Discovery of PDKI, One of the Missing Links in Insulin Signal Transduction

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Abstract
Historically, two strategies have been used to dissect the insulin signal transduction pathway. One was to start at the insulin receptor and work down the signal transduction pathway from the plasma membrane. The other was to select a physiological action of insulin, namely the mechanism by which insulin stimulates glycogen synthesis, and then work backwards towards the receptor. The hope was that eventually the groups working down from the top of the insulin signalling pathway would meet up with those working upwards from the bottom of the pathway. This has now happened, and in this lecture I will describe the recent advances that have linked the research from both ends of the insulin signal transduction pathway. I will also discuss how these findings have enabled pharmaceutical companies to embark on novel programmes to develop improved therapies for the treatment of diabetes in the future.

Introduction
Insulin is a hormone that is secreted by the β-cells of the pancreas following food ingestion by animals. Its function is to stimulate the uptake of nutrients (glucose, amino acids, fatty acids) and their conversion into storage macromolecules (glycogen, protein, lipids) in skeletal muscle, adipose tissue and liver. Type I diabetes is characterized by the failure to synthesize or secrete insulin, while in Type II diabetes (which affects 85% of diabetic subjects) the disease is caused by the target tissues becoming resistant to the effects of insulin. Diabetes is the third most prevalent disease in the Western world, affecting at least 2% of the population, and its incidence is likely to
double over the next 30 years. Although diabetes can be treated with daily injections with insulin and/or regimes of drugs and nutritional control, diabetic patients are likely to suffer from many long-term complications, including kidney and heart disease as well as loss of sight; life expectancy is reduced on average by 5–10 years (reviewed in [1]). Therefore there is a great need for more effective drugs to treat diabetes, and, in order to develop them, it is essential to first understand the molecular mechanisms by which insulin exerts its physiological effects. In this lecture I describe work that has led to the identification of an insulin signal transduction pathway that mediates many of the effects of insulin in cells. These findings raise exciting opportunities for the development of novel therapies that mimic the effects of insulin by modulating the protein kinases and phosphatases in this cascade.

Work during the late 1980s and early 1990s (reviewed in [2–6]) revealed that the interaction of insulin with its receptor leads within seconds to the recruitment to the plasma membrane of a family of lipid kinases known as Class IA phosphoinositide 3-kinases (PI 3-kinases) (Figure 1). Here, these enzymes phosphorylate the lipid phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P$_2$] at the D-3 position of the inositol ring to generate PtdIns(3,4,5)P$_3$. It was suggested that PtdIns(3,4,5)P$_3$, and perhaps its immediate breakdown product PtdIns(3,4)P$_2$ (reviewed in the Colworth Lectures of Peter Downes [2] and Len Stephens [4]), were likely to be the key second messengers of insulin signal transduction, as potent, although not entirely specific, inhibitors of PI 3-kinase termed wortmannin [7] and LY294002 [8] were found to prevent most of the cellular responses to insulin, including stimulation of glucose transport [9], inhibition of lipolysis [10,11], stimulation of glycogen synthesis [12] and protein synthesis [13,14], and effects on gene expression [15]. These concepts were strengthened by the finding that the overexpression of dominant-negative forms of PI 3-kinase also prevented insulin responses [16], while the overexpression of constitutively activated forms of PI

**Figure 1**

Mechanism by which insulin stimulates the formation of the second messenger PtdIns(3,4,5)P$_3$

Insulin binds to its receptor, which is a tyrosine kinase, and causes the receptor to phosphorylate itself at a number of tyrosine residues. Certain of these phosphorylated tyrosine residues are recognized by the phosphotyrosine-binding (PTB) domain of the insulin receptor substrate (IRS) family of adaptor proteins, which then interacts with the insulin receptor. The insulin receptor then phosphorylates the IRS adaptors on tyrosine residues. Particular phosphotyrosine residues in the IRS subunit then interact with an SH2 (Src homology 2) binding domain situated in the regulatory p85 subunit of PI 3-kinase. This recruits PI 3-kinase from the cytosol to the cell membrane, bringing it into the vicinity of its physiological substrate PtdIns(4,5)P$_2$. PtdIns(4,5)P$_2$ is phosphorylated by the p110 catalytic subunit of PI 3-kinase at the D-3 position of the inositol ring to generate the second messenger PtdIns(3,4,5)P$_3$. A specific 5-phosphatase can convert PtdIns(3,4,5)P$_3$ into PtdIns(3,4)P$_2$, which is also predicted to be a second messenger, as it is not present in unstimulated cells. A specific 3-phosphatase, termed PTEN, can convert PtdIns(3,4,5)P$_3$ back into PtdIns(4,5)P$_2$ [110].

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3-kinases induced insulin-mediated processes in the absence of insulin [17,18]. This indicated that PtdIns(3,4,5)P$_3$/PtdIns(3,4)P$_2$ triggered the activation of downstream signal transduction pathways that would mediate physiological processes controlled by insulin.

