PKHRL1
GSK-3 α	101 L A G T S S F 102	human	inactivates GSK-3 activity
GSK-3 β	102 P F T S S F 103	human	inactivates GSK-3 activity
14B kinase II	111 E R L G T G 112	human	activates transcription activity of NF- κ B
IRS-1	300 R S R T E S S 301	mouse	protects IRS-1 from the action of PTase
mTOR	240 E T R T D S Y 241	mouse	inhibits mTOR activity
PDE-3B	100 P F R R S S 101	mouse	inactivates PDE-3B activity
PKC-2	400 M G R N S F 401 402 R P K N T S V 403	bovine	activates PKC-2 activity
Rac1	100 R R L P L S Y 101	human	inhibits Rac1-GDP binding
17ERF	44 R R G S S Y 45	human	enhances telomerase activity

lization that the protein kinase GSK-3 is a major target of PKB, and because a detailed substrate-specificity study was undertaken that defined the consensus PKB phosphorylation site as Arg-X-Arg-Y-Z-Ser/Thr-Hyd, where X is any amino acid, Y and Z are small residues other than Gly and Hyd is a bulky hydrophobic residue (67). Database searches identified a large number of proteins with this consensus site and played a very significant role in the identification of a vast array of substrates involved in many different cellular processes, such as regulation of glucose metabolism in insulin-response tissues, cell survival, transcription, protein synthesis, cell cycle progression and cell transformation. A current list of identified PKB substrates is shown in Table 1.

Insulin signaling

PKB can regulate glucose metabolism at several levels and, therefore, has had a major impact on our understanding of insulin signaling (see Figure 4). The realization that PKB activation is PI 3-kinase dependent

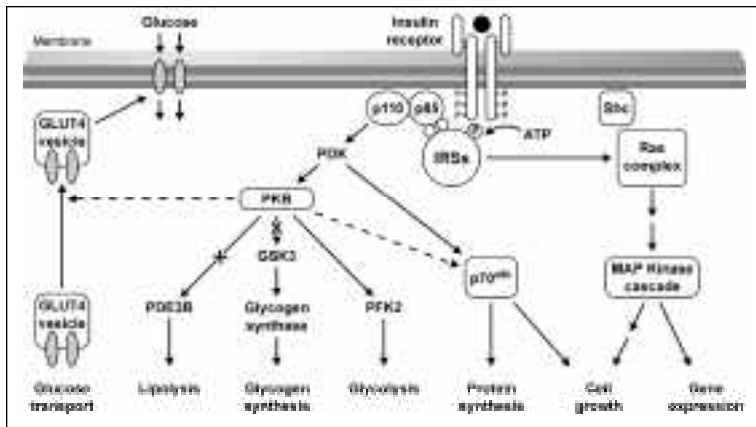


Figure 4. A current summary of the major insulin signaling pathways

was a major breakthrough indicating that this kinase is a major player in signal transduction. The second major advance was the discovery that PKB regulated GSK3 (the first physiological substrate) in an insu-

lin-dependent manner (13). This information essentially linked insulin receptor activation, through a protein kinase cascade, to the regulation of glycogen metabolism. Serine21 in GSK3 and Serine9 in GSK3 are phosphorylated by PKB and this serves to negatively regulate GSK3 activity and to maintain glycogen synthase in a dephosphorylated and active state (13). Thus, insulin stimulation of glycogen synthesis is mediated through the inhibition of GSK3 coupled with simultaneous activation of the relevant glycogen synthase phosphatase. The identification of PDK1 as a regulator of several protein kinases activated by insulin provided a further layer of complexity to insulin signaling (see Figure 5).

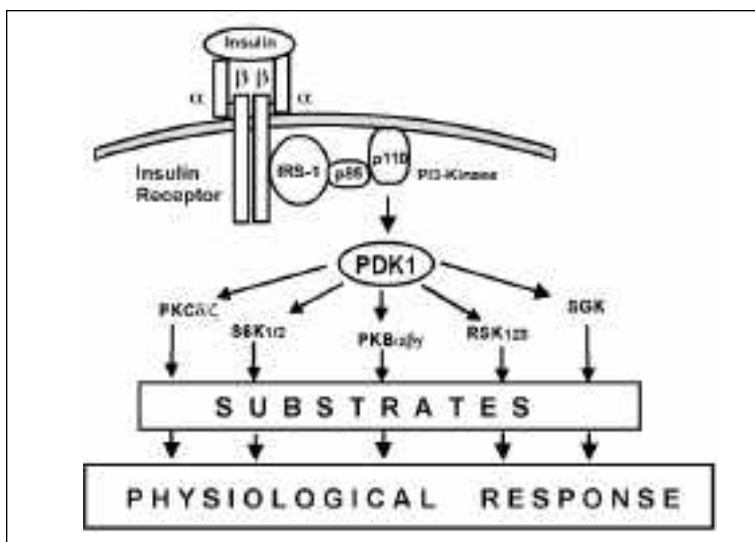


Figure 5. Phosphoinositide dependent protein kinase 1 (PDK1) is a central player in insulin signaling

