REVIEW ARTICLE

Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation

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While a plethora of extracellular molecules exist that modulate cellular functions via binding to membrane receptors inside the cell, their actions are mediated by relatively few signalling mechanisms. One of these is activation of phosphatidylinositol 3-kinase (PI-3K), which results in the generation of a membrane-restricted second messenger, polyphosphatidylinositides containing a 3′-phosphate. How these molecules transduced the effects of agonists of PI-3K was unclear until the recent discovery that several protein kinases become activated upon exposure to 3′-phosphorylated inositol lipids. These enzymes include protein kinase B (PKB)/AKT and PtdIns(3,4,5)P_3-dependent kinases 1 and 2, the first two of which interact with 3′-phosphorylated phosphoinositides via pleckstrin homology domains. Once targeted to the membrane by this motif, PKB becomes phosphorylated at two residues, which relieves intermolecular inhibition, allowing the activated complex to dissociate and modify its targets. Identification of these substrates is the subject of intensive research, since at least one must play a key role in suppressing apoptosis, as demonstrated by expression of activated alleles of PKB. The generation of effective trans-dominant mutants, coupled with genetic analysis of the protein kinase in simpler organisms, should help in elucidating outstanding questions in the functions, targets and regulation of this important mediator of PI-3K signalling.

ORIGINS OF AKT

The AKR strain of mice exhibit a high incidence of leukaemias and lymphomas from spontaneous thymoma [1]. A retrovirus termed AKT8 was isolated from one of these lines derived from a spontaneous thymoma. This virus was demonstrated to uniquely transform only mink lung cells (CCL64) in culture, while virus inoculated into newborn mice was shown to be tumorigenic [2]. The non-viral DNA component transduced from the mouse genome was subsequently identified, and two human homologues, *AKT1* and *AKT2*, cloned [3]. The location of the human *AKT* locus was mapped to chromosome 14q32, proximal to the immunoglobulin-heavy-chain locus [4], a region frequently affected by translocations and inversions in human T-cell leukaemia/lymphoma, mixed-lineage childhood leukaemia and clonal T-cell proliferations in ataxia telangiectasia, supporting a role for this oncogene in formation of a variety of tumours [5]. Analysis of a panel of human tumours revealed a 20-fold amplification of *AKT1* in a primary gastric adenocarcinoma. *AKT2*, on the other hand, was mapped to chromosome region 19q13.1-q13.2 and shown to be amplified and overexpressed in several ovarian carcinoma and pancreatic cancer cell lines [6,7]. A recent large-scale study of *AKT2* alterations in ovarian and breast tumours revealed amplification in 12.1% ovarian and 2.8% breast carcinomas [8]. Furthermore, amplification of *AKT2* was especially frequent in undifferentiated tumours, suggesting that *AKT2* alterations may be associated with tumour aggressiveness.

CLONING OF PROTEIN KINASE B (c-Akt)

Some 15 years after the identification of the AKT8 retrovirus the cellular homologue of v-Akt was cloned independently by several groups and found to be a 57 kDa protein serine/threonine kinase [9–11]. Different strategies were utilized in the identification of this novel kinase: degenerate oligonucleotide-based PCR against protein kinase catalytic domains [9]; low-stringency library screening with a cAMP-dependent kinase probe [10] and sequencing of a human cDNA hybridizing with v-*akt* DNA [11]. The kinase shows most similarity to protein kinase A (PKA) and protein kinase C (PKC) and has thus has been termed both protein kinase B (PKB) [9] or **R**elated to **A**- and **C**-kinase (RAC-PK; [10]) as well as c-Akt [11]. The current nomenclature for this kinase has not been clearly resolved, but the name RAC is no longer used to avoid confusion with the small GTP-binding protein p21rac [12]. Mammalian genomes contain three genes encoding PKBs (termed α/AKT1, β/AKT2 and γ) (see Figure 1). PKBβ and PKBγ are approx. 82% identical with the α-isoform, although PKBγ lacks 23 amino acids at the C-terminus compared with the others [13]. Homologues have also been identified in the nematode worm *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster*, demonstrating wide evolutionary conservation [14,15].

Sequence analysis of PKB/Akt and v-Akt cDNAs revealed that the viral gene is a fusion between a truncated tripartite viral group-specific antigen gag (p12, p15, Δp30) and PKB/AKT1 (Figure 1). These two domains are joined by a 21-amino-acid-

Abbreviations used: PI-3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKA, protein kinase A; PKC, protein kinase C; RAC-PK, related to A- and C-kinase; PH, pleckstrin homology; PtdIns, phosphoinositide; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; IL, interleukin; ERK, extracellular-signal-regulated kinase; Btk, Bruton tyrosine kinase; PDK, PtdIns(3,4,5)P_3-dependent kinase; MAPKAP, mitogen-activated protein kinase-activated protein; SH2, Src homology 2; gag, group-specific antigen; GLUT, glucose transporter; GSK, glycogen synthase kinase; PFK2, 6-phosphofructose-2-kinase; IGF, insulin-like growth factor; 4E-BP1, 4E-binding protein; PHAS, pH- and acid-stable; BCR-ABL, breakpoint cluster region–Abelson; ICE, interleukin-1 converting enzyme; SCID, severe-combined immunodeficiency; CEF, chicken embryo fibroblast; AV16, avian sarcoma virus 16; MDCK, Madin–Darby canine kidney; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end-labelling; $p70^{S6K}$, p70 S6-kinase.

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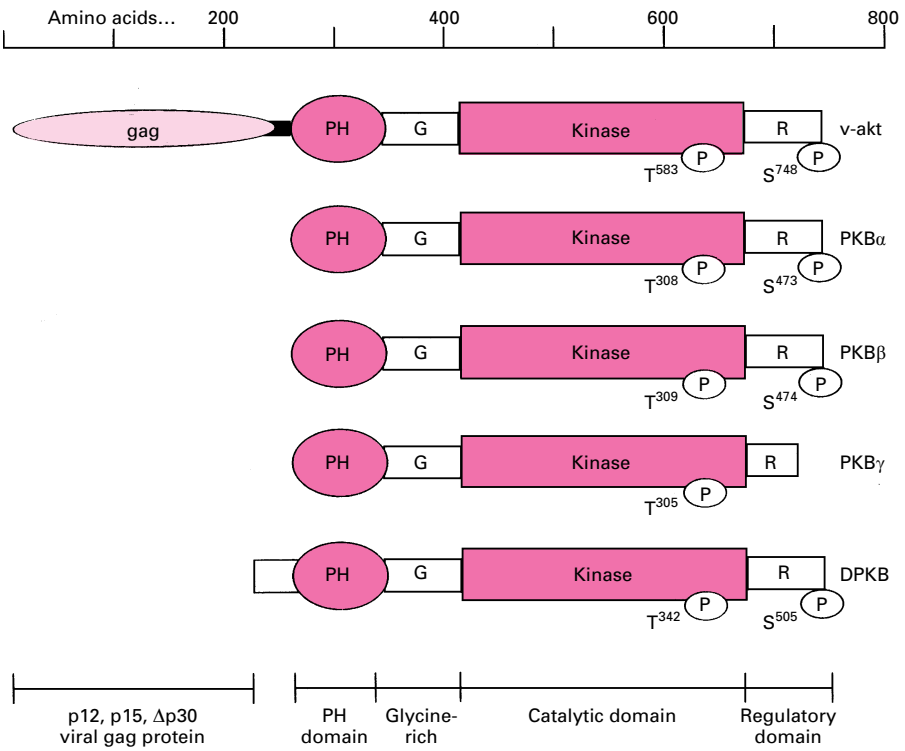


Figure 1 Schematic representation of PKB structure

The domain structure and size of v-Akt, PKB isoforms (α, β and γ) and *Drosophila* PKB (DPKB) are indicated. v-Akt consists of a fusion of PKB with a tripartite viral gag protein (pink) and is myristoylated, targeting it to the membrane. The N-terminus of PKB contains a PH domain ('PH') and glycine-rich region ('G'). The C-terminus has a regulatory role ('R') and shows similarity to protein kinase C isoforms. The positions of activating phosphorylation sites in all PKB isoforms are indicated (see the text for details). It should be noted that PKBγ lacks the most C-terminal serine phosphorylation site, owing to truncation.

long peptide encoded by sequences from the 5′-untranslated sequence of PKB/AKT1 plus three extra nucleotides at the recombination breakpoint. This structure immediately suggests a mechanism for oncogenic activation of PKB. The viral gag protein is myristoylated at its N-terminus and normally targeted to the plasma membrane. Indeed, while PKB is localized primarily in the cytosol (90 %), v-Akt is myristoylated and dispersed among various cellular compartments, with 40 % being localized to the plasma membrane, 30 % nuclear and 30 % cytosolic [16]. This has dramatic consequences for regulation and function, as discussed below. Analysis of the translated sequence of PKB cDNA reveals a multi-domain protein. The N-terminus contains an Src homology 2 (SH2)-like domain that has been subsequently redefined as a pleckstrin homology (PH) domain [17,18]. The central catalytic domain shows considerable similarity to PKA and PKC, as mentioned above, while the C-terminal tail shows similarity to a regulatory region present in members of the PKC family [19]. How these various domains play a role in regulation of kinase activity will be discussed in detail below.

