The Tenth Datta Lecture

PDK1, one of the missing links in insulin signal transduction?

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Abstract The initial steps in insulin signal transduction occur at the plasma membrane and lead to the activation of phosphatidylinositide (PtdIns) 3-kinase and the formation of PtdIns(3,4,5,)P_3 in the inner leaflet of the plasma membrane which is then converted to PtdIns(3,4)P_2 by a specific phosphatase. Inhibitors of PtdIns 3-kinase suppress nearly all the metabolic actions of insulin indicating that PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 are key 'second messengers' for this hormone. A major effect of insulin is its ability to stimulate the synthesis of glycogen in skeletal muscle. By 'working backwards' from glycogen synthesis, we have dissected an insulin-stimulated protein kinase cascade which is triggered by the activation of PtdIns 3-kinase. The first enzyme in this cascade is termed 3-phosphoinositide-dependent protein kinase (PDK1), because it is only active in the presence of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. PDK1 then activates protein kinase B (PKB) which, in turn, inactivates glycogen synthase kinase-3 (GSK3), leading to the dephosphorylation and activation of glycogen synthase and hence to an acceleration of glycogen synthesis. We review the evidence which indicates that the phosphorylation of other proteins by PKB and GSK3 is likely to mediate many of the intracellular actions of insulin.

Key words: Insulin; Protein kinase B; c-Akt; GSK3; PI(3,4,5)P_3; Inositol phospholipid

1. Insulin and diabetes

Diabetes is characterised by the failure to synthesise, secrete or respond to insulin and is the third most prevalent disease in the Western world, affecting at least 3% of the population of Europe and North America and about 100 million worldwide. In at least 75% of diabetics, the level of circulating insulin is normal, or even above normal, and the disease is caused by the major target tissues for insulin (muscle, fat, liver) becoming resistant to this hormone. In order to understand the causes of this disease, it is therefore important to elucidate the mechanism by which insulin signals to the cell interior. However, this apparently simple problem has proved to be remarkably difficult to solve. Although Banting and Macleod received the Nobel Prize for Medicine in 1923 for the discovery of insulin, and Fred Sanger and Dorothy Hodgkin were awarded the 1958 and 1964 Nobel Prizes for Chemistry for elucidating the amino-acid sequence and three-dimensional structure of this hormone, respectively, the signalling mechanism has remained elusive for 75 years.

2. From the insulin receptor to the formation of phosphatidylinositide (3,4,5) triphosphate

A famous cartoon published in 1979 and reproduced in Fig. 1 nicely summarises the frustrations of investigators who were engaged in studying insulin transduction in the late 1970s. However, 3 years later a major breakthrough occurred when Ron Kahn and his colleagues discovered that the insulin receptor [1], like the epidermal growth factor receptor [2], was a protein tyrosine kinase. The binding of insulin to the α-subunit of the receptor activates the protein tyrosine kinase associated with the β-subunit which then phosphorylates itself at multiple tyrosine residues. Another crucial finding first made by Ron Kahn and Morris White during the 1980s and developed during the 1990s, was that IRS1 (insulin receptor substrate-1) [3] and a close relative IRS2 [4] are key substrates for the insulin receptor. IRS1 and IRS2 interact with a particular phosphotyrosine residue on the β-subunit and are then phosphorylated by the receptor at many tyrosine residues. This allows them the interact with and recruit SH2-domain-containing proteins to the plasma membrane [5]. One of these proteins is phosphatidylinositide (PtdIns) 3-kinase, an enzyme that was first described by Lew Cantley [6] and whose physiological role is to convert a minor inositol phospholipid in the plasma membrane, termed phosphatidylinositide (PtdIns) 4,5 bisphosphate [PtdIns(4,5)P_2], to PtdIns 3,4,5 trisphosphate [PtdIns(3,4,5)P_3] [7] (Fig. 2). The advent of two relatively specific (but structurally dissimilar) inhibitors of PtdIns 3-kinase, wortmannin [8] and LY 294002 [9], was critical in demonstrating that this enzyme plays an essential role in mediating nearly all the metablic actions of insulin. Thus, evidence has been accumulating the PtdIns(3,4,5)P_3, or a metabolite derived from it, may be long sought after second messenger for insulin, raising the question of how this compound exerts its effects on cell function.