In parallel to these observations, it was reported that insulin-mediated activation of PKB also promoted glucose uptake by affecting the GLUT1, GLUT3 and GLUT4 glucose transporters (68, 69). Apparently, activated PKB induces the expression of GLUT1 and 3 and the translocation of GLUT4 to the plasma membrane. However, these results are somewhat controversial and we still need a detailed mole-

cular description of the mechanism for stimulating membrane recruitment. Recently, GLUT4-containing vesicles were found to have a specific association with PKB (70). Direct phosphorylation of the transporter is still a possible mechanism. Furthermore PKB has been implicated in the regulation of two further classical insulin responses: 6-phosphofructose-2-kinase (PFK-2) and phosphodiesterase 3B (PDE3B). Deprez et al. (71) proposed that PKB mediates the insulin-induced activation of heart PFK-2 by phosphorylating Ser466 and Ser483. Insulin inhibits triglyceride hydrolysis apparently by activating PDE3B through phosphorylation of Ser302. The data so far available indicate that PKB phosphorylates the critical PDE regulatory site (72).

Cell survival

Considerable evidence has accumulated indicating that PKB plays a major role in growth factor-mediated cell survival and inhibition of apoptosis in response to several different stimuli (see Figure 6). PI 3-

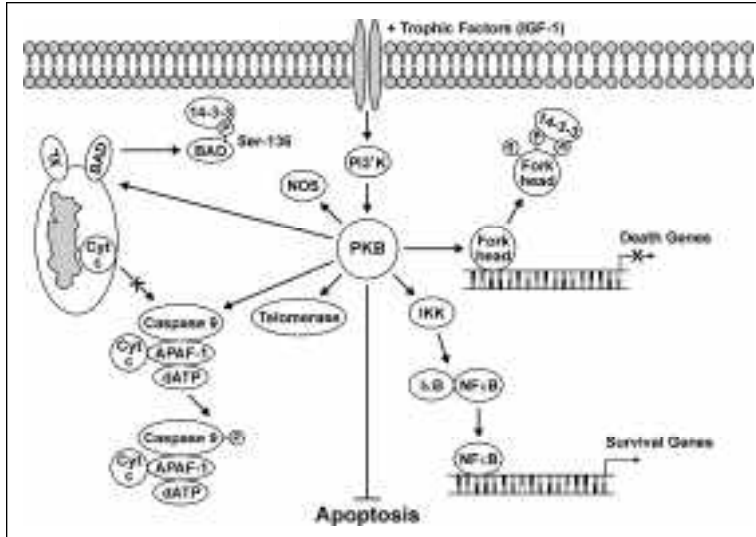


Figure 6. PKB regulates cell survival through the phosphorylation of many proteins involved in the regulation of apoptosis.

kinase was first implicated in the suppression of apoptosis in a study by Yao and Cooper (73). These results suggested that PKB is a critical regulator of cell survival. The idea was first tested in a study with cultured cerebellar granule cells that investigated the signaling molecules utilized by IGF-1 and PI 3-kinase to promote cell survival (74). The results revealed that constitutively active PKB promoted cell survival in the absence of IGF-1. Numerous studies have now shown that PKB blocks apoptosis induced by apoptotic stimuli, such as removal of growth factors, UV irradiation, matrix detachment, cell cycle arrest, DNA damage, and treatment of cells with anti-Fas antibody or TGF (reviewed in 14-16). Subsequently, targets were sought that were relevant to the survival-promoting effects of PKB. This was aided by work defining the substrate specificity of PKB (67). Many of the proteins of the apoptosis machinery contain PKB consensus sites (15). Interestingly we demonstrated that NGF activates all three isoforms of PKB in PC12 cells (75) but little attention has been paid to the role of the different isoforms in cell survival signaling.

One mechanism by which PKB may promote survival is through the inhibition of a component of the cell-death machinery. Among molecules central to the regulation of cell death in eukaryotes are members of the BCL-2 family of proteins (15). Several members of the BCL-2 family (BCL-2, BCL-XL, MCL-1, A1 and BAG-1) promote survival, whereas others (BCL-XS, BAD, BAX and BAK) promote cell death (15). BCL-2 family members homo- and hetero-dimerise and the balance between particular homo- and heterodimers is critical in the maintenance of cell survival or the induction of apoptosis. The fact that BAD is phosphorylated *in vivo* on a consensus PKB site (76) ushered in an era of research that dramatically changed our understanding of the regulation of cell death. It is now appreciated that PKB can regulate apoptosis at multiple levels. Several direct substrates, including BAD (76), caspase 9 (77), the Forkhead family of transcription factors (78-82) and the NF- κ B regulator kinase (83 – 86), play critical roles in mediating cell-death.