PKB expression

PKBα and PKBβ are widely expressed, with highest levels in brain, thymus, heart and lung [9,10,19]. Expression of the γ-isoform is more restricted (high in brain and testes, lower in heart, spleen, lung and skeletal muscle), but all tissues contain at least one form of PKB. Little is currently known concerning regulation of expression of PKB; however, it appears to be up-regulated as cells become more terminally differentiated. For example, PKB expression is low in a multipotent fibroblast cell line (10T1/2), but is increased when these cells are transformed with *MyoD* to induce differentiation into myocytes [20]. Furthermore, differentiation of pluripotent P19 embryonal carcinoma (EC) cells with retinoic acid results in dramatic induction of PKB after 3 days, while terminally differentiated derivatives of P19 embryonal carcinoma cells (MES-1, END-2, EPI-7) constitutively express high levels of PKB (P. J. Coffer, unpublished work). Finally, 3T3-L1 fibroblasts spontaneously differentiate into adipocytes when transfected with a constitutively active PKB [21,22]. Thus expression of PKB appears to be tightly connected with the terminal differentiation of various cell types. *Drosophila* PKB expression shows no spatial restriction during embryogenesis, but is maternally as well as zygotically expressed [15,23].

Phosphatidylinositol 3-kinase (PI-3K) mediates PKB activation

Despite the fact that PKB had oncogenic potential, its normal regulation and function were, until recently, unclear. About 4 years after the kinase was cloned, several groups concurrently identified PKB as a downstream target of PI-3K activation [24–26]. PI-3K phosphorylates phosphoinositides (PtdIns) at the 3-position of the inositol ring, generating PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Studies were primarily based on two observations: (i) that growth-factor-induced PKB activation could be completely blocked by addition of wortmannin, an inhibitor of PI-3K, and (ii) that growth-factor-receptor point mutations that prevent the activation of PI-3K also inhibit PKB. PKB was

found to be rapidly and transiently activated by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin, and this activation was paralleled by phosphorylation of the kinase itself [24,25].

Phosphorylation appears to be critical for kinase activity, as treatment of active PKB immune complexes with phosphatase abolishes the ability of the kinase to phosphorylate *in vitro* substrate [24,26,27] (see below). This phosphorylation can be observed on SDS/PAGE as a slower migrating form of PKB in much the same way as extracellular-signal-regulated mitogen-activated protein kinases (ERK MAP kinases) p44ERK1/p42ERK2. While the inhibitory effects of wortmannin on PKB activation must be cautiously interpreted in view of potential non-specific effects of this pharmacological reagent, using the PDGF receptor as a paradigm, two tyrosine residues, Tyr^{740} and Tyr^{751}, were demonstrated to mediate PKB activation [24,25]. Phosphorylation of both these tyrosine residues generates high-affinity binding sites for the p85 regulatory subunit of PI-3K [28]. Furthermore, co-transfection of a dominant-negative form of PI-3K (Δp85; [29]) also inhibited PKB activation [24]. It was later shown that introduction of constitutively active mutants of the catalytic subunit of PI-3K was sufficient to activate PKB in cells [30,31]. These studies strongly implicated PKB as a downstream effector of growth-factor-stimulated PI-3K activation in a variety of cell types. Subsequently PKB has been shown to be activated by a wide variety of stimuli including haemopoietic cytokines [IL (interleukin)-2, IL-3, IL-4, IL-5], chemokines [formylmethionyl-leucylphenylalanine, IL-8, RANTES (regulated on activation, normal T-cell expressed and secreted)], heat shock, hyperosmolarity, hypoxia, integrins, the T-cell antigen receptor and nerve growth factor [32–40].

While activation of PI-3K appears to induce PKB universally, the reverse does not hold. For example, heat-shock-mediated activation of PKB is insensitive to inhibition by wortmannin in some cell types [32]. Agents which raise intracellular cAMP levels can also activate both PI-3K and PKB activity [38,39]. However, it has been shown in 293 cells that pharmacological reagents that elevate cAMP levels, such as forskolin, can activate PKB in a PI-3K-independent manner [41]. PKB containing activating mutations at the serine/threonine phosphorylation sites (see below) is refractive to activation by elevated intracellular levels of cAMP. Thus it was suggested that cAMP modulates the same phosphorylation events that are stimulated by PI-3K-dependent PKB-activating stimuli. In contradiction, however, insulin and forskolin were observed to have synergistic effects on PKB activation, suggesting alternative mechanisms [41]. Since most physiological agonists that elevate cAMP levels also strongly activate PI-3K, it is still unclear as to the relevance of these observations. Finally, in rat epididymal fat cells, isoprenaline ('isoproterenol') (acting through α_3-adrenoreceptors), but not cAMP analogues, increases the activity of PKB, although to a lesser extent than insulin [42]. In this system wortmannin abolishes PKB activation by insulin, but has no effect on the activation seen in response to isoprenaline. Activation of PKB by isoprenaline was not accompanied by an electrophoretic mobility shift. Thus it appears that, while activation of PI-3K is the major factor regulating activation of PKB, several alternative mechanisms may exist that are utilized by specific stimuli.

Interestingly, activation of PKB can be down-regulated by certain stress stimuli which increase intracellular ceramide [43,44]. Thus agents which activate acidic sphingomyelinase (such as tumour necrosis factor-α) may antagonize PKB activity. The level at which this occurs is presently unclear, but appears to be between PI-3K and PKB. These data are of particular relevance in light of the anti-apoptotic functions of PKB (see under 'Anti-apoptotic signalling mediated by PKB' below) and may represent a mechanism for cross-talk between survival and apoptotic stimuli.

Mechanisms of activation of PKB

The finding that PKB activity is induced in a PI-3K-dependent manner immediately suggested that the 3′-phosphorylated lipid products of PI-3K mediated the activation. Indeed, incubation of purified PKB with purified 3′-phosphorylated phospholipids resulted in various extents of activation [45,46]. Others have found binding in the absence of activation [47]. These lipids [such as PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3] specifically associate with PH domains in a number of proteins [48]. Since certain mutations of the PH domain of PKB rendered it inactive, the mechanism of activation of the protein kinase could simply occur via recruitment to the plasma membrane following formation of 3′-phosphorylated phosphatidylinositides. As mentioned above, activation of PKB is accompanied by its phosphorylation. Since dephosphorylation of the kinase causes its inactivation, phosphorylation appears to be a required step in the induction process. Phosphorylation could occur via transphosphorylation by a protein kinase(s) acting on PKB or by autophosphorylation of PKB following its activation (a common phenomenon for protein kinases). The issue has been resolved by the identification of two phosphorylation sites on PKBα and PKBβ and the identification of at least one protein kinase activity that targets these sites [49] (see Scheme 1).