3. The stimulation of glycogen synthase by insulin

Two approaches can be employed to elucidate signal transduction pathways. One way is to start from the receptor at the plasma membrane and to work step by step from the outside to the inside of the cell, while the second is to select a physiological action of the hormone and then work backwards towards the receptor. The latter method was used by Earl Sutherland when he discovered that cyclic AMP is the second

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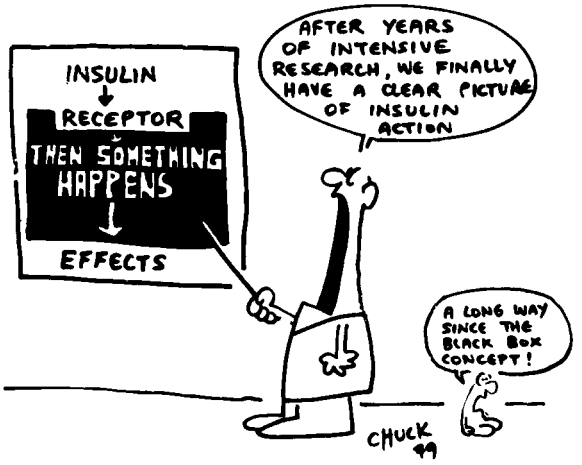

Fig. 1. Cartoon drawn by Pierre de Metz and published in Trends in Biochemical Sciences in 1979 that summarized the lack of progress in understanding insulin signal transduction at that time. It was known that insulin interacted with a specific receptor in the plasma membrane, but how this led to the physiological effects of the hormone was obscure.

messenger that mediates the adrenergic stimulation of glycogenolysis [10], and this is the approach that we have adopted.

The principal metabolic actions of insulin are to stimulate the uptake of nutrients (glucose, amino acids, fatty acids) into cells, to accelerate the rate at which they are converted to storage macromolecules (protein, glycogen, triglycerides) and to inhibit the degradation of these macromolecules. The system that we selected for analysis 24 years ago was the synthesis of glycogen in mammalian skeletal muscle, which is stimulated by insulin within minutes. The reason for this choice was that glycogen synthesis was the only system where any biochemical information was available. In the early 1960s Joe Larner and his colleagues had discovered that glycogen synthase, the enzyme which catalyses the last step in the pathway of glycogen synthesis, was inactivated by phosphorylation and reactivated by dephosphorylation [11], the third enzyme shown to be regulated in this manner. Larner also showed that insulin stimulates glycogen synthase by promoting its dephosphorylation [12], the first enzyme whose activity was shown to be regulated by this hormone. It was therefore already obvious, even in the 1960s, that insulin must exert this effect via inhibition of a protein kinase and/or activation of a protein phosphatase. Following the identification of cyclic AMP-dependent protein kinase (termed PKA) in 1968 [13], PKA was shown to phosphorylate glycogen synthase in vitro and to decrease its activity [14,15], suggesting that PKA might be the target for insulin. However, the level of cyclic AMP was unaffected by insulin under conditions where insulin stimulated glycogen synthesis [12], which led to a suggestion that insulin might exert its effects via a novel second messenger, which antagonised the activation of PKA by cyclic AMP [16].

We started working on this problem in late 1973 and, within a few months, had discovered that PKA was not only protein kinase capable of phosphorylating glycogen synthase. We identified another enzyme that phosphorylated a residue on glycogen synthase distinct from that phosphorylated by PKA [17], a finding which led our laboratory (and several others) to purify and characterise novel glycogen synthase kinases. Two years later the area had become very confused and the number of residues phosphorylated by PKA [18], the number of glycogen synthase kinases and their identities, and amount of phosphate bound covalently to glycogen synthase [19] were all extremely controversial topics. Ed Krebs added fuel to the flames by reporting that some proteins which are not phosphorylated in their native states become good substrates when they are unfolded and denatured [20], raising the possibility that some phosphorylations might be artefacts of no physiological relevance! A cartoon drawn by Peter Roach, which he presented at the 1976 IUB meeting in Hamburg, admirably summarized the situation at the time (Fig. 3). In retrospect, it is clear that everybody depicted in the cartoon was partially correct and the confusion was caused by the unexpected complexity of the system. Glycogen synthase was the paradigm for the phenomenon of 'multisite' phosphorylation [21]; i.e. the phosphorylation of a protein at multiple sites by two or more protein kinases. We now know that 'multisite phosphorylation' is an extremely common phenomenon that is used for a variety of purposes, such as signal integration.

The full complexity of the regulation of glycogen synthase has only been revealed over the past 10 years. The enzyme is phosphorylated at nine serine residues in vivo by a minimum of six protein kinases [22,23] (Fig. 4). Phosphorylation of four residues, sites 2a, 2b, 3a and 3b, leads to decreases in the activity of glycogen synthase, but the effects are cumulative so that almost complete inhibition occurs if all four serines are phosphorylated [23,24]. Fluctuations in the level of phosphorylation of these residues allows the activity of glycogen synthase to respond to the level of muscle glycogen and the contractile state of the tissue, as well as to the hormones adrenalin and insulin.

