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Phosphoinositide-Regulated Kinases and Phosphoinositide Phosphatases

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I. Introduction

Stimulation of cells with growth factors, survival factors, and insulin within seconds leads to the recruitment to the plasma membrane of a family of lipid kinases known as Class 1 phosphoinositide 3-kinase (PI 3-kinases, reviewed in refs 1 and 2). In this location PI 3-kinases phosphorylate the glycerophospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) at the D-3 position of the inositol ring, converting it to PtdIns(3,4,5)P_3, which is then converted to PtdIns(3,4)P_2 through the action of the SH2-containing inositol phosphatases (SHIP1 and SHIP2) or back to PtdIns(4,5)P_2 via the action of the lipid phosphatase termed Phosphatase and TENsin homologue deleted on chromosome TEN (PTEN, Figure 1). Recent evidence indicates that PtdIns(3,4,5)P_3 and perhaps PtdIns(3,4)P_2 are likely to play a key role in regulating cell growth and survival, differentiation and cytoskeletal changes, as well as mediating many, if not all, of the known physiological responses to insulin. PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 mediate cellular effects by interacting with proteins that possess a certain type of pleckstrin homology domain (PH domain).[3] In many cases, this recruits these proteins to the plasma membrane. A number of types of PH domain-containing proteins that interact with PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 have been identified. These include the serine/threonine protein kinases 3-phosphoinositide-dependent protein kinase (PDK1) and protein kinase B (PKB also known as Akt), tyrosine kinases of the Tec family,[4,5] numerous adaptor molecules such as the Grb2-associated protein (GAB1[6]), the dual adaptor of phosphotyrosine and 3-phosphoinositides DAPP1,[7−10] and the tandem PH domain-containing proteins (TAPP1 and TAPP2[11]) as well as GTP/GDP exchange[12,13] and GTPase-activating proteins[14] for the ARF/Rho/Rac family of GTP-binding proteins (Figure 2). In this review we will focus on recent research aimed at understanding the mechanism by which activation of PI 3-kinase, and hence formation of PtdIns(3,4,5)P_3, enables PDK1 to phosphorylate and activate a group of serine/threonine protein kinases that belong to the AGC subfamily of protein kinases. These include isoforms of Protein kinase B (also known as Akt),[15] p70 ribosomal S6 kinase (S6K),[16] serum and glucocorticoid-induced protein kinase (SGK),[17−19] and protein kinase C (PKC) isoforms[20] (Figure 2). It is believed that the activation of these kinases mediates many of the effects of PI 3-kinase on promoting cell survival and mediating the physiological responses of cells and tissues to insulin. We will also discuss the importance of the PTEN and the SHIP phosphoinositide phosphatases that play key roles in regulating the cellular concentration of PtdIns(3,4,5)P_3 and thus regulate the activity of the downstream effector pathways activated by these phosphoinositides.

II. The Serine/Threonine Kinase PKB Interacts with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2

There are three isoforms of PKB (termed PKBα, PKBβ, and PKBγ) which possess >85% sequence identity and are widely expressed in human tissues.[15] Stimulation of cells with agonists that activate PI 3-kinase invariably induces a large activation of PKB isoforms with a half-time for maximal activation occurring within 1−2 min. There is a strong body of evidence that PKB lies downstream of PI 3-kinase, as the activation of PKB is prevented by inhibitors of PI 3-kinase such as wortmannin or LY294002 or

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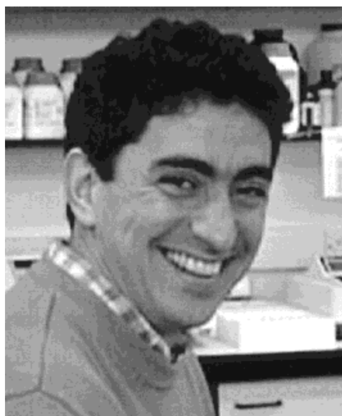

Ricardo M. Biondi was born in Buenos Aires, Argentina, in 1966. He graduated in Biology (1991) and obtained his Ph.D. (1996) degree at the University of Buenos Aires. During a postdoctoral stay at Pasteur Institut in Paris, he developed his interests on how protein kinases really interact with their substrates. For this reason he moved to Dundee and joined the Division of Signal Transduction Therapy as a postdoctoral research scientist, where he has worked with both Dr. Dario R. Alessi and Sir Professor Philip Cohen. In Dundee he has been investigating the involvement of protein kinase–substrate docking interactions as specificity determinants in the insulin/growth factor signaling pathways.

Dario R. Alessi was born in Strasbourg, France, in 1967. He was educated in Brussels, Belgium, and studied for his First Degree in Biochemistry and Biotechnology at the University of Birmingham (U.K.) and graduated in 1988. He carried out his Ph.D. degree jointly supervised by Professor Ian Trayer at the University of Birmingham and Dr. David Trentham at the National Institute of Medical Research in London. His Ph.D. research focused on the organic synthesis of spin-labeled nucleotide probes to be used in structural investigations of biological problems and was awarded his Ph.D. degree in 1991. In 1991 he moved to the MRC Protein Phosphorylation Unit at the University of Dundee to work under Sir Professor Philip Cohen, studying the role of protein phosphatases and protein kinases regulating diverse physiological processes. In 1997 he became a principal investigator at the MRC Protein Phosphorylation unit in Dundee, and since then the focus of the work in his laboratory has been to study signal transduction processes that are triggered by PI 3-kinase in cells.

