REVIEW ARTICLE

The PI3K–PDK1 connection: more than just a road to PKB

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Phosphoinositide 3-kinases (PI3Ks) generate specific inositol lipids that have been implicated in the regulation of cell growth, proliferation, survival, differentiation and cytoskeletal changes. One of the best characterized targets of PI3K lipid products is the protein kinase Akt or protein kinase B (PKB). In quiescent cells, PKB resides in the cytosol in a low-activity conformation. Upon cellular stimulation, PKB is activated through recruitment to cellular membranes by PI3K lipid products and phosphorylation by 3′-phosphoinositide-dependent kinase-1 (PDK1). Here we review the mechanism by which PKB is activated and the downstream actions of this multifunctional kinase. We also discuss the evidence that PDK1 may be involved in the activation of protein kinases other than PKB, the mechanisms by which this activity of PDK1 could be regulated and the possibility that some of the currently postulated PKB substrates targets might in fact be phosphorylated by PDK1-regulated kinases other than PKB.

Key words: AGC kinase, Akt, apoptosis, lipid, phosphorylation.

INTRODUCTION

The identification of the pleckstrin homology (PH) domain as a specialized lipid-binding module has been a major breakthrough in the understanding of the mechanism by which membrane-bound lipids convey signals to the cytosol [1]. PH domains are present in a wide variety of proteins which, as a consequence of their interaction with lipids, undergo changes in their subcellular localization, conformation, activation state and/or interaction with other proteins.

Agonist-stimulated phosphoinositide 3-kinases (PI3Ks) generate specific inositol phospholipids that are recognized by a subset of PH domains [2–4]. Protein kinase B (PKB) was among the first proteins known to contain a PH domain, a few years before the function of this domain came to light. The PH domain of PKB specifically binds PI3K lipid products, and a firm link between PI3K and PKB signalling has now been established. Advances in this research area have been fast and extensive. Here we review the most recent progress made in this field, and refer readers to previous reviews in the *Biochemical Journal* for a more extensive background of the earlier discoveries on PKB [5,6].

PI3Ks GENERATE THE ACTIVATING SIGNALS FOR PKB

Lipids made by PI3Ks: 3′-phosphoinositides (3′-PIs)

Inositol-containing lipids consist of a glycerol backbone with fatty acids attached at positions 1 and 2, and an inositol 1-phosphate group at position 3. If this inositol ring carries no additional phosphates, this lipid is called **p**hospha**tid**yl**in**os**i**tol (PtdIns; Figure 1).

In cells, all free –OH groups of the inositol ring of PtdIns – apart from those at the 2′ and 6′ position – can be phosphorylated, in different combinations. A phosphorylated derivative of PtdIns is referred to as a **p**hospho**i**nositide (PI).

PI3Ks phosphorylate the 3′-OH position of the inositol ring in inositol phospholipids, generating 3′-PIs. Inside cells, they produce three lipid products, namely PtdIns3*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. As will be detailed below, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are the lipids that are crucial for the activation of PKB.

Resting cells contain substantial levels of PtdIns3*P*, but hardly any PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3. Stimuli that induce tyrosine (Tyr) kinase activity in cells almost invariably lead to the generation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [7]. This Tyr kinase activity can be provided by receptors with intrinsic Tyr kinase activity (Figure 2) or by non-receptor Tyr kinases [such as kinases from the Src or JAK (Janus kinase) family]. Non-receptor Tyr kinases have been implicated in the activation of PI3Ks by B- and T-cell antigen receptors, many cytokine receptors and co-stimulatory molecules (such as CD28), as well as by cell–cell and cell–matrix adhesion. Likewise, activation of (some) serpentine receptors that are coupled to heterotrimeric G-proteins leads to PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 production [7,8].

Class I PI3Ks generate the lipids that activate PKB

There are multiple isoforms of PI3Ks which can be divided into three classes. Only the class I PI3Ks have been shown to activate PKB in cells and are described in more detail below (for more detailed reviews of PI3K structure and classification, see [2,8–10]).

Abbreviations used: BAD, **B**cl-2/Bcl-X_L-**a**ntagonist, causing cell **d**eath; *BRCA1*, breast cancer susceptibility gene-1; CAMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; ΔPH-PKBα, PKBα with the PH domain removed; eNOS, endothelial nitric oxide synthase; FH, forkhead; GSK, glycogen synthase kinase; IAP, inhibitor of apoptosis; IGF-1, insulin-like growth factor-1; I-κB, cytosolic inhibitor of NF-κB (see below); IKK, I-κB kinase; ILK, integrin-linked kinase; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; MSK, mitogen- and stress-activated protein kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κB; PDE-3B, phosphodiesterase-3B; PDK1, 3′-phosphoinositide-dependent kinase-1; PFK2; 6-phosphofructose-2-kinase; PH, pleckstrin homology; PI, phosphoinositide; PI3K, phosphoinositide 3-kinase; PIF, PDK1-interacting fragment of PRK2; PKA, protein kinase A or cAMP-dependent kinase; PKB, protein kinase B; PKC, protein kinase C; PKG, protein kinase G or cyclic GMP-dependent protein kinase; PP2A, protein phosphatase 2A; PRK2, PKC-related kinase 2; p90-RSK, 90 kDa ribosomal S6 kinase; S6K, S6 kinase; SGK, serum- and glucocorticoid-induced protein kinase; SH2, Src homology 2; Tyr kinase, tyrosine kinase; VEGF, vascular endothelial growth factor.

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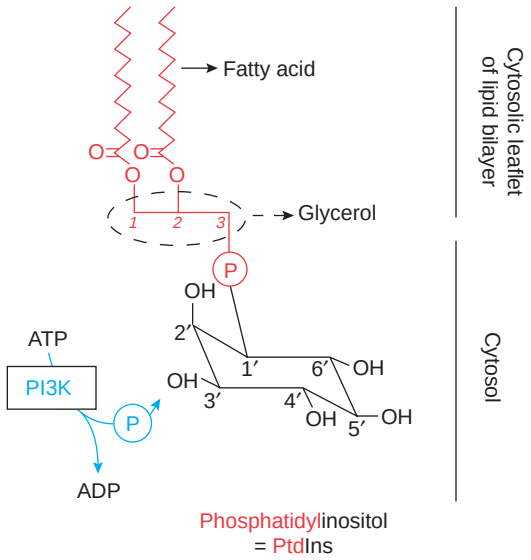

Figure 1 Simplified representation of PtdIns and the point of action of PI3K

By convention, the numbers indicating the carbon atoms in the inositol ring carry a prime, in contrast with the carbon atoms in glycerol itself.

In cells, the preferred substrate of class I PI3Ks appears to be PtdIns(4,5)P_2. The resulting PtdIns(3,4,5)P_3 is then thought to gives rise, via the action of 5′-inositol phosphatases, to PtdIns(3,4)P_2 [11]. All mammalian class I PI3Ks show a similar *in vitro* sensitivity to inhibition by wortmannin ($IC_{50} \approx 5$ nM) and LY294002 ($IC_{50} \approx 1\ \mu$M), two structurally unrelated, cell-permeable low-molecular-mass compounds.

Class I PI3Ks are heterodimers made up of a ≈ 110 kDa catalytic subunit (p110) and an adaptor/regulatory subunit. These enzymes also bind to the monomeric G-protein Ras, but the physiological significance of this interaction in PI3K signalling is not entirely clear. Class I PI3Ks linked to Tyr kinases (Figure 2) and heterotrimeric G-protein-coupled receptors (not shown) are referred to as class I_A and class I_B PI3Ks respectively. The latter PI3Ks appear only to be present in mammals, and the p110γ catalytic subunit complexed with a 101 kDa regulatory protein (p101) is the only class I_B PI3K identified to date.

Class I_A PI3Ks are very diverse in mammals (Table 1): they have three catalytic p110 isoforms (p110α, p110β and p110δ; each encoded by a separate gene) and seven adaptor proteins (generated by expression and alternative splicing of three different genes: p85α, p85β and p55γ). A single class I_A catalytic/adaptor heterodimer is present in the fruitfly *Drosophila melanogaster* (Dp110/p60) and the nematode *Caenorhabditis elegans* (AGE-1/AAP-1). The slime mould (*Dictyostelium discoideum*) has three PI3K catalytic subunits (PI3K1, PI3K2 and PI3K3) with homology with class I_A PI3Ks. No class I PI3K family members have been found in yeast, which is consistent with the absence of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in these organisms. Plant cells do not contain PtdIns(3,4,5)P_3, but have significant levels of PtdIns(3,4)P_2 [12]. No class I PI3Ks have been identified in plants thus far.

Specific PH domains selectively bind PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3

PH domains are globular protein domains of about 100 amino acids found in over 150 proteins to date. Some PH domains bind phospholipids with high affinity. Residues in PH domains essential for high-affinity binding to PIs have recently been identified [13,14]. These residues lie at the N-terminus, in a $KX_{7-13}R/KXR\clubsuit$ motif, where X is any amino acid and ♣ is a hydrophobic amino acid. The basic amino acids in this motif direct interactions with the inositol phosphate groups of PIs. PH domains that lack these residues bind PIs with low affinity [13].

A subset of PH domains preferentially binds to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 over other PIs [1,4,13]. Most PH domains that interact with PtdIns(3,4,5)P_3 also bind PtdIns(3,4)P_2, although frequently with lower affinity. At present there are no known examples of PH domains that interact with PtdIns(3,4)P_2 only. However, the observation that some stimuli (such as ligation of integrins in platelets) increase PtdIns(3,4)P_2 levels without any increase in PtdIns(3,4,5)P_3 [15] indicates that PtdIns(3,4)P_2 may induce signalling pathways distinct from those induced by PtdIns(3,4,5)P_3, possibly by interaction with specific PH domains.

