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The Croonian Lecture 1998. Identification of a protein kinase cascade of major importance in insulin signal transduction

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THE ROYAL SOCIETY

The Croonian Lecture 1998. Identification of a protein kinase cascade of major importance in insulin signal transduction

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Diabetes affects 3% of the European population and 140 million people worldwide, and is largely a disease of insulin resistance in which the tissues fail to respond to this hormone. This emphasizes the importance of understanding how insulin signals to the cell's interior. We have recently dissected a protein kinase cascade that is triggered by the formation of the insulin 'second messenger' phosphatidylinositide (3,4,5) trisphosphate (PtdIns(3,4,5)P_3) and which appears to mediate many of the metabolic actions of this hormone. The first enzyme in the cascade is termed 3-phosphoinositide-dependent protein kinase-1 (PDK1), because it only activates protein kinase B (PKB), the next enzyme in the pathway, in the presence of PtdIns(3,4,5)P_3. PKB then inactivates glycogen synthase kinase-3 (GSK3). PDK1, PKB and GSK3 regulate many physiological events by phosphorylating a variety of intracellular proteins. In addition, PKB plays an important role in mediating protection against apoptosis by survival factors, such as insulin-like growth factor-1.

Keywords: insulin; IGFl; diabetes; protein kinase; apoptosis; inositol phospholipid

1. INTRODUCTION

Although it has been known for 100 years that proteins contain covalently bound phosphate, it was only in the mid-1950s that the biological significance of this phenomenon began to be recognized. Just over 40 years ago, Edmond Fischer and Edwin Krebs discovered that glycogen phosphorylase was regulated by reversible phosphorylation. This enzyme, which mobilizes glycogen for energy production during muscle contraction, is present in an inactive form in resting muscle, and is converted to an active form during contraction. Fischer and Krebs showed that activation involves the transfer of phosphate from ATP to a serine residue on glycogen phosphorylase, in a phosphorylation reaction catalysed by phosphorylase kinase, the first protein kinase to be identified. Phosphorylase kinase is activated by calcium ions, explaining how the mobilization of glycogen is coupled to the onset of muscle contraction (Cohen 1980). When contraction ceases, glycogen phosphorylase is reconverted to the inactive, dephosphorylated species by protein phosphatase-1 (Cohen 1978).

The general significance of these findings was not appreciated for many years and phosphorylation was still thought of as a control mechanism specific to the regulation of glycogen metabolism when I first started to work in this field in late 1969. The subsequent explosion of this area has therefore been remarkable, since it is now clear that the reversible phosphorylation of proteins regulates most aspects of cell life. Its functions include the control of metabolism, secretion, the expression of genes, the immune response, cell proliferation and differentiation, fertilization and even the acquisition of memory.

About one-third of mammalian proteins contain covalently bound phosphate, while protein kinases and phosphatases (together with a myriad of regulatory and targetting subunits that control and direct them to the correct subcellular locations (Hubbard & Cohen 1993; Egloff *et al*. 1997)) account for perhaps 5% of all human gene products. The phosphorylation of proteins on serine, threonine and tyrosine residues alters their shape and hence their ability to function. However, phosphorylation can affect the functioning of protein in other ways, for example by altering the rate at which they are degraded, or their ability to migrate from one cellular compartment to another. The simplicity, flexibility and reversibility of phosphorylation explains why this covalent modification has been adopted by eukaryotic cells as its major control mechanism.

2. REVERSIBLE PROTEIN PHOSPHORYLATION AND HUMAN DISEASE

In view its importance in cell regulation, it should come as no surprise to learn that abnormal protein phosphorylation is a cause or consequence of many diseases. For example, at least 15 hereditary disorders result from genetic alterations in various protein kinases and phosphatases. These include forms of muscular dystrophy, leukaemia and lymphoma, severe combined immunodeficiency syndromes and leprechaunism.

Several toxins and tumour promoters that are a threat to the health of man and his domestic livestock are inhibitors of phosphatases that dephosphorylate serine and threonine residues in proteins. These include okadaic acid (Cohen *et al*. 1990), the toxin responsible for diarrhetic

seafood poisoning (DSP), which is produced by marine micro-organisms and accumulates in the digestive glands of shellfish. This substance has not only poisoned many thousands of people (including in 1976 the President of the French Republic), but continues to have devastating effects on the shellfish industry worldwide, causing losses amounting to millions of pounds each year.

Another toxin that inhibits the same class of protein phosphatases is microcystin, a cyclic heptapeptide produced by toxic blue-green algae (MacKintosh *et al.* 1990, 1995). This substance is an extremely powerful hepatotoxin and the most potent liver carcinogen known to man. It poses a potential threat to water supplies because toxic algal blooms affect 75% of reservoirs worldwide, and this problem is becoming more serious because the growth of algae has accelerated as a result of the enrichment of water with nitrates from fertilizers and phosphates from detergents. Several years ago our Unit developed a simple method for measuring the level of microcystin, based on phosphatase inhibition, which is thousands of times more sensitive than those used previously (MacKintosh & MacKintosh 1994). This method has been adopted by the Thames Water Authority and several reservoirs were stopped from supplying water to the London area in 1994 because of the high level of microcystin detected by the use of this technique.

Several serious diseases, such as pseudotuberculosis and the bubonic plague, are caused by bacteria of the genus *Yersinia*. The bubonic plague has been responsible for pandemics such as 'the Black Death', which killed one-quarter of the population of Europe and 42 million people worldwide in the 12th and 13th centuries. The essential virulence agent for these diseases is a protein phosphatase encoded in the genome of *Yersinia* (Bliska *et al.* 1991), which causes the uncontrolled tyrosine dephosphorylation of many proteins and leads rapidly to death.

The abnormal phosphorylation of proteins is also the cause or consequence of major diseases such as cancer, inflammation and diabetes. However, before discussing recent progress in understanding how cells respond to insulin, which is defective in diabetes, I must first briefly introduce the concept of 'signal transduction'.