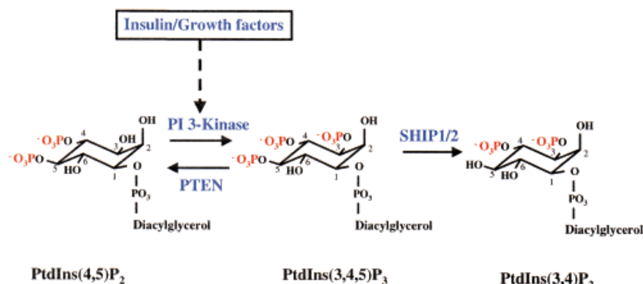

Figure 1. Mechanism by which agonists stimulate the formation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. Agonists bind to their receptors, and this results in the recruitment of PI 3-kinase from the cytosol to the cell membrane, bringing it into the vicinity with its physiological substrate PtdIns(4,5)P_2. PtdIns(4,5)P_2 is phosphorylated by the p110 catalytic subunit of PI 3-kinase at the D3 position of the inositol ring to generate the second messenger PtdIns-(3,4,5)P_3. A specific 5-phosphatases termed SHIP1 or SHIP2 converts PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2, which is also predicted to be a second messenger. A specific 3-phosphatase termed PTEN can convert PtdIns(3,4,5)P_3 back to PtdIns(4,5)P_2.

by the overexpression of a dominant negative regulatory subunit of PI 3-kinase.[21-23] Furthermore, overexpression of a constitutively active mutant of PI 3-kinase induces PKB activation in unstimulated cells[24] (see below) as does deletion of the PTEN phosphatase which results in increased cellular levels of PtdIns(3,4,5)P_3.[25-29] These observations indicate that PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 generation in cells is sufficient to induce the activation of PKB isoforms.

All PKB isoforms possess an N-terminal PH domain followed by a kinase catalytic domain and then a C-terminal tail. Although pleckstrin homology domains were first noticed in various proteins predicted to have a role in signal transduction in 1993,[30-33] it was not until 1995 that it became apparent that PH domains on certain proteins could function as a phosphoinositide-binding module, enabling proteins to interact with cell membranes (reviewed in ref 3). It was then demonstrated by several groups that PKB or its isolated PH domain bound in vitro with high affinity to PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2.[34,35] Furthermore, it was also shown that following stimulation of cells with agonists that activate PI 3-kinase, PKB was translocated to the plasma membrane, where PtdIns(3,4,5)P_3 is located, and that translocation was prevented by inhibitors of PI 3-kinase or by deletion of the PH domain of

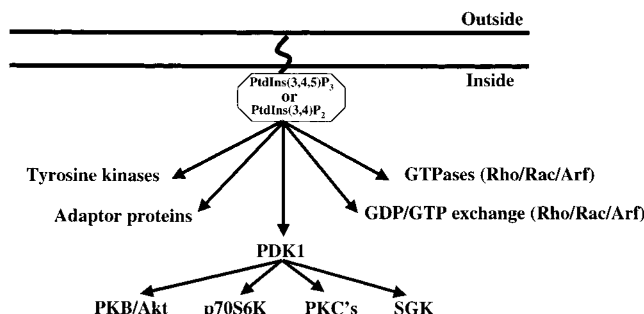

Figure 2. Signal transduction pathways known to be triggered by PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. Apart from PDK1 and PKB, a number of other proteins possess PH domains that interact with PtdIns(3,4,5)P_3 and/or PtdIns-(3,4)P_2. These include certain adaptor proteins, tyrosine kinases belonging to the TEC family, as well as 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 to define the physiological proccess that are regulated by each branch of this pathway.

PKB.[36−38] These findings strongly indicate that PKB interacts with PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2 in vivo.

III. PKB Is Activated by Phosphorylation of Thr308 and Ser473

Importantly, PKB is not directly activated by its interaction with either PtdIns(3,4,5)P_3 or PtdIns(3,4)-P_2.[35,39] Although some authors claimed that PKB could be activated by binding to PtdIns 3P[22] or through its interaction with PtdIns(3,4)P_2,[34,40,41] these results have not been reproduced and are now not thought to be physiologically relevant.[42] As the activated form of PKB was inactivated by serine/threonine phosphatases,[21,43] this indicated that PKB might be activated by phosphorylation on a serine/threonine residue(s). ^{32}P-cell-labeling experiments revealed that insulin or IGF1 stimulation of cells that potently activates PI 3-kinase induced the phosphorylation of PKBα at two residues, Thr308 and Ser473, and that inhibitors of PI 3-kinase prevented the phosphorylation of both residues.[44] Thr308 is located in the "T-loop" (also known as 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. Ser473 is located outside of the catalytic domain in a motif Phe-Xaa-Xaa-Phe-Ser-Tyr (where Xaa is any amino acid) that is present in most AGC kinases and is termed the hydrophobic motif. The phosphorylation of PKBα at both Thr308 and Ser473 is likely to be required to activate PKBα maximally as mutation of Thr308 to Ala abolished PKBα activation by insulin or IGF1, whereas mutation of Ser473 to Ala reduced the activation of PKBα by ~85%. The mutation of both Thr308 or Ser473 to Asp (to mimic the effect of phosphorylation by introducing a negative charge) increased PKBα activity substantially in unstimulated cells, and this mutant could not be further activated by insulin.[44] Attachment of a membrane-targetting domain to PKBα resulted in it becoming highly activated in unstimulated cells and induced a maximal phosphorylation of Thr308 and Ser473.[36,45] These observations indicated that recruitment of PKB to the membrane of cells could be sufficient for it to become activated at that location by the phosphorylation of Thr308 and Ser473. PKBβ is activated by phosphoryation of the equivalent residues (Thr309 and Ser474), and a splice variant of PKBγ that does not possess a residue equivalent to Ser473 of PKBα (because it terminates at residue 454) is also activated when overexpressed in cells solely via phosphorylation of Thr305 (the residue equivalent to Thr308 of PKBα).[46] A longer splice variant of PKBγ has been identified that like the other PKB isoforms possesses a hydrophobic motif, and it is likely that PI 3-kinase activation induces the phosphorylation of the residue equivalent to Ser473 of PKBα in the long splice variant of PKBγ (Ser472).[47]