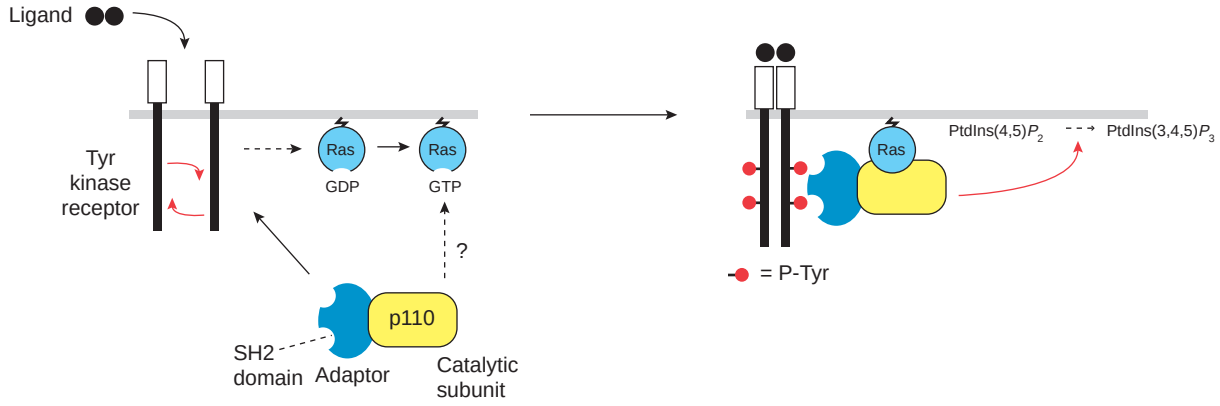

Figure 2 Recruitment/activation of class I_A PI3Ks to receptor Tyr kinases

The p110 catalytic subunit in class I_A PI3Ks exists in complex with an adaptor protein that has two Src-homology 2 (SH2) domains. The latter bind to phosphorylated Tyr residues – in a specific context of surrounding amino acids – that are generated by activated Tyr kinases in receptors and various adaptor proteins. This is thought to allow the translocation of cytosolic PI3Ks to the membranes where their lipid substrates reside. A receptor with intrinsic Tyr kinase activity is shown to dimerize upon binding of its cognate ligand and to transphosphorylate on Tyr residues, creating recognition/docking sites for the SH2 domains of class I_A PI3Ks.

Table 1 Nomenclature of orthologues of mammalian PI3K, PKB and PDK1 in other eukaryotes

'–' Indicates that no orthologue has been found as yet; '(–)' indicates that no homologue has been found in the fully sequenced genome. Between square brackets ([ ]) are proteins that are most related to the mammalian kinase but which are most likely not true orthologues. The GenBank® accession numbers for class I PI3Ks and human PKBs are listed in [10] and Table 3, and can be retrieved at the following URL: http://www2.ncbi.nlm.nih.gov/genbank/query_form.html. The accession number for AAP-1, the *C. elegans* class I_A adaptor subunit that has been shown to act in the AGE-1/DPK1/AKT pathway (C. Wolkow and G. Ruvkun; personal communication) is AF209707. Accession numbers for non-human PKBs are as follows: Dakt1 or *Drosophila* PKB (Z26242), *C. elegans* AKT-1 [the *akt-1* gene gives rise to two splice variants indicated as AKT-1a (AF072379) and AKT-1b (AF072380)] and AKT-2 (AF072381), *D. discoideum* PKB (U15210), *S. cerevisiae* Ypk1 (M21307) and Ykr2 (P18961). Accession numbers for PDK1s are: human PDK1 (AF017995), mouse (AF086625), *D. melanogaster* PDK1 or DSTPK61 (Y07908), *C. elegans* PDK1 [the *pdk-1* gene gives rise to two splice variants indicated as PDK-1a (AF130406) and PDK-1b (AF130407)], *S. cerevisiae* PKH1 (S69675) and PKH2 (Q12236), *S. pombe* KSG1 (X99280) and plant PDK1 (AF132742).

	Class I PI3K subunits			
Species	Catalytic	Adaptor	PKB	PDK1
Mammals	p110α, p110β, p110γ, p110δ	p85α, p85β, p55γ	PKBα, PKBβ and PKBγ	PDK1
D. melanogaster	Dp110	p60	Dakt1 or PKB	PDK1 or DSTPK61
C. elegans	AGE-1	AAP-1	AKT-1a/b, AKT-2	PDK1a/b
D. discoideum	PI3K1, PI3K2 and PI3K3	–	Akt or PKB	–
S. cerevisiae	(–)	–	[Ypk1, Ykr2]	PKH1, PKH2
S. pombe	–	–	–	KSG1
Plants	–	–	–	PDK1

PKB AND PDK1: SERINE/THREONINE PROTEIN KINASES WITH 3′-PI-BINDING PH DOMAINS

PKB/Akt

PKB was identified as a protein kinase with high homology with the protein kinases A and C, and was therefore termed PKB. It is the cellular homologue of the viral oncoprotein v-Akt, and is therefore also referred to as c-Akt or Akt. Another name given to PKB, RAC (**R**elated to **A** and **C**) kinase, is no longer used in order to avoid confusion with the small G-protein Rac. The discovery and cDNA cloning of PKB/Akt/RAC has been reviewed in [5].

PKB/Akt is a 57 kDa Ser/Thr kinase with a PH domain that preferentially binds PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 over other PIs [16,17].

Mammals have three closely related PKB genes, encoding the isoforms PKBα, PKBβ and PKBγ. PKBβ and PKBγ show 81 and 83% amino acid identity with PKBα respectively. All PKB isoforms show a broad tissue distribution and consist of an N-terminal PH domain, a kinase domain and a C-terminal regulatory tail (Figure 3). Two specific sites, one in the kinase domain (Thr^{308} in PKBα) and the other in the C-terminal regulatory region (Ser^{473} in PKBα), need to be phosphorylated for full activation of these kinases (Figure 3; see below).

PKB is cytosolic in unstimulated cells, and some of it translocates to the plasma membrane upon activation of PI3K, where it becomes activated [18–20]. Active PKB then appears to detach from the plasma membrane and to translocate through the cytosol to the nucleus [18,19]. The mechanism of this translocation is unclear.

PH domain-containing PKB homologues have been identified (Table 1) in fruitflies (*Drosophila* PKB or Dakt1; [21,22]), *Dictyostelium* [23] and *C. elegans* (AKT-1 and AKT-2; the *akt-1* gene gives rise to two splice variants indicated as *akt-1a* and *akt-1b*; [24]). *C. elegans* AKT-1 has phosphorylation sites equivalent to both Thr^{308} and Ser^{473}, whereas AKT-2 apparently has only the site equivalent to Thr^{308}, raising the possibility that these proteins are differentially regulated [24].

The kinases in *Saccharomyces cerevisiae* (a budding yeast) most related to PKB (termed Ypk1 and Ykr2; [25,26]) do not possess a PH domain and are more likely to be homologues of the serum- and glucocorticoid-induced protein kinases (SGKs) [27,28] than homologues of PKB. Thus far, no PKB homologues have been reported in *Schizosaccharomyces pombe* (a fission yeast) or plants.

PDK1

PDK1 is a 63 kDa Ser/Thr kinase ubiquitously expressed in human tissues. It consists of an N-terminal kinase domain and a C-terminal PH domain (Figure 3). *In vitro*, its PH domain binds PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 with higher affinity than other PIs such as PtdIns(4,5)P_2. Its affinity for PIs in general appears to be significantly higher than that of PKBα.

PDK1 was first identified by its ability to phosphorylate Thr^{308} of PKBα *in vitro* [17,29,30]. As this activity was absolutely dependent on the inclusion of PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 in the reaction mixture, this kinase was given the name 3′-**p**hosphoinositide-**d**ependent **k**inase-**1** [29].

As purified or recombinant PDK1 only phosphorylated Thr^{308} of PKBα and not Ser^{473}, it was assumed that the phosphorylation of Ser^{473} would be catalysed by a distinct protein kinase, tentatively termed PDK2 [29]. Recent evidence (discussed below) suggests that PDK1 itself, rather than a distinct kinase, may phosphorylate PKB on Ser^{473} *in vivo*.

PDK1 seems to exist in an active, phosphorylated configuration under basal conditions and appears to be refractive to additional activation and phosphorylation upon cell stimulation with agonists which activate PI3K [31–33].

In unstimulated cells, overexpressed PDK1 is mainly cytosolic, with some localization at the plasma membrane [34,35]. PH domain mutants of PDK1 that do not interact with 3′-PIs are entirely cytosolic, indicating that the membrane association of PDK1 is dependent upon a functional PH domain [35]. Using a surface-plasmon-resonance-based binding assay, PDK1 was found to interact with PtdIns(4,5)P_2 with significant affinity, raising the possibility that the association of PDK1 at the membranes of unstimulated cells could be mediated by interaction with PtdIns(4,5)P_2 rather than with PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 ([35]; note that PtdIns(4,5)P_2 is always present in cells, in contrast with PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which are nominally absent in unstimulated cells). However, it should be noted that PDK1 was found not to interact significantly with PtdIns(4,5)P_2 in other studies employing either a lipid-vesicle binding assay or a protein/lipid overlay assay [17,36]. It is also controversial whether PDK1 translocates to the plasma mem-