3. PROTEIN PHOSPHORYLATION AND SIGNAL TRANSDUCTION

Signal transduction is the process by which cells respond to extracellular agonists, such as hormones, growth factors and cytokines. These substances circulate in the blood at nanomolar concentrations (or even lower) and yet, without entering their target cells, they can elicit remarkable alterations in cell behaviour on the seconds-to-minutes time-scale. The generalized mechanism by which this is achieved is illustrated schematically in figure 1. Signals bind to specific receptors on the outer surface of the cell membrane, triggering the activation of transmembrane signalling systems that control the production of chemical mediators termed 'second messengers'. These substances regulate the activities of protein kinases and phosphatases, thereby altering the level of phosphorylation of intracellular proteins. The effect of a signal is amplified enormously through these devices, and since each 'second messenger'-dependent protein kinase (or phosphatase) normally catalyses the phosphorylation (or dephosphorylation) of many intracellular proteins, this explains how any one signal can produce such a diversity of responses (Cohen 1982, 1985). The amplification and diversity of a response are frequently enhanced when several protein kinases become linked together in a sequence to form a protein kinase 'cascade'. In these situations, each protein kinase in the cascade can also become a target for regulation by other protein kinases and phosphatases that are themselves controlled by different signals, leading to signal integration. Moreover, once activated, protein kinases can migrate to different regions of the cell, allowing the signal to 'reach' different compartments. The MRC Protein Phosphorylation Unit is involved in dissecting a number of protein kinase cascades the activation of which is abnormal in major diseases like cancer, arthritis and diabetes. In this paper I concentrate on the last of these three topics.

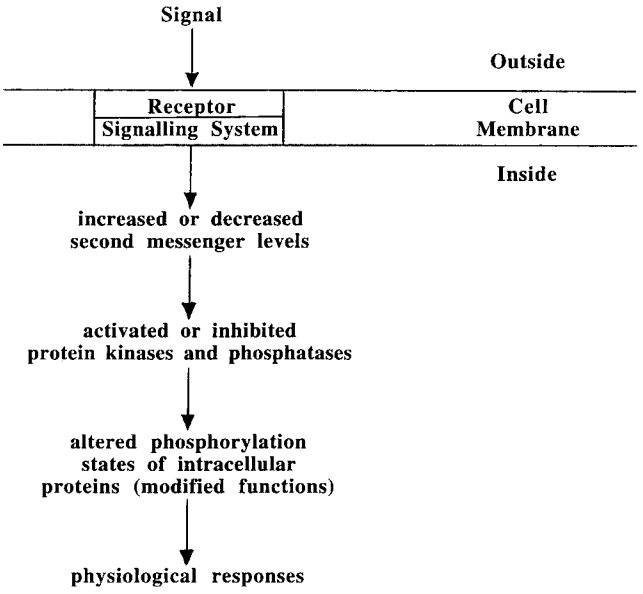

Figure 1. Generalized signal transduction pathway by which hormones and other extracellular signals regulate cell functions.

4. INSULIN AND DIABETES

Insulin is a protein produced and secreted by the β-cells of the pancreas, whose principal functions are to regulate the uptake of nutrients (glucose, amino acids, fatty acids) and the production of storage macromolecules (protein, glycogen, triglycerides) by muscle, liver and adipose tissue. Diabetes is characterized by the failure to synthesize, secrete or respond to insulin and is the third most prevalent disease in the Western world. It affects some 3% of the population of Europe and North America and 140 million worldwide. However, due to long-term complications, such as increased risk of heart disease, kidney failure and blindness, the treatment of diabetes was estimated to account for 12% of total health-care expenditure in the USA in 1992. In most people with diabetes, the level of insulin in the circulation is normal, or even above normal, at least in the early stages of the disease, and the primary defect is the failure of the tissues to respond to this hormone. To understand the causes of diabetes, and hence to be able to improve its

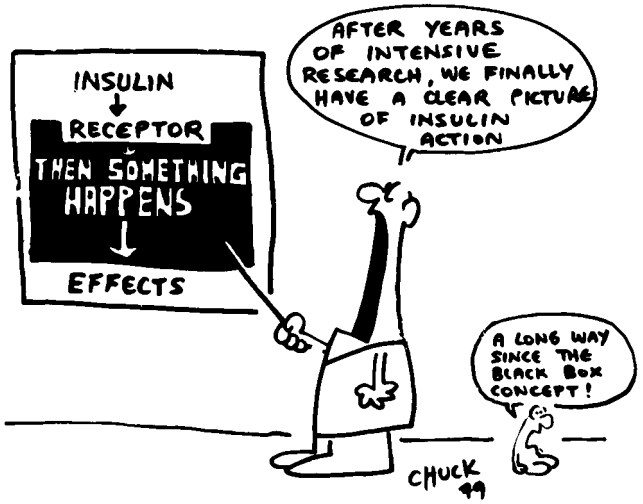

Figure 2. Cartoon drawn by Pierre de Metz and published in *Trends in Biochemical Sciences* in 1979 that summarized the lack of progress in understanding insulin signal transduction at that time. It was known that insulin interacted with a specific receptor in the plasma membrane, but how this led to the physiological effects of the hormone was obscure.