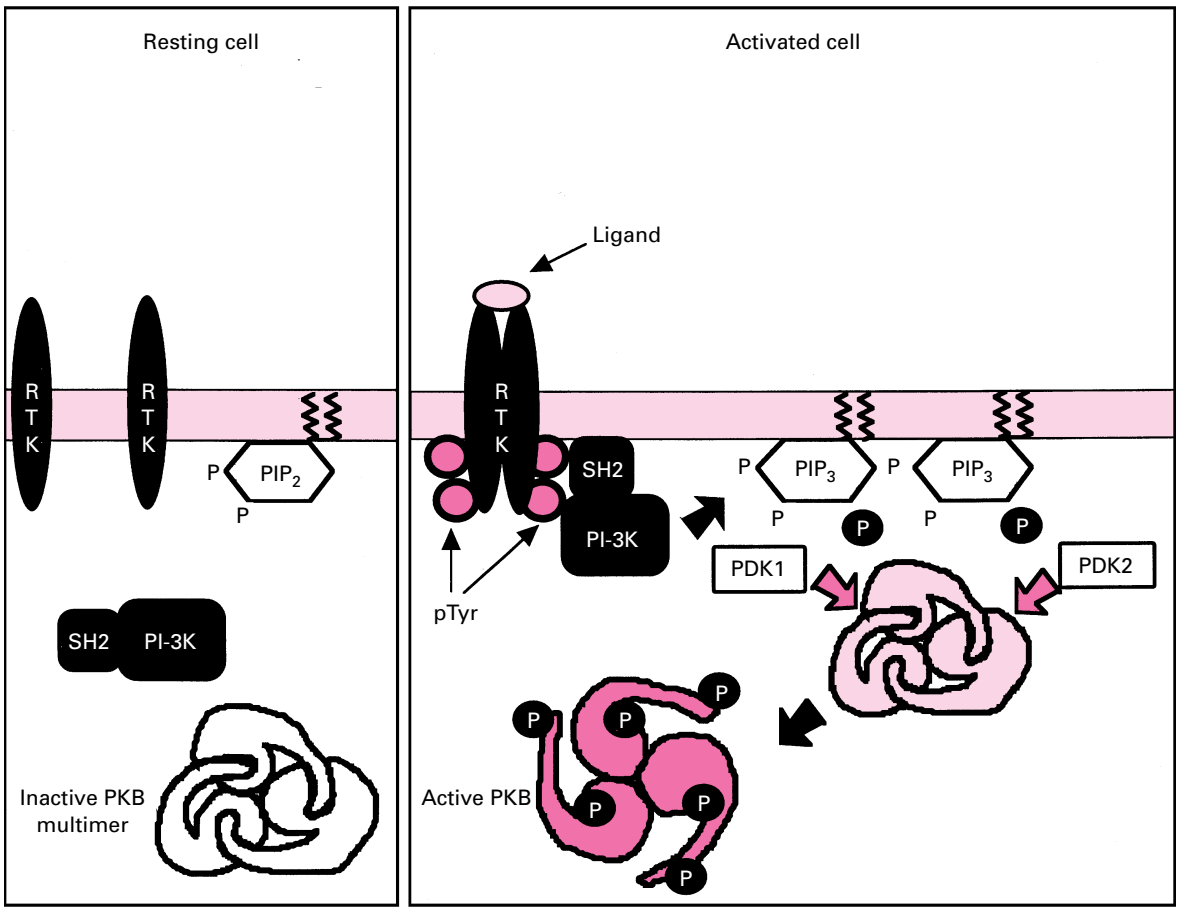
Upon stimulation of PI-3K activity, PKBα becomes phosphorylated at two residues, Thr^{308} within the P-loop of the protein kinase domain and Ser^{473}. Mutagenesis of each residue to alanine revealed that both are required for full activation [49]. Furthermore, substitution with aspartic acid residues rendered PKB partially active and independent of agonists or inhibition by PI-3K antagonists such as wortmannin. The phosphorylation sites are conserved in PKBβ (Thr^{309}, Ser^{474}), although, due to a C-terminal truncation, PKBγ lacks a Ser^{473} homologue (PKBγ Thr^{305} is in an analogous context to the P-loop site in PKBα) [50] (see Figure 1).

PKB complexes

PKB exists as a multimer in cells. It has been demonstrated that the PH domain of PKB mediates interaction between PKB molecules, playing a role in regulation [51]. Transfection of COS-1 cells with an epitope-tagged C-terminally truncated PKB allows co-immunoprecipitation of full-length PKB. Deletion of the PH domain on the epitope-tagged PKB abolishes this interaction. Furthermore, the PH-domain interaction appears to be highly specific, as determined by the failure of the PKBα PH domain to bind to PKBβ in transfected COS cells. Utilizing the yeast two-hybrid system, it was further shown that this PH-domain-dependent interaction required no further adaptor proteins. Surprisingly, the addition of recombinant PKB PH-domain protein to immune complex PKB kinase assays resulted in a significant enhancement of kinase activity.

Bruton tyrosine kinase (Btk) has been reported to associate with PKC family members through its PH domain, although the role of this association is unknown [52]. Similarly, Kikkawa and co-workers have shown that *in vitro* PKB can associate with PKCs, although with no measured effect on activity [53,54]. The functional relevance of these observations *in vivo* remains to be resolved.

Expression of an epitope-tagged kinase-defective mutant of PKB is able to 'fish out' endogenous PKB, since the mutant associates with endogenous wild-type PKB (J. Jin, L. Ruel, B.



Scheme 1 Model of mechanism of activation of PKB by growth factors

In resting cells PI-3K and PKB are cytoplasmic and inactive (via intermolecular inhibition of the protein kinase domain). Upon receptor tyrosine kinase (RTK) activation, the receptor autophosphorylates on tyrosine residues, some of which form docking sites for SH2 domains within the regulatory subunit of PI-3K, recruiting it to its source of phosphoinositides in the membrane. Phosphorylation of these lipids at the 3′-position of their inositol rings acts to recruit PH-domain-containing proteins such as PKB and the PDKs. The assembly of these proteins within the same microdomain facilitates phosphorylation of PKB at the two activatory sites, which relieves the intermolecular inhibition. Activated PKB dissociates from the membrane and moves to the nucleus and other subcellular compartments. Abbreviations: PIP_2, PtdIns(4,5)P_2; PIP_3, PtdIns(3,4,5)P_3.

Staveley, A. Manoukian and J. R. Woodgett, unpublished work). Using this type of hybrid-association analysis we have probed the composition of the multimer as well as the mechanism of activation. Expression of epitope-tagged versions either ATP-binding site (K179A) or T308A/S473/A mutants in cells results in negligible kinase activity recoverable by anti-epitope immunoprecipitation. Co-expression of either of these mutants with wild-type PKB allows recovery of normally regulated PKB in the anti-epitope immunoprecipitates. Surprisingly, the same is true when the ATP-binding-site mutant and the activation-site mutants are co-expressed (J. Jin, L. Ruel, B. Staveley, A. Manoukian and J. R. Woodgett, unpublished work). That is, whereas the two types of mutation independently incapacitate PKB, a complex containing both regains activation potential. Our interpretation of these data is that, in resting cells, the PKB multimer is held in an inactive conformation via intermolecular interactions. Phosphorylation of the activatory phosphorylation sites on one molecule releases the inhibitory effect on a partnering molecule. Since the phosphorylation occurs via 'third party' kinases, the PDKs (see under 'PKB kinases'), the phosphorylated PKB molecule does not need intrinsic kinase activity to deregulate its partner. Likewise, the partner does not actually require phosphorylation of its own sites (T308/S473) for activation; rather phosphorylation must occur on the regulatory sites of the PKB molecule that is inhibiting its activity. Phosphorylation essentially causes intermolecular derepression of the PKB complex.

Phosphorylation at the activatory sites in the PKB complex is both necessary and sufficient for activation. How then is the oncogenic form, v-Akt, activated? Substitution of the two phosphorylation sites with alanine inactivates the oncogenic, gag-fused mutant, indicating that the oncogene is still dependent on phosphorylation for activity. v-Akt is constitutively phosphorylated at the two activatory phosphorylation sites, suggesting that the increased membrane localization of this mutant allows interaction with the T308/S473 kinases. Since v-Akt activity is decreased by wortmannin treatment, these kinases must be as dependent on the presence of 3′-phosphorylated polyphosphoinositides as wild-type PKB.