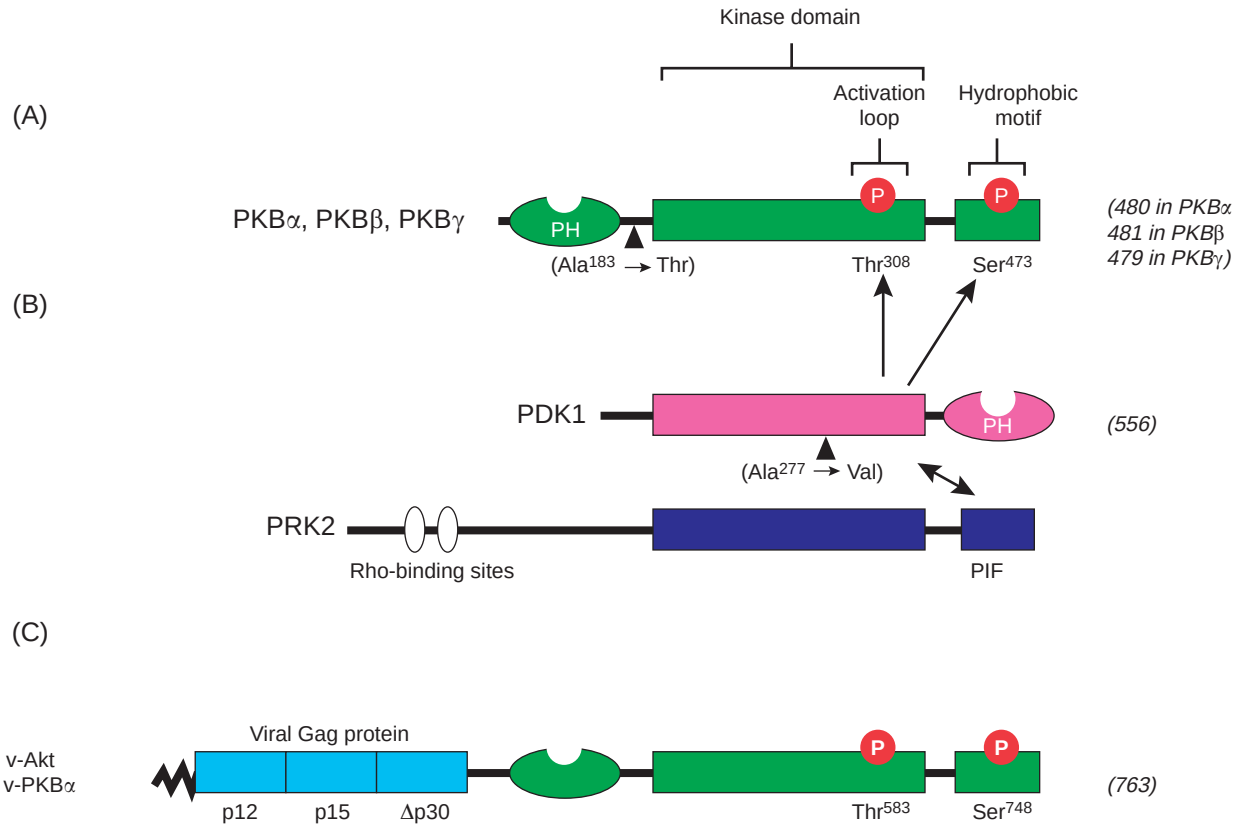

Figure 3 Schematic overview of the protein architecture of PKB family members and PDK1

The total number of amino acids in the human proteins is given in italics and in parentheses. (**A**) Ala → Thr, location of an activating mutation in *C. elegans* AKT-1 [24]. Note that this Ala is not conserved in mammalian PKBs. Note: originally it was thought that (rat) PKBγ lacked 23 residues at the C-terminus (in comparison with PKBα and β) and thus did not contain the regulatory Ser phosphorylation site equivalent to Ser^{473} of PKBα [168]. However, subsequent work revealed that human PKBγ [175,176] and rat PKBγ (M. Deak and D. R. Alessi, unpublished work) do indeed possess the regulatory C-terminal Ser phosphorylation site found in other PKB isoforms, suggesting that the original PKBγ was either a splice variant or not a full-length clone. (**B**) Single long arrows, phosphorylation of PKBs by PDK1; arrowhead (▲), Ala → Val activating mutation in *C. elegans* and human PDK1 [37]; thick double-headed arrow, binding of PIF (= PDK1-interacting fragment) of PRK2 with the kinase domain of PDK1. (**C**) v-Akt consists of the tripartite Gag protein (p12, p15 and Δp30) fused to PKBα via a 21-amino-acid spacer (20 amino acids which are based on the 5′-untranslated region of PKBα; one amino acid is encoded by three nucleotides absent from both the *gag* and *PKB*α genes). The zig-zag line represents N-terminal myristoylation in Akt.

brane in response to growth-factor stimulation. One study reported that PDK1 translocated to the membranes of endothelial cells in response to platelet-derived growth factor (PDGF) [34], but one of us (D. R. A.) has been unable demonstrate any translocation of PDK1 to the membrane either in endothelial cells or in other cell lines (see [34,35], where the possible reasons for this discrepancy are discussed). PDK1 appears to be excluded from the nucleus in both stimulated and unstimulated cells [34,35].

There is a PH domain-containing PDK1 homologue (Table 1) in fruitflies [31], *C. elegans* (PDK1, which has two splice variants, PDK1a and PDK1b; [37]), fission yeast (called KSG1; [38]) and plants [39]. Budding yeast has two PDK1 homologues (referred to as PKH1 and PKH2) which lack a PH domain [26,40].

The *Drosophila* and *C. elegans* PDK1 homologues possess the PH domain motif required for high-affinity PI-binding. This motif is absent from the PH domains of fission-yeast and plant PDK1 [13,14] and, indeed, the PH domain of plant PDK1 interacts only weakly with PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 [39].

ACTIVATION OF PKB BY PI3K AND PDK1

It should be stressed that the overall activation mechanism of PKB is complex and not completely understood, not least because it is difficult to mimic lipid-dependent phenomena in the test tube. Furthermore, the subcellular distribution of endogenous PKB and PDK1 has not been firmly established, and the evidence on the localization of these enzymes has been gathered using ectopic expression studies only (see above).

Biochemical studies on the activation mechanism of PKB

Below, we summarize the experimental data accumulated in this field and present a model for activation of PKB that is compatible with these observations (Figure 4).

Initial studies indicated that PKB might be activated directly by PtdIns3*P* [41] or PtdIns(3,4)P_2 [42], but subsequent work failed to reproduce these results. The activation of PKBα by insulin and growth factors is accompanied by its phosphorylation on Thr^{308} in the kinase domain (in the so-called activation- or T-loop; see below) and Ser^{473} in the C-terminal regulatory domain (in the so-called hydrophobic motif; see below) [43]. Activation of PKB and phosphorylation of both these residues are abolished if the cells are incubated with PI3K inhibitors prior to stimulation with agonists [43]. Phosphorylation of both of these residues is essential for maximal activation of PKBα [43]. The PKBβ and PKBγ isoforms are also activated in response to agonists which activate PI3K, by phosphorylation of the residues equivalent to Thr^{308} and Ser^{473} [44]. Mutation of either of these residues of PKBα to Ala does not prevent the other residue from becoming

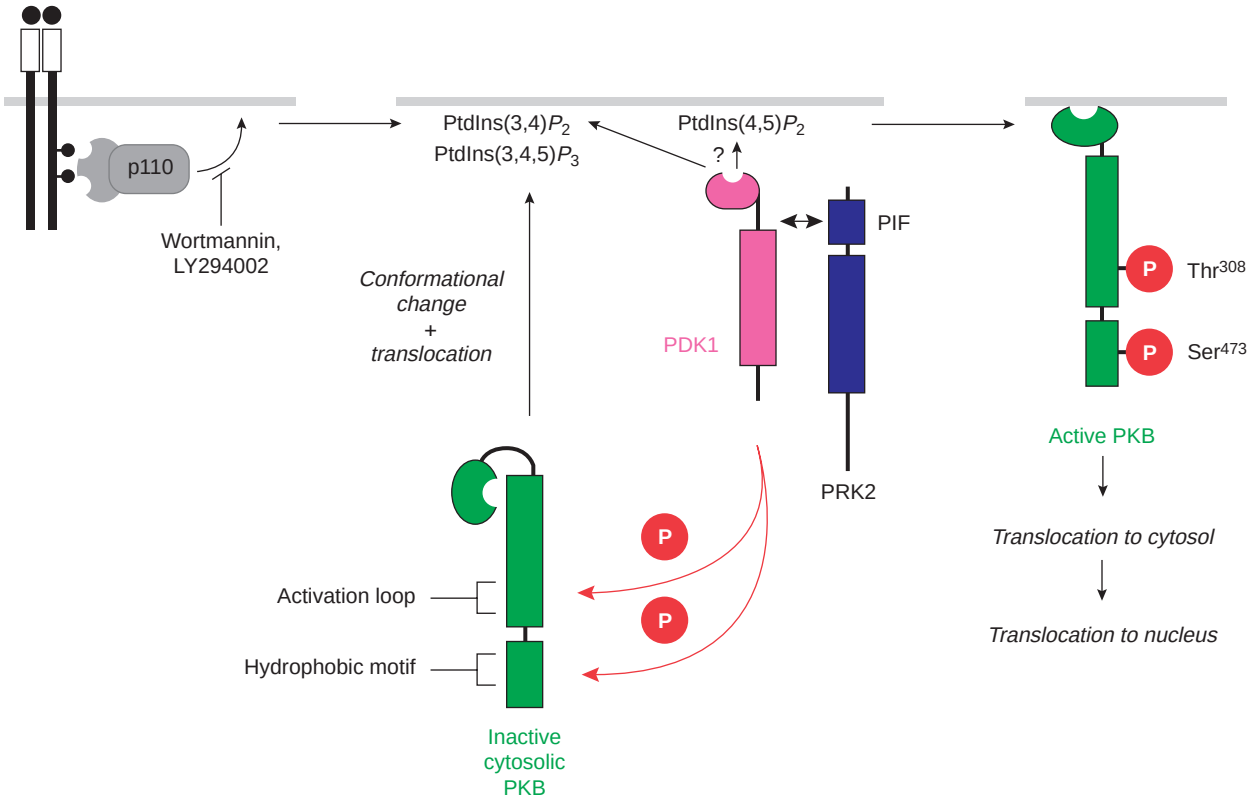

Figure 4 Model of the activation mechanism of PKB by PI3K and PDK1

See text for details.

phosphorylated in response to insulin, indicating that the phosphorylation of these residues can occur independently from each other [43].