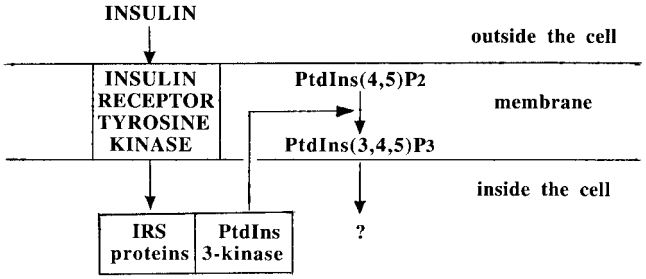

Figure 3. Molecular mechanism by which insulin stimulates the formation of PtdIns(3,4,5)P_3. Insulin binds to the α-subunit of the insulin receptor, activating the protein tyrosine kinase associated with the β-subunit. The β-subunit then phosphorylates itself at a number of residues, including Tyr-960. The phosphorylation of Tyr-960 triggers an interaction between the receptor and isoforms of the insulin receptor substrate (IRS), allowing the receptor to phosphorylate IRS at multiple tyrosine residues. Particular phosphotyrosine residues in IRS proteins then interact with the SH2 domain of the p85 subunit of class 1 PtdIns 3-kinases, thereby recruiting the p110 catalytic subunit to the plasma membrane where it converts the inositol phospholipid PtdIns(4,5)P_2 to PtdInsI(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.

treatment, it is therefore critical to elucidate the mechanism by which insulin signals to the cell interior. However, the solution to this apparently simple problem has proved to be remarkably elusive. The Nobel Prize for Medicine was awarded in 1923 for the discovery of insulin a couple of years earlier by George Banting and Charles Best (the 1955 Croonian Lecturer), while Fred Sanger (the 1975 Croonian Lecturer) and Dorothy Hodgkin were awarded the 1958 and 1964 Nobel Prizes for Chemistry for elucidating its amino acid sequence and three-dimensional structure. However, the insulin signalling mechanism remained unsolved for over 50 years. A famous cartoon from 1979 reproduced in figure 2 nicely summarized how little we knew about this process 18 years ago.

5. FROM THE INSULIN RECEPTOR TO THE FORMATION OF PHOSPHATIDYLINOSITIDE (3,4,5) TRISPHOSPHATE

Real progress in understanding insulin signal transduction started to be made in the early 1980s. Ron Kahn discovered that the insulin receptor (Kasuga *et al*. 1982), like the epidermal growth factor receptor (Ushiro & Cohen 1980), was a protein tyrosine kinase, and he and Morris White identified the insulin receptor substrate proteins (IRS) (Myers *et al*. 1994; Sun *et al*. 1995) as key targets of the receptor. The phosphotyrosine residues on IRS proteins then interact with the SH2-domain (Pawson & Gish 1992) of the p85 regulatory subunit of class 1 phosphatidylinositide (PtdIns) 3-kinases, thereby recruiting the latter to the plasma membrane. Class 1 PtdIns 3-kinases, first described by Lew Cantley (Whitman *et al*. 1988), then phosphorylates a minor inositol phospholipid in the plasma membrane, termed PtdIns (4,5) bisphosphate at the D-3 position of the inositol ring to produce PtdIns (3,4,5) trisphosphate (PtdIns(3,4,5)P_3) (Vanhaesbroeck *et al*. 1997) (figure 3), which is subsequently converted to PtdIns(3,4)P_2 by the action of a 5′phosphatase(s) (Woscholski & Parker 1997). The level of PtdIns(3,4,5)P_3 *in vivo* increases 10- to 100-fold within 30 s of stimulation with insulin, followed by a similar rise in PtdIns(3,4)P_2 (Vanhaesbroeck *et al*. 1997; Woscholski & Parker 1997). The importance of PtdIns 3-kinases in insulin signal transduction became clear when inhibitors of these enzymes, wortmannin (Ui *et al*. 1995) and LY 294002 (Vlahos *et al*. 1994), were shown to suppress nearly all the metabolic actions of insulin. This suggested that PtdIns(3,4,5)P_3 or a metabolite derived from it, such as PtdIns(3,4)P_2, might be the long sought after 'second messenger' for insulin, raising the question of how this compound exerted its effects on cell function.

6. THE STIMULATION OF GLYCOGEN SYNTHESIS 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 exploited by Earl Sutherland (the 1970 Nobel Laureate for Medicine) when he discovered that cyclic AMP (the original second messenger) mediates the adrenergic stimulation of glycogenolysis (Robison *et al*. 1971), and is the approach that we have adopted.

We selected glycogen synthesis in skeletal muscle as our model system in the early 1970s because it was the only one where any biochemical information was available about insulin signal transduction. In the early 1960s, Joe Larner and his colleagues had discovered that glycogen synthase, the enzyme that catalyses the last step in the synthesis of glycogen, was inactivated by phosphorylation and reactivated by dephosphorylation (Friedman & Larner 1963). Larner also showed that insulin stimulates

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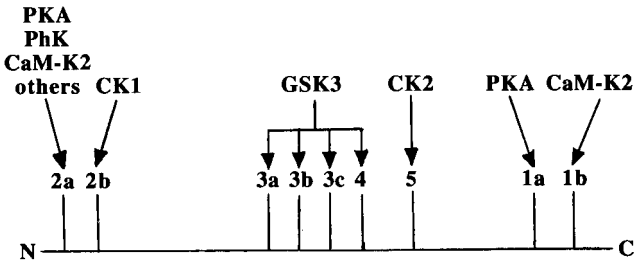

Figure 4. Glycogen synthase in rabbit skeletal muscle is phosphorylated on nine serine residues *in vivo* by at least six protein kinases. The nine serine residues are located at residues 7 (site 2a), 10 (2b), 640 (3a), 644 (3b), 648 (3c), 652 (4), 656 (5), 697 (1a) and 710 (1b). Abbreviations: PKA, 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.

glycogen synthase by promoting its dephosphorylation (Craig & Larner 1964), the first time any enzyme had been shown to be regulated by this hormone. It was therefore obvious that insulin must exert its effect via inhibition of a protein kinase and/or activation of a protein phosphatase. Cyclic AMP-dependent protein kinase (PKA) was identified in 1968 (Walsh *et al*. 1968) and shown to phosphorylate and inhibit glycogen synthase *in vitro* (Schlender *et al*. 1969; Soderling *et al*. 1970). This finding suggested that the inhibition of PKA might underlie the insulin-induced activation of glycogen synthase, but the level of cyclic AMP in skeletal muscle was not affected by insulin (Craig & Larner 1964).