PKB kinases

PDK1

The mutational analysis of T308 and S473, as well as their local sequence context, suggested that the two sites were independently

regulated [49]. In the case of PKBγ, activation must only be dependent upon the P-loop site, since the C-terminal regulatory site is absent. An ATP-binding site/kinase-inactive PKB in cells retained agonist-dependent phosphorylation of Thr^{308} and Ser^{473} when expressed, suggesting that autonomous kinase activities were responsible for PKB phosphorylation at these sites. Using glutathione S-transferase–PKB as an affinity column, Alessi et al. purified a 67 kDa protein kinase from rabbit skeletal muscle [56]. This enzyme specifically phosphorylated Thr^{308}, partially activated PKB (30-fold) *in vitro* and was dependent for activity on PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [54]. In view of its dependency on these lipids, it was termed PtdIns(3,4,5)P_3-dependent protein kinase-1 (PDK1). A similar (perhaps identical) protein kinase to PDK1 was partially purified from rat brain [57]. Cloning of PDK1 revealed it to be ubiquitously expressed and, like PKB itself, to contain a PH domain but, in this case, C-terminal to its kinase domain [58,59]. Overexpression of PDK1 in cells was sufficient to partially activate PKB and mutation of the PH domain in either PKB or PDK1 significantly reduced activation of PKB [58,60]. Interestingly, treatment of cells with agonists of PI-3K did not result in changes in PDK1 activity or phosphorylation state, suggesting that it is constitutively active and its function is instead regulated by subcellular localization. Scanning of the gene databanks revealed PDK1 to be most similar (54% identity within the kinase domain) to a *Drosophila* protein kinase termed DSTPK61 [58]. This kinase can also phosphorylate Thr^{308} of PKB in a PtdIns(3,4,5)P_3-dependent-manner. DSTPK61 has been implicated in sexual differentiation of *Drosophila* and in oogenesis and spermatogenesis. The DSTPK61 gene undergoes sex-specific splicing, which yields distinct 5′- and 3′-untranslated regions in male and female flies. The role of this sex-specific regulation in PKB functions in *Drosophila* is currently unclear, but may relate to differential regulation of apoptosis (see below).

PDK2

What of the protein kinase that targets PKB at Ser^{473} (operationally termed PDK2)? The phosphorylation status of this residue is unaffected in cells overexpressing PDK1, indicating that this residue is autonomously regulated and is not an autophosphorylation site dependent on Thr^{308} phosphorylation [56]. *In vitro*, the site can be phosphorylated by mitogen-activated protein kinase-activated protein (MAPKAP) kinase-2, an enzyme that is regulated by the p38 MAP kinase pathway [49]. However, MAPKAP kinase-2 is very unlikely to physiologically phosphorylate Ser^{473}, since agents that induce MAPKAP kinase-2 activity, such as tumour necrosis factor-α and IL-1, do not induce PKB activation (or Ser^{473} phosphorylation; J. Jin and J. R. Woodgett, unpublished work) and a variety of PKB agonists, including insulin, insulin-like growth factor (IGF)-1 and PDGF do not significantly activate MAPKAP kinase-2. The identity of the physiologically relevant Ser^{473} kinase is currently unknown, but it is likely to be regulated in an analogous manner to PDK1, since phosphorylation of the two activatory sites on PKB is usually co-ordinated. It is therefore tempting to speculate that PDK2 will contain a lipid-binding domain such as the PH domain in PKB and PDK1.

Synopsis of the activation process

The following model of PKB activation is consistent with the data generated to date (Scheme 1). In resting cells there are detectable but low levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. The levels are insufficient to allow recruitment of all of the elements required for activation of the PKB multimer to the plasma membrane. However, addition of membrane targeting domains to PKB presumably stabilizes the protein kinase within the correct proximity for interaction with the transiently associating PDK1 and PDK2 molecules that are attracted to the membrane by the basal levels of polyphosphorylated PtdIns molecules. Hence, the gag–PKB or membrane-targeted PKB complexes slowly accumulate the necessary phosphorylation events for its activation in the absence of a signal.

Growth-factor activation of wild-type PKB requires catalytic activation of PI-3K via the association of SH2 domains on its 85 kDa regulatory subunit with specific phosphotyrosine residues on activated receptors. This localizes the 110 kDa catalytic domain to the plasma membrane, where it produces PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. This 'microdomain' of PtdIns(3,4)P_2 and PtdIns(3,4)P_3 would attract the two PDKs as well as the PKB multimer to form an activatory complex, resulting in the generation of biphosphorylated PKB complexes. The phosphorylated activation sites de-repress the kinase activity of the complex, and this form of the protein kinase is fully active and independent of phospholipids (as revealed by the properties of the double aspartate mutant). Immunofluorescence studies have revealed that, following activation, transfected PKB concentrates in the nucleus ([61,62]; J. Jin and A. Manoukian, unpublished work). Similar effects can be observed with endogenous PKB using antibodies specific for the phosphorylated form of Ser^{473} in mammalian cells and *Drosophila* embryos (J. Jing, J. R. Woodgett and A. Manoukian, unpublished work). The process is reversed upon dephosphorylation of the two regulatory sites, which causes intramolecular inhibition, rendering the enzyme complex inactive until it is recycled to the membrane.

This model begs the question of why the mechanism is so complicated? Since PKB contains a PH domain, why should it be regulated as a multimeric complex by two additional PH- domain-containing kinases? The data suggest that formation of multi-protein activation structure at the membrane is requisite, and a clue to the complexity issue may be gleaned from the membrane-targeting mutants which are signal-independent. It would appear that the basal concentrations of activatory phospholipids are too high to guarantee that the enzyme is inactive under resting conditions. By requiring the interaction of three different types of molecules, the chances of all three simultaneously co-localizing are substantially reduced. Still, it is not really clear why two PDKs are necessary, especially since PKBγ harbours only one activation site. Perhaps Ser^{473} acts as a 'turbo-charger' to create a second tier of kinase activity. This would imply the existence of two classes of activated PKB within a cell and may allow distinct thresholds of responses depending on the strength of the activatory signal. Further, depending on the proportion of PKB molecules in a multimer, there will be differing degrees of activation. For example, if the PKB complex is a trimer, one of the molecules might be fully dephosphorylated and therefore exerting an inhibitory effect. The trimeric model allows 64 possible phosphorylation states of the two activatory sites in a complex, all but one (the fully dephosphorylated state) having some level of activity, resulting in a smooth spectrum of possible activity levels that will be acutely tuned to the original signal intensity and rate of dephosphorylation of the sites.

It is possible that PKB represents only one target of the PDKs and that other signalling molecules are regulated by these two enzymes. In this scenario, the PDKs could be a processing factory for the PI-3K pathway and modify the functions of additional enzymes unrelated to PKB.

Another, as yet unresolved, question is whether membrane-'anchored' PKB is functionally identical with activated wild-

type protein. In the case of gag–PKB/v-Akt, which is oncogenic, a significant fraction of the protein kinase is soluble. However, other mutants, such as those with GTPase farnesylation sequences such as the CAAX box, are physically restricted to the membrane. Indeed, despite the fact that this mutant is 'catalytically activated', it acts as a dominant negative protein by holding the endogenous PKBs to the membrane (presumably by forming complexes with these molecules) [63]. Translocation of the activated protein kinase to the nuclear compartment suggests that its primary targets may exist there (see below), and while plasma-membrane tethering may result in formation of active PKB, its exclusion from soluble or nuclear targets would likely restrict its biological activity. Conversely, the double aspartate mutant, PKB_{DD}, presumably never interacts with membranes (at least in resting cells) and therefore will not encounter membrane-localized substrates. Comparison of the biology and the substrates targeted by PKB–CAAX and PKB_{DD} with wild-type PKB may provide insights into the critical substrates for particular processes.