In the 1960s it was established from the work of Joseph Larner that insulin induced the dephosphorylation and activation of glycogen synthase [19], the enzyme that catalyses the final step in glycogen synthesis, namely the conversion of UDP-glucose into glycogen. In 1980, Philip Cohen demonstrated that a kinase termed glycogen synthase kinase 3 (GSK3) phosphorylated and inhibited glycogen synthase [20,21], and in 1983 he showed that the insulin-induced dephosphorylation of glycogen synthase occurred mainly from the residues phosphorylated by GSK3 [22]. This could be explained if insulin stimulated the protein phosphatase that dephosphorylated glycogen synthase and/or if insulin could inhibit the activity of GSK3. Much of the work in the late 1980s/early 1990s focused on the protein phosphatase, PP1G, that was associated with glycogen and that dephosphorylated glycogen synthase [23]. However, despite intensive research, it is still unclear whether insulin induces the activation of PP1G or another phosphatase. Although Philip Cohen predicted in his Colworth Lecture in 1979 [24] that insulin could function by inducing the inactivation of GSK3, it was not until 1992 that Jim Woodgett and Bill Benjamin were first able to demonstrate that GSK3 itself was inhibited by insulin [25]. This was followed in 1994 by experiments carried out by the groups of Philip Cohen [26] and Chris Proud [27] demonstrating that the inactivation of GSK3 in response to insulin was prevented by inhibitors of PI 3-kinase (Figure 2).

Insulin also stimulates protein synthesis, thereby promoting the conversion of amino acids into protein [13]. GSK3 not only phosphorylated glycogen synthase, but also phosphorylated and

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**Figure 2**

**Mechanism by which insulin stimulates glycogen synthesis and protein synthesis**

GSK3 in non-stimulated cells is active and phosphorylates glycogen synthase at three C-terminal residues (located 30, 34 and 38 amino acids from the C-terminal end of the protein), leading to inhibition of this enzyme. GSK3 also phosphorylates the α-subunits of eIF2B at Ser-540, thereby reducing the efficiency with which this protein can stimulate the initiation of protein translation. Within 2-5 min of a cell being stimulated by insulin, GSK3 is inhibited, resulting in the dephosphorylation of both glycogen synthase and eIF2B through the action of protein phosphatases. This results in the activation of glycogen synthesis and protein synthesis. As the inhibition of GSK3 following insulin stimulation is prevented by inhibitors of PI 3-kinase, this indicates that PI 3-kinase lies upstream of GSK3 in this signalling pathway.
inhibited a guanine nucleotide exchange factor that regulates the initiation stage of protein translation, termed eIF2B (eukaryotic initiation factor 2B). Chris Proud’s group demonstrated that insulin induced the dephosphorylation of eIF2B at the site phosphorylated by GSK3 [27–29], therefore showing that the inactivation of GSK3 underlies a mechanism by which insulin promotes protein synthesis as well as glycogen synthesis (Figure 2).

As summarized in Figure 2, these findings indicated that insulin activates PI 3-kinase (within 30 s), which leads to the inactivation of GSK3 (within 2–5 min), and this results in the dephosphorylation and activation of glycogen synthase and eIF2B (within 5–15 min), thereby activating glycogen synthesis and protein synthesis. These results defined the next challenge in the insulin signal transduction pathway, which was to discover how the phosphoinositide lipid [PtdIns(3,4,5)P3] could induce the inactivation of a protein kinase (GSK3) in a cell.

**Mechanism of inactivation of GSK3**

There are two closely related isoforms of GSK3, termed GSK3α and GSK3β, both of which are inhibited by insulin. GSK3α and GSK3β isolated from insulin-stimulated cells or tissues could be 're-activated' by incubation with serine/threonine-specific protein phosphatases, indicating that inhibition of GSK3 resulted from serine/threonine phosphorylation [26,27]. Work carried out by Philip Cohen’s group demonstrated that insulin induced the phosphorylation of GSK3α at Ser-21 and of GSK3β at Ser-9 [30]. Consistent with this finding, we showed that a mutant form of GSK3β in which Ser-9 was changed to Ala could no longer be inactivated by insulin [31]. These observations indicated that insulin must activate a protein kinase that phosphorylates GSK3α at Ser-21 and GSK3β at Ser-9. The phosphorylation of GSK3 was inhibited by wortmannin [26,27], it was predicted that activation of the insulin-stimulated protein kinase that phosphorylates GSK3 would also be prevented by inhibitors of PI 3-kinase. Furthermore, as GSK3 isoforms are phosphorylated within 2–5 min following insulin stimulation (Figure 2), it was likely that the relevant insulin-stimulated protein kinase would be activated at least as rapidly.