PDK1 phosphorylates PKBα on Thr^{308} [29] and the equivalent Thr residue in PKBβ and PKBγ [44]. This phosphorylation is enhanced over 1000-fold in the presence of lipid vesicles containing low amounts of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 but not PtdIns(4,5)P_2 or any other PI tested [29]. The requirement for PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 in this reaction is mediated (at least in part) by the interaction of these lipids with (1) the PH domain of PKB, which may alter the conformation of PKB so that Thr^{308} becomes accessible to PDK1, and (2) the PH domain of PDK1, which most likely co-localizes PDK1 with its PKB substrate at the surface of the lipid vesicles. These conclusions are supported by the following observations (all based on *in vitro* experimentation):

- in the absence of PtdIns(3,4,5)P_3, full-length PKB is not phosphorylated by PDK1. Removal of the PH domain of PKBα (ΔPH-PKBα), however, allows this phosphorylation to occur [30,31]. A full-length point mutant of PKBα that cannot interact with PtdIns(3,4,5)P_3 is also not phosphorylated by PDK1 in the presence of PtdIns(3,4,5)P_3 [30], and PtdIns(3,4,5)P_3 is still required for the phosphorylation of PKBα by ΔPH-PDK1 [31].
- the interaction of PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 with PDK1 does not appear to *directly* activate PDK1, as the rate at which it phosphorylates ΔPH-PKB or other substrates which do not interact with 3′-PIs (see below) is not further increased by PtdIns(3,4,5)P_3. It may therefore appear somewhat surprising to find that PH domain mutants of PDK1 that do not interact with PtdIns(3,4,5)P_3 phosphorylate PKB at only 5% of the rate of wild-type PDK1 in the presence of phospholipid vesicles containing PtdIns(3,4,5)P_3 [31]. However, this observation is likely to be explained by the requirement for PDK1 and PKB to co-localize on PtdIns(3,4,5)P_3-containing lipid vesicles in order for PDK1 to phosphorylate PKB efficiently. It is also possible that PtdIns(4,5)P_2 could function to localize PDK1 to the lipid/aqueous interphase, but PtdIns(3,4,5)P_3 would *still* be required to recruit and induce the appropriate conformational change in PKB before PDK1 can activate it. As described below, PDK1 can become directly activated by PtdIns(3,4,5)P_3 when complexed to a C-terminal fragment of protein kinase C (PKC)-related kinase 2 (PRK2) (see below).

PDK2 is possibly a ‘modified’ PDK1

A major outstanding question is the identity of the kinase that phosphorylates PKB on Ser^{473}. It has been claimed that integrin-linked kinase (ILK) is capable of phosphorylating Ser^{473} of PKBα *in vitro*, and when overexpressed in cells [45]. A recent study indicates that ILK may not directly phosphorylate PKB at Ser^{473}, but rather promotes phosphorylation of this site by an indirect mechanism [45a]. It should also be noted that ILK is an unusual kinase as it lacks certain motifs present in the kinase domain of other protein kinases (such as the conserved Mg^{2+}-binding DFG motif in subdomain VII of the kinase) [46].

Recent findings have shown that PDK1 can interact with a fragment of the C-terminus of PRK2 [47]. This PRK2 fragment has been termed ‘**P**DK1-**i**nteracting **f**ragment’ (PIF; Figure 3B). Remarkably, the interaction of PDK1 with PIF converts PDK1 from an enzyme that phosphorylates PKBα only on Thr^{308}

into a kinase that phosphorylates *both* Thr^{308} and Ser^{473} of PKBα. Furthermore, the interaction of PIF with PDK1 converts the latter from a form that is not activated by PtdIns(3,4,5)P_3 directly into a form that is activated $\approx$ 3-fold by PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2, but not by PtdIns(4,5)P_2 [47]. The major kinase activity from brain extracts that phosphorylates Ser^{473} of PKBα in a PtdIns(3,4,5)P_3-dependent manner has been partially purified and is immunoprecipitated with a PDK1 antibody [47]. These findings could explain the observation by Stokoe et al. [30] that the ability of a partially purified PDK1 from brain cytosol to phosphorylate and activate ΔPH-PKB was still enhanced in the presence of PtdIns(3,4,5)P_3, suggesting that the PDK1 in this preparation could have been complexed to PRK2 or a related protein.

The physiological relevance of these findings awaits further investigation, but these data suggest that PDK1 in complex with another protein(s) (which may be PRK2, a proteolytic fragment of PRK2 or a related protein) may mediate the phosphorylation of PKBα on Ser^{473} rather than a distinct enzyme. PDK1 can form complexes with various PKC family members [48] and p70-S6 kinase (p70-S6K) [49,50], and it is possible that these interactions modulate PDK1 activity towards PKB (and other substrates; see below).

Model for activation of PKB

Taken together, the data mentioned above are compatible with the following model for activation of PKB (Figure 4). PKB exists in the cytosol of unstimulated cells in a low-activity conformation. Upon activation of PI3K, PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 are synthesized at the plasma membrane and PKB interacts through its PH domain with these lipids. This induces (1) the translocation of PKB from the cytosol to the inner leaflet of the plasma membrane and (2) a conformational change which converts PKB into a substrate for PDK1, perhaps by exposing the Thr^{308} and Ser^{473} phosphorylation sites. PDK1 – which may already be membrane-localized by virtue of its PH domain bound to, for example, basal levels of PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 [or PtdIns(4,5)P_2] – then phosphorylates and activates PKB. PDK1 in this location of the cell may also be complexed with a PKC-related kinase or an equivalent protein, not only enabling it to phosphorylate PKB on both Thr^{308} and Ser^{473}, but also inducing responsiveness of PDK1 to PtdIns(3,4,5)P_3/PtdIns(3,4)P_2.

Attachment of a membrane-targeting motif to the N-terminus of either full-length or ΔPH-PKBα is sufficient to induce activation of PKB, and its phosphorylation on Thr^{308} and Ser^{473} in unstimulated cells [18,51,52]. These findings help to explain why the oncogenic form of PKB (v-Akt) is highly active, even in unstimulated cells: a large fraction of this kinase is located at the plasma membrane owing to fusion at its N-terminus with the myristoylated Gag viral protein (Figure 3). It is possible that basal concentrations of PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 are sufficient to induce the opening up of membrane-targeted PKB, allowing its phosphorylation and activation. These observations also indicate that there must be a significant amount of PDK1 present at the membranes of unstimulated cells. However, if basal levels of PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 exist in cells and PDK1 is already at the plasma membrane, why is wild-type PKB then not phosphorylated and activated all the time? One possible explanation lies in the fact that PKB interacts with PtdIns(3,4,5)P_3 with over 10-fold lower affinity than PDK1, and that the levels of PtdIns(3,4,5)P_3 in unstimulated cells are too low to recruit wild-type PKB to the membranes. Alternatively, the addition of an epitope-tag or Gag protein to the N-terminus of PKB might affect its general activation characteristics.

Genetic studies in *C. elegans*

Genetic studies in the nematode worm *C. elegans* have confirmed that PDK1 is a downstream target of AGE-1 (the *C. elegans* class I_A PI3K catalytic subunit) and an upstream activator of PKB. In the worm, AGE-1, PDK1 and the two PKB isoforms, AKT-1 and -2, function in an insulin/insulin-like growth factor-1 (IGF-I) receptor-mediated signalling pathway that regulates metabolism, development and longevity [24,37]. Inactivating mutants in PDK1 prevent the activation of AKT-1 and AKT-2, as well as physiological processes known to be downstream of these kinases [37]. A high copy number of wild-type *akt-1*, but not *akt-2*, can bypass the need for PI3K, indicating that the *akt-1* gene is most potent in the insulin/IGF-I pathway. *Both* genes, however, need to be inactivated in order to give a phenotype equivalent to the loss of the insulin receptor, indicating that the activities of AKT-1 and AKT-2 are probably redundant.

This work also revealed activating mutations in PDK1 and AKT-1 [24,37]. In PDK1 the mutation results in the replacement of a conserved Ala in the kinase domain (the equivalent of Ala^{277} in human PDK1) with a Val (Figure 3). The equivalent substitution also activates human PDK1 2-fold *in vitro*, which demonstrates that the activity of PDK1 *can* be increased above its basal level [37]. As mentioned above, biochemical experimentation in mammalian cells has thus far failed to detect any activation of PDK1 above its basal level during cellular stimulation, and it is at present not clear whether physiological stimuli can mimic the activatory mutations in PDK1 that were revealed by these genetic screens [31–33].

The activating mutation in AKT-1 leads to an Ala^{183} → Thr substitution in the region between the PH domain and the kinase domain (Figure 3). Although this Ala is not conserved in mammalian PKB isoforms, these observations suggest that the linker region between the PH and kinase domain might play an important role in PKB activity.

The activated *C. elegans* PDK1 mutant no longer needs PI3K in order to fulfil its function in the organism, but still requires AKT-1 and AKT-2. The requirement for PI3K is also no longer seen for the activated AKT-1 mutant. Likewise, increased expression of wild-type *akt-1* also relieves the requirement for PI3K. These observations suggest that 3′-PI lipids might not be required for the activation of PDK1/AKT-1/AKT-2 in *C. elegans*. However, in the absence of AGE-1 PI3K, PtdIns(3,4)P_2 might still be provided by the other *C. elegans* PI3Ks. This includes the *C. elegans* class II PI3K which, by analogy with mammalian PI3Ks, might produce PtdIns(3,4)P_2 by 3′-phosphorylation of PtdIns4*P* [8]. Alternatively, PtdIns(3,4)P_2 might be generated by phosphorylation of PtdIns by the *C. elegans* class III PI3K and a PtdIns3*P* 4-kinase, similar to what has been observed in integrin-stimulated platelets [15]. A compensatory overexpression of the *C. elegans* Class II and III PI3Ks upon inactivation of the class I PI3K can also not be excluded at present. In addition, the activating mutations in PDK1 and AKT-1 might sensitize these proteins to PtdIns(3,4)P_2 such that they are activated by sub-threshold levels that would not normally activate wild-type PDK1 and AKT-1.