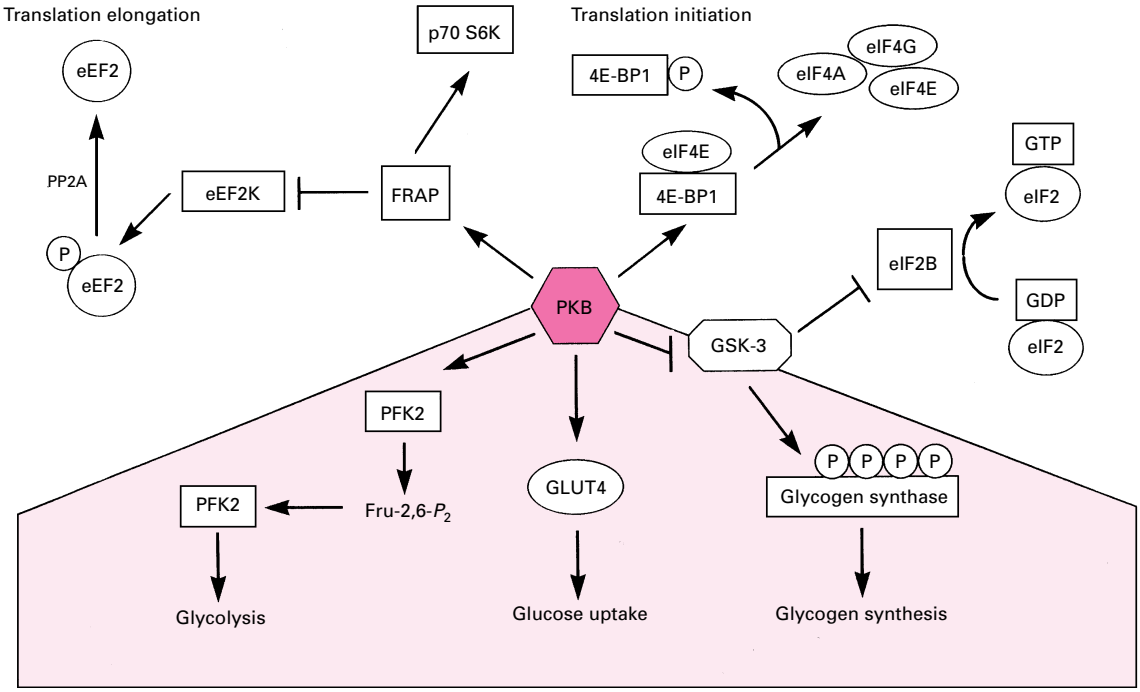
BIOLOGICAL FUNCTIONS OF PKB

Since PKB activity is stimulated by a variety of growth factors and insulin, much effort has focused on the acute metabolic effects associated with its activation (these targets are summarized in Scheme 2). However, a role for the enzyme in protecting cells from apoptosis has recently been defined in mammals and *Drosophila*. The linkage between these functions remains to be established.

Tools for modulating PKB activity

Several approaches have been developed to assess the role of PKB is various cellular processes. Most involve expression of activated alleles or putative dominant negative mutants. While constitutively active mutants exist, they are known to affect the growth potential of cells or to induce differentiation. Currently there are no pharmacological inhibitors of PKB, and attempts to generate transdominant negative mutants have been irreproducible. One consequence of the intermolecular repression model of regulation (see under 'Mechanisms of activation of PKB') is that ATP-binding-site mutants or activation-site mutants retain competency to either activate their partners in a complex or be activated themselves respectively. Such mutants may reduce the overall activation capacity of a PKB complex, but will be incapable of full inhibition unless expressed at extremely high levels. For inhibition to occur, mutants containing both ATP-binding-site and activation-site substitutions would be required. Overexpression of all of these mutants may interfere with other PH-domain-containing proteins (of which there are in excess of 100, some of which are highly specific for 3-phosphorylated phosphoinositides). If the PDKs are required for activating other proteins, overexpression of PKB mutants might titre out not only their PKB activation capacity but also other PDK-dependent processes.

For these reasons, data using mutants of PKB should be interpreted with caution and will likely require revisiting once more effective and specific means of manipulating the activity levels of the protein kinase are found. With these caveats in



Scheme 2 Metabolic functions regulated by PKB

Metabolic functions of PKB (red) can be divided into those that regulate protein synthesis and those that regulate glycogenesis (pink). While a role for PKB has been implicated in many of these processes, a direct link requires further confirmation (see the text for details). Protein synthesis: PKB-mediated phosphorylation of 4E-BP1 (PHAS-I) leads to its dissociation from eIF-4E, resulting in eIF4F complex-formation and increased initiation of translation. Activation of $p70^{S6K}$ by PKB may also lead to increased translation of a specific subset of mRNAs, while inhibition of GSK-3 activity results in the dephosphorylation and activation of eIF2B, leading to increased peptide-chain initiation. Inhibition of eEF2 phosphorylation by rapamycin results in activation of overall peptide-chain elongation. Glucose metabolism: phosphorylation of PFK2 mediated by PKB results in production of fructose 2,6-bisphosphate (Fru-2,6-P_2) and activation of glycolysis, while inhibition of GSK-3 results in increased glycogen synthesis by regulating glycogen synthase phosphorylation. Furthermore, activation of PKB can mediate the glucose uptake via translocation of GLUT4 transporters to the cell surface.

mind, there are several processes in which a role for PKB has been implicated, including intermediary metabolism and protein synthesis.

Metabolism

Glucose transport

One of the major metabolic responses invoked by insulin challenge is the stimulation of glucose transport in muscle and adipose tissue by recruiting glucose transporters (GLUT1 and GLUT4) to the cell surface from intracellular pools. However, the molecular mechanisms regulating this process remain ill-defined. Inhibitors of PI-3K have been reported to block the ability of insulin to stimulate glucose uptake [64–66], while overexpression of active forms of PI-3K at least partially mimic the effect of insulin [67–70]. Several groups have recently provided a possible link between activation of PKB and the stimulation of GLUT4 translocation [21,71,72]. As mentioned above, expression of constitutively active membrane-targeted variants of PKB in 3T3-L1 fibroblasts results in spontaneous differentiation into adipocytes [21,22]. This may be due to the activation of p70 S6-kinase ($p70^{S6K}$), which has also been implicated in this process [24,73]. Subcellular fractionation of 3T3-L1 adipocytes reveals that GLUT4 is mostly located in the low- and high-density microsomes [71]. The largest concentration of endogenous PKB is found in the cytosol, while gag–PKB and the p85 subunit of PI-3K are enriched in membrane fractions. Furthermore, constitutively active PKB induces glucose uptake associated with increased GLUT1 expression and GLUT4 translocation to the plasma membrane [21,71]. The increased glucose influx is associated with lipogenesis. Surprisingly, however, insulin-stimulated glycogen synthesis is inhibited in the presence of active PKB in 3T3-L1 adipocytes [21]. This inhibition of insulin-stimulated glycogenesis may be explained in that persistent activation of this pathway could result in desensitization. Interestingly, PDGF, while able to activate PI-3K in 3T3-L1 adipocytes, is unable to stimulate GLUT4 translocation [74,75]. In these cells PDGF is also unable to induce phosphorylation of PKB, although activity measurements were not performed [76].

Activation of PI-3K by PDGF occurs only in plasma membranes, whereas insulin also induces activation in low-density microsomes, where GLUT4 expression is predominant [75,76]. Furthermore, okadaic acid, a potent stimulator of GLUT4 transport, induces PKB phosphorylation as efficiently as insulin without activation of PI-3K. These observations demonstrate a parallel between the ability of agents to promote GLUT4 translocation and activate PKB, suggesting an important function for PKB in this process. These data further suggest that compartmentalization may play a critical role in restricting which agonists can activate PKB. Recently, insulin treatment of 3T3-LI adipocytes has been reported to increase the association of PKBβ with GLUT4-containing vesicles, suggesting that specific isoforms may play distinct roles and interact with different substrates [77]. The distribution rather than the mere presence of PI-3K and its lipid products, the protein kinase(s) upstream of PKB and the PKBs themselves will thus regulate the outcome of receptor stimulation.