In 1995 Franke and colleagues [32] reported that a protein kinase termed protein kinase B (PKB; also known as Akt) was activated rapidly following stimulation of cells with platelet-derived growth factor, and that its activation was inhibited by wortmannin or by the overexpression of a dominant-negative form of PI 3-kinase [32]. PKB had been independently discovered 4 years previously by the groups of Brian Hemmings [111] and Paul Coffer and Jim Woodgett [112] as a kinase whose catalytic domain was homologous with cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), and also by Philip Tsichlis and colleagues [113] as a retroviral oncogene. Burgering and Coffer [33] and Richard Roth and colleagues [34] demonstrated that insulin also induced the PI 3-kinase-dependent activation of PKB, with half-maximal activation occurring within 1 min of a cell being stimulated with insulin. Darren Cross, a Ph.D. Student in Philip Cohen’s laboratory, had already identified an insulin-stimulated protein kinase in rat L6 myotubes that could phosphorylate and inactivate GSK3 isoforms. Moreover, the activation of this protein kinase, like the inhibition of GSK3, was prevented by inhibitors of phosphatidylinositol (PI) 3-kinase. Darren Cross then demonstrated that the activity that he had discovered was immunoprecipitated with antibodies raised against PKB provided by Mirjana Andjelkovic and Brian Hemmings. Furthermore, the immunoprecipitated PKB phosphorylated GSK3α at Ser-21 and GSK3β at Ser-9, leading to the inhibition of these enzymes [30]. Furthermore, the GSK3 isoforms were also inhibited when co-transfected with PKB in cells [31], and a dominant-negative PKB was subsequently shown to inhibit the insulin-induced inactivation of GSK3 [35]. These findings provided strong evidence that PKB was situated 'upstream' of GSK3 in the insulin signal transduction pathway.

**PKB is a key mediator of insulin signal transduction responses**

As virtually all insulin-stimulated processes, like the activation of PKB, are suppressed by inhibitors of PI 3-kinase, it was speculated as early as 1995 that PKB could perhaps be a key mediator of many insulin signalling processes in cells. Many groups addressed the question of what roles PKB might play in the insulin signalling pathway by overexpressing constitutively active mutants of PKB in insulin-responsive cell lines. These results demonstrated that overexpression of activated PKB not only activated glycogen and protein synthesis as expected, but also stimulated the uptake of nutrients into cells, such as glucose and
amino acids [36,37], as well as stimulating certain changes in gene expression that are induced by insulin [36,38]. It was unlikely that all of these processes were mediated by the phosphorylation of GSK3, suggesting that PKB phosphorylates other cellular substrates. To develop a strategy to identify other substrates of PKB, we determined the key specificity determinants surrounding Ser-9 of GSK3β that enabled PKBa to phosphorylate this site. We found that a peptide that encompassed Ser-9 of GSK3β was a good substrate for PKB and, by synthesizing variant versions of this peptide, we established that the minimum sequence motif required for efficient phosphorylation by PKB was Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa are preferably small residues other than glycine, and Hyd is a bulky hydrophobic residue (Phe, Leu). The requirement for Arg at positions three and five N-terminal to the phosphorylation site was critical, as changing either of these residues, even to Lys, abolished phosphorylation of the peptide by PKB [39]. More recently, Cantley and colleagues [40] have confirmed that this motif is accurate by screening a random peptide library to isolate peptides phosphorylated by PKB and by determining the optimal amino acids at positions N-terminal and C-terminal to the site of phosphorylation.

The determination of this PKB phosphorylation motif has led to the identification of over 20 proteins that possess this motif and whose function is known or postulated to be regulated by insulin and other agonists that activate PKB (reviewed in [41]); some novel substrates are proposed in [40]. Many (but not all) of these proteins have been shown to be phosphorylated at the predicted residue by PKB in vitro or following overexpression of PKB in cells. In some cases it has been shown that these proteins are phosphorylated at the site of PKB phosphorylation in vivo in response to insulin, and that phosphorylation is prevented by inhibitors of PI 3-kinase. Although these studies indicate that PKB has the potential to phosphorylate numerous proteins, the possibility cannot yet be ruled out that a kinase other than PKB, which possesses a similar substrate specificity and is activated downstream of PI 3-kinase, mediates these phosphorylations. It is also likely that the overexpression of activated mutants of PKB in cell lines for many hours, or even days, may force PKB to phosphorylate proteins that it does not normally phosphorylate, and therefore to evoke physiological responses that are not normally mediated by PKB. Much of the future work in this area will be aimed at developing pharmacological reagents and genetic and biochemical approaches that not only identify novel substrates for PKB, but also verify whether these substrates are indeed phosphorylated physiologically by PKB.

**Discovery that PKB interacts with PtdIns(3,4,5)P₃**

PKB contains an N-terminal pleckstrin homology domain (PH domain) followed by a kinase catalytic domain and then a C-terminal tail. The kinase domain belongs to a group of protein kinases that are known as the AGC family, and which includes PKA, PKC and the p70 ribosomal S6 kinase (S6K) isoforms [42,43]. There are three isoforms of PKB (termed PKBα, PKBβ and PKBγ), which possess > 85% sequence identity and are widely expressed in human tissues [41]. Although PH domains were first noticed in various proteins predicted to have a role in signal transduction in 1993 [44–47], it was not until 1995 that it became apparent that PH domains on certain proteins, such as spectrin, could function as a phosphoinositide-binding module, enabling proteins to interact with cell membranes [48]. In 1996, in collaboration with Steve James and Pete Downes, we demonstrated that endogenous PKB isolated from unstimulated or insulin-stimulated L6 myotubes bound to lipid vesicles made up of a 1:1 mixture of phosphatidylserine and phosphatidylcholine containing a low molar fraction of PtdIns(3,4,5)P₃. However, lipid vesicles containing PtdIns(4,5)P₃ or PtdIns3P failed to interact with PKB [49]. This was the first evidence that PKB could interact with PtdIns(3,4,5)P₃ in vitro. Subsequent work performed by us and others demonstrated that, following stimulation of cells with insulin, PKB was translocated to the plasma membrane, where PtdIns(3,4,5)P₃ is located, and that translocation was prevented by inhibitors of PI 3-kinase or by deletion of the PH domain of PKB [50–52]. These findings established that PKB interacts with PtdIns(3,4,5)P₃ and/or PtdIns(3,4)P₃ in vivo.