Following survival factor treatment BAD is phosphorylated on two serine residues, Ser116 and Ser136, embedded in 14-3-3 consensus

binding sites causes BAD to dissociate from BCL-XL/BCL-2, and to associate with 14-3-3 proteins (87). BAD phosphorylation is promoted by a PI 3-kinase/PKB pathway upon stimulation by PDGF or IGF-1 (76). PKB is a potent Ser136 kinase *in vitro*, and *in vivo*. Recent results show that PKB primarily triggers BAD phosphorylation at Ser136 and that phosphorylation at this site is sufficient to promote survival. More recent data suggest that several protein kinases target the multiple sites on BAD and contribute to cell survival (15, 88). The degree of importance of PKB in cell survival signaling was expanded by the observation that PKB phosphorylated and inactivated caspase 9 (77). This result explains how growth factors can block cell death subsequent to increases in cytoplasmic cytochrome C (89). Increases in the concentration of cytochrome C in the cytoplasm result in the nucleation of a multienzyme complex, the apoptosome, comprising Apaf-1, cytochrome C, dATP, and the aspartyl-directed protease caspase 9. Inactivation of caspase 9 by PKB blocks the activation of this complex, even in conditions promoting cytochrome C release from the mitochondria. Genetic studies by the groups of Ruffkun and Kenyon, using the worm *C.elegans* to study the control of diapause formation and longevity provided critical data identifying DAF-16, a Forkhead family member, as a target of the PI 3-kinase/PKB pathway (90, 91). These observations, linked to the fact that many apoptotic stimuli also induce *de novo* gene expression as an essential part of the apoptotic program, prompted many laboratories to investigate whether the Forkhead transcription factors are phosphorylated by PKB (78-82). Mammalian cells produce three isoforms of these transcription factors (FKHR, FKHL1 and AFX). Significantly, these three isoforms have been identified at the sites of chromosomal rearrangements in certain human tumors (92). PKB phosphorylates all members of the Forkhead family on several sites (78-82). Thus far, three sites have been mapped and the major site of phosphorylation by PKB is the second C-terminal site in the DNA-binding domain. Phosphorylation of Forkhead isoforms at each of the three sites is induced upon exposure to IGF-1. The fact that PKB and translocate (43) to the nucleus following stimulation maybe a critical component of this signaling mechanism, however, it has also been suggested that PKB phosphorylates these transcription factors in the cytoplasm.

Growth regulation and cancer

Following our report on the molecular cloning of PKB in 1991, the group of Tsichlis made the dramatic observation that the oncogene from the AKT8 retrovirus corresponds to the mouse PKB (cAkt) fused to the viral gag protein (20). The AKT8 retrovirus was first isolated some 15 years earlier by Staal and colleagues, who proceeded to do some very insightful work without identifying or cloning the v-Akt gene (18, 19). This observation placed PKB in an elite, the proto-oncogene group of serine/threonine specific protein kinases, which includes c-Raf, c-Mos, Pim1, Cot1, Aurora 2, MEK and PKC epsilon (93). Since the mechanism of PKB activation has been investigated in considerable detail, it is now possible to rationalise why v-Akt is oncogenic. Attachment of the gag sequence introduces an N-terminal myristic acid that targets PKB to membrane (43, 44, 52). We demonstrated that this is sufficient to promote the phosphorylation of PKB on Thr308 and Ser473 and produce a constitutively active form of the kinase (43). This could have two possible effects: a) constitutive phosphorylation of substrates such as the Forkhead transcription factors, BAD, and possibly GSK3, thereby blocking apoptosis, or b) phosphorylation of as yet unidentified substrates capable of bringing about cell transformation.

The oncogenic potential of the PI 3-kinase/PKB pathway has been emphasised recently by the discovery that the catalytic subunit of PI 3-kinase is an oncogene (94), and the fact that the tumor suppressor gene PTEN is an upstream negative regulator of PKB (33-35). Significantly the avian sarcomavirus PI 3-kinase derived oncogene (v-p3k) is a fusion between the catalytic subunit of the lipid kinase and the viral gag gene (94). This AVI6 oncogene has the potential to induce haemangiosarcomas in chickens and transform chicken embryo fibroblasts. CEFs transformed by v-p3k have elevated PIP2 and PIP3 and enhanced PKB activity. A further connection between PI 3-kinase, PKB and cellular transformation can be found in the realization that Ras, a small GTP-binding protein mutated in many human cancers, is an activator of PI 3-kinase (95).