However, from the standpoint of insulin's action in this system, a key discovery was the finding that most of the phosphate released from glycogen synthase in response to this hormone is removed from the tryptic peptide-containing sites 3a and 3b [25]. Since the protein kinase in muscle extracts which is most active in phosphorylating these residues is glycogen synthase kinase-3 (GSK3) [26,27], it therefore appeared that insulin must either induce a decrease GSK3 activity or

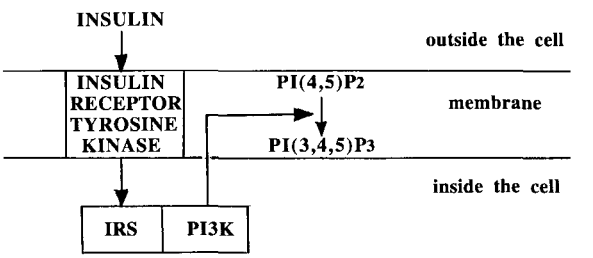

Fig. 2. Molecular mechanism by which insulin stimulates the formation of PtdIns(3,4,5)P_3. The insulin binds to the α-subunit of the insulin receptor, activating the protein tyrosine kinase associated with the β-subunit. The β-subunit then phosphorylates itself at a number of residues, including Tyr-960. The phosphorylation of Tyr-960 triggers an interaction between the receptor and IRS [71], allowing the receptor to phosphorylate IRS1/IRS2 at multiple tyrosine residues. Particular phosphotyrosine residues in IRS1/IRS2 then interact with the p85 subunit of PtdIns 3-kinase, thereby recruiting the p110 catalytic subunit of PtdIns 3-kinase to the plasma membrane where it converts the inositol phospholipid PtdIns(3,4)P_2 to PtdIns(3,4,5)P_3. PI(3,4,5)P_3 is subsequently converted to PtdIns(3,4)P_2 by a specific 5′-phosphatase.

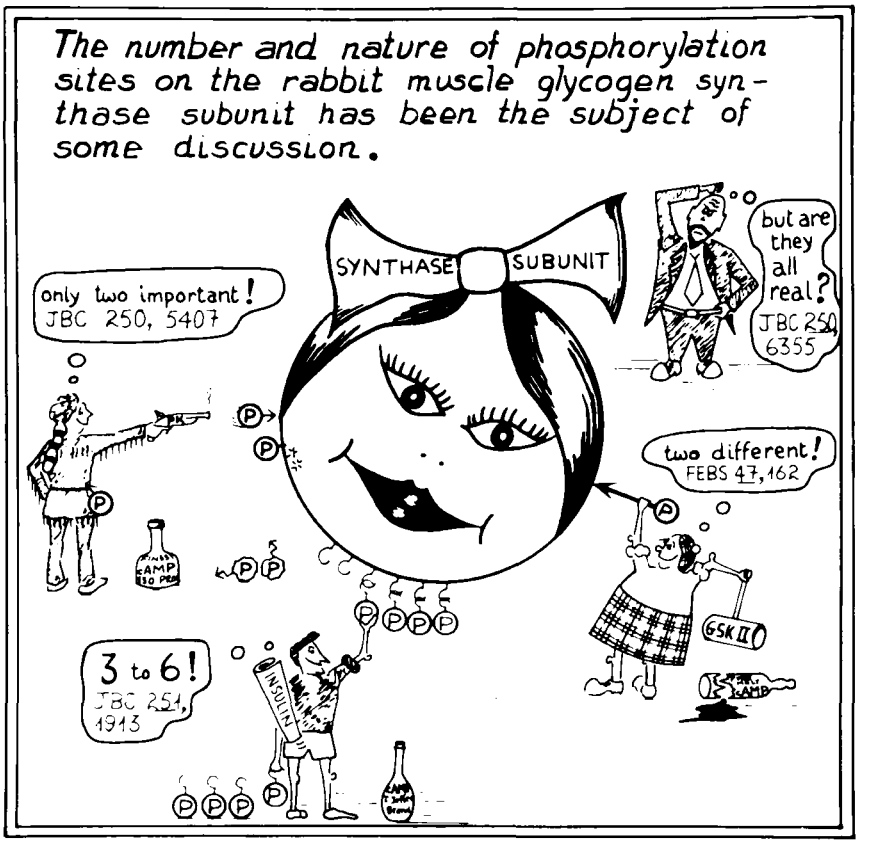

Fig. 3. Cartoon drawn and presented by Peter Roach in his poster (1976 IUB meeting Hamburg) that summarized the controversy surrounding the regulation of glycogen synthase. The participants in the debate are: West, Tom Soderling (then at Vanderbilt University, Nashville, Tennessee); South, Joe Larner (University of Virginia, Charlottesville); North East, Ed Krebs (University of Washington, Seattle; who has subsequently removed his beard – a dephosphorylation?!) and myself.

activate the protein phosphatase which dephosphorylates these sites. Importantly, these findings implied that inhibition of PKA was not, after all, the target for the action of insulin on glycogen synthase.