**Discovery that PKB is activated by phosphorylation**

Although some authors claimed that PtdIns(3,4)P₃ could activate PKB directly [53,54], we found that PKB was not activated by its interaction with either PtdIns(3,4,5)P₃ or PtdIns(3,4)P₃ [49]. Moreover, the activated form of PKB was inacti-
ated by serine/threonine phosphatases [30,33], suggesting that PKB could be activated by phosphorylation on a serine/threonine residue(s). To explore this possibility, we performed 32P cell labelling experiments using L6 myotubes. These studies revealed that insulin induced the phosphorylation of PKB at two residues, Thr-308 and Ser-473, and that inhibitors of PI 3-kinase prevented the phosphorylation of both residues [55]. Thr-308 is located in the 'activation loop' between subdomains VII and VIII of the kinase catalytic domain, situated at the same position as the activating phosphorylation sites found in many other protein kinases. Ser-473 is located outside the catalytic domain in a motif that is present in most AGC kinases, termed the hydrophobic motif. In collaboration with Mirjana Andjelkovic and Brian Hemmings, we demonstrated that the mutation of Thr-308 or Ser-473 to Ala inhibited the insulin-induced or insulin-like growth factor-1 (IGF-1)-induced activation of PKBz by over 85%. Conversely, mutation of Thr-308 or Ser-473 to Asp (to mimic the effect of phosphorylation by introducing a negative charge) increased PKBz activity by 5–10-fold in unstimulated cells, and this mutant could not be activated further by insulin [55]. Attachment of a membrane-targeting domain to PKBz resulted in it becoming highly activated in unstimulated cells, and induced maximal phosphorylation of Thr-308 and Ser-473 [50,56]. These observations indicated that recruitment of PKB to the membrane of cells was sufficient for it to become activated at that location by the phosphorylation of Thr-308 and Ser-473. We also demonstrated that PKBβ is activated by phosphorylation of the equivalent residues (Thr-309 and Ser-474), and that a splice variant of PKBβ that does not possess a residue equivalent to Ser-473 of PKBz (because it terminates at residue 454) was also activated in vivo solely via phosphorylation of Thr-305 (the residue equivalent to Thr-308 of PKBz) [57].

Figure 3

Mechanism of activation of PKB

Stimulation of cells with insulin activates PI 3-kinase and triggers the production of PtdIns(3,4,5)P3 (PIP3) at the plasma membrane [12]. PKB then interacts with PtdIns(3,4,5)P3 and/or PtdIns(3,4)P2 (PIP2) through its PH domain, and is thus recruited from the cytosol to the plasma membrane. The interaction of PKB with PtdIns(3,4,5)P3 alters the conformation of the kinase, so that Thr-308 becomes accessible for phosphorylation by PDK1. PKB is also phosphorylated at the membranes at Ser-473 by an as yet uncharacterized protein kinase, which is termed here PDK2. Phosphorylation of PKB at Thr-308 and Ser-473 activates it. The interaction of PDK1 with PtdIns(3,4,5)P3 through its PH domain does not activate PDK1, but is thought to play an important role in localizing PDK1 at the membrane so that it can phosphorylate the PKB which is recruited to this location.
Identification of the PtdIns(3,4,5)P₃-dependent protein kinase (PDK1) that activates PKB

The next step was to identify a protein kinase(s) that could phosphorylate PKB at Thr-308 and Ser-473. We speculated that the observations described above could be accounted for if a protein kinase(s) existed that phosphorylated PKB only in the presence of PtdIns(3,4,5)P₃. We were able to detect and purify [58] and subsequently clone [59] an activity in cell extracts that phosphorylated PKB at Thr-308 and activates PKB (D. R. Alessi, unpublished work), consistent with the cellular situation, where there is typically a 100-fold excess of PtdIns(4,5)P₂ over PtdIns(3,4,5)P₃. The requirement for PtdIns(4,5)P₂ or PtdIns(3,4)P₂ in the PDK1-catalysed activation of PKB is mediated, at least in part, by their interaction with the PH domain of PKB. These inositol lipid ‘second messengers’ appear to alter the conformation of PKB, so that Thr-308 becomes accessible to PDK1 (Figure 3). These conclusions are supported by the following three observations. Firstly, in the absence of PtdIns(3,4,5)P₃, full-length PKB is not phosphorylated by PDK1, but removal of the PH domain of PKB results in its phosphorylation and activation by PDK1 [59]. Secondly, a PKBa mutant that cannot interact with PtdIns(3,4,5)P₃ is not phosphorylated by PDK1 [60,61]. Thirdly, PtdIns(3,4,5)P₃ is still required for the activation of PKB by a truncated form of PDK1 that lacks the PH domain [59,61].

PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ also interact with the PH domain of PDK1, and with somewhat higher affinity than with the PH domain of PKBα [62]. It is thought that the interaction of PDK1 with these lipids enables PDK1 and PKB to co-localize at membranes that contain PtdIns(3,4,5)P₃ and/or PtdIns(3,4)P₂ [61,62]. The activation of PKB by PDK1 was enhanced over 1000-fold in the presence of lipid vesicles containing a low molar fraction of PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂ [59]. The activation was stereospecific for the D-enantiomers and for the naturally occurring form of PtdIns(3,4,5)P₃ (the 1-stearoyl, 2-arachidonyl derivative), which was significantly more effective than the dipalmityl derivative [58,61]. This may be due to the loose packing of unsaturated fatty acid backbones in the phospholipid bilayer, resulting in greater exposure of the headgroup of PtdIns(3,4,5)P₃ and hence allowing more efficient interactions with cytosolic proteins. This may explain why inositol phospholipids, several of which form specific interactions with PH domains or other lipid-binding domains, tend to contain fatty acids with unsaturated backbones, whereas other, non-signalling, phospholipids generally possess more saturated fatty acid moieties. Neither PtdIns(4,5)P₂ nor any other inositol phospholipid tested can replace PtdIns(3,4,5)P₃ in the PDK1-catalysed activation of PKB [58,61]. Even the presence of a 100-fold molar excess of PtdIns(4,5)P₂ over PtdIns(3,4,5)P₃ did not impair PDK1 from phosphorylating and activating PKB (D. R. Alessi, unpublished work), consistent with the cellular situation, where there is typically a 100-fold excess of PtdIns(4,5)P₂ over PtdIns(3,4,5)P₃.

How is PKB phosphorylated at Ser-473?

Michayla Williams in our laboratory generated mouse embryonic stem (ES) cells in which both copies of the PDK1 gene were disrupted to prevent the expression of PDK1, and tested how this affected phosphorylation of PKB at Ser-473 in...
response to agonists that activate PI 3-kinase. These cells (termed PDK1−/− ES cells) were viable, and proliferated at a similar rate to wild-type ES cells expressing PDK1 (termed PDK1+/+ ES cells). IGF-1 stimulation of PDK1−/− ES cells activated PI 3-kinase to a greater degree than in the control PDK1+/+ ES cells; however, PKB was not activated significantly in the PDK1−/− ES cells, whereas it was activated robustly in the control PDK1+/+ ES cells. As expected, IGF-1 stimulation of PDK1−/− ES cells failed to induce phosphorylation of PKB at Thr-308 in its T-loop, providing the first genetic evidence in mammalian cells that PDK1 mediates this phosphorylation. Importantly, however, PKB was still phosphorylated at Ser-473 in response to IGF-1 stimulation, and this was inhibited by the PI 3-kinase inhibitors wortmannin and LY294002 [66]. This indicated that, at least in PDK1−/− ES cells, there is a kinase distinct from PDK1 that can mediate phosphorylation of Ser-473. However, it is possible that phosphorylation of PKB at Ser-473 could be regulated differently in other cell types. It will therefore be important in the future to prepare cell lines other than ES cells that lack PDK1 and to establish the effect that this has on Ser-473 phosphorylation in response to a variety of agonists. The availability of a specific PDK1 inhibitor (which has not yet been developed) would also be extremely valuable, to corroborate data regarding the inability of PDK1 to regulate the phosphorylation of PKB at its hydrophobic motif in PDK1 knockout cell lines. In this regard it should be noted that Hemmings and colleagues have found that the non-specific kinase inhibitor staurosporine (which was presumed, but not demonstrated, to inhibit PDK1) prevented the phosphorylation of PKB at Thr-308, but not Ser-473, in 293 cells [67]. This provides pharmacological evidence that PDK1 is not essential for the phosphorylation of PKB at Ser-473 in these cells. The identity of the elusive kinase that phosphorylates PKB at Ser-473 is not known. Although it has been reported that the integrin-linked kinase (ILK) [68], MAPKAP (mitogen-activated protein kinase-activated protein) kinase-2 [69,70] and conventional PKC isoforms [71] may mediate the phosphorylation of PKB at Ser-473 under certain cellular situations, there is no decisive evidence that any of these enzymes mediate the phosphorylation of PKB isoforms at their hydrophobic motif following insulin stimulation of muscle, liver or adipose cells.