Our work on this problem was initiated in late 1973 and within months we had discovered another protein kinase that phosphorylated glycogen synthase at a residue distinct from that targetted by PKA (Nimmo & Cohen 1974). We (and others) then identified further glycogen synthase kinases, but the full complexity of the situation was only revealed many years later when it became clear that glycogen synthase is phosphorylated at nine serine residues *in vivo* by a minimum of six protein kinases (Poulter *et al*. 1988; Nakielny *et al*. 1991) (figure 4). In 1983, we showed that the insulin-induced dephosphorylation of glycogen synthase occurred mainly at serine residues phosphorylated by glycogen synthase kinase-3 (GSK3) (Parker *et al*. 1983), an enzyme that we had identified a few years earlier (Embi *et al*. 1980; Rylatt *et al*. 1980). These observations implied that the inhibition of PKA did not underlie the stimulation of glycogen synthase by insulin. Rather, it appeared that insulin must either induce a decrease in GSK3 activity and/or activate protein phosphatase-1, the enzyme that dephosphorylates these sites.

7. 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 that phosphorylated a number of proteins including ATP-citrate lyase, the enzyme that converts citrate to acetyl CoA. The activity of the kinase decreased when adipocytes were stimulated for a few minutes with insulin, and this enzyme was subsequently identified by Jim Woodgett as the α-isoform of GSK3 (Hughes *et al*. 1992). Chris Proud described an insulin-inhibited protein kinase that phosphorylated eIF2B, a guanine nucleotide exchange factor essential for the initiation of protein synthesis; this protein kinase was identified as the β-isoform of GSK3 (Welsh & Proud 1993). Subsequently, we showed that insulin decreases the activities of both GSK3 isoforms in skeletal muscle (Cross *et al*. 1994, 1997).

In unstimulated cells, GSK3 is phosphorylated stoichiometrically at one tyrosine residue (Tyr-279 in the GSK3α isoform and Tyr-216 in GSK3β) and the dephosphorylation of these residues *in vitro* is accompanied by inactivation (Hughes *et al*. 1993). It had therefore been suggested that the inhibition of GSK3 by insulin might result from activation of a protein tyrosine phosphatase. However, the insulin-induced inhibition of both GSK3 isoforms could be reversed by the serine–threonine-specific protein phosphatases, implying that inhibition was caused by increased serine or threonine phosphorylation and not by decreased tyrosine phosphorylation (Welsh & Proud 1993; Cross *et al*. 1994). We established, subsequently, that this was indeed the case and that the inactivating phosphorylations occur at Ser21 (GSK3α) and Ser9 (GSK3β) (Cross *et al*. 1995; Shaw *et al*. 1997).

We initially identified two insulin-stimulated protein kinases that phosphorylated GSK3α at Ser-21 and GSK3β at Ser-9 (Sutherland *et al*. 1993; Sutherland & Cohen 1994). One 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 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 (Alessi *et al*. 1995), while the activation of p70 S6 kinase can be prevented by the immunosuppressant drug rapamycin (Chung *et al*. 1992). We therefore used these compounds to try and determine which insulin-stimulated protein kinase mediated the inhibition of GSK3 by insulin. Surprisingly, these and other experiments revealed that neither MAPKAP-K1 nor p70 S6 kinase were rate limiting for the inhibition of GSK3 by insulin (Cross *et al*. 1995). However, inhibition was suppressed by inhibitors of PtdIns 3-kinase (Cross *et al*. 1995), indicating that GSK3 was inactivated by a novel insulin-stimulated protein kinase whose activation was dependent on PtdIns 3-kinase.

To identify the relevant insulin-stimulated protein kinase(s), we subjected lysates from L6 cells to anion exchange chromatography and assayed the fractions for protein kinases capable of phosphorylating a synthetic peptide related to the amino acid sequence surrounding Ser21 in GSK3α and Ser9 in GSK3β (Cross *et al*. 1995). We called this peptide 'Crosstide' after Darren Cross, the PhD student who was carrying out these experiments. These studies revealed three major peaks of Crosstide kinase activity which had the properties we were looking for, i.e. they could only be detected if the cells were

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stimulated with insulin, and their appearance was prevented if the cells were preincubated with inhibitors of PtdIns 3-kinase, but not if the cells were preincubated with PD 98059 and rapamycin. It looked as though we would now have to spend a considerable time purifying and characterizing these protein kinases but, at this juncture, a stroke of luck occurred with the arrival of Brian Hemmings from Basel to examine one of my wife's PhD students. After telling Brian about our results, he informed us that an enzyme termed protein kinase B (PKB) had just been shown to be activated by insulin and other growth factors via a PtdIns 3-kinase-dependent mechanism (Franke *et al.* 1995; Burgering & Coffer 1995). PKB had been cloned several years earlier by Brian while he was searching for novel homologues of PKA, and by Jim Woodgett who had been trying to detect novel isoforms of protein kinase C (PKC). Brian had called it RAC (related to A and C) kinase (Jones *et al.* 1991), while Jim had used the name protein kinase B (PKB) because it was similar (in between) PKA and PKC (Coffer & Woodgett 1991). The name PKB is used hereafter to avoid any confusion with the GTP-binding protein RAC.

Fortunately, Brian had raised an immunoprecipitating antibody to the α-isoform of PKB, so that we were able to show very rapidly that two of the peaks of Crosstide kinase activity corresponded to PKBα (Cross *et al.* 1995); more recently the other peak of Crosstide kinase activity was identified as PKBγ (Walker *et al.* 1998).

We showed that PKB phosphorylates each GSK3 isoform *in vitro* and at the relevant serine residues (Cross *et al.* 1995), and several further lines of evidence were consistent with PKB lying 'upstream' of GSK3 *in vivo*. First, PKB is activated by insulin with a half-time of 1 min, slightly faster than the inhibition of GSK3, which occurs with a half-time of 2 min (Cross *et al.* 1995, 1997). Second, GSK3 is inhibited when cotransfected into mammalian cells with PKB, or with PDK1 (one of the activators of PKB; see below) (Shaw *et al.* 1997). It was therefore clear that we now needed to find out how PKB was activated.