CAN PKB BE ACTIVATED INDEPENDENTLY OF PI3K?

Several reports have indicated that PKB can be activated in cells by a mechanism independent of PI3K activation, for example in response to heat shock, or increases in intracellular Ca^{2+} or cAMP [53–57].

Konishi et al. [54] reported that PKB is activated by heat shock in NIH3T3 fibroblasts and that this response was not inhibited by wortmannin. Using the same as well as other cells,

we confirmed that PKB is activated by heat shock as well as oxidative stress, but in our hands this activation was completely suppressed by the PI3K inhibitors wortmannin and LY294002 [58].

Agonists which increase Ca^{2+} levels in cells have been reported to activate PKB in a PI3K-independent manner through the Ca^{2+}/calmodulin-dependent protein kinase kinase (CAMKK) [56]. This study reported that CAMKK phosphorylates PKB on Thr^{308} in the absence of PtdIns(3,4,5)P_3. However, another group found that CAMKK is not capable of inducing the phosphorylation of PKB [32] and others have not been able to induce PKB activation in neuronal, kidney or fibroblast cell lines by agonists which increase intracellular Ca^{2+} levels (D. R. Alessi, M. Shaw and P. Cohen, unpublished work).

It has also been reported that *transfected* PKB can be partially activated ($\approx$ 2-fold) in a PI3K-independent manner by agents that increase cAMP levels [57]. We have been unable to measure any activation of *endogenous* PKB in several cell lines by such stimuli despite being able to measure large increases in cAMP and cAMP-dependent protein kinase/protein kinase A (PKA) activity in these cells (M. Shaw, P. Cohen and D. R. Alessi, unpublished work).

It should be noted that the yeast PDK1 homologue, Pkh1, which does not interact with phosphoinositides, only phosphorylates PKB on Thr^{308} in the presence of PtdIns(3,4,5)-P_3/PtdIns(3,4)P_2. This indicates that Thr^{308} of PKB may be exposed only when its PH domain interacts with PtdIns-(3,4,5)P_3/PtdIns(3,4)P_2. It is therefore not clear how PKB could become phosphorylated at Thr^{308} by agonists that do not increase PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 levels in cells.

DOWNSTREAM OF PKB (FIGURE 5)

The minimum sequence motif required for efficient phosphorylation of small peptide substrates by PKB is RXRXXS/T✤, where X is any amino acid, and ✤ is a bulky hydrophobic residue [phenylalanine (F) or leucine (L)] [59]. All three PKB isoforms possess indistinguishable substrate specificity towards synthetic peptides [44]. Most of the sequences surrounding the phosphorylation sites in the proposed PKB substrates discussed below (underlined) conform to the above consensus motif.

Table 2 summarizes the criteria that have been used to define PKB substrates. It should be stressed that, for many of these proteins, considerable evidence that they are indeed phosphorylated by PKB in cells is still lacking. In several of the studies mentioned below, PKB and/or its substrates have been overexpressed in cells. While this type of experiment shows that PKB *can* phosphorylate these targets, they do not prove that this also occurs under physiological conditions in cells. Some studies also make use of dominant-negative mutants of PKB. Only two types of dominant-negative PKB have been shown to be effective in cells at preventing agonist-induced phosphorylation of glycogen synthase kinase 3 (GSK3, a well-characterized PKB substrate [60]; see below). These are triple PKB mutants (termed AAA-PKB) in which the activating phosphorylation sites Thr^{308} and Ser^{473}, as well as Lys^{179} in subdomain II of the kinase domain, have been mutated to Ala [61,62], and a mutant (termed Caax-PKB) in which the membrane-targeting signal of a Ras isoform, Ki-Ras (the so-called Caax motif), has been attached to its C-terminus [63]. Although many groups have reported that a kinase-dead PKB in which only Lys^{179} has been mutated to Ala acts as a dominant-negative protein, it does not prevent agonist-induced GSK3 inactivation in several cell types ([63] and D. R. Alessi, unpublished work). When using dominant-negative PKB constructs we recommend that it is verified that these prevent agonist-induced phosphorylation of GSK3. However, great care should be taken when interpreting the results of overexpression

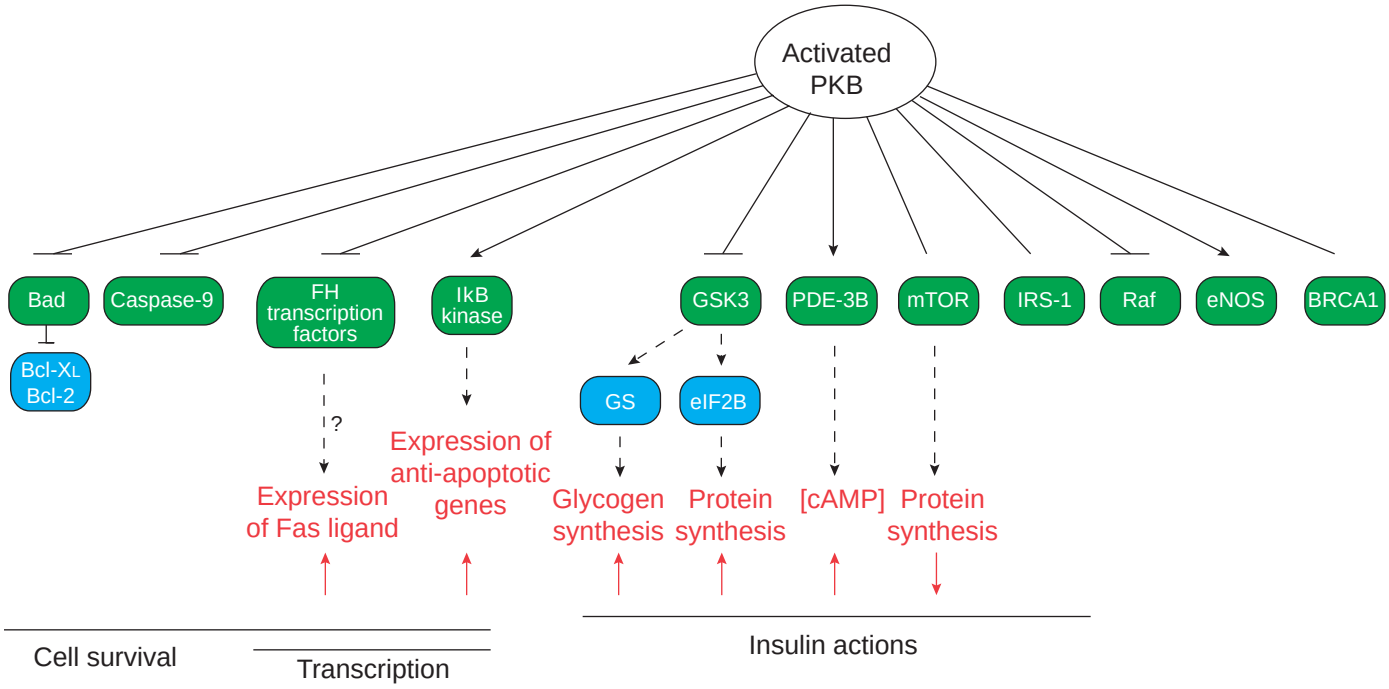

Figure 5 Targets of PKB phosphorylation

A solid line indicates direct phosphorylation by PKB, whereas a broken line indicates a signalling link which does not necessarily involve direct phosphorylation events. A horizontal bar and an arrowhead indicate, respectively, an inhibitory and stimulatory impact of PKB-mediated phosphorylation. The absence of such symbols indicates that the impact of PKB-mediated phosphorylation is unclear at the moment. GS, glycogen synthase; eIF2B, eukaryotic initiation factor-2B (a GDP/GTP exchange factor for the translation initiation factor eIF2). It is important to mention that, for several of the targets shown, considerable evidence that they are indeed phosphorylated by PKB in cells is lacking.

Table 2 Criteria used to determine whether a substrate is phosphorylated by PKB

All the substrates listed are phosphorylated by PKB *in vitro*. Abbreviations used: Transf., substrate is transfected; Endog., endogenous substrate; Y, yes; NR, not reported; Dom.Neg., dominant negative. Column 1 implies that the substrate in cells is phosphorylated by extracellular signals which activate PI3K. Column 2 implies that phosphorylation of a substrate in response to agonists which activate PI3K in cells is prevented by the PI3K inhibitors wortmannin or LY294002. Column 3 implies that the site at which PKB phosphorylates the substrate has been mapped *in vitro*, or *in vivo* in response to stimuli which activate PI3K. (pm), the site has been determined by peptide-mapping procedures; (mut), the phosphorylation site has been predicted and this residue mutated to prevent phosphorylation; (pAb), the phosphorylation site has been established using phosphospecific antibodies. Column 4 implies that overexpression of a constitutively active form of PKB leads to phosphorylation of the substrate in cells or that a dominant-negative mutant of PKB prevents the phosphorylation of the substrate in response to agonists which activate PI3K. (179) is a PKB mutant in which Lys^{179}, which lies in subdomain II of the kinase domain, has been mutated to Ala; (AAA) is a triple PKB mutant in which Lys^{179}, Thr^{308} and Ser^{473} have been changed to Ala; (AA) is the double PKB mutant in which Thr^{308} and Ser^{473} have been mutated to A; (Caax) is a mutant of PKB possessing a Ras membrane-targeting motif at its C-terminus [63]. Column 5 implies that a stable interaction between PKB and the substrate has been detected in cells. Column 6 indicates whether genetic evidence has been obtained to support the substrate being downstream of PKB.