There are three reports which indicate that under certain physiological conditions PKB can be activated independently from PI 3-kinase. First, Ca^{2+} levels in cells have been reported to activate PKB through the Ca^{2+}/calmodulin-dependent protein kinase kinase (CAMKK).[48] This study reports that CAMKK phosphorylates PKB on Thr308 in the absence of PtdIns-(3,4,5)P_3. However, another group found that CAMKK is not capable of inducing the phosphorylation of PKB,[49] and others have not been able to induce PKB activation in neuronal, kidney, or fibroblast cell lines by agonists which increase intracellular Ca^{2+} levels (Shaw, M.; Cohen, P. Unpublished observations). Second, it has also been reported that when PKB is overexpressed in 293 cells, it can be partially activated (~2-fold) in a PI3K-independent manner by agents that increase cAMP levels.[50] Physiological relevance of this requires further investigation, and in this regard we have been unable to detect the activation of *endogenously* expressed PKB in several cell lines by such stimuli, despite being able to measure large increases in cAMP- and cAMP-dependent protein kinase/PKA activity in these cells (Shaw, M.; Alessi, D. R. Unpublished observations). Third, another study claimed that oxidative stress and heat shock treatment of cells could activate PKB and that this activation was not inhibited by wortmannin.[51] However, a subsequent study inicated that activation of PKB by these stressors was inhibited by PI 3-kinase inhibitors.[52]

IV. PKB May Be a Key Mediator of Cell Survival, Cell-Cycle Regulation, and Insulin Responsiveness

Soon after PKB was found to be activated downstream of PI 3-kinase, it was postulated that PKB could function as a mediator of PI 3-kinase-dependent signaling processes in cells. Several groups addressed the question of what roles PKB might play by overexpressing constitutively active mutants of PKB in cell lines. These results demonstrated that overexpression of activated PKB strongly inhibited apoptosis induced by many cellular insults that induce cell death (reviewed in refs 53 and 54). Overexpression of active forms of PKB in insulin-responsive cells induced many processes that are normally activated by insulin such as glycogen synthesis and protein

Table 1[a]

substrate	physiological role of phosphorylation
GSK3α/GSK3β	stimulates glycogen[43] and protein[202] synthesis in insulin responsive tissues
PFK2	stimulates glycolysis in heart tissue[203]
PDE3B	insulin-mediated reduction of cyclicAMP levels in adipocytes[73]
FKHR	regulation of transcription and cell survival[147,149,204]
BAD	promotion of cell survival by inhibiting apoptosis[69]
mCaspase 9	promotion of cell survival by inhibiting apoptosis[164]
IKKα	NF-κB activation by tumor necrosis factor[166,167]
eNOS	VEGF or shear-induced activation of eNOs promoting angiogenesis[157,158]
Raf	inhibition of the Raf-MEK-ERK signaling pathway[75]
mTOR	stimulation of protein synthesis[205,206]
BRCA1	regulation nuclear localization[165]
HTERT	activation of the human telomerase reverse transcriptase subunit[207]
IRS	inhibition of IRS to activate downstream insulin-mediated pathways[208]
$P21^{CIP1/WAF1}$	forces cytoplasmic localization of $P21^{CIP1/WAF1}$ preventing growth arrest[71]

[a] List of proteins that may be phosphorylated physiologically by PKB. The potential role that phosphorylation of these proteins plays in mediating physiological processes is stated. Abreviations: GSK3, glycogen synthase kinase 3; PFK2, 6-phosphofructo-2-kinase; PDE 3B, phosphodiesterase 3B; FKHR, forkhead transcription factor; mCaspase9, mouse caspase 9; IKKα, IkB kinase-α; eNOS, endothelial nitric oxide synthase; mTOR, mammalian target of Rapamycin; BRCA1, breast cancer susceptibility gene-1; hTERT, human telomerase reverse transcriptase subunit; IRS, insulin receptor subunit, cell-cycle-inhibitor protein. Although there is evidence that PKB phosphorylates mouse caspase 9 at Ser196, as this site is not conserved on the human form of this enzyme,[201] the physiological relevance of this observation is not certain at the moment.

synthesis,[55,56] stimulated uptake of nutrients such as glucose and amino acids,[57,58] and also induced certain changes in gene expression that are induced by insulin and growth factors.[57,59,60] Furthermore, the overexpression of active forms of PKB in cells has also been shown to promote differentiation of some cells[61−63] while inhibiting the differentiation of others.[64,65] It is likely that these processes are mediated by PKB phosphorylating a number of key regulators of cellular function, and much of the work in this area is aimed at identification and characterization of these substrates.

It is well established that the minimum sequence motif required in a peptide substrate to be efficiently phosphorylated by PKB lies in the motif 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).[66,67] The requirement for the Arg at positions 3 and 5 residues N-terminal to the phosphorylation site is critical as changing either of these residues even to Lys abolished phosphorylation of peptide substrates by PKB.[66]

The identification 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 ref 15), and some novel substrates have been proposed by Cantley and colleagues.[67] In Table 1 we list the best characterized PKB substrates and state what role that phosphorylation may play in regulating the activity of these proteins, which has also been reviewed elsewhere.[15,68−70] A very recent substrate for PKB to be discovered is the inhibitor of the cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$. PKB phosphorylates this protein at Thr145, thereby preventing it from entering the nucleus of cells where it needs to be in order to arrest the growth of cells.[71] This mislocalization of a major regulator of cell-cycle progression may be a general mechanism by which various oncogenes such as Her2/neu that activate PKB can enable cells to proliferate under conditions whereby their growth should be arrested.[71,72] Although the presence of an Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr-Hyd motif in a protein is certainly an important determinant in enabling a protein to be phosphorylated by PKB, it is not currently known whether there are other sites on PKB substrates that would act as “docking sites”, enabling PKB to interact with these and hence enhance the rate at which these are phosphorylated. In this regard, it should be noted that several PKB substrates including phosphodiesterase 3B,[73] endothelial nitric oxide synthase,[74] Raf1,[75] and $p21^{Cip1/WAF1}$ [71] have been reported to physically interact with PKB isoforms. It is interesting to note that Thr145 of $p21^{Cip1/WAF1}$ does not lie in a optimal PKB phosphorylation consensus. Although the Arg residues 5 and 3 residues before the site of phosphorylation are conserved, the residue following Thr145 is a Ser rather than a large hydrophobic residue. One could speculate that the interaction of PKB with $p21^{Cip1/WAF1}$ (as well as with other PKB substrates), through specific PKB binding motif(s), may be required for them to be phosphorylated efficiently by PKB in vivo. This will require further investigation.