4. The inhibition of GSK3 by insulin is likely to be mediated by protein kinase B

The discovery that GSK3 is inhibited by insulin was made by investigators studying the mechanism by which insulin stimulates fatty acid synthesis and protein synthesis. Bill Benjamin identified a 'multifunctional' protein kinase which phosphorylates a number of proteins, including ATP-citrate lyase, the enzyme which converts citrate to acetyl CoA. The activity of this kinase was found to decrease by 50% when adipocytes were stimulated for a few minutes with insulin, and was subsequently identified by Jim Woodgett and Bill Benjamin as the α-isoform of GSK3 [28]. Similarly, Chris Proud described a protein kinase which phosphorylates eIF2B, a guanine nucleotide exchange factor essential for the initiation of protein synthesis, and found a 70% decrease in its activity when a chinese hamster ovary cell line that overexpresses the insulin receptor was stimulated with insulin; this enzyme was identified as the β-isoform of GSK3 [29]. Subsequently, the activities of the α- and β-isoforms of GSK3 were both shown to decrease by about 40% when rat L6 myotubes (a skeletal muscle cell line) [30] or rat skeletal muscle in situ [31] were stimulated with insulin (Fig. 5).

In unstimulated cells, GSK3 is phosphorylated stoichiometrically at one tyrosine (Tyr-279 in GSK3α, Tyr-216 in GSK3β) and the dephosphorylation of this residue leads to inactivation [32]. It had therefore been suggested that the inhibition of GSK3 by insulin might results from the activation of a protein tyrosine phosphatase. However, the inhibition of both GSK3 isoforms by insulin can be reversed by incubation with PP2A, a serine/threonine-specific protein phosphatase, indicating that inhibition resulted from serine/threonine phosphorylation and not from tyrosine dephosphorylation [29,30].

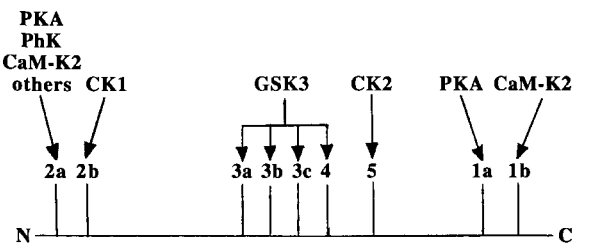

Fig. 4. Glycogen synthase in rabbit skeletal muscle is phosphorylated on nine serine residues in vivo. The serine residues are located at residues 7 (site 2a), 10 (2a), 640 (3a), 644 (3b), 648 (3c), 652 (4), 656 (5), 697 (1a) and 710 (1b). Abbreviations: PKA, cyclic AMP-dependent protein kinase; PhK, phosphorylase kinase; CaM-K2, calmodulin-dependent protein kinase-2; CK1, casein kinase-1; GSK3, glycogen synthase kinase-3; CK2, casein kinase-2. Most of the phosphate released from glycogen synthase in response to insulin is removed from the tryptic peptide-containing sites 3a, 3b and 3c.