Glycogen synthesis

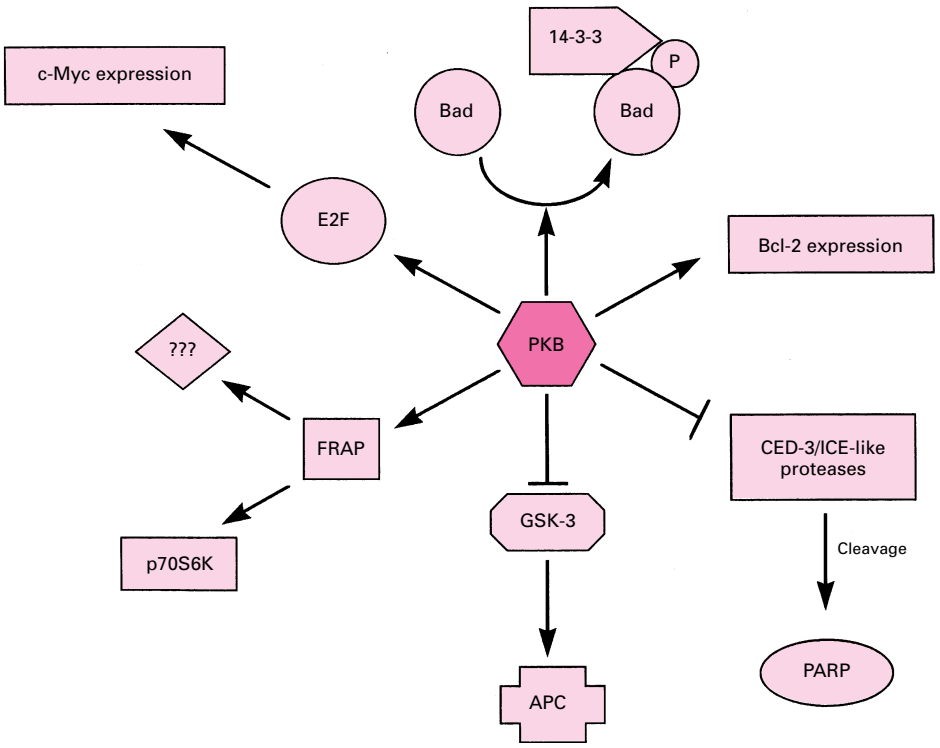
Apart from regulation of glucose-transporter function, insulin stimulation also results in the phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3) and activation of glycogen synthesis [78–80]. Work by Hemmings, Alessi and co-workers has demonstrated that GSK-3 is a substrate of PKB, at least *in vitro* [81]. Treatment of L6 myoblasts with insulin results in a 40–50 % inhibition of GSK-3, a process inhibited by PI-3K inhibitors. PKB from insulin-treated L6 myotubes inactivated GSK-3α and GSK-3β *in vitro*, and this inhibition was reversed by the serine/threonine-specific protein phosphatase PP2A1. Following insulin treatment, the two isoforms of GSK-3 become phosphorylated at Ser^{23} and Ser^{9} respectively. Using a synthetic peptide based around the latter residue, Cross et al. identified the major Ser^{9} kinase in skeletal-muscle extracts as PKB [81]. In support of this, overexpression of PKB in cells reduced GSK-3 activity by a phosphorylation-dependent mechanism. These data would predict that insulin enhancement of glycogen synthesis in adipocytes should be regulated by PKB. However, as mentioned above, constitutively active PKB mutants did not increase the rate of glycogen synthesis in transfected 3T3-L1 adipocytes [27]. An explanation for this inconsistency has been recently proffered by work of Kadowaki and co-workers [82]. In 3T3-L1 adipocytes, expression of gag–PKB did not affect either basal or insulin-stimulated glycogen synthase activity, while wortmannin resulted in complete inhibition of insulin-induced glycogen synthase activity. In contrast, expression of gag–PKB in L6 myocytes revealed an enhanced basal level of glycogen synthase activity essentially comparable with that normally induced by insulin stimulation. This demonstrates a distinct difference between the mechanisms of glycogen synthesis in these two cell types. Indeed, GSK-3 is expressed in L6 myocytes, but not 3T3-L1 adipocytes [83], correlating with the ability of PKB to induce glycogen synthesis only in L6 myocytes. Further work has suggested that insulin activation of glycogen synthase in 3T3-LI adipocytes occurs by at least two distinct mechanisms (including inhibition of GSK-3 and activation of a phosphatase) which are temporally regulated during adipogenesis [84].

Glycolysis

6-Phosphofructose 2-kinase (PFK2) is responsible for generating fructose 2,6-bisphosphate, a key allosteric activator of 6-phosphofructose-1-kinase, the rate-limiting enzyme in mammalian glycolysis. Phosphorylation of PFK2 by insulin, for example, results in enhanced activation. It has been shown that $p70^{S6K}$, MAPKAP kinase-1 and PKB can all phosphorylate and activate PFK2 *in vitro* [85]. However, activation in isolated rat cardiomyocytes by insulin is sensitive to wortmannin, but not to rapamycin or PD098059, suggesting that a PI-3K-mediated pathway is the major one [86]. Thus phosphorylation of PFK2 by PKB may explain how glycolysis is stimulated by insulin.

Protein synthesis

The stimulation of protein synthesis is another important response to insulin observed in a variety of cell types, but predominantly in skeletal muscle and adipose tissue [87]. A critical event is the phosphorylation of 4E-binding protein [4E-BP1; PHAS (pH- and acid-stable)-I] and its dissociation from eukaryotic initiation factor 4E, leading to mRNA translation. This phosphorylation occurs at multiple residues and appears to involve PI-3K and $p70^{S6K}$, since it is wortmannin- and rapamycin-sensitive [88–90]. Since PKB activation is also wortmannin-sensitive and can contribute to $p70^{S6K}$ activation [24], it may play a role in the regulation of insulin-mediated protein synthesis. Indeed expression of gag–PKB in 3T3-L1 or L6 cells results in a much higher phosphorylation of 4E-BP1 even after treatment with wortmannin [82,91]. Furthermore, this resulted in an increase in the rate of protein synthesis in both basal and insulin-treated cells. Thus, through combined activation of $p70^{S6K}$, causing ribosomal phosphorylation and increased translation,



Scheme 3 Anti-apoptotic signalling mediated by PKB

Regulation of programmed cell death is critical in the control of development and homoeostasis of multicellular organisms. PKB (red) has been recently implicated as a critical component of anti-apoptotic signalling through overexpression of various mutant constructs (see the text for details). Phosphorylation of Bad directly by PKB results in its dissociation from Bcl-2 and association with 14-3-3 proteins. Expression of catalytically active PKB mutants promotes the expression of Bcl-2 and c-Myc, possibly through enhanced E2F *trans*-activation. The inhibition of ICE-like proteases (caspases) also results in cellular protection. Inhibition of GSK-3 by PKB regulates adenomatous polyposis coli and β-catenin, which modulates cell adhesion, providing a potential link with anoikis. Abbreviation: APC, adenomatous polyposis coli.

and phosphorylation of 4E-BP1 (either directly or indirectly), PKB may induce increased rates of protein synthesis.

The situation is likely to be more complicated, however, since PDK1 has recently been shown to be capable of inducing $p70^{S6K}$ activation independently of PKB [92,93]. Thus PI-3K-mediated $p70^{S6K}$ activation may well occur via alternative pathways. In support of this, certain agonists can differentially induce PKB and $p70^{S6K}$ [94].

3T3-L1 adipocytes expressing constitutively active myristoylated PKB exhibited a greater-than-20-fold increase in leptin levels [95]. The induction appears to be due to a non-transcriptional mechanism, since levels of mRNA in these cells remain unchanged. Leptin is an adipostatic hormone playing a role in energy balance, and its expression is regulated by insulin. It is tempting to speculate that control of leptin protein synthesis is indeed regulated in adipocytes by the mechanisms outlined above.

Anti-apoptotic signalling mediated by PKB

Cell-survival pathways

Programmed cell death or apoptosis is fundamental in the regulation of development and the control of tissue homoeostasis under conditions of cellular stress [96]. While addition of growth factors and cytokines to cells tends to promote mitogenesis, the removal of these survival factors can result in the induction of programmed cell death. Until recently the molecular mechanisms of induction of apoptosis have remained unclear. However, work utilizing inhibitors of PI-3K has implicated this signalling pathway in regulating the balance between mitogenesis and apoptosis [97–100]. These studies have led to the investigation of the role of PKB in the regulation of both these processes in a variety of cell systems.

Initial studies focused on the mechanism by which growth factors promote the survival of cerebellar neurons [101]. Addition of survival factors such as IGF-1 provide a signal that inhibits apoptosis of neurons independently of p21ras-ERK activation [101]. IGF-1 also stimulates PI-3K, and inhibition of the lipid kinase utilizing the specific inhibitor LY294002 blocks neuronal survival. Transfection of catalytically inactive PKB or the PH domain of PKB alone into cerebellar neurons resulted in increased apoptosis, and this could not be rescued by addition of survival factors. Furthermore, overexpression of wild-type PKB enhanced survival of neurons, and this was not inhibited by LY294002. A protective role for the protein kinase in primary neuronal cultures and in neuronal differentiation has also been demonstrated [102–104].