It should be noted that although all the PKB substrates listed in Table 1 have been shown to be phosphorylated by PKB in vitro, the evidence that the endogenous forms of these proteins are phosphorylated in vivo in response to agonists that activate PI 3-kinase is in many cases lacking. Furthermore, as no specific PKB inhibitors have been developed, it is very difficult to rule out that the phosphorylation of the PKB substrate protein being studied is mediated by a PKB isoform rather than by another PI 3-kinase-activated protein kinase distinct from PKB (such as SGK). Although dominant negative forms of PKB have been used extensively to dissect the signaling networks that are regulated by PKB, great caution in our opinion should be employed when using these reagents. This is becuase it is not clear what is the mechanism by which dominant negative PKB mutants are functioning in cells. For example, it is possible that dominant negative PKB is working by interacting with and inhibiting the upstream

protein kinase(s) that activate PKB, and this in turn could also affect the phosphorylation of other AGC kinases, such as SGK. It is also likely that the overexpression of constitutively active or dominant negative mutants of PKB in cell lines for many hours or even days may indirectly evoke physiological responses that are not normally mediated by PKB. For these reasons, the future work in this area will need to be aimed at developing pharmacological reagents and genetic and biochemical approaches that not only identify novel roles for PKB, but also verify which cellular functions are indeed mediated physiologically by PKB.

V. Identification of the 3-Phosphoinositide-Dependent Protein Kinase (PDK1) That Activates PKB

An enzyme was purified[39,76] and subsequently cloned[77,78] that phosphorylated PKBα at Thr308 only in the presence of lipid vesicles containing PtdIns-(3,4,5)P_3 or PtdIns(3,4)P_2 that was termed 3-phosphoinositide-dependent protein kinase-1 (PDK1). It was found to be composed of an N-terminal catalytic domain and a C-terminal PH domain that interacted with PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2.[78,79] The activation of PKB by PDK1 was enhanced over 1000-fold in the presence of lipid vesicles containing a low mole fraction of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2.[77] The activation was stereospecific for the physiological D-enantiomers of these lipids. Furthermore, the naturally occurring form of PtdIns(3,4,5)P_3 (the 1-stearoyl, 2-arachidonyl derivative) was significantly more effective than the dipalmitoyl derivative.[39,78] It has been postulated that the packing of the unsaturated fatty acids of natural PtdIns(3,4,5)P_3 in the phospholipid bilayer of cellular membranes is loose, causing greater exposure of the headgroup of PtdIns-(3,4,5)P_3 and hence allowing more efficient interactions with PH domain-containing 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 nonsignaling phospholipids generally possess more saturated fatty acid moieties. Neither PtdIns(4,5)P_2 nor any other inositol phospholipid other than PtdIns-(3,4)P_2 can replace PtdIns(3,4,5)P_3 in the PDK1-catalyzed activation of PKB.[39,78] Even the presence of a 100-fold molar excess of PtdIns(4,5)P_2 over PtdIns(3,4,5)P_3 did not prevent PDK1 from phosphorylating and activating PKB (Alessi, D. R. Unpublished results), consistent with the cellular situation where there is typically $>$100-fold excess of PtdIns-(4,5)P_2 over PtdIns(3,4,5)P_3.

The requirement for PtdIns(3,4,5)P_3 or PtdIns(3,4)-P_2 in the PDK1-catalyzed 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 Thr308 becomes accessible to PDK1 (Figure 3). These conclusions are supported by the finding that in the absence of PtdIns(3,4,5)P_3, full-length PKB is not phosphorylated by PDK1 but removal of the PH domain of PKB results in its phosphorylation and activation by PDK1 in vitro.[77] Furthermore, a PKBα mutant that cannot interact with PtdIns(3,4,5)P_3 is not phosphorylated by PDK1,[76] but PtdIns(3,4,5)P_3 is still required for the activation of PKBα by a truncated form of PDK1 which lacks the PH domain.[77,78]

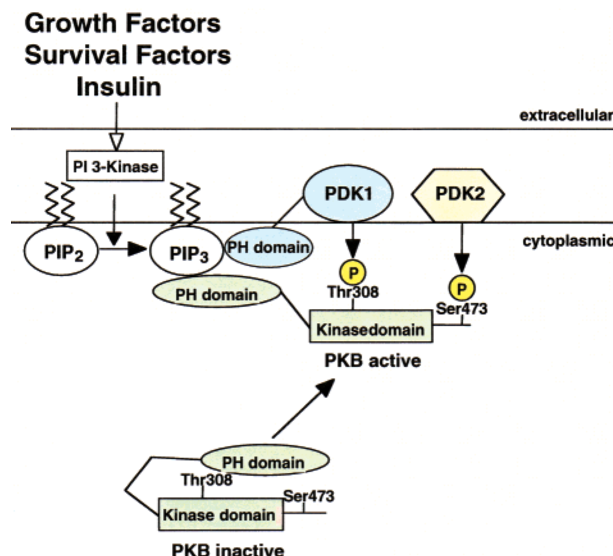

Figure 3. Mechanism of activation of PKB. Stimulation of cells with agonists that result in the activation of PI 3-kinase trigger the production PtdIns(3,4,5)P_3 at the plasma membrane. PKB then interacts with PtdIns(3,4,5)-P_3 (and/or PtdIns(3,4)P_2) through its PH domain and is thus recruited from the cytosol to the plasma membrane. The interaction of PKB with PtdIns(3,4,5)P_3 alters its conformation so that Thr308 becomes accessible for phosphorylation by PDK1. PKB is also phosphorylated at the membranes at Ser473 by an as yet uncharacterized protein kinase that is termed here PDK2. Phosphorylation of PKB at Thr308 and Ser473 activates PKB. The interaction of PDK1 with PtdIns(3,4,5)P_3 through its PH domain does not activate PDK1 but is thought to play an important role in enabling PDK1 to be located at the membrane so that it can phosphorylate PKB that is recruited to this location. PIP2 stands for PtdIns(4,5)P_2, whereas PIP3 indicates PtdIns(3,4,5)P_3. (Reprinted with permission from Alessi, D. R. *Biochem. Soc. Trans.*, in press. Copyright 2001 The Biochemical Society.)

PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 interact with the PH domain of PDK1, with somewhat higher affinity than with the PH domain of PKBα.[79] 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)P_3 and/or PtdIns(3,4)P_2 and that this plays a key role in enabling PDK1 to phosphorylate PKB efficiently. These conclusions are supported by the finding that the rate of activation of PKBα by PDK1 in vitro in the presence of lipid vesicles containing PtdIns(3,4,5)P_3 is lowered considerably if the PH domain of PDK1 is deleted. Furthermore, the mutant of PKB that lacks its PH domain is also a very poor substrate for PDK1 compared to wild-type PKB as it is unable to interact with lipid vesicles containing PtdIns(3,4,5)P_3. It is controversial as to whether PDK1 translocates to the plasma membrane of cells in response to agonists that activate PI 3-kinase. Two reports[79,80] and J. Tavare (University of Bristol, personal communication) indicate that a small proportion of PDK1 is

associated with the membrane of unstimulated cells, and they do not observe any further translocation of PDK1 to membranes in response to insulin stimulation that activates PI 3-kinase and PKB. However, other groups have reported that PDK1 translocates to cellular membranes in response to agonists that activate PI 3-kinase.[81,82] It is possible, as suggested by Van Obberghen and colleagues,[82] that unless the basal levels of PtdIns(3,4,5)P_3 in unstimulated cells are maintained at a very low level, it may be hard to observe translocation of PDK1 to membrane of cells as it possesses a high affinity for PtdIns(3,4,5)P_3, and thus, a significant amount of PDK1 could co-localize with low amounts of PtdIns(3,4,5)P_3 at the plasma membranes of unstimulated cells. Indeed, there is evidence that PDK1 is likely to be located at cell membranes of unstimulated cells as the expression of a membrane-targeted PKB construct in such cells is active and fully phosphorylated at Thr308.[36,45] This observation indicates that a significant amount of endogenous PDK1 as well as PtdIns(3,4,5)P_3 must be present at the membrane of these cells to enable PKB that is fixed at this location to become phosphorylated and activated.

VI. How Is PKB Phosphorylated at Ser473?

A major outstanding question is to characterize the mechanism by which PKB is phosphorylated at its hydrophobic motif. There is currently considerable controversy on whether PKB is phosphorylated at this residue by a distinct upstream kinase that has not yet been identified (sometimes referred to as PDK2) or whether PKB, after it becomes phosphorylated at Thr308, is able to phosphorylate itself at Ser473. In this section we will review the evidence for and against each of these models.

We[39,46,77,83] and others[76,78] originally postulated that PKB would not be phosphorylated at Ser473 by autophosphorylation or by PDK1 as it was found that PDK1 only phosphorylated PKB significantly at Thr308 in vitro and that PKB phosphorylated at Thr308 (which is partially activated) was not able to autophosphorylate itself at Ser473 even in the presence of lipid vesicles containing PtdIns(3,4,5)P_3. This was also supported by the finding discussed above that a catalytically inactive mutant of PKB or a mutant of PKB in which Thr308 is changed to either Ala or Glu was still phosphorylated on Ser473 following stimulation of 293 cells with IGF1.[44] However, some caution is required when interpreting this latter result as it could be argued that either the kinase dead PKB transfected into cells is not 100% "dead" and still could possess the ability to autophosphorylate itself at Ser473 or that this phosphorylation could be mediated by the wild-type endogenous PKB present in these cells.

This notion was recently challenged by Toker and Newton, who reported that PDK1 in vitro was capable of inducing the PKB to phosphorylate itself at Ser473.[84] This analysis was carried out using a very sensitive phosphospecific antibody that recognizes PKB phosphorylated at Ser473, and thus, it is uncertain whether the phosphorylation observed at Ser473 in these experiments occurred at trace levels or at a significant stoichiometry. Further evidence that PKB may be able to phosphorylate itself at Ser473, at least in vitro, is the finding that PKBα when incubated with PtdIns(3,4,5)P_3 and PDK1 complexed to a C-terminal fragment of PKC-related kinase-2, termed PDK1 interacting fragment (PIF), became phosphorylated at both Thr308 and Ser473.[83] Although we originally interpreted this result to imply that PDK1 was phosphorylating both Thr308 and Ser473 of PKB, the subsequent observation that catalytically inactive mutants of PKB are only phosphorylated at Thr308 and not at Ser473 by PDK1 when complexed to PIF[85] may indicate that PKB is phosphorylating itself at Ser473 under these conditions. It is therefore possible that the PIF peptide might be able to bind to PKB and following phosphorylation of Thr308 enable PKB to phosphorylate Ser473 by a mechanism that has not been characterized. It should be noted that this conclusion is not definitive as it could be argued that the catalytically inactive PKB could be misfolded and therefore not recognized by PDK1 complexed to PIF. Furthermore, we have also not been able to detect a significant interaction between PKB and PIF by co-immunoprecipitation or surface plasmon resonance binding studies, although it could be argued that these proteins are able to interact with each other weakly.