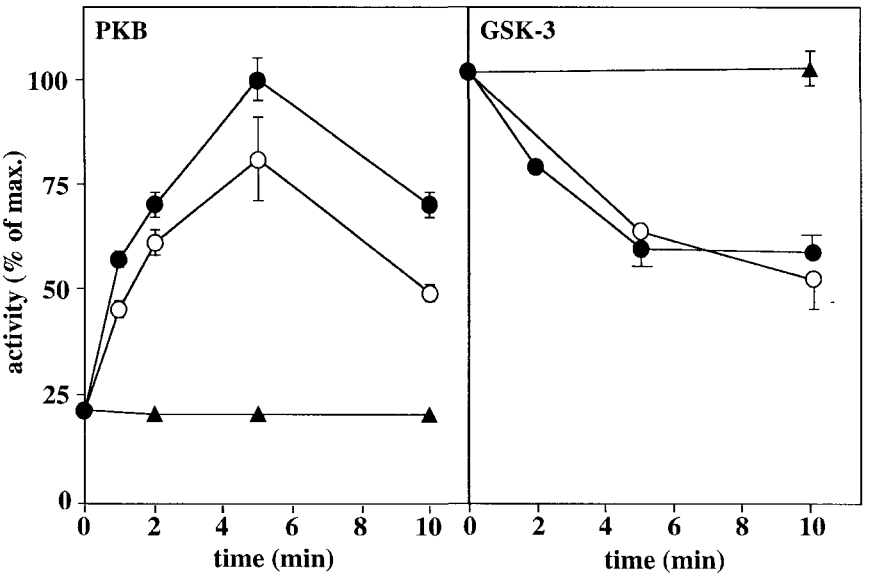

Fig. 5. The activation of PKB and the inhibition of GSK3 by insulin in L6 myotubes is prevented by inhibitors of PtdIns 3-kinase, but is unaffected by rapamycin or PD 98059. L6 myotubes were preincubated with 100 nM wortmannin (closed triangles), with 100 nM rapamycin plus 50 μM PD 98059 (open circles) or with no additions (closed circles), then stimulated with 0.1 μM insulin for the times indicated. The cells were lysed and PKB and GSK3 immunoprecipitated from the lysates and assayed. Similar results have been obtained in rat skeletal muscle in vivo [31].

This finding was confirmed subsequently by the isolation of tryptic phosphopeptides and amino-acid sequencing [33]. These studies showed that, in L6 myotubes, insulin induces the phosphorylation of Ser-21 in GSK3α and the equivalent residue (Ser-9) in GSK3β, without altering the ^{32}P-labelling of the phosphotyrosine-containing tryptic peptide.

We initially identified two insulin-stimulated protein kinases which phosphorylate GSK3α specifically at Ser-21 and GSK3β at Ser-9, and which leads to the inactivation of both isoforms in vitro [34,35]. One of these enzymes was **MAP** kinase-activated protein kinase-1 (MAPKAP-K1, also known as p90^{rsk}), a component of the classical MAP kinase cascade, while the other was p70 S6 kinase, the enzyme which mediates the phosphorylation of ribosomal protein S6 [36]. S6 phosphorylation is induced by insulin (and other growth factors) and has been implicated in stimulating the translation of mRNA transcripts which contain a polypyrimidine tract at their 5′ transcriptional start site; these transcripts mainly encode ribosomal proteins and protein synthesis elongation factors [37].

The insulin-induced activation of the MAP kinase cascade, and hence the activation of MAPKAP-K1 can be prevented by prior exposure of cells to the drug PD 98059 [38] or to cyclic AMP-elevating agents (such as 8-bromo cyclic AMP), while the activation of p70 S6 kinase can be prevented by the immunosuppressant drug rapamycin [36]. We therefore used these compounds to determine which of these insulin-stimulated protein kinases mediated the inhibition of GSK3 by insulin. Surprisingly, these experiments revealed that neither MAPKAP-K1 nor p70 S6 kinase were rate limiting for the inhibition of GSK3 by insulin, because the inhibition of GSK3 was unaffected when L6 myotubes were incubated in the presence of both PD 98059 and rapamycin under conditions which prevented the activation of MAPKAP-K1 and p70 S6 kinase [33]. However, the inhibition of GSK3 by insulin was suppressed by wortmannin (Fig. 5) or by LY 294002 [33], indicating that inactivation was catalysed by a protein kinase whose activation by insulin is dependent on PI 3-kinase activity.

We detected an insulin-stimulated protein kinase in extracts prepared from L6 myotubes whose activation was unaffected by rapamycin or PD 98059, but prevented by wortmannin, and which phosphorylated GSK3α at Ser-21 and GSK3β at Ser-9 [33]. Immunological studies identified this enzyme as the α-isoform of a protein kinase whose catalytic domain is most similar to PKA and protein kinase C (PKC) and which has therefore been termed protein kinase B (PKB) [39] or RAC (related to A and C) kinase [40]. It had already been shown that PKB was activated by insulin and other growth factors and that activation could be prevented by wortmannin or LY 294002 or by overexpression of a dominant negative mutant of PtdIns 3-kinase [41,42]. Mutation of tyrosine residues in the PDGF receptor that (when phosphorylated) bind to PtdIns 3-kinase, also prevented the activation of PKBα [41,42]. Furthermore, expression of a constitutively activated form of PtdIns 3-kinase triggered the activation of PKBα [43–45].

PKB is activated by insulin with a half time of about 1 min in L6 myotubes [33] (Fig. 5) or rat skeletal muscle [31], maximal activation occurring after 5 min. This is consistent with it being 'upstream' of GSK3 which is inhibited with a half time of 2 min in L6 myotubes (Fig. 5). GSK3 is also inhibited when co-transfected with PKB into human embryonic kidney 293 cells (D.R. Alessi and P. Cohen, unpublished work).