8. PROTEIN KINASE B IS ACTIVATED BY PHOSPHORYLATION; IDENTIFICATION OF PDK1

The activated form of PKBα can be inactivated by PP2A, but not by protein tyrosine phosphatases (Cross *et al.* 1995; Andjelkovic *et al.* 1996), suggesting that activation is triggered by phosphorylation of a serine and/or threonine residue(s). We found that the activation of PKBα in L6 myotubes induced by either insulin or insulin-like growth factor-1 (IGF1) was accompanied by the phosphorylation of Thr308 in the catalytic domain and Ser473 close to the C-terminus. Several lines of evidence showed that the phosphorylation of either residue produced partial activation, that the phosphorylation of both residues was required for full activation and that neither site was phosphorylated by PKBα itself (Alessi *et al.* 1996*a*).

There are three isoforms of PKB. PKBβ is activated by the phosphorylation of Thr309 and Ser474, the residues equivalent to Thr308 and Ser473 of PKBα (Meier *et al.* 1997), but PKBγ is activated solely by the phosphorylation of Thr395, the residue equivalent to Thr308 (Walker *et al.* 1998) because it terminates at residue 454 and therefore lacks Ser473 of PKBα.

The insulin-induced or IGF1-induced phosphorylation of Thr-308 and Ser-473, like the activation of PKB, is prevented by inhibitors of PtdIns 3-kinase (Alessi *et al.* 1996), suggesting that the protein kinase(s) responsible for phosphorylating PKB at these sites might require PtdIns(3,4,5)P_3 for activity. These observations led us to detect a protein kinase that phosphorylates PKBα at Thr308 (Alessi *et al.* 1997*a*), PKBβ at Thr309 and PKBγ at Thr305 (Walker *et al.* 1998), only in the presence of lipid vesicles containing micromolar concentrations of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. We therefore termed this enzyme 3-phosphoinositide-dependent protein kinase-1 (PDK1).

We purified PDK1 to near homogeneity (Alessi *et al.* 1997*b*) and after sequencing a number of tryptic peptides used them to interrogate protein sequence databases. This allowed almost the entire sequence to be assembled from ESTs, and revealed that PDK1 comprises an N-terminal catalytic domain and a C-terminal pleckstrin homology (PH) domain (Alessi *et al.* 1997*b*). It is therefore the 'mirror image' of PKB, in which a PH domain preceeds the catalytic domain. The database search also revealed that, surprisingly, the nucleotide sequence encoding a *Drosophila* homologue of PDK1 had also recently been deposited in the database. Even more surprisingly, it had been deposited by another investigator (Mary Bownes) working in Scotland. I return to this near the end of the paper.

9. ROLE OF 3-PHOSPHOINOSITIDES IN THE PDK1-MEDIATED ACTIVATION OF PKB

The activation of PKB is stereospecific for the D-enantiomers of PtdIns(3,4,5)P_3, and the naturally occurring form (the 1-stearoyl, 2-arachidonyl derivative) is more effective than the dipalmitoyl derivative (Alessi *et al.* 1997*a*). Since unsaturated fatty acids discourage tight packing of adjacent phospholipid molecules, this arrangement may allow for more efficient interaction between PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 and their protein effectors, perhaps explaining why inositol phospholipids have such unusual fatty acid compositions. Neither PtdIns(4,5)P_2 nor any other inositol phospholipid tested can replace PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 in the PDK1-catalysed activation of PKB. PKB itself is not activated (or inhibited) directly by any inositol phospholipid.

The findings described above gave rise to the name PDK1 (3-phosphoinositide-dependent protein kinase-1) (Alessi *et al.* 1997*a*), but subsequent work indicated that the requirement for PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 is mediated, at least in part, by the interaction of these inositol phospholipids with the PH domain of PKB. These 'second messengers' appear to alter the conformation of PKB so that Thr308 becomes accessible to PDK1. This conclusion is supported by the observation that a PKBα mutant that cannot interact with PtdIns(3,4,5)P_3 is not phosphorylated by PDK1 (Alessi *et al.* 1997*b*), and by the finding that PKBα lacking the PH domain is phosphorylated by PDK1 independently of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 (Stokoe *et al.* 1997; Alessi *et al.* 1997*b*).

Consistent with this model, PtdIns(3,4,5)P_3 is still required for the activation of PKBα by a truncated form of PDK1 that lacks the C-terminal PH domain (Stokoe *et al*. 1997).

The PH domains of PKB (James *et al*. 1996) and PDK1 (Currie *et al*. 1999) both bind PtdIns(3,4,5)P_3, PtdIns(3,4)P_2 and PtdIns(4,5)P_2 *in vitro*, and PDK1 appears to interact with these inositol phospholipids more strongly than PKB. Moreover, the rate of activation of PKBα is reduced about 30-fold if the PH domain of PDK1 is deleted (Alessi *et al*. 1997*b*) or mutated so that it cannot bind PtdIns(3,4,5)P_3. These observations suggest that the PH domain of PDK1 plays an important role in the activation process by localizing this enzyme to the same lipid vesicles as PKB.

10. RECRUITMENT TO THE PLASMA MEMBRANE IS SUFFICIENT TO TRIGGER THE PHOSPHORYLATION AND ACTIVATION OF PKB

PKBα is located in the cytosol of unstimulated cells, but rapidly translocates to the plasma membrane following cell stimulation with IGF1 (Andjelkovic *et al*. 1997) and other agonists (Ahmed *et al*. 1997; Wijkander *et al*. 1997). Translocation is prevented by inhibitors of PtdIns 3-kinase or if the PH domain of PKB is deleted (Andjelkovic *et al*. 1997). Moreover, attachment of a membrane-targetting sequence to the N-terminus of PKBα is sufficient to induce maximal activation of PKB (Kohn *et al*. 1996), and its phosphorylation at Thr308 and Ser473 (Andjelkovic *et al*. 1997), in unstimulated cells. These findings suggest that a key role of PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 in the activation of PKB is to recruit this enzyme to the plasma membrane where it is phosphorylated by PDK1 and by an as yet unidentified Ser473 kinase (hereafter termed PDK2). Consistent with this mechanism, a significant proportion of the PDK1 localizes to the plasma membrane (even in unstimulated cells) when overexpressed in human embryonic kidney 293 cells (Currie *et al*. 1999). This membrane association may reflect the ability of PDK1 to bind inositol phospholipids more strongly than PKB.