	Column 1		Column 2		Column 3			Column 4		Column 5	Column 6
	Phosphorylation of substrate after PI3K activation		Effect of PI3K inhibitors on substrate phosphorylation		Identification of phosphorylation site *in vitro* and in cells			Effect of phosphorylation of substrate in cells obtained by overexpression of:		Interaction of PKB with substrate	Genetic evidence
Substrate	Transf.	Endog.	Transf.	Endog.	*In vitro*	Transf.	Endog.	Active-PKB	Dom.Neg.PKB		
GSK3	Y	Y	Y	Y	Y(pm)	Y(pm)	Y(pm)	Y	Y(Caax)	Y	None
PFK2	Y	Y	Y	Y	Y(pm)	Y(pm)	Y(pm)	Y	Y(AAA)	NR	None
PDE-3B	Y	Y	Y	Y	Y(mut)	Y(mut)	NR	Y	Y(AA)	Y	None
FKHR	Y	Y	Y	Y	Y(pAb)	Y(pAb)	Y(pAb)	Y	NR	NR	Y
BAD	Y	Y	Y	Y	Y(pAb)	Y(pAb)	Y(pAb)	Y	Y(179)	Y	None
hCaspase-9	Y	NR	NR	NR	Y(pm)	Y(pm)	NR	NR	NR	NR	None
IKKα	Y	NR	NR	NR	Y(mut)	NR	NR	NR	NR	Y	None
eNOS	Y	Y	Y	Y	Y(mut)	Y(mut)	NR	Y	Y(179)	Y	None
mTOR	Y	Y	Y	Y	(pAb)	Y(pAb)	Y(pAb)	Y	NR	NR	None
IRS	Y	Y	NR	NR	NR	NR	NR	NR	NR	Y	None
BRCA1	Y	Y	NR	Y	Y(mut)	NR	NR	NR	Y(179)	NR	None
Raf	Y	Y	NR	Y	Y(mut)	NR	Y(pAb)	Y	Y(179)	Y	None

of dominant-negative forms of PKB, as they might bind substrates, preventing them from becoming phosphorylated by a distinct kinase that is its 'natural' kinase under normal conditions. In addition, these dominant-negative PKBs may function by binding PDK1, preventing it from activating kinases other than PKB (discussed below).

1. Targets of PKB in promoting cell survival

Overexpression of PKB has an anti-apoptotic effect in many cell types, resulting in a *delay* of cell death [64–66]. This might be important for the cancers in which PKB is overexpressed; this is the case for PKBα and PKBγ in breast-cancer cells [67,68] and for PKBβ in pancreatic [69] and ovarian [70] carcinomas. Certain mutated forms of PKB are oncogenic in young chickens [70a]. An overactivation of PKB (without gene amplification) may also be important in disease. This is exemplified by human cancers in which the PTEN tumour suppressor gene is mutated and inactivated. PTEN encodes a 3′-phosphatase that converts PtdIns(3,4)P_2 into PtdIns4P, and PtdIns(3,4,5)P_3 into PtdIns(4,5)P_2. The inactivation of PTEN results in increased levels of 3′-PIs, leading to elevated PKB activity [71–77], which might contribute to transformation. Another example are mice that lack the Tyr kinase lyn, and in which the B cells possess a higher PKB activity than wild-type cells. This may contribute to the hyperproliferation seen in these B cells and in the development of auto-immune diseases [78,79].

Thus far, no satisfactory explanation has been provided as to how PKB delays cell death. Research in this area has mainly focused on finding direct links between PKB and the cell-death machinery, and alternative explanations have so far been largely unexplored. For example, control of cellular and mitochondrial metabolism and function by insulin and other growth/survival factors is likely to be crucial for cell survival, and some of the PKB targets mentioned in the paragraphs below could be important in this regard. It is also intriguing that inducible inhibition of PI3K (leading to a reduction in endogenous PKB activity) blocks cellular proliferation but does *not* induce apoptosis [80], suggesting that PKB might play a role in cell-cycle regulation and/or surveillance mechanisms. Indeed, PKB has been shown to activate the transcription factor E2F [81,82], a crucial regulator of cell-cycle checkpoints, and to increase cyclin D1 levels [83,84].

Below, we describe components of the apoptotic machinery which have been reported to be targets of PKB. These include the Bcl-2 family protein BAD (**B**cl-2/Bcl-X_L-**a**ntagonist, causing cell **d**eath), human caspase-9 and transcriptional regulation of apoptotic (e.g. Fas ligand) and anti-apoptotic genes.

(a) BAD

This protein forms a heterodimer with the anti-apoptotic proteins Bcl-2 or Bcl-X_L and thereby prevents them from exerting their anti-apoptotic function. When phosphorylated on Ser^{112} or Ser^{136}, BAD no longer interacts with Bcl-2 or Bcl- X_L, allowing them to inhibit apoptosis. PKB can phosphorylate BAD on Ser^{136}, and this might be one way by which PKB contributes to cell survival ([85] and references cited therein). It is important to mention, however, that not all cell types express BAD and that cell survival can be regulated independently of both PKB activation and BAD phosphorylation [86–89]. Other kinases that can phosphorylate BAD include PKA and kinases activated by the classical mitogen-activated protein kinase (MAPK) pathway [86,90,91], such as the 90 kDa ribosomal S6 kinase (p90-RSK) [92].

PKB does not not seem to affect the expression of Bcl-2, Bcl-X_L and the pro-apoptotic protein Bax [66,89,93], although one group reported increased expression of the Bcl-2 protein after PKB overexpression [94].

(b) Human caspase-9

Caspase-9 is a protease crucial in the initiation and possibly later stages of apoptosis [95,96]. Human caspase-9 has been reported to be phosphorylated and inhibited by PKB [97]. It is not yet clear how important and/or general this is for PKB-mediated regulation of apoptosis, as the residue which PKB phosphorylates in human caspase-9 is not conserved in the mouse, rat and monkey homologues [98]. Consistent with this is the observation that mouse caspase-9, unlike human caspase-9, is not phosphorylated by PKB *in vitro* [98]. Evidence has also been presented that PKB promotes cell survival by intervening early on in the apoptosis cascade, *before* cytochrome *c* release from the mitochondria and caspase-9 activation, possibly by maintaining the integrity of the mitochondrial membrane [89].

(c) Forkhead (FH) transcription factors

Recently, two transcription-based mechanisms by which PKB can interfere with cell death have been reported. The first is via three members of the large family of forkhead transcription factors. These are FKHR, FKHRL1 and AFX, which have been shown to be directly phosphorylated on three residues by PKB [99–103]. A link between PKB and FH transcription factors was first established in *C. elegans*, where the insulin receptor/PI3K/PDK1/AKT pathway suppresses the action of the DAF16 gene which encodes a transcription factor belonging to the FH family [24,37,104].

In serum-starved mammalian cells, FH transcription factors reside predominantly in the nucleus, whereas upon cellular stimulation, they are found mainly in the cytosol. This differential subcellular localization is regulated by (amongst others) PKB [99]. The currently held view is that phosphorylation of FH transcription factors by PKB prevents them from stimulating gene transcription [103]. This phosphorylation promotes the export of FKHR, FKHRL1 and AFX from the nucleus to the cytosol, where they interact with the 14-3-3 proteins, effectively holding them in the cytoplasm, away from their target genes in the nucleus [99,100,102]. In one study [99], FH transcription factors have been implicated in expression of the Fas ligand, which can induce cell death upon autocrine or paracrine production. Upon phosphorylation by PKB, FH transcription factors are retained in the cytosol and therefore the Fas ligand is not expressed, allowing the cells to survive [99]. It remains to be established how general this link with Fas signalling is in different cell systems. Phosphorylation of nuclear targets by PKB is consistent with its documented translocation from the cytosol to the nucleus upon activation [18,19].

(d) IκB kinases (IKKs)

A second transcription-dependent anti-apoptotic action of PKB may operate via the transcription factor NF-κB (nuclear factor-κB) [105–108]. When bound to its cytosolic inhibitor, IκB, NF-κB is sequestered in the cytoplasm. Upon its phosphorylation by **IκB k**inase**s** (IKKs), IκB is degraded. This allows NF-κB to move to the nucleus and activate the transcription of (among others) anti-apoptotic proteins such as inhibitor-of-apoptosis (IAP) proteins c-IAP1 and c-IAP2 [109,110]. PKB has been reported to associate with, and activate, IKKs [105–108]. The mechanism by which PKB might activate these kinases is unclear, but one study [107] claims that PKB directly phosphorylates and activates the α form of the IKKs. It should be noted, however, that the predicted site of phosphorylation on IKKα does not lie in an optimal consensus sequence for PKB phosphorylation (see

above), as it possesses a glycine residue in its C-terminus rather than a hydrophobic residue [59,107]. In addition, no evidence has been presented that this residue of IKKα becomes phosphorylated in cells in response to extracellular signals which activate PI3K.

2. Role of PKB in insulin signal transduction

Nearly all the physiological responses of a mammalian cell to insulin are prevented by PI3K inhibitors [6], and the picture is rapidly emerging that PKB could mediate many of the cellular effects of insulin [63,111–116]. This conclusion is based on the overexpression of constitutively activated forms of PKB in insulin-responsive cells having the same effect as insulin [113, 116–118]. However, the role of PKB in mediating a number of these insulin effects is controversial (see [119,120]). Consistent with PKB playing a role in insulin signalling, PKB activation by insulin has been found to be diminished in adipocytes from human patients suffering from Type 2 diabetes [121,122].

The targets of PKB that could be involved in its action downstream of insulin are GSK3 [60], **p**hospho**die**sterase-3B (PDE-3B), **m**ammalian **t**arget **o**f **r**apamycin (mTOR) [123,124], the FH family member FKHR [101,103,125] and **i**nsulin **r**eceptor **s**ubstrate-**1** (IRS-1) [126,127] (reviewed in [128]; Figure 5).