5. The mechanism of activation of protein kinase B

The activated form of PKB can be inactivated by PP2A, but not by protein tyrosine phosphatases [33,46], suggesting that the activation of PKB is triggered by phosphorylation of a serine and/or threonine residue(s). We found that the activation of PKBα in L6 myotubes was accompanied by the phosphorylation of two residues, Thr-308 and Ser-473 [47].

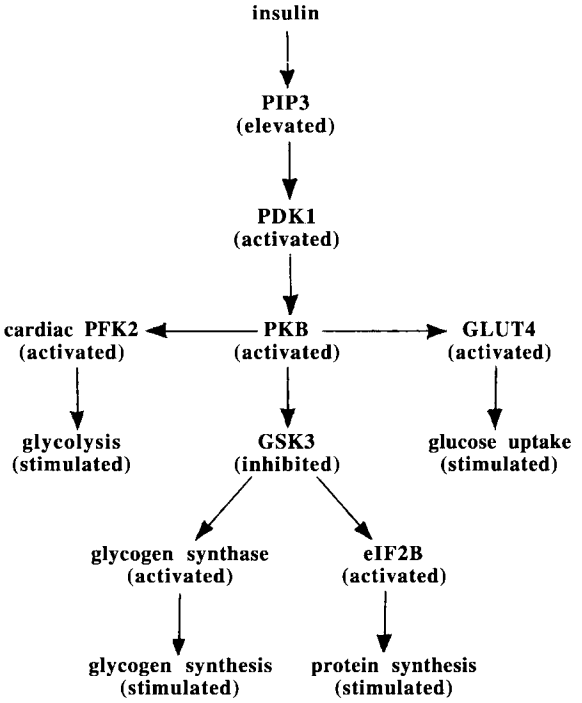

Fig. 6. The signal transduction pathway through which PKB and GSK3 may mediate many of the metabolic effects of insulin. The steps involved in the stimulation of PtdIns(3,4,5)P_3 formation by insulin are shown schematically in Fig. 2. Although transfection of 3T3-L1 adipocytes with a constitutively active form of PKB mimics the action of insulin in stimulating the translocation of GLUT4 from an intracellular compartment to the plasma membrane, GLUT4 itself is not thought to be a physiological substrate for PKB.

Thr-308 is located in the 'activation loop' between subdomains VII and VIII of the kinase catalytic domain, which lies nine residues 'upstream' of a conserved Ala-Pro-Glu motif; i.e. the same position as the activating phosphorylation sites found in many other protein kinases, such as MAP kinase. Ser-473 is not located in the catalytic domain but is very close to the C-terminus in a Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr motif that is present in several protein kinases that participate in growth factor-stimulated protein kinase cascades, such as MAPKAP-K1, p70 S6 kinase and PKC [48]. However, the significance of this observation is not yet clear.

Several lines of evidence indicate that the phosphorylation of Thr-308 and Ser-473 are both required for the full activation of PKBα [47]. First, the mutation of either Thr-308 or Ser-473 to Asp (to try and mimic the effect of phosphorylation by introducing a negative charge) increases the basal activity of PKBα about 5-fold, but the effects are synergistic, so that activation is 18-fold when both sites were mutated to Asp. Secondly, the phosphorylation of Ser-473[(2)] in vitro causes a 5-fold increase in PKBα activity, but activation is 25-fold when the phosphorylation of Ser-473 is combined with the mutation of Thr-308 to Asp. These observations indicate that the phosphorylation of Thr-308 and Ser-473 act synergistically to generate a high level of PKBα activity in vivo. PKB which has been partially activated by mutation of Thr-308 to Asp cannot phosphorylate itself at Ser-473. Similarly, PKB which has been partially activated by phosphorylation of Ser-473 or by mutation of Ser-473 to Asp cannot phosphorylate itself at Thr-308 [47]. These observations indicate that the enzyme(s) which phosphorylates Thr-308 and Ser-473 in vivo is unlikely to be PKB itself.

6. The mechanism of activation of PKB; identification of a PtdIns(3,4,5)P_3-dependent protein kinase-1 (PDK1)

The insulin- or IGF-1-induced phosphorylation of Thr-308 and Ser-473, like the activation of PKB, is prevented by wortmannin, suggesting that the protein kinase(s) responsible for phosphorylating PKB might be dependent on PI(3,4,5)P_3 for activity [47]. We detected an enzyme which phosphorylates PKB at Thr-308 (but not Ser-473) and increases its activity 30-fold in vitro, and purified it about 500 000-fold from skeletal muscle extracts to near homogeneity [49]. This enzyme is only active in the presence of lipid vesicles containing PI(3,4,5)P_3 or PI(3,4)P_2 and has therefore been termed 3-phosphoinositide-dependent protein kinase-1 (PDK1). PDK1 is not inhibited by wortmannin at concentrations 10-fold higher than those which inhibit PtdIns 3-kinase completely [49]. Its apparent molecular mass is 67 kDa, and its catalytic domain is most similar to the subfamily of protein kinases that include PKA and PKB (D. Alessi, unpublished work).