Discovery that PDK1 activates other kinases

As mentioned above, PKB belongs to the AGC family of protein kinases. These kinases possess over 40% identity within their kinase catalytic domain, and all members of this family of kinases require phosphorylation of their T-loop residue in order to be activated. The amino acid sequences C-terminal to the T-loop phosphorylation site are highly conserved in all AGC kinases [41]. Most AGC kinase family members also possess a region homologous with the hydrophobic motif of PKB that encompasses Ser-473 and is located ~160 amino acids C-terminal to the T-loop residue lying outwith the catalytic regions of these enzymes. The hydrophobic motif of most AGC kinases is characterized by the motif: Phe-Xaa-Xaa-Phe-Ser/Thr/Tyr/Phe (where Xaa is any amino acid and the Ser/Thr residue is equivalent to Ser-473 of PKB). Atypical PKC isoforms and PRK (PKC-related kinase) isoforms, instead of possessing a Ser/Thr residue in their hydrophobic motif, have an acidic residue. PKA, in contrast, only possesses the Phe-Xaa-Xaa-Phe moiety of the hydrophobic motif, as the PKA amino acid sequence terminates at this position [72].

Similar to PKB, the activation of other members of the AGC family of protein kinases that occurs in response to insulin and other agonists is prevented by inhibitors of PI 3-kinase or by the overexpression of dominant-negative forms of PI 3-kinase. These include the S6K isoforms (S6K1 and S6K2) [73], serum- and glucocorticoid-induced protein kinase (SGK) isoforms (SGK1, SGK2 and SGK3) [74-76], as well as novel and atypical PKC isoforms [77]. Work performed over the past 5 years in many laboratories has revealed that the activation of PI 3-kinase induces the phosphorylation of these AGC kinases at their T-loop residue and their hydrophobic motif. The phosphorylation of S6K and SGK isoforms at both the T-loop and hydrophobic motif is required to activate these kinases. In the case of novel PKC isoforms, it is thought that phosphorylation of the T-loop motif activates these enzymes, while phosphorylation of the hydrophobic motif may play a role in stabilizing these kinases in an active conformation [78,79]. Atypical PKC isoforms are thought to be activated solely by the phosphorylation of their T-loop residue, as they possess an acidic residue instead of a phosphorylatable Ser/Thr residue in their hydrophobic motif.
The finding that the T-loop residues of all AGC kinases are so conserved suggested that a common upstream protein kinase, i.e. PDK1, might phosphorylate these residues. We and others found that the AGC kinases activated downstream of PI 3-kinase, namely S6K1 [80,81], SGK isoforms [74–76] and novel and atypical PKC isoforms [82,83], were phosphorylated specifically at their T-loop residue by PDK1 in vitro or following the overexpression of PDK1 in cells (Figure 4). Moreover, AGC kinases that were not activated in a PI 3-kinase-dependent manner in cells, such as the p90 ribosomal S6 kinase (p90RSK) isoforms [84,85], conventional PKC isoforms, PRK isoforms [86–89] and even PKA [90], were also proposed to be physiological substrates for PDK1, as they could all be phosphorylated by PDK1 at their T-loop residue in vitro or following overexpression of PDK1 in cells (Figure 4).

In order to obtain firmer evidence that PDK1 mediates the activation of these AGC kinases in vivo, we tested whether these enzymes could be activated in PDK1−/− ES cells. We found that S6K1 was substantially active in wild-type PDK1+/+ ES cells, and was activated a further 2-fold by IGF-1. However, there was no detectable S6K1 activity in unstimulated or IGF-1-treated PDK1−/− ES cells, despite normal expression of S6K1 in these cells. Interestingly, and in contrast with the effect on PKB, IGF-1 failed to induce phosphorylation of S6K1 at its hydrophobic motif [66]. PDK1 could phosphorylate the hydrophobic motif of S6K1 directly, but it is also possible that PDK1 controls the expression of another protein kinase that phosphorylates the hydrophobic motif of S6K1. Furthermore, as a mutant of S6K1 in which the T-loop residue was changed to Ala was phosphorylated very poorly at its hydrophobic motif in cells [91,92], it is possible that the lack of T-loop phosphorylation of S6K1 either hinders the phosphorylation of the hydrophobic motif of S6K1 by an upstream kinase or promotes the dephosphorylation of this site by a protein phosphatase.

In ES cells lacking PDK1 (PDK1−/− cells), the intracellular levels of endogenously expressed PKCζ, PKCβI, PKCγ, PKCδ, PKCε, and PRK1 are vastly reduced compared with those in control ES cells (PDK1+/+ cells) [93], consistent with the notion that PDK1 phosphorylates these enzymes and that this plays an essential role in the proposed post-translational stabilization of these kinases [78,79]. In contrast, the levels of PKCζ and PRK2 protein were only moderately reduced in the PDK1−/− ES cells. We were able to demonstrate that PKCζ expressed in ES cells lacking PDK1 is not phosphorylated at its T-loop residue, in contrast with that in wild-type cells [93]. These observations provided the first genetic evidence that PKCζ is a physiological substrate for PDK1. Interestingly, we found that PRK2 was still phosphorylated at its T-loop in PDK1−/− cells, although at a lower level than in PDK1+/+ ES cells. This might indicate that, in addition to PDK1 phosphorylating PRK2, either PRK2 may be able to phosphorylate itself at its T-loop residue or another protein kinase, distinct from PDK1, might be able to phosphorylate this residue [93].