In summary, PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 appear to play three roles in the activation of PKB. First, they alter the conformation of PKB so that the phosphorylation sites become accessible to PDK1 and PDK2. Second, they recruit PKB to the plasma membrane where PDK1 and PDK2 are located (figure 5). Third, they bind to PDK1 and may facilitate the activation of PDK1 (and perhaps PDK2) *in vivo*.

11. EVIDENCE THAT THE PDK1–PKB–GSK3 CASCADE MEDIATES MANY OF THE METABOLIC ACTIONS OF INSULIN

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, in outline, a pathway that is likely to contribute to the stimulation of glycogen synthesis by insulin. However, evidence is now accumulating which suggests that the PDK1–PKB–GSK3 'cascade' may play a much wider role and mediate many of the metabolic actions of insulin.

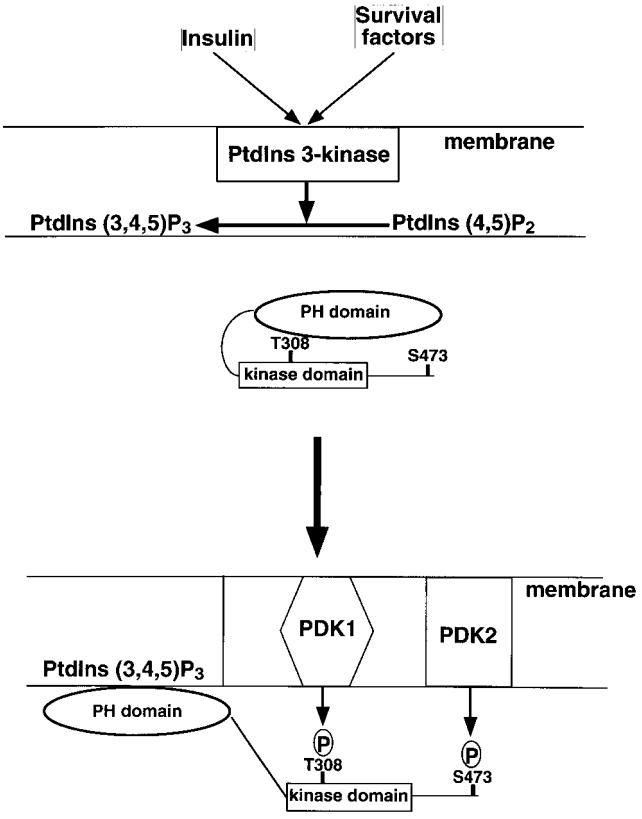

Figure 5. The mechanism of activation of PKB. The stimulation of cells with insulin or a survival factor, such as IGF1, activates PtdIns 3-kinase and elevates the level of PtdIns(3,4,5)P_3 at the plasma membrane. PtdIns(3,4,5)P_3 and/or its immediate breakdown product PtdIns(3,4)P_2 then interacts with the PH domain of PKB, which has two effects. First, it alters the conformation of PKB in such a way that Thr308 and (perhaps) Ser473 become accessible for phosphorylation by PDK1 and PDK2, respectively. Second, PKB is recruited from the cytosol to the plasma membrane where PDK1 and PDK2 are located. The phosphorylation of PKB at Thr308 and Ser473 (indicated by the circled letter Ps) activates PKB.

GSK3 phosphorylates numerous proteins *in vitro* at serine or threonine residues, and the inhibition of GSK3 by insulin is therefore likely to induce the dephosphorylation of numerous proteins *in vivo*. In particular, the inhibition of GSK3 appears to underlie the insulin-induced dephosphorylation and activation of eIF2B and may therefore contribute to the stimulation of protein synthesis (Welsh & Proud 1993), as well as glycogen synthesis (figure 6). GSK3 phosphorylates IRS-1 *in vitro*, impairing the ability of the insulin receptor to phosphorylate IRS-1 at tyrosine residues (Eldar-Finkelman & Krebs 1997). This could represent a feedback activation loop for enhancing the insulin signal, but more work is needed to establish whether it really operates *in vivo*.

PKB phosphorylates serine and threonine residues in proteins and peptides that lie in Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd motifs, where Xaa is any residue, Yaa and Zaa are small side chains other than Gly, and Hyd is a bulky hydrophobic residue such as Phe or Leu (Alessi *et al*. 1996*b*). This motif not only surrounds the serine

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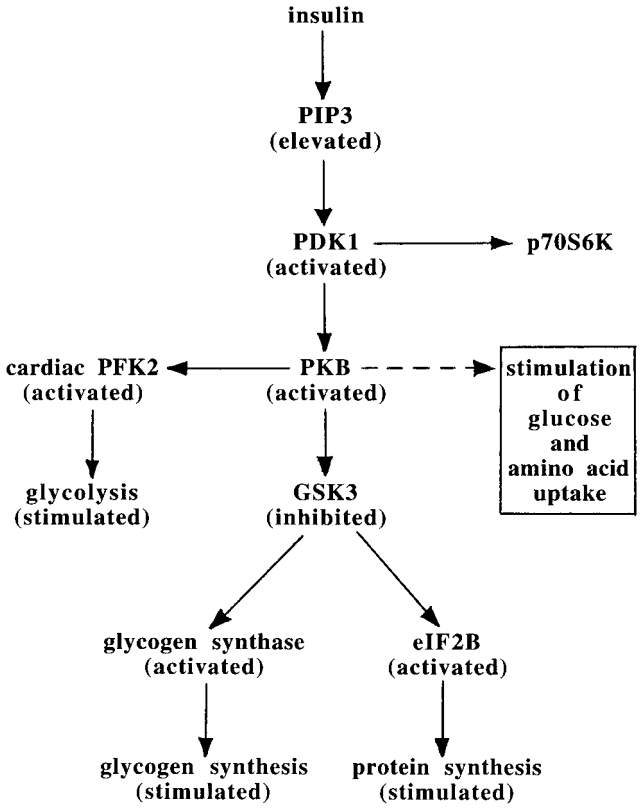