The concentration of PI(3,4,5)P_3 or PI(3,4)P_2 required for half maximal activation of PDK1 in vitro is 1–2 μM and activation is stereospecific; only the D-enantiomers are effective and many other inositol phospholipids have no effect [49]. PtdIns(3,4,5)P_3 derivatives containing unsaturated fatty acids are much more potent activators of PDK1 than derivatives containing saturated fatty acids [49]. Since unsaturated fatty acids discourage tight packing of adjacent phospholipid molecules, it is possible that this arrangement allows for more efficient interaction between membrane inserted PtdIns(3,4,5)P_3 and its effectors, perhaps explaining the biological significance of the unusual fatty acid composition of inositol phospholipids (the D-enantiomer of sn-1-stearoyl, 2-arachidonyl PtdIns(3,4,5)P_3 is likely to be the major species that is present in vivo).

The structure of PKB comprises an N-terminal pleckstrin homology (PH) domain of about 100 residues, which is followed by the catalytic domain and a C-terminal tail. PH domains in other proteins are known to be capable of interacting with inositol phospholipids, and we and others found that PKB (via its PH domain) bound with micromolar affinity to lipid vesicles containing PI(3,4,5)P_3 or PI(3,4)P_2, the interaction with PI(4,5)P_2 being about 10-fold weaker [50,51]. However, neither PI(3,4,5)P_3 nor PI(3,4)P_2 have any effect on the activity of PKBα under conditions where these lipids activate PDK1 strongly [49,50].

Our results disagree with recent reports from other laboratories which claimed that PKBα is activated directly by PtdIns(3,4)P_2 [51–53]. Contamination of PKBα preparations with trace PDK1 activity might explain this discrepancy; recall that PKA was originally discovered as a trace contaminant in purified preparations of phosphorylase kinase (one of its 'downstream' targets). It has also been reported that PKBα is inhibited by PtdIns(3,4,5)P_3 [51,53], but none of the four PtdIns(3,4,5)P_3 derivatives we tested inhibited the basal PKBα

(2) The phosphorylation of Ser-473 is catalysed by MAPKAP kinase-2 in vitro, but this protein kinase does not mediate the insulin-induced phosphorylation of Ser-473 for reasons discussed in [47].

activity at all, while all four were capable of activating PDK1. It is possible that the synthetic PtdIns(3,4,5)P_3 used in these studies contained impurities that inhibited PKBα.

Although PKBα is not activated directly by PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2, the interaction with these lipids may facilitate the activation of PKBα by PDK1 and/or the Ser-473 kinase, either by a conformational change or by recruiting PKBα to the plasma membrane. Indeed, we have recently demonstrated that transfected PKBα does translocate to the plasma membrane in response to IGF-1 in 293 cells [54]. However, neither of these mechanisms appear to be essential for the activation of PKBα in vivo, because insulin is reported to activate a mutant PKB lacking the PH domain at least as well as wild-type PKB [54–56]. Recruitment of PKBα to the plasma membrane may therefore play a different role. For example, it may facilitate the phosphorylation of membrane-associated substrates.

With the discovery of PDK1, the two different approaches used to dissect the insulin signal transduction pathway (the 'outside to inside' and 'inside to outside' approaches) have finally met one another to produce the outline of a pathway that may explain many of the metabolic actions of insulin (Fig. 6). PDK1 is likely to be a major target for the insulin second messenger PtdIns(3,4,5)P_3 and is therefore a landmark in our understanding of insulin signal transduction. It remains to be seen whether drugs can be developed which activate PDK1 (by mimicking PtdIns(3,4,5)P_3) and whether such drugs will be efficaceous for the treatment of diabetes.

GSK3 is a multifunctional protein kinase and its inhibition is likely to stimulate the dephosphorylation of a number of proteins in vivo. In particular, the inhibition of GSK3 appears to underlie the dephosphorylation and activation of eIF2B and may therefore contribute to the stimulation of protein synthesis initiation [29], as well as to the stimulation of glycogen synthesis (Fig. 6). GSK3 is inhibited potently and relatively specifically by millimolar concentrations of lithium ions [57] and, consistent with this finding, lithium ions mimic the action of insulin in stimulating glycogen synthesis [58]. It will be interesting to see if lithium ions also mimic the effect of insulin on protein synthesis. Perhaps drugs which inhibit GSK3 specifically will mimic a number of the actions of insulin and also prove to be useful in the treatment of diabetes.