Figure 4

PDK1 plays a central role in activating several, but not all, AGC kinases

The AGC kinases that are likely to be phosphorylated physiologically by PDK1 are indicated by uninterrupted arrows. PKA, MSK1, PKCζ, AMPK, and the AGC kinase family members are not likely to be phosphorylated in vivo by PDK1, as these enzymes are active in mouse ES cells that lack PDK1 (see the main text for details). Note that there is evidence that PRK2, in addition to being phosphorylated at its T-loop residue by PDK1, may also become phosphorylated at this site by a PDK1-independent mechanism [93].
p90RSK isoforms possess two kinase catalytic domains, and are phosphorylated and activated by the classical mitogen-activated protein kinase family members ERK1 (extracellular-signal-regulated kinase 1) and ERK2 following stimulation of cells with agonists that activate these kinases [94]. The N-terminal kinase domain on p90RSK is an AGC kinase and requires to be phosphorylated at its T-loop residue in order to be activated by ERK1/ERK2 [95]; PDK1 can phosphorylate this residue [84,85]. Stimulation of PDK1⁺⁺ ES cells with phorbol esters potently activated ERK1/ERK2 and p90RSK. However, although ERK1 and ERK2 were activated in cells lacking PDK1, p90RSK isoforms were not significantly activated, even though they were expressed at normal levels [66]. This provides strong genetic evidence that PDK1 mediates the phosphorylation of the T-loop residue of the N-terminal kinase domain of p90RSK. In contrast, PKA, which was reported to be phosphorylated at its T-loop by PDK1, was active and phosphorylated at its T-loop in PDK1⁻⁻ ES cells to the same extent as in wild-type ES cells [66]. This argues that PDK1 is not rate-limiting for the phosphorylation of PKA in ES cells. It is possible that PKA phosphorylates itself at its T-loop residue in vitro, as it has been shown to possess the intrinsic ability to phosphorylate its own T-loop when expressed in bacteria. Furthermore, two other AGC kinases that we have tested in PDK1⁻⁻ ES cells, namely mitogen- and stress-activated protein kinase-1 (MSK1) and the AMP-activated protein kinase (AMPK), were also activated normally, indicating that PDK1 is not rate-limiting for the activation of all AGC kinases in vitro [66] (Figure 4).

**Mechanism of regulation of PDK1 activity**

The work described above established a central role for PDK1 in activating not only PKB, but also several other AGC kinases that are predicted to possess very distinct physiological roles. A major challenge is to understand the mechanism by which PDK1 activity is regulated in cells to enable it to phosphorylate its AGC kinase substrates in a co-ordinated manner. Initial experiments focused on whether insulin could activate PDK1 directly. We [59] and others [81] observed that when PDK1 was isolated from unstimulated or insulin-stimulated cells, it possessed the same activity towards PKB or S6K1. Furthermore, although we found that PDK1 was phosphorylated at five serine residues in 293 cells, insulin or IGF-1 did not induce any change in the phosphorylation state of any of these sites [96]. Only one of these phosphorylation sites, namely Ser-241, was essential for PDK1 activity. Ser-241 is located in the T-loop of PDK1, and as PDK1 expressed in bacteria is phosphorylated at Ser-241, it is likely that PDK1 can phosphorylate itself at this residue [96]. Although PDK1 becomes phosphorylated on tyrosine residues following stimulation of cells with peroxovanadate (a tyrosine phosphatase inhibitor) [63,97], we [96] and our collaborators [63] have not detected tyrosine phosphorylation of PDK1 following stimulation of 293 cells with insulin.

Taken together, these observations suggested that PDK1 might not be activated directly by insulin. Instead, one intriguing solution that might explain how PDK1 could phosphorylate a number of AGC kinases in a regulated manner is if PDK1, instead of being activated by an agonist, is 'constitutively active' in cells, and that it is the substrate, rather than PDK1 itself, that is converted into a form that can be phosphorylated by PDK1. In the case of PKB, it is the interaction of PKB with PtdIns(3,4,5)P³ that converts it into a substrate for PDK1. However, with regard to other AGC kinases that are activated downstream of PI 3-kinase, such as S6K, SGK and PKC isoforms, which do not interact with PtdIns(3,4,5)P³ and whose phosphorylation by PDK1 in vitro is not enhanced by this inositol lipid, it is not obvious how PtdIns(3,4,5)P³ can regulate the phosphorylation of these enzymes in vivo. Work performed by Riccardo Biondi in our laboratory indicates that the hydrophobic motifs of S6K1, SGK (R. Biondi and D. R. Alessi, unpublished work), atypical PKC isoforms and PRK isoforms [86] can interact directly with a hydrophobic pocket in the kinase domain of PDK1, that has been termed the 'PIF-binding pocket' [72]. Initial experiments suggest that the interaction of S6K1 and SGK with PDK1 is significantly enhanced if these enzymes are phosphorylated at their hydrophobic motifs (R. Biondi and D. R. Alessi, unpublished work). It is therefore possible that PtdIns(3,4,5)P³ does not activate PDK1, but induces the activation of an unknown protein kinase(s) that phosphorylates S6K1 and SGK at their hydrophobic motif. Similarly, Frodin and colleagues [98] have shown that PDK1 interacts directly with the hydrophobic motif of the N-terminal kinase domain of p90RSK, and that this interaction is required for the phosphorylation of p90RSK by PDK1. As for
S6K and SGK isoforms, the affinity of PDK1 for p90RSK is increased substantially following phosphorylation of the hydrophobic motif of the N-terminal AGC-like kinase domain of p90RSK, which is thought to be mediated by the C-terminal p90RSK kinase domain following its phosphorylation by ERK1/ERK2. This suggests that the activation of p90RSK first involves the ERK1/ERK2 catalysed phosphorylation and activation of the C-terminal kinase domain of p90RSK. The C-terminal kinase domain of p90RSK then phosphorylates the hydrophobic motif of the N-terminal AGC-like kinase domain. This in turn converts p90RSK into a form that can interact with PDK1, enabling PDK1 to phosphorylate the T-loop residue of the N-terminal kinase domain, thereby activating it [98].