Mouse embryonic stem (ES) cells in which both copies of the PDK1 gene were disrupted to prevent the expression of PDK1 have recently been generated.[86] These cells (termed PDK1$^{-/-}$ ES cells) were viable and proliferated at a similar rate to the wild-type ES cells expressing PDK1 (termed PDK1$^{+/+}$ ES cells). IGF1-stimulation of PDK1$^{-/-}$ ES cells activated PI 3-kinase to a greater level than in the control PDK1$^{+/+}$ ES cells, but PKB was not activated significantly in the PDK1$^{-/-}$ ES cells whereas it was activated robustly in the control PDK1$^{+/+}$ ES cells. As expected, IGF1-stimulation of PDK1$^{-/-}$ ES cells failed to induce phosphorylation of PKB at Thr308 in its T-loop, providing the first genetic evidence in mammalian cells that PDK1 mediates this phosphorylation. Importantly, however, PKB was still phosphorylated at Ser473 in response to IGF1 stimulation, and this was inhibited by the PI 3-kinase inhibitors wortmannin and LY294002.[86] This indicated that, at least in PDK1$^{-/-}$ ES cells, PDK1 is not required for the phosphorylation of PKB at Ser473. One explanation for these data is that a kinase distinct from PDK1 and PKB can mediate phosphorylation of Ser473. An alternative possibility could be that PKB might be regulated by a PIF-like molecule in vivo that, in the absence of PDK1 and phosphorylation of PKB at Thr308, can bind to PKB and enable phosphorylation of Ser473. It is possible that phosphorylation of PKB at Ser473 could be regulated differently in other cell types or in response to different agonists. It will therefore be important in the future to prepare other cell lines which lack PDK1 and to establish the effect that this has on Ser473 phosphorylation in response to a variety of agonists. The availability of a specific PDK1 inhibitor (which has not yet been developed) would also be a extremely valuable tool with which 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 found that the nonspecific kinase inhibitor staurosporine (which was presumed, but not demonstrated, to inhibit PDK1) prevented the phosphorylation of PKBα at Thr308 but not Ser473 in 293 cells.[87] This provides pharmacological evidence that PDK1 is not essential for the phosphorylation of PKBα at Ser473 in these cells. Blenis and colleagues made the very interesting observation that addition of *N*-α-tosyl-L-phenylalanylchloromethyl ketone (TPCK) to cells prevented insulin-stimulated PKB activation and its phosphorylation at both Thr308 and Ser473, without affecting activation of PI 3-kinase.[88] This study indicates that TPCK may inhibit PDK1 (and phosphorylation of PKBα at Ser473) in vivo, but as TPCK does not inhibit PDK1 in vitro from phosphorylating PKB,[88] further investigations are required to establish how this drug is inhibiting PKB activation in cells.

Interestingly, other than PDK1 or PKB, three other protein kinases have been reported to mediate the phosphorylation of PKB at its hydrophobic motif, namely, the integrin-linked kinase (ILK), MAPKAP kinase-2, and conventional PKC isoforms. Dedhar and colleagues[89] reported that ILK was capable of directly phosphorylating PKB at Ser473 as well as being able to interact with PtdIns(3,4,5)P_3 despite not possessing a PH domain. We (Alessi, D. R.; Dowler, S. Unpublished data) and others[90] have not been able to reproduce these results, and more recently it has been reported that ILK may regulate the phosphorylation of PKB at Ser473 by an indirect mechanism as the overexpression of both a wild-type and a catalytically inactive mutant of ILK was capable of inducing phosphorylation of PKB at Ser473 in unstimulated cells.[90]

MAPKAP kinase-2 is activated by phosphorylation by the p38 MAP kinase following exposure of cells to cellular stresses and cytokines. We originally purified MAPKAP kinase-2 from rabbit skeletal muscle as an activity that could phosphorylate PKBα at Ser473 in vitro.[44] However, as an inhibitor of p38 termed SB203580 that prevented MAPKAPK2 activation in cells did not affect phosphorylation of PKBα at Ser473 in many cell lines[52] including in ES cells (Lizcano, J. Unpublished results) and agonists such as sodium arsenite and UV irradiation that activated MAPKAP kinase-2 did not induce phosphorylation of PKBα[52] at Ser473, this was taken as evidence that MAPKAPK2 did not phosphorylate this residue of PKBα physiologically. However, a recent study demonstrates that in neutrophils stimulated with FMLP, SB 203580 is capable of inhibiting phosphorylation of PKB at Ser473 at low concentrations of the SB inhibitor (<1 μM) that inhibit p38 without affecting phosphorylation of PKB at Thr308.[91] These results indicate a potential role for MAPKAPK2 in controlling the phosphorylation of PKBα at Ser473 in neutrophils but do not rule out the possibility that in neutrophils p38 is regulating the activity of a kinase distinct from MAPKAPK2 that mediates or influences indirectly phosphorylation of Ser473. In other primary T-cells it has been demonstrated that high concentrations of SB203580 can inhibit phosphorylation of PKBα at both Thr308 and Ser473, and this finding was interpreted as implying that SB203580 might be capable of directly inhibiting PDK1 at these high concentrations.[92] By contrast, one of us (Alessi, D. R. Unpublished results) and our colleagues in Dundee[93] have not found SB203580 to inhibit PDK1 significantly in vitro. Akkerman and colleagues[94] obtained some evidence that activation of conventional PKC isoforms in platelets can result in a PI 3-kinase-independent phosphorylation of PKB at Ser473 specifically and not Thr308, but as purified PKCα does not apparently directly phosphorylate PKB at Ser473, the authors suggest an additional cofactor is required for this reaction to occur. This pathway may be specific to platelets as the phosphorylation of PKB at Ser473 by virtually all agonists investigated in other cell lines was inhibited by wortmannin/LY294002. Moreover, in most cell lines, phorbol esters which activate conventional PKC isoforms do not induce PKB activation or its phosphorylation at Ser473.

In summary, despite the effort to discover the kinase responsible for the phosphorylation of PKB at Ser473, no single candidate seems to be indisputable. It should be kept in mind that the sequence surrounding the actual phosphorylation site is very unusual (Phe-Xaa-Xaa-Phe-Ser-Tyr), and it has been previously pointed out that the protein kinase responsible for this phosphorylation should display a unique selection for hydrophobic determinants.[95] It should also be considered that the hydrophobic motif is unlikely to be exposed to the aqueous solvent. In fact, based on the crystal structure of PKA, the most simple model would predict that the hydrophobic motif would be burried in a hydrophobic pocket in the kinase domain of PKB (equivalent to the PIF binding pocket on PDK1) in a position unsuitable for recognition by protein kinases. Hence, the putative kinase catalytic site could possess a high affinity binding for the hydrophobic motif in order to compete for the interaction to its own catalytic core. Alternatively, it is tempting to speculate that the mechanism of phosphorylation of this site could require another protein that would enhance the efficiency at which Ser473 is phosphorylated, perhaps by releasing the hydrophobic motif from its pocket.