Insulin-related pathways appear to be conserved in evolution. As discussed above, genetic studies in *C. elegans* have established an insulin/IGF-1-receptor-mediated signalling pathway that regulates metabolism, development and longevity [24,37]. An analogous pathway is being uncovered in fruitflies, involving the mammalian insulin-receptor homologue Inr, the IRS homologue Chico, Dp110/p60 PI3K and *Drosophila* PKB [129–132]. This signalling pathway regulates cell size, cell number and, ultimately, the size of organism [133,134].

3. Raf protein kinase

The Raf protein kinase is activated by translocation to the plasma membrane by its interaction with activated Ras. Raf phosphorylates and activates MAPK kinase, which in turn leads to the activation of MAPK, which regulates many physiological processes such as proliferation, differentiation and apoptosis (reviewed in [135]). Recent studies indicate that PKB can inhibit the Raf protein kinase by phosphorylating it at Ser^{259}. This leads to interaction of Raf with 14-3-3 proteins resulting in an inhibition of the Raf–MAPK signal transduction pathway [136]. This cross-talk pathway may not operate ubiquitously, as PKB does not inhibit Raf in undifferentiated myoblast precursor cells, but it does when these cells are differentiated into skeletal-muscle myotubes [137]. It should also be noted that, in a significant number of cell lines, PI3K inhibitors (and therefore blockade of PKB) either have no effect on agonist-induced Raf activation or in some cases actually inhibit the activation of Raf, suggesting that PKB can contribute to Raf activation under certain circumstances [138–140].

4. Endothelial nitric oxide synthase (eNOS)

Maintained production of NO by endothelial cells has been implicated in many biological effects, such as gene regulation and angiogenesis. PKB becomes activated in endothelial cells in response to VEGF (vascular endothelial growth factor) or shear stress (the pressure coming from the blood flow). PKB thereby phosphorylates and activates eNOS [141–145], and this underlies the sustained production of NO by endothelial cells. PKB-mediated NO synthesis may also be part of the mechanism by which VEGF, produced by tumour cells, induces angiogenesis of surrounding blood vessels, thereby promoting increased blood flow to the tumour.

5. BRCA1, the breast-cancer-susceptibility-gene-1 (*BRCA1*) product

BRCA1 encodes a nuclear phosphoprotein of 220 kDa. It is thought to be a tumour suppressor that plays a role in transcriptional regulation and DNA repair [146]. Evidence has been presented that PKB phosphorylates BRCA1 in a region that is important for its nuclear translocation [147]. The impact of this phosphorylation on BRCA1 is not clear, but it may interfere with the nuclear translocation of BRCA1 and thus its biological activity.

PDK1 PHOSPHORYLATES AND ACTIVATES OTHER KINASES BESIDES PKB: IMPLICATIONS FOR ASSIGNMENT OF A PROTEIN AS A PKB SUBSTRATE

T-loop and hydrophobic motifs in AGC kinases

Amino acid sequences very similar to those surrounding Thr^{308} and Ser^{473} in PKB are conserved in all members of the AGC family of Ser/Thr protein kinases which includes PK**A**, protein kinase **G** (PK**G**), and PK**C** isoforms as well as all PKB isoforms, p70-S6Ks, p90-RSKs, SGKs and **m**itogen- and **s**tress-activated protein **k**inases (MSKs).

The residues equivalent to Thr^{308} of PKBα lie in a segment of the kinase domain between subdomains VII and VIII, known as the *activation loop* or *T-loop* (Table 3). The residues equivalent to Ser^{473} of PKBα appear to be unique to the AGC subfamily of

Table 3 Alignment of the amino acid sequences surrounding the T-loop and the hydrophobic motif of AGC kinases

All the sequences and accession numbers pertain to human proteins. The underlined residues correspond to those that become phosphorylated.

	Activation or T-loop	AGC hydrophobic motif	NCBI accession number*
Consensus...	TFCGTXXYXAPE L D	FXXFSY YTF	
PKBα	TFCGTPEYLAPE	FPQFSY	Y15056
PKBβ	TFCGTPEYLAPE	FPQFSY	P31751
PKBγ	TFCGTPEYLAPE	FPQFSY	AF135794
SGK1	TFCGTPEYLAPE	FLGFSY	AAD41091
SGK2	TFCGTPEYLAPE	FLGFSY	AF169034
SGK3	TFCGTPEYLAPE	FLGFSY	AF169035
PKCα	TFCGTPDYIAPE	FEGFSY	4506067
PKCβI	TFCGTPDYIAPE	FAGFSY	4506069
PKCβII	TFCGTPDYIAPE	FEGFSF	P05127
PKCγ	TFCGTPDYIAPE	FGGFTY	P05129
PKCδ	TFCGTPDYIAPE	FAGFSF	5453970
PKCζ	TFCGTPNYIAPE	FEGFEY	4506079
PKCι	TFCGTPNYIAPE	FEGFEY	4506071
PRK1	TFCGTPEFLAPE	FLDFDF	AAC50209
PRK2	TFCGTPEFLAPE	FRDFDY	AAC50208
p70-S6Kα	TFCGTIEYMAPE	FLGFTY	AAA36410
p70-S6Kβ	TFCGTIEYMAPE	FLGFTY	4506739
p90-RSK1	SFCGTVEYMAPE	FRGFSF	I38556
p90-RSK2	SFCGTVEYMAPE	FRDFSF	P51812
p90-RSK3	SFCGTIEYMAPE	FRGFSF	CAA59427
MSK1	SFCGTIEYMAPD	FQGYSF	AAC31171
MSK2	SFCGTIEYMAPE	FQGYSF	AAC67395
PKA	TLCGTPEYLAPE	FSEF†	P22612
PDK1	SFVGTAQYVSPE	‡	AF017995

* The protein sequences listed can be accessed in the NCBI database at the following URL: http://www.ncbi.nlm.nih.gov/Entrez/protein.html

† The PKA protein terminates at this position.

‡ PDK1 does not possess a hydrophobic motif.

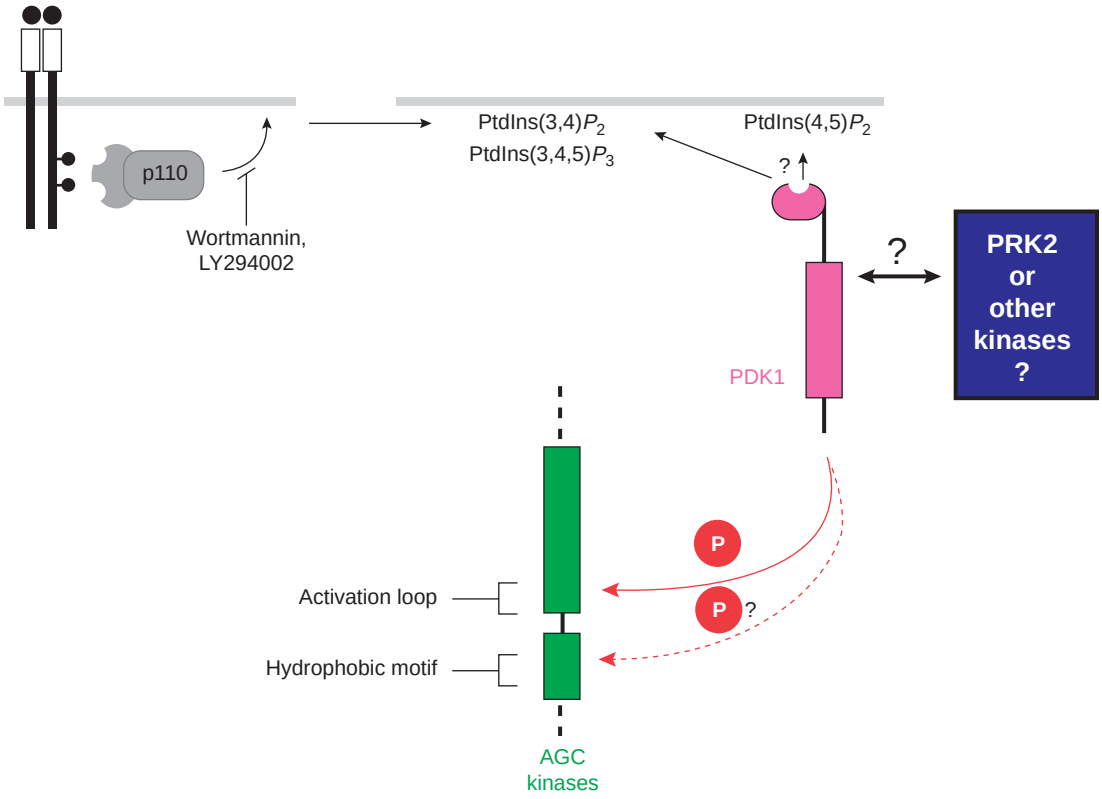

Figure 6 Phosphorylation of AGC kinases by PDK1

See text for details.

protein kinases and lie in a *hydrophobic motif* (Table 3) located C-terminally to the catalytic domain in a region that displays high homology between different AGC family members.

It is now clear that phosphorylation of the residues in the activation loop and hydrophobic motif plays an important role in the regulation of the activity of all AGC kinase family members. In the case of PKB, p70-S6K, p90-RSK and SGK, phosphorylation of the hydrophobic motif is required for maximal activity. In the case of conventional PKC isoforms, mutation of the phosphorylation residues in the hydrophobic motif has no effect on PKC activity. Instead, phosphorylation on these residues functions to stabilize the kinase [148]. Acidic residues (Asp or Glu) rather than Ser/Thr are found in the hydrophobic motif of the atypical isoforms of PKC (PKCζ, PKCι and PKCλ) and the PKC-related kinases (PRK1 and PRK2), perhaps mimicking a constitutively phosphorylated state. Unlike other AGC protein kinases, PKA does not possess a residue equivalent to Ser^{473} of PKB. Instead, its amino acid sequence terminates with the sequence -FSEF, corresponding to the first part of the hydrophobic motif -FXXFS/TY/F in other AGC kinases (Table 3). Nevertheless, this C-terminal region of PKA plays an important role, as its mutation or deletion greatly diminishes PKA activity [149].