PRK isoforms and atypical PKC isoforms possess a hydrophobic motif in which the residue equivalent to Ser-473 is Asp or Glu, and these enzymes can, in principle, interact with PDK1 as soon as they are expressed in a cell [86]. However, it is possible that the interaction of PRK isoforms and atypical PKC isoforms with PDK1 could be regulated through the interaction of these enzymes with other molecules. For example the interaction of PRK2 with Rho-GTP [89] or of PKCζ with hPar4 and hPar6 [99] might induce a conformational change in these enzymes that controls their interaction with PDK1.

Perspectives and concluding remarks

The discovery of PDK1 was a landmark in the study of the insulin signalling pathway, as it was the first time that researchers working from the top of the insulin signalling pathway met up with those working upwards from the bottom of the pathway. Elucidation of the mechanism by which PKB is activated by PDK1 in cells also provided the first example of how the second messenger of the insulin pathway, PtdIns(3,4,5)P₃, could activate downstream signalling processes. It should be noted, however, that in addition to PDK1 and PKB there are now known to be many other widely expressed proteins that possess PH do-

Figure 5

Are all insulin signal transduction processes triggered by the PDK1/AGC kinase pathway?

Apart from PDK1 and PKB, a number of other proteins possess PH domains that interact with PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂. These include certain adaptor proteins, tyrosine kinases belonging to the Tec family, and GDP/GTP exchange proteins and GTPase-activating proteins for the ARF/Rho/Rac family of small GTP-binding proteins. A key question for future research is whether all insulin-dependent processes are regulated through the PDK1/AGC kinase branch of this pathway, or whether there are other PI 3-kinase-dependent, but PDK1-independent, branches that could regulate certain key insulin-dependent processes. We hope to be able to address this question in the future by studying insulin responses in mice that lack the expression of PDK1 specifically in muscle, liver and adipose tissue.
maintains that interact with PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 (Figure 5). These include adaptor molecules such as GAB1 (Grb2-associated binder-1) [100], DAPK1 (dual adaptor for phosphotyrosine and 3-phosphoinositides) [101–104] and TAPP1/2 (tandem PH-domain-containing protein 1/2) [105], GTP/GDP exchange [106,107] and GTPase-activating proteins [108] for the ADP-ribosylation factor (ARF)/Rho/Rac family of GTP-binding proteins, and tyrosine kinases of the Tec family [109]. It is not known whether any insulin-dependent signal transduction responses are triggered by the binding of these proteins to PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2. One of the challenges in the future will be to establish whether all insulin-induced signalling processes are mediated through the PDK1/AGC kinase pathway, or whether other PtdIns(3,4,5)P_3-dependent, but PDK1-independent, signalling pathways play a role in mediating some of insulin’s responses. Another challenge will be to identify the protein kinase(s) that mediate the PtdIns(3,4,5)P_3-dependent phosphorylation of the hydrophobic motif of PKB and other AGC kinase family members, and to establish how this protein kinase(s) is regulated by PtdIns(3,4,5)P_3. New genetic and pharmacological approaches are also desperately needed in order to dissect the roles of each individual AGC kinase in mediating insulin-dependent responses, because information obtained by overexpression of constitutively active and dominant-negative mutants of AGC kinases is not providing physiologically reliable results.

Although our knowledge and understanding of the insulin signal transduction pathway is far from being complete, we now have a better framework for the development of novel drugs to treat diabetes, which could ultimately benefit patients suffering from this disorder. For example, a drug that could mimic PtdIns(3,4,5)P_3 would enable PDK1 to activate PKB in tissues of diabetic subjects, and such a drug would be expected to promote glucose uptake, glycogen synthesis and protein synthesis in these patients. An inhibitor of GSK3 would mimic the effects of insulin on this enzyme, and should promote glycogen synthesis and protein synthesis. Although work is under way in pharmaceutical and biotechnology companies to develop such drugs, there is no guarantee that a compound capable of mimicking PtdIns(3,4,5)P_3 or inhibiting GSK3 will not have toxic side effects. It is therefore still essential that the insulin signalling pathway is characterized in much greater detail, and that novel components of this pathway are identified, as they could be the key anti-diabetic drug targets of the future. If such drugs are orally effective, it is possible that they could be used to treat Type I as well as Type II diabetes, and so replace the need for daily insulin injections.

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