VII. Discovery that PDK1 Activates Other Kinases

As mentioned previously, 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 kinase family 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.[15] Most AGC kinase family members also possess a region of homology to the hydrophobic motif of PKBα that encompasses Ser473 and is located ~160 amino acids C-terminal to the T-loop residue lying outside the catalytic regions of these enzymes. The hydrophobic motif of most AGC kinases is characterized by a conserved motif: Phe-

Xaa-Xaa-Phe-Ser/Thr-Tyr/Phe (where Xaa is any amino acid and the Ser/Thr residue is equivalent to Ser473 of PKB). Atypical PKC isoforms (PKCζ, PKCλ, PKCι) and the related PKC isoforms (PRK1 and PRK2) instead of possessing a Ser/Thr residue in their hydrophobic motif have an acidic residue. PKA, in contrast, only possess the Phe-Xaa-Xaa-Phe moiety of the hydrophobic motif as the PKA amino acid sequence terminates at this position.[85]

In addition 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. Examples of these are S6K isoforms (S6K1 and S6K2[16]), SGK isoforms (SGK1, SGK2, and SGK3[17−19]), as well as novel and atypical PKC isoforms.[96] Work performed over the past 5 years by 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.[97,98] 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 at their hydrophobic motif. Novel PKC isoforms are also dependent upon PI 3-kinase activity for their T-loop and hydrophobic motif phosphorylation.[96] Recent work has revealed that basal levels of PtdIns(3,4,5)P_3 in cells that are maintained through integrin−matrix interactions play a key role in enabling the novel PKCδ isoform to be phosphorylated at the T-loop and hydrophobic motif as removal of basal PtdIns(3,4,5)-P_3 in unstimulated cells, by the overexpression of PTEN or culturing the cells in suspension, results in the dephosphorylation of PKCδ at its T-loop and hydrophobic motif.[99]

The finding that the T-loop residues of all AGC kinases were so conserved suggested that a common upstream protein kinase such as PDK1 might phosphorylate these residues (Figure 4). It was found that the AGC kinases activated downstream of PI 3-kinase, namely, S6K1,[49,100] SGK isoforms,[17−19] and novel and atypical PKC isoforms[101,102] were phosphorylated specifically at their T-loop residue by PDK1 in vitro or following the overexpression of PDK1 in cells. Moreover, AGC kinases that were not activated in a PI 3-kinase-dependent manner in cells, such as the p90 ribosomal S6K (p90RSK) isoforms,[103,104] conventional and related PKC isoforms,[105−108] and even PKA,[109] 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.

To obtain firmer evidence that PDK1 mediates the activation of these AGC kinases in vivo, we tested whether these enzymes could be activated in

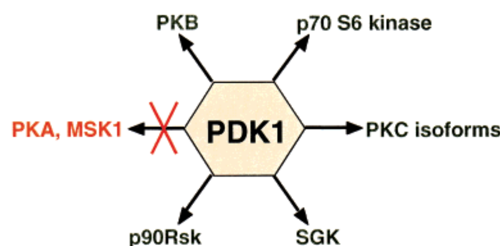

Figure 4. PDK1 plays a central role in activating several but not all AGC kinases. The AGC kinases that are likely to be phosphorylated physiologically by PDK1 are shown in green. PKA and MSK1[86,209] AGC kinase family members shown in red are not likely be phosphorylated in vivo by PDK1 as these enzymes are active in mouse embryonic stem cells that lack PDK1 (see 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.[112] (Reprinted with permission from Alessi, D. R. *Biochem. Soc. Trans.*, in press. Copyright 2001 The Biochemical Society.)

PDK1$^{-/-}$ES cells. We found that S6K1 was substantially active in wild-type PDK1$^{+/+}$ ES cells and activated a further 2-fold by IGF1. However, there was no detectable S6K1 activity in unstimulated or IGF1-treated PDK1$^{-/-}$ ES cells despite normal expression of S6K1 in these cells. In contrast to PKB, IGF1 failed to induce phosphorylation of S6K1 at its hydrophobic motif.[86] Although PDK1 could phosphorylate the hydrophobic motif of S6K1 directly and evidence to support this has been proposed,[110] it is also possible that PDK1 controls the activation or 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,[110,111] 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, the intracellular levels of endogenously expressed PKCα, PKCβI, PKCγ, PKCδ, PKCϵ, and PRK1 are vastly reduced compared to wild-type ES cells,[112] 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.[97,98] In contrast, the levels of PKCζ and PRK2 protein were only moderately reduced in the PDK1$^{-/-}$ ES cells. PKCζ expressed in ES cells lacking PDK1 is not phosphorylated at its T-loop residue, in contrast to wild-type cells,[112] providing genetic evidence that PKCζ is a physiological substrate for PDK1. Interestingly, PRK2 was still phosphorylated at its T-loop in PDK1$^{-/-}$ cells, although to 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.[112]