Further studies have revealed that this model applies to a variety of cell systems. In mesenchymal cells, induction of apoptosis by c-Myc is effectively suppressed by PDGF and IGF-1 [105], while survival is also inhibited by preincubation of cells with LY294002 [106]. In Rat-1 fibroblasts, overexpression of a constitutively active mutant of PI-3K delayed the onset of apoptosis while, surprisingly, an oncogenic mutant of p21ras (V12) resulted in both enhanced apoptosis and an increase in PI-3K activity [107]. This suggests that p21ras triggers both a pro-

apoptotic and PI-3K-dependent anti-apoptotic pathway. The suggestion is that, in the absence of additional factors, the pro-apoptotic pathway will dominate. The use of p21ras partial-loss-of-function mutants has allowed further dissection of the role of specific signalling pathways downstream of p21ras. These mutants, in the context of an active p21ras(V12) background, activate either the MAP kinase or PI-3K downstream signalling pathways. Expression of p21ras(V12-C40), which activates only PI-3K and not MAP kinase, induced PKB activity and was effective in protecting cells against c-Myc-induced death after serum withdrawal. Furthermore, p21ras(V12-S35), which is a potent activator of MAP kinase, but not PI-3K, induced apoptosis in overexpressing cells and did not activate PKB. The expression of gag–PKB, a membrane-targeted constitutively active mutant similar to v-Akt, resulted in substantial apoptotic protection. In contrast with the work of Dudek et al. [101], the overexpression of wild-type PKB alone did not effect the level of apoptosis. The contradictory nature of p21ras in both inhibition and induction of apoptosis is potentially regulated downstream, depending on cell type and environment to generate the correct response in the appropriate circumstance, analogous to c-Myc itself. Irradiation of Rat-1 cells with UV-B also induces apoptosis that is protected by either IGF-1 or introduction of constitutively active mutants of either PI-3K or PKB [108].

Cytokine-mediated survival

PKB has also been demonstrated to play a role in cytokine-mediated cell survival. Addition of IL-3 to 32D cells results in the rapid induction of PKB activity, while mutants of PKB interfere with IL-3-dependent proliferation [37]. Overexpression of wild-type PKB protects 32D cells from apoptosis induced by IL-3 withdrawal. This appears also to be true not only for cell lines, but also in primary human leucocytes, where PKB is rapidly activated by survival factors [39] and rescue from cytokine-depletion-mediated apoptosis is sensitive to wortmannin (P. J. Coffer, unpublished work).

IL-2 controls T-cell survival, clonal expansion and induces progression of T-cells through G1 into S-phase of the cell cycle. IL-2 has also been demonstrated to activate PKB via PI-3K, and this results in membrane translocation of PKB [33,36]. Activation of PKB appears to be responsible for the activation of $p70^{S6K}$ in T-cells via a pathway possibly involving the FRAP kinase [36]. Through the induction of Bcl-2, IL-2 inhibits apoptosis, and through the combination of Bcl-2 and c-Myc it stimulates progression through the cell cycle. Expression of catalytically active PKB mutants in the pre-B-cell line BAF/3, containing an IL-2 receptor defective in PI-3K activation, results in expression of Bcl-2 and c-Myc and inhibition of apoptosis [33]. Thus activation of PKB by IL-2 is responsible for the rescue from apoptosis and activation of cell cycle progression.

Although the mechanisms by which PKB contributes to regulation of apoptosis have become better defined (see below; summarized in Scheme 3), relatively little is known concerning its role in the regulation of cell-cycle progression. One recent study has demonstrated that the cell-cycle regulator E2F is a component of the downstream proliferative machinery regulated by PKB [109]. One of the key events in G1-phase is the activation of E2F, which in turn then binds to promoters and *trans*-activates various genes critical for cell-cycle progression, such as cyclin. Brennan et al. have shown that dominant-negative PI-3K and pharmacological inhibitors of PI-3K both abrogate IL-2 induction of E2F almost completely [109]. Furthermore, expression of gag–PKB also induced a strong transcriptional activation of E2F, suggesting that these proliferation effects are indeed mediated via PKB. However, constitutively active PI-3K was sufficient to induce transcriptional activity of E2F, but was not sufficient to induce DNA synthesis and G1-phase/S-phase transition in Kit255 cells. Thus IL-2-induced DNA synthesis requires the integration of a network of signals that include the activation of PKB.

How does PKB protect cells?

Apoptosis requires the regulation of positive signals such as the induction of the apoptosis inhibitor Bcl-2 and negative signals such as the induction of interleukin-1-converting-enzyme (ICE)-like protease (caspase) activity. Studies in fibroblasts have demonstrated that PKB does not induce the Bcl-2/Bcl-X_L, but does inhibit the Ced3/ICE-like proteases that specifically cleave the poly(ADP-ribose) polymerase, thus promoting survival [110] (Scheme 3). A similar, but varying, observation was made in the lymphoid cell line BAF/3 transfected with the IL-2 receptor [33]. Expression of catalytically active mutants of PKB promoted the expression of Bcl-2 and c-Myc and inhibited apoptosis induced by cytokine deprival.

Bcl-2 interacts with several partners as well as itself. The ability to protect cells from activation of the caspase machinery is critically dependent upon the ratio of anti-apoptotic Bcl-2 like factors (Bcl-2, Bcl-X_L, Mcl-1 and Bag) to pro-apoptotic factors (such as Bcl-X_S, Bax, Bad and Bak). These latter proteins can stifle the protective effects of the Bcl-2 factors by dimerizing with them. One of these, Bad, is regulated by phosphorylation at two serine residues (Ser^{112} and Ser^{136}) [111,112]. Certain haemopoietic cell lines that are dependent upon IL-3 as a survival factor induce phosphorylation of Bad at the two serine residues when treated with IL-3. Phosphorylation of Bad is accompanied by association of the protein with 14-3-3 proteins which recognize phosphoserine in the context of specific amino acid sequences [113]. Under these circumstances Bad phosphorylation correlates with survival, and agents that induce modification of Ser^{112} and Ser^{136} should provide a survival signal.

The first protein kinase reported to phosphorylate Bad at the protective sites was c-Raf, although this kinase additionally phosphorylates other residues on Bad [114]. Several groups have since shown that Ser^{136} can be specifically phosphorylated by PKB *in vitro* and in transfected cells [115–117]. With the use of ion-exchange chromatography, PKB was found to be the major Ser^{136} kinase in PDGF-treated fibroblasts, although other protein kinases, such as calmodulin-dependent protein kinase-II and p90RSK, can also phosphorylate Ser^{136} *in vitro*.

Is phosphorylation of Bad the mechanism by which PKB mediates its protective effect on cells? While this event may play an important role in the survival signalling process, it is likely that there are other contributing factors. For example, Bad expression is rather restricted, and the other pro-apoptotic members related to Bad (of broader tissue expression) have yet to be shown to be regulated by phosphorylation (or PKB). In addition, PKB activation and Bad phosphorylation do not always correlate in haemopoietic cell lines [118]. For example, granulocyte–macrophage-colony stimulating factor stimulates Bad phosphorylation even when PKB activation is blocked. IL-4 actives PKB and promotes cell survival, but does not induce Bad phosphorylation. As mentioned above, following activation, PKB largely translocates to the nucleus, whereas the Bcl-2 family of proteins are usually cytoplasmic or associated with the mitochondria. While this will not prevent interaction between activated PKB and Bad, it does suggest there are other primary targets within the nucleus, possibly transcription factors that mediate transcriptional induction of other survival genes. Fur-

thermore, the physiological relevance of Bad phosphorylation by PKB has yet to be demonstrated in non-transfected cells (i.e. under conditions where the proteins are not overexpressed).