Figure 6. The PDK1–PKB–GSK3 cascade may mediate many of the metabolic effects of insulin. The steps involved in the formation of PtdIns(3,4,5)P_3 are shown schematically in figure 3. The transfection of 3T3-L1 adipocytes and L6 myotubes with a constitutively active form of PKB mimics the action of insulin on glucose and amino acid transport, but how PKB mediates these effects is unknown.

residues in GSK3α and GSK3β, whose phosphorylation is induced by insulin, but also the serines whose phosphorylation underlies the insulin-induced activation of the cardiac isoform of 6-phosphofructo-2-kinase (PFK2). These residues are phosphorylated by PKB *in vitro*, and their phosphorylation *in vivo* is prevented by inhibitors of PtdIns 3-kinase (but not by rapamycin and PD 98059) (Lefebvre *et al*. 1996; Deprez *et al*. 1997). PFK2 catalyses the formation of fructose 2, 6 bisphosphate, an allosteric activator of 6-phosphofructo-1-kinase (PFK1), which is the rate-limiting enzyme in glycolysis. Thus the phosphorylation of PFK2 by PKB probably explains how insulin stimulates glycolysis in the heart (figure 6). In transfection-based experiments, PKB also mimics other actions of insulin, such as the enhancement of glucose uptake in adipocytes (Kohn *et al*. 1996) and L6 myotubes (Hajduch *et al*. 1998) that results from the translocation of GLUT4 from an intracellular compartment to the plasma membrane, as well as the increased rate of amino acid uptake into L6 myotubes (Hajduch *et al*. 1998).

The recent finding that the activation of PKBα (Andjelkovic *et al*. 1997) and PKBβ (Meier *et al*. 1997) by IGF1 involves first their recruitment to the plasma membrane and then their translocation to the nucleus suggests that PKB may also have nuclear targets, such as transcription factors. This is supported by the finding that, in transfection-based experiments, PKB mimics the ability of insulin to induce the expression of leptin, the obesity gene product (Barthel *et al*. 1997).

12. OTHER PROTEIN KINASES ARE LIKELY TO BE SUBSTRATES FOR PDK1 AND PDK2 *IN VIVO*

Thr308 of PKBα lies between kinase subdomains VII and VIII, while Ser473 is situated beyond the catalytic domain in a Phe-Xaa-Xaa-Phe-Ser/Thr-Phe/Tyr motif. Amino acid sequences similar to those surrounding Thr308 and Ser473 are found in many protein kinases that play important roles in cell signalling, such as the isoforms of PKC (Newton 1995), Rho-dependent protein kinases (ROK) (Leung *et al*. 1996) and p70 S6 kinase (p70S6K) (Pullen & Thomas 1997) (figure 7). Moreover, the residues equivalent to Thr308 and Ser473 are nearly always separated by 160–165 amino acids. It is therefore tempting to speculate that these protein kinases are all phosphorylated by PDK1, PDK2 or closely related enzymes. For example, agonists that activate phospholipase Cγ elevate the level of diacylglycerol, which interacts with PKCs recruiting them to the plasma membrane where they may be phosphorylated by PDK1 and/or PDK2. Diacylglycerol binding may also change the conformation of PKC isoforms so that they become accessible to PDK1 and PDK2. Similarly, the binding of GTP-Rho to ROK may permit phosphorylation by PDK1 and PDK2. In this scenario, PKB, PKC and ROK would all be regulated by analogous mechanisms in which their interaction with an effector would make the phosphorylation sites accessible to PDK1 and PDK2 as well as promoting recruitment to the plasma membrane where these activating kinases seem to be located.

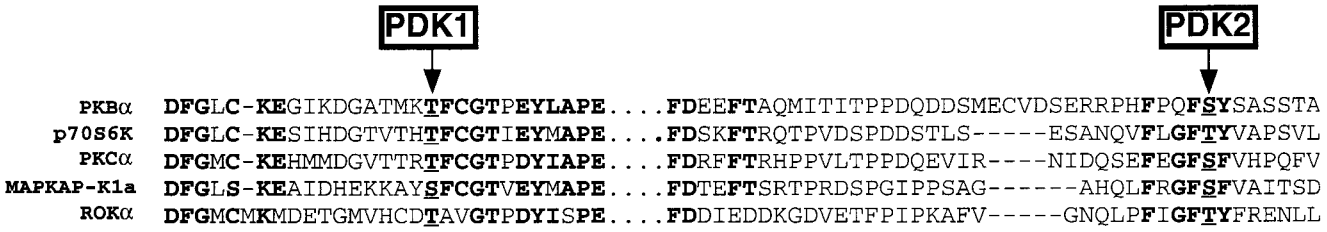

Figure 7. Alignment of the phosphorylation site sequences of PKBα with the corresponding regions of p70 S6 kinase (p70S6K), PKCα, Rho-dependent protein kinase α (ROKα) and the N-terminal kinase domain of MAPKAP-K1a (also known as p90^{rsk}). Conserved amino acids surrounding the residues equivalent to Thr308 and Ser473 of PKBα are shown in bold type. p70 S6 kinase, PKCα and MAPKAP-K1a are known to be phosphorylated *in vivo* at the residues equivalent to Thr308 and Ser473 of PKBα. It is speculated that these residues are phosphorylated by PDK1, PDK2 or closely related enzymes.