PKB phosphorylates serine residues in proteins and peptides which lie in Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd motifs, where Hyd is a bulky hydrophobic residue (Phe, Leu) [59]. This motif is not only found at the serine residues in GSK3α and GSK3β whose phosphorylation is induced by insulin, but also surrounding the serines whose phosphorylation underlies the activation of 6-phosphofructo-2-kinase (PFK2) by insulin in cardiac muscle. These serine residues are phosphorylated by PKB in vitro, and their phosphorylation in vivo is prevented by wortmannin (but resistant to rapamycin and PD 98059) [60,61]. PFK2 catalyses the formation of fructose 2,6,bisphosphate, a key allosteric activator of 6-phosphofructo-1-kinase, the rate-limiting enzyme in glycolysis. Thus, the phosphorylation of PFK2 by PKB may explain how cardiac glycolysis is stimulated by insulin (Fig. 6).

Insulin stimulates glucose uptake into adipose tissue and muscle by promoting the translocation of the glucose transporter GLUT4 from an intracellular location to the plasma membrane. This effect is prevented by inhibition of PtdIns 3-kinase and mimicked by constitutively active forms of PtdIns 3-kinase. Transfection of a constitutively active PKB into 3T3-L1 adipocytes also mimics the effect of insulin by promoting translocation of GLUT4 to the plasma membrane and glucose uptake [62]. The PKB-catalysed phosphorylation of an (as yet unidentified) protein may therefore underlie the stimulation of glucose uptake by insulin (Fig. 6).

The activation of p70 S6 kinase is not only prevented by rapamycin, but also by wortmannin. PKB has been shown to induce the activation of p70 S6 kinase in co-transfection experiments [41], suggesting that PKB may also lie on the signalling pathway by which insulin induces the phosphorylation of ribosomal protein S6, and hence the translation of certain mRNA transcripts [37]. However, PKB does not phosphorylate p70 S6 kinase directly and how it triggers this effect is unknown.

PKB is the cellular homologue of v-Akt, a protein encoded in the genome of the AKT-8 acute transforming retrovirus isolated from a rodent T-cell lymphoma [63] and has therefore also been termed c-Akt. It is therefore intriguing that the PKBβ isoform is overexpressed in 12% of ovarian cancers, 10% of pancreatic cancers and 3% of breast cancers [40,64,65]. It has recently been found that PKB provides a survival signal that protects cells from apoptosis induced by ultraviolet radiation, by the withdrawal of the survival factor insulin-like growth factor-1 from neuronal cells, and by the withdrawal of serum factors while myc is being articially expressed [66–68]. The activation of PKB by gene amplification (and other mechanisms) may therefore contribute to the generation of malignancies that are able to flourish in the absence of extracellular survival signals. The development of specific inhibitors of PKB isoforms could be efficaceous for the treatment of some cancers.

7. Concluding remarks and perspective

The discovery of PDK1, its role in the activation of PKB and the realisation that PKB and GSK3 are likely to play many key roles in vivo (Fig. 6), represent significant advances in our understanding of insulin signal transduction. Nevertheless, many important questions still remain to be answered. For example, is it PtdIns(3,4,5)P_3, PtdIns(3,4)P_2 or both of these molecules that activates PDK1 in vivo? This information is crucial in order to understand the physiological role of the 5′-phosphatase which convers PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2. PDK1 presumably activates the PKBβ and PKBγ isoforms, but does it activate other protein kinases as well? What is the identity of the protein kinase which phosphorylates PKBα at Ser-473 in vivo and is this enzyme, like PDK1, also activated directly by PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2? Ser-473, and the amino-acid sequence surrounding it, is conserved in PKBβ but intriguingly, is absent in PKBγ [69]. How many proteins do PKB and GSK3 phosphorylate in vivo and how many of the intracellular actions of insulin do these protein kinases really mediate? Although the insulin-induced inhibition of GSK3 in skeletal muscle (40%, Fig. 5) is, in theory, almost sufficient to account for the observed (2-fold) stimulation of glycogen synthase, it remains entirely possible that insulin also induces the activation of the glycogen-associated form of protein phosphatase-1 (PP1G) which is likely to dephosphorylate glycogen synthase in skeletal muscle. Indeed, evidence that PP1G may be activated by insulin has been presented [70]. Finally, it should be

admitted that the genetic evidence needed to establish that GSK3 and cardiac PFK2 are really physiological substrates for PKB, and that glycogen synthase and eIF2B are really physiological substrates for GSK3, is still lacking. Drugs which inhibit PKB and GSK3 specifically would be immensely valuable in helping to elucidate their physiological roles, in addition to having therapeutic potential.

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