PDK1 phosphorylates T-loop motifs in AGC kinases

PDK1 has now been shown to play a central role in activating many of the AGC subfamily members (reviewed in [150,151]). Apart from phosphorylating PKB on Thr^{308}, PDK1 phosphorylates the equivalent residues on PKC isoforms [48,152,153], p70-S6K [32,154], the three isoforms of SGK [27,28,155] and PKA [156] (Figure 6).

PDK1 itself is a member of the AGC subfamily of protein kinases and, like other members of this family, has to be phosphorylated at its T-loop (residue Ser^{241}; Table 3) in order to be active [33]. As PDK1 expressed in bacteria is active and phosphorylated on Ser^{241} [33], it is possible that PDK1 can phosphorylate itself at this site, leading to its own activation. Ser^{241} is also very resistant to dephosphorylation by protein phosphatase-2A (PP2A) [33], and PDK1 dephosphorylated by PP2A is likely to be able to rephosphorylate itself at this residue when performing subsequent kinase assays in the presence of MgATP. This explains why PDK1 was found not to be inactivated by phosphatase treatment in early work on this kinase [29].

Could PDK1 phosphorylate the hydrophobic motif of AGC kinases other than PKB?

As the hydrophobic motif is highly conserved all AGC kinases (Table 3), it has been speculated that a common kinase may be capable of phosphorylating this motif in all AGC kinases. To date a confusing picture has emerged as to the possible identity of such upstream kinase(s). The finding that PDK1 has the ability to phosphorylate PKBα at its hydrophobic motif suggested that PDK1 might be able to phosphorylate other AGC kinase family members on this residue.

Recent evidence suggests that conventional isoforms of PKC are capable of intramolecular autophosphorylation at their hydrophobic motif once they become phosphorylated at their T-loop site by PDK1 [157]. Consistent with this, catalytically inactive mutants of conventional PKC isoforms, when transfected in cells, are not phosphorylated at their hydrophobic motif, and inhibitors of PKC also prevent phosphorylation of the hydrophobic motif. Recent work has also implicated an

atypical PKC isoform (PKCζ) in mediating the phosphorylation of the novel PKC isoform (PKCδ) at its hydrophobic motif [158].

In contrast with conventional PKC isoforms, catalytically inactive forms of p70-S6K [159] and PKB [59] when introduced in cells are still phosphorylated at their hydrophobic motifs in response to stimuli which activate PI3K, suggesting that these residues are not phosphorylated by an intramolecular autophosphorylation reaction. A recent study provides evidence that PDK1 activity is required for the IGF1-induced phosphorylation of the hydrophobic motif as well as the T-loop of p70-S6K *in vivo* [50], but it is not yet established whether PDK1 directly phosphorylates this residue in cells. *In vitro*, the PDK1-induced phosphorylation of p70-S6K on these residues is not dependent upon 3′-PIs, yet PI3K inhibitors block this activity of PDK1 in transfected cells. Perhaps the sensitivity of PDK1 to PI3K lipids in cells is conferred by the interaction of PDK1 with other proteins, as discussed above [47].

Recent data indicate that mTOR can phosphorylate p70-S6K directly at its hydrophobic motif *in vitro* [160,161]. In addition, treatment of cells with rapamycin (which inhibits mTOR) blocks p70-S6K phosphorylation at this motif [160,161]. It is unlikely, however, that mTOR phosphorylates the p70-S6K hydrophobic motif site *in vivo*. This is based on the observation that a p70-S6K mutant that lacks its N-terminal 46 amino acids is still activated and phosphorylated at its hydrophobic motif in a PI3K-dependent manner under conditions where mTOR is inactive (i.e. in the presence of rapamycin) [162]. Secondly, the negative effect of rapacymin on p70-S6K phosphorylation in cells might be due to a rapamycin-stimulated PP2A-like activity [163–165] which could dephosphorylate p70-S6K.

Regulation of PDK1 activity – conversion of AGC kinases into PDK1 substrates

PDK1 does not appear to be directly activated or inhibited by any extracellular signal tested to date [31–33]. It is likely that PDK1 will instead be controlled both by substrate-directed mechanisms (discussed below) and by PDK1-interacting proteins, which will not only regulate PDK1′s activity, substrate specificity and cellular localization, but may also enable PDK1 to become responsive to PI3K lipid messengers. Indeed, the interaction of PDK1 with PIF converts PDK1 into a kinase that is capable of phosphorylating both Thr^{308} and Ser^{473} of PKB *and* is directly activated by PtdIns(3,4,5)P_3/PtdIns(3,4)P_2 [47]. PDK1 can form complexes with various PKC family members [48] and p70-S6K [49,50], and it is possible that these interactions modulate PDK1 activity towards its substrates. There have been also reports on an association between PKC family members with p70-S6K [49,166] or PKB [167,168], providing further evidence that AGC family members can form multimeric complexes in cells.

AGC kinases can be classified into four groups based on the mechanism by which they might be converted into a substrate for PDK1 phosphorylation at their T-loop.

Group 1: lipid-dependent substrates

This group includes PKB, which is converted into a PDK1 substrate following its interaction with PtdIns(3,4,5)P_3, as well as certain PKC isoforms, which may be converted into PDK1 substrates through their interaction with phorbol esters, diacylglycerol, phosphatidylserine, phosphatidylcholine or PtdIns(3,4,5)P_3 [48,152,153].

Group 2: phosphorylation-dependent substrates

This group includes p70-S6K [32,154] and SGK isoforms [27,28,155], whose T-loop phosphorylation is highly enhanced following phosphorylation of the hydrophobic motif. p70-S6K and SGK are activated by a class I PI3K-dependent mechanism *in vivo*, and it is possible that a key regulatory phosphorylation event controlling the activity of these kinases is the phosphorylation of the hydrophobic motif. This hydrophobic motif phosphorylation does not significantly activate these enzymes, but instead converts them into a conformation which can be phosphorylated at the T-loop by PDK1, leading to their further activation.

Group 3: Rho-dependent substrates

These include PRK1 and PRK2, whose interaction with Rho-GTP through their N-terminal Rho-binding domains results in a conformational change that enables PDK1 to interact with these kinases and phosphorylate their T-loop [169].

Group 4: substrates which are constitutively phosphorylated at their T-loop residue in cells (the phosphorylation of this site is not influenced by PI3K or other known inputs)

This group includes PKA [156] and p90-RSK [170,171]. It is possible that these enzymes become phosphorylated by PDK1 as soon as they are synthesized and then are regulated post-PDK1 phosphorylation by other mechanisms. For example, PKA is regulated by the formation of complexes with other protein subunits [172] and p90-RSK is activated by phosphorylation on other residues than the PDK1-phosphorylation sites by the classical MAPK pathway [173].

Implications for the assignment of a protein as a PKB substrate

AGC kinases such as p70-S6K, SGK and atypical PKCs are activated in many cells by the same stimuli as PKB and have a similar substrate specificity to PKB. Without the availability of a specific inhibitor of PKB or mammalian cell lines lacking all PKB isoforms, it will not be possible to rule out the possibility that the proposed PKB substrates listed in Table 2 and Figure 5 will not be instead phosphorylated by a distinct PI3K-activated AGC kinase *in vivo*. An example is the insulin-induced phosphorylation of 6-phosphofructo-2-kinase (PFK2) in heart. Previous evidence suggested that PKB may directly phosphorylate and activate the cardiac-specific isoform of PFK2 by phosphorylating two serine residues [174]. However recent work showed that a dominant-negative mutant of PDK1, but not a dominant-negative form of PKB, prevented the insulin-induced activation of PFK2 [61]. Since PDK1 does not phosphorylate PFK2 *in vitro* (Mark Rider, personal communication), these results raise the possibility that an AGC kinase member, distinct from PKB and other insulin-stimulated protein kinases, mediates PFK2 phosphorylation and activation by insulin.

As mentioned above, the possibility that PDK1 regulates kinases other than PKB makes interpretation of experiments in which dominant-negative or wild-type PKBs are overexpressed not unambiguous: these PKBs might interact with PDK1 and thus prevent it from activating other AGC kinases. They could also bind a substrate, preventing it from becoming phosphorylated by an AGC kinase other than PKB, which is the substrate natural kinase under normal conditions.

PERSPECTIVE

A picture is now emerging of the intracellular mechanisms through which PI3K and PDK1 activate PKB, and of the way in which this protein kinase in turn regulates many physiological processes. The mechanism through which PKB becomes phos-

phorylated on Ser^{473} *in vivo* remains to be established. It is also crucial to determine whether the three mammalian isoforms of PKB each have unique physiological roles.

Unravelling the mechanism by which PKB is activated by PI3K in cells has provided important insights into the mechanism by which other AGC kinases are regulated. A key focus for future research will be the determination of the mechanisms by which the activity and substrate selection of PDK1 is regulated. Further challenges involve the identification of additional specific substrates for PKB and other AGC subfamily members, and the development of strategies to distinguish whether a physiological process is mediated by PKB, rather than a related AGC kinase.

Much of the work discussed in this review has been deduced by biochemical analysis and transient transfection experiments in mammalian cell lines. Development of genetic models for these pathways in *Drosophila*, *C. elegans* and *Dictyostelium* is well under way and, apart from providing stronger evidence for the model of the PI3K/PDK1/PKB signal transduction pathway discussed here, is yielding important information on the physiological roles of these pathways. It is likely that many key advances in this area will be made in these model organisms.

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