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PDK1 has recently been shown to phosphorylate p70S6K at Thr252 (Alessi *et al*. 1998), the residue equivalent to Thr308 of PKBα (figure 7). The phosphorylation of Thr252 activates p70 S6 kinase partially, but activation is greatly enhanced if Thr412 (the residue equivalent to Ser473 of PKBα) is mutated to Glu. This suggests that, as in PKB, the phosphorylation of both sites is required for the full activation of p70S6K. p70S6K mediates the insulin-induced phosphorylation of ribosomal protein S6, whose phosphorylation 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 (Jeffries *et al*. 1994).

13. PKB AND THE PREVENTION OF APOPTOSIS

PKB is the cellular homologue of a viral oncogene v-Akt, a protein kinase encoded in the genome of a transforming retrovirus isolated from a rodent T-cell lymphoma (Bellacosa *et al*. 1991). The finding that overexpression of v-Akt is required to maintain the transformed state, that PKBβ is overexpressed in about 15% of ovarian cancers (Cheng *et al*. 1992) and 12% of pancreatic cancers (Cheng *et al*. 1996), and that PKBα is overexpressed in about 3% of breast cancers (Bellacosa *et al*. 1995), indicates that PKB has the potential to become oncogenic in high copy number. One reason for this is that PKB provides a signal that protects cells from apoptosis induced by a number of agents and this may explain why the overexpression of PKB enables cancer cells to survive and proliferate in the absence of suvival factors, in situations where cells expressing normal levels of PKB would undergo apoptosis (reviewed in Marte & Downward 1997).

One of the cellular targets that PKB may phosphorylate to protect cells from apoptosis is BAD (Datta *et al*. 1997). This protein, in its dephosphorylated form, interacts with the BCl family member Bcl_{XL} and induces apoptosis of some cells. However, after BAD is phosphorylated at Ser 136 by PKB, it dissociates from Bcl_{XL} and apoptosis is prevented (Datta *et al*. 1997). Ser136 lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser motif. However, BAD has a restricted tissue distribution, suggesting that PKB may be able to arrest the apoptotic pathway in other ways.

A gene encoding a *Drosophila* homologue of PDK1 (termed DSTPK61) (Alessi *et al*. 1997*b*) was identified by Mary Bownes at the University of Edinburgh because it is differentially spliced in male and female *Drosophila* giving rise to transcripts that have the same coding sequence but different 5′ and 3′ untranslated regions. The levels of these transcripts vary in different tissues of male and female flies implying that the level of expression of the PDK1 protein varies in these cells in a sex-specific manner. The lower level of expression of PDK1 may result in a lower level of activation of PKB, raising the intriguing possibility that such cells may undergo more rapid apoptosis. It is tempting to speculate that this mechanism could underlie certain sexual characteristics. For example, male *Drosophila* develop a special muscle for holding the female abdomen during mating. This muscle is not produced in females because the progenitor cells undergo apoptosis at an early stage of development. This may result from a low level of expression of DSTPK61.

14. THE DEVELOPMENT OF SPECIFIC PROTEIN KINASE INHIBITORS FOR THE TREATMENT OF DIABETES AND OTHER DISEASES

The idea that inhibitors of protein kinases and phosphatases might be used therapeutically only became a reality in 1991, with the discovery by Stuart Schreiber of how cyclosporin acted to suppress the immune system. This drug, which has revolutionized approaches to organ transplantation, binds to an intracellular protein, termed cyclophilin, and the cyclosporin–cyclophilin complex then inhibits a protein phosphatase, termed PP2B or calcineurin (Liu *et al*. 1991), that had been identified in my laboratory ten years earlier (Stewart *et al*. 1982). More recently, several specific protein kinase inhibitors with therapeutic potential have been developed. These include PD 98059, developed by Alan Saltiel and his colleagues at Parke-Davis, which inhibits the growth factor-stimulated MAP kinase cascade by preventing the activation of MAP kinase kinase (Alessi *et al*. 1995). PD 98059 causes reversion of the transformed phenotype of many Ras-transformed cell lines (Dudley *et al*. 1995), suggesting that more potent inhibitors of the MAP kinase pathway may be of value in the treatment of cancer. Another compound SB 203580, developed by John Lee, Peter Young and their colleagues at SmithKline Beecham, suppresses the production and some of the actions of pro-inflammatory cytokines and, in animal models, is efficaceous in the treatment of inflammatory conditions such as rheumatoid arthritis (Badger *et al*. 1996). This compound is a specific inhibitor of a MAP kinase family member termed SAPK2a/p38 (Cohen 1997).

Drugs that modulate the PDK1–PKB–GSK3 cascade have yet to be developed. However, specific inhibitors of GSK3, or compounds that stimulate the PDK1-dependent activation of PKB by mimicking the action of $PtdIns(3,4,5)P_3$, may well be efficacious in the treatment of diabetes. Conversely, specific inhibitors of PKBβ that do not inhibit other PKB isoforms may be effective in the treatment of ovarian and pancreatic cancer, because they may cause these cancer cells to apoptose. A specific inhibitor of PKBβ is unlikely to cause diabetes because PKBα is the dominant isoform in skeletal muscle and liver and it is also a significant isoform in adipose tissue (Walker *et al*. 1998).

A Division of Signal Transduction Therapy has recently been set up in the Biochemistry Department at Dundee with the backing of five major pharmaceutical companies. One of its major aims is to facilitate the development of many more specific inhibitors of protein kinases and phosphatases with therapeutic potential for the treatment of many diseases, including diabetes. Like, PD 98059, rapamycin and SB 203580, these inhibitors are likely to become invaluable reagents for identifying the physiological roles of particular protein kinases and the signal tranduction pathways in which they operate.

I thank the postgraduate students, postdoctoral fellows and collaborators who have contributed to the work of my laboratory over the past 27 years. I am indebted to Michael Rosemeyer (my PhD supervisor) for the excellent training in protein chemistry, to Edmond Fischer (my postdoctoral advisor) for introducing me to the field of reversible protein phosphorylation and to Peter Garland, who recruited me to Dundee and created

the atmosphere that allowed my early work to flourish. I would also like to thank the British Diabetic Association, the Louis-Jeantet Foundation, the MRC and the Royal Society for the financial support which made these studies possible.

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