Anoikis

An additional mammalian system in which a role for PI-3K has been implicated is in the regulation of epithelial- and endothelial-cell apoptosis during the induction of cell death upon detachment from matrix, a process termed 'anoikis' [119]. This mechanism normally ensures that cells do not survive outside the context of their normal environment. In Madin–Darby canine kidney (MDCK) cells it has been demonstrated that detachment from the matrix leads to a rapid decrease in the levels of PI-3K products and PKB activity, and vice versa for attachment [120]. Introduction of active mutants of p21ras, PI-3K or PKB protects MDCK cells from apoptosis in suspension, while inhibition of PI-3K with LY294002, or PKB by transient expression of the PH domain alone [51], resulted in enhanced apoptosis of adhered cells [120]. These data suggest a model whereby through engagement of integrins by extracellular-matrix interactions, PI-3K becomes activated and provides a protective signal through activation of PKB. Epithelial-cell detachment results in PI-3K, and thus PKB, inhibition and the apoptotic pathway is triggered. Most importantly, the activation of p21ras by oncogenic mutation, results in the activation of PI-3K in epithelial cells in suspension and thus overrides the normal death signal. These data provide an explanation for how transformed cells are able to grow in the absence of contact with the extracellular matrix and demonstrate a critical role for PKB. The fact that PKB has been found overexpressed in a variety of human tumours may well be due to the fact that it can promote survival of transformed cells under conditions where normally apoptosis would prevent malignant growth.

Transformation

As discussed above, PKB was initially identified as the cellular counterpart of the v-*akt* oncogene and has been found overexpressed in various tumours. The mechanisms by which PKB can transform cells are, however, not clear, although several recent studies have provided the first clues to its transforming potential. Recent data have demonstrated that transfection of NIH 3T3 cells with gag–PKB was insufficient to transform cells. Active Ras mutants capable of activating the Raf/ERK pathway, but incapable of activating PI-3K (Ras V12 E38), were also transformation incompetent [121]. However, co-transfection of these constructs results in a synergistic transformation capability, suggesting that although PKB by itself is a very weak oncogene, it can provide a transforming signal which complements that of the Raf pathway.

The breakpoint cluster region–Abelson (BCR/ABL) oncogenic tyrosine kinase plays an essential role in the pathogenesis of chronic myelogenous leukemia and Philadelphia acute lymphoblastic leukaemia. This kinase activates PI-3K by a mechanism requiring the binding of BCR/ABL to the p85 subunit of PI-3K [122]. Pharmacological inhibition of PI-3K suppresses BCR/ABL-dependent colony formation of murine bone-marrow cells, suggesting a critical role for this pathway in mediating the oncogenic capacity of BCR/ABL. This effect appears to be mediated by PKB, since a kinase-dead PKB mutant inhibited BCR/ABL-dependent transformation of murine bone-marrow cells *in vitro* and suppressed leukaemia development in severe-combined-immunodeficiency (SCID) mice. Furthermore, a mutant of PKB that has been said to be constitutively active (E40K; [33]) markedly enhanced growth-factor-independent colony formation and leukaemia in SCID mice infected with transformation-defective BCR/ABL mutants. PKB was further found to activate c-Myc and Bcl-2 expression in murine bone-marrow cells, suggesting that the mechanisms by which PKB induces transformation is tightly linked with its ability to regulate apoptosis. These studies demonstrate an essential role for PKB in BCR/ABL leukaemogenesis.

Interestingly, recent analysis of the avian sarcoma virus 16 (AV16) genome has revealed that it encodes an oncogene derived from the cellular gene for the catalytic subunit of PI-3K [123]. AV16 induces haemangiosarcomas in chickens and transforms cultured chicken embryo fibroblasts (CEFs). The viral gene, named v-p3k, is a fusion between its cellular counterpart, c-p3k and the viral gag gene. This is, of course, reminiscent of the PKB and gag fusion that is found in v-akt [11]. CEFs transformed with either v-p3k or c-p3k showed elevated levels of both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 as well as enhanced activation of PKB. Thus, as is observed in the p21ras-ERK signalling pathway, several components of the PI-3K signalling pathway have now also been identified as transforming oncogenes.

Genetic analysis of the PKB pathway

Activation of regulatory survival mechanisms are thought to be critical in embryonic development and longevity of adult tissues. Indeed a *C. elegans* PI-3K homologue has recently been implicated in mediating longevity [124]. Analysis of the role of *C. elegans* PKB homologues will shed light on whether this kinase mediates these survival effects [14]. A *Drosophila* homologue of PKB was identified by two groups [15,23]. Recently a mutation in the *Drosophila* gene was isolated which has shed light on the physiological functions of the protein kinase during development [125]. The mutation consists of a single amino acid change in the highly conserved DFG motif in subdomain VII and results in substitution of isoleucine for phenylalanine. This completely inactivates the protein kinase (introduction of an analogous mutation in mammalian PKB has a similar effect), but does not alter protein expression nor phosphorylation at the activation sites (in the case of the *Drosophila* PKB, T342 and S505; Figure 1). The mutation can be efficiently rescued by transgenic expression of wild-type *Drosophila* PKB and to a lesser, but significant, extent by bovine PKB. The inactive protein therefore does not encode a transdominant negative mutant (consistent with the intermolecular-regulation model; see under 'PKB complexes' above).

Loss of PKB function in fruitflies results in embryonic lethality [124]. The gene is maternally expressed (i.e. RNA from the mother is deposited into the egg during oogenesis and directs synthesis of the protein during the early stages of development until zygotic transcription is initiated following nucleation). Germ-line clone analysis demonstrated that, in the absence of zygotic transcription of PKB, there was a severe defect in cuticle formation. This effect is similar to deregulated apoptosis [126]. Further analysis showed that the mutant embryos exhibited ectopic apoptosis, as judged by Acridine Orange staining, which preferentially stains blebbing membranes, and terminal deoxynucleotidyltransferase-mediated dUTP nick-end-labelling (TUNEL) assays (which detects DNA ends). The phenotype could be suppressed by expression of baculoviral P35, an inhibitor of caspase activation, indicating the effect is not simply a consequence of abrogated development [125]. Expression of a dominant interfering mutant of the catalytic subunit of PI-3K phenocopied the effect of the PKB mutation, indicating that

PI-3K signalling acts via PKB to repress apoptosis in flies, a similar situation to that occuring in mammals (A. Manoukian, B. Staveley, L. Ruel, J. Jin and J. R. Woodgett, unpublished work; see above).

TUNEL staining of normal embryos reveals that a small fraction of cells naturally die by apoptosis. Indeed, studies of cell-death regulatory genes (such as ced9 and ced4 in *C. elegans*) have previously demonstrated the occurrence of controlled cell death during development [127,128]. Expression of activated mutants of PKB in *Drosophila* inhibits this normal death but does not interfere with viability (J. Jin, L. Ruel, B. Staveley, A. Manoukian and J. Woodgett, unpublished work). Together these data indicate that activation of PKB via the PI-3K pathway modulates cell death. Since essentially all of the cells in a PKB mutant embryo undergo apoptosis, PI-3K signalling appears to be necessary to actively repress cell death in cycling cells. This would provide a failsafe mechanism to ensure that incomplete mitogenic signalling does not result in viable cells. The repression of normal cell death by activation of the PI-3K/PKB pathway suggests that this signalling system can veto death under certain circumstances and implies that death may be induced by selective repression of the pathway. The mechanism by which this occurs is, as yet, unknown.

CONCLUSIONS

Clearly there has been rapid progress in elucidating the biological regulation and functions of PKB/c-Akt. While the kinase was first cloned in 1991, most of the work describing the enzyme has been published since 1997. This explosive growth in the field is due in large part to the established research on PI-3K and the finding that PKB was activated by PI-3K signalling. As a consequence, much effort has been focused on understanding the role of PKB in providing effector functions for the lipid kinase.

Many questions remain, including the relationship between the roles of PKB in cell death and intermediate metabolism. The advent of improved tools to dissect the function of the protein kinase, as well as genetic analysis in simpler organisms, should shed light on the relevant upstream and downstream components and the relative importance of its functions in different tissues and stages. Most importantly, the next wave of research should determine the therapeutic promise of modulation of the PKB pathway. For example, tumour cells may exhibit selective sensitivity to inhibition of PKB. The protein kinase is potentially a more selective target than PI-3K, since the latter class of proteins clearly play important roles in other cellular processes such as vesicle fusion. On the other hand, if PKB signalling is important for insulin action, antagonists may cause complications in blood sugar homoeostasis. Despite the remarkable progress of recent years, there is much to be discovered about PKB biology, and its established role in regulating metabolism and apoptosis ensures this field will continue to flourish.

Note added in proof (received 24 August 1998)

Integrin-linked kinase (ILK) has recently been shown to phosphorylate PKB at serine-473 [129]. Since ILK activity is dependent on PI-3K activation, this protein kinase may represent PDK2 [129].

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