In the case of PTEN, its role in PKB was unsuspected. Initially isolated as a tumor suppressor gene mutated in many cancers, most impor-

tantly in the rare hereditary breast and thyroid cancer predisposition syndrome, Cowden's disease, the PTEN gene was thought to encode a dual-specificity tyrosine phosphatase with preference for acid substrates (reviewed in 96). This led to the dramatic discovery that PTEN prefers phospholipids as substrates and specifically dephosphorylates the D3 position of the PI(3,4)P₂ and PI(3,4,5)P₃ products of PI 3-kinase (33, 34). It was recognised immediately that PTEN could function as a negative regulator of the PKB signaling pathway. Normally, PKB activity is low in the absence of growth factors. PTEN-deficient tumor cell lines, as well as immortalised fibroblasts and tumors derived from PTEN-deficient mice, show high levels of PKB phosphorylation. Furthermore, PTEN^{-/-} fibroblasts are resistant to several anti-apoptotic stimuli, consistent with negative regulation of apoptosis by PKB. All these data clearly implicate PKB in the development of cancer. Further data to support this idea come from the study of PKB expression in human tumors. PKB was found to be amplified in some human gastric adenocarcinomas (19), whereas PKB amplification was found in human ovarian carcinomas (97). Further examples of PKB amplification and/or overexpression were found in pancreatic carcinomas (98, 99). PKB has been found to be upregulated in estrogen receptor-deficient breast cancer and in androgen-independent prostate cancer cell lines (22).

Our recent work has revealed a new player in this complex signaling pathway (100). We have isolated a novel protein, CTMP, that appears to function as a tumor suppressor because it can negatively regulate PKB by inhibiting kinase activation. The growth of v-Akt tumors could be blocked or significantly inhibited by overexpressing CTMP. Furthermore, TCL-1, a proto-oncogene involved in prolymphocytic leukaemia, was found recently to stimulate PKB activity (101, 102). Here, a new paradigm appears to be emerging that involves a novel group of adaptor proteins that modulate PKB activation and activity.

Thus, a considerable body of information now directly or indirectly implicates PKB in the development of cancer. Our task in the future will be to develop therapeutics that can successfully ablate the kinase without significantly affecting glucose metabolism.

Concluding remarks

Over the past 3 years, our appreciation of PKB as a key component in several signaling pathways has increased dramatically. The nature of these pathways, which impinge on such diseases as diabetes, neurodegeneration and cancer, mean that PKB will remain the focus of intense research activity in the coming years. Obviously, there are many unanswered questions regarding PKB function. The unfolding of the PKB story, nonetheless, illustrates the need for basic research programmes focusing on fundamental mechanisms, which will eventually lead to a better understanding of cellular regulatory processes. Such an approach linked to the new techniques encompassed by genomics and proteomics, should provide us with the information and tools to develop the therapeutics needed to manage some of the major diseases currently afflicting the western population.

We should also keep in mind that the current estimate of protein kinases in the human genome is around 2000 (a significant number at the start of the new millennium). Of the known, or intensely studied, protein kinases, probably less than 5 – 10% can be ascribed functions in a known signaling pathways. In other words, we still have to discover 90% of the signaling pathways encoded by the human genome and to delineate their functions. Despite the immense amount of work ahead, many of the major paradigms are now mapped out and these provide a firm framework to discover new signaling pathways.

Thanks and Acknowledgements

My career in science has taken several twists and turns and many journeys across the Atlantic with a long stable stay in Basel. During the course of these transitions over the years, I have met and collaborated with many. I especially acknowledge the early crew from my laboratory who struggled with the orphan kinase! These include Fernando Pittossi, Pam Jones, Teresa Jakubowicz and, especially, Mirjana Andjelkovic. Mirjana arrived in Basel from Belgrade before the situation became so desperate in the Balkans and suffered through many

turmoils and restrictions. She came for 6 months and eventually stayed for 8½ years. These co-workers were followed by Peter Cron, Evan Ingely, Tom Millward, Mathias Frech, Roger Meier, Jongsun Park, Daniela Brodbeck, Michel Maira, Ivana Galetic, Michelle Hill, Zhang Zho Yang, Derek Brazil and Jianhua Feng.

In addition I wish to acknowledge several others who have had a significant impact on my career over the years. These include Earl Stadtman at the NIH, Philip Cohen in Dundee, Paul Nurse and Peter Parker both at the ICRF in London, Stuart Stone, Jan Hofsteenge and Max Burger in Basel. I would like to thank the following for help with producing this manuscript: Mirjana Andjelkovic, Ivana Gelatic, Jongsun Park, Jianhua Feng, Mike Rothnie, Pat King and Gabi Gruber. Research in my laboratory has been generously funded by the Novartis Research Foundation and in part by the Swiss Cancer League and EMBO.

Finally, I thank my wife, Maja, and son, Arthur, (7 years), who kept me focused on getting the job done. As my son once said: «What's this PKB you keep talking about? Is it your protein or what?». Not any more!

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