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

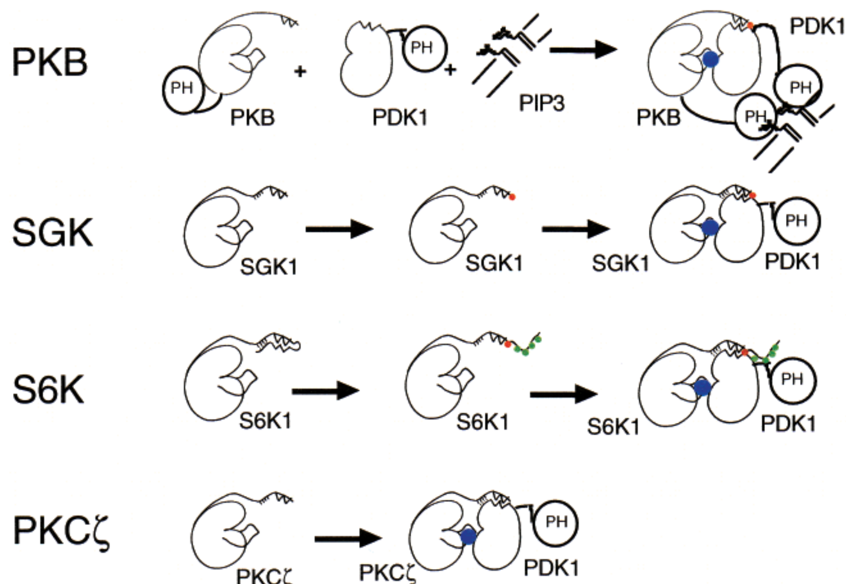

Figure 5. Summary of the model by which PDK1 can recognize, interact, and then phosphorylate different AGC kinase substrates. PKB does not require the PIF-binding pocket on PDK1 to become phosphorylated by PDK1. It is the binding of PDK1 and PKB to the second messenger PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 at the membrane of cells which is believed to co-localize these enzymes and hence enable PDK1 to phosphorylate PKB. SGK is a poor substrate for PDK1 until it is phosphorylated at its hydrophobic motif. This converts it into a form that can interact with the PIF binding pocket of PDK1 and hence permits PDK1 to interact with it and to phosphorylate SGK at its T-loop site. S6K is similarly converted into a form that can interact with the PIF binding pocket of PDK1 through a combination of the phosphorylation of the C-terminal Ser-Pro/Thr-Pro in its autoinhibitory domain[16] and by phosphorylation of its hydrophobic motif. Thus, for SGK and S6K, it is the phosphorylation of these enzymes at their C-terminal residues that is rate limiting for the phosphorylation of these kinases at their T-loop by PDK1. PKCζ, in contrast, might be constitutively phosphorylated at its T-loop motif in cells as the hydrophobic motif of PKCζ which possesses a Glu residue instead of a Ser/Thr residue at the site of phosphorylation can directly interact with the PIF binding pocket on PDK1, in principle as soon as it is expressed in a cell,[105] resulting in PKCζ becoming phosphrylated at its T-loop site. Blue circles indicate phosphorylation of T-loop residue, red circles phosphorylation of the hydrophobic motif, and green circles phosphorylation of the Ser-Pro/Thr-Pro residues in the C-terminal autoinhibitory domain of S6K. Hydrophobic motifs are marked with two triangles.

the same extent as in wild-type ES cells.[86] 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 vivo as it has been shown to possess the intrinsic ability to phosphorylate its own T-loop when expressed in bacteria.

VIII. Mechanism of Regulation of PDK1 Activity

The work described above establishes 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 coordinated manner. This problem has also been addressed in three recent reviews.[113−115] Initial experiments focused on whether insulin or growth factors could directly activate PDK1. When isolated from unstimulated or insulin-stimulated cells, PDK1 possessed the same activity toward PKB or S6K1.[49,77] Furthermore, although PDK1 is phosphorylated at 5 serine residues in 293 cells, insulin or IGF1 did not induce any change in the phosphorylation state of PDK1.[116] Only one of these phosphorylation sites, namely, Ser241, was essential for PDK1 activity. Ser241 is located in the T-loop of PDK1, and as PDK1 expressed in bacteria is phosphorylated at Ser241, it is likely that PDK1 can phosphorylate itself at this residue.[116] Although PDK1 becomes phosphorylated on tyrosine residues following stimulation of cells with peroxovanadate (a tyrosine phosphatase inhibitor),[80,117] no tyrosine phosphorylation of PDK1 has been detected following stimulation of cells with insulin.[80,116]

Taken together, these observations have suggested that PDK1 might not be activated directly by insulin. Instead, one intriguing possibility 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 substrates that are converted into forms that can interact with PDK1 and thus become phosphorylated at their T-loop. In the case of PKB, it is the interaction of PKB with PtdIns(3,4,5)P_3 that converts it into a substrate for PDK1 (Figures 3 and 5). In the case of 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)P_3 and whose phosphorylation by PDK1 in vitro is not enhanced by PtdIns(3,4,5)P_3, it is not obvious how PtdIns(3,4,5)-P_3 can regulate the phosphorylation of these enzymes in vivo. Recent studies indicate that the hydrophobic motif of S6K1, SGK1 (Biondi, R. M.; Kieloch, A.; Currie, R. A.; Deak, M.; Alessi, D. R. *EMBO J.* **2001**, *20*, in press.) and atypical (PKCζ) and related PKC (PRK2) isoforms[105] can interact with a hydrophobic pocket in the kinase domain of PDK1 that has been

termed the "PIF-binding pocket",[85] which provides a docking interaction required for their efficient phosphorylation. These experiments indicate that the interaction of S6K and SGK with PDK1 is significantly enhanced if these enzymes are phosphorylated at their hydrophobic motifs (Biondi, R. M.; Kieloch, A.; Currie, R. A.; Deak, M.; Alessi, D. R. *EMBO J.* **2001**, *20*, in press.). It is therefore possible that PtdIns(3,4,5)P_3 does not activate PDK1 but instead induces phosphorylation of S6K and SGK isoforms at their hydrophobic motifs (Figure 5). A similar observation has been made by Frodin and Gammeltoft,[118] who demonstrated that phosphorylation of the hydrophobic motif of p90RSK (which is induced following phosphorylation of p90RSK by ERK1/ERK2[119]) promotes its interaction with PDK1, therefore enhancing the ability of PDK1 to phosphorylate p90RSK at its T-loop motif. It should be noted that PI 3-kinase is not known to physiologically regulate p90RSK activity and it is the phosphorylation of p90RSK by ERK1/ERK2 that regulates its activation.

Related PKC isoforms and atypical PKC isoforms possess a hydrophobic motif in which the residue equivalent to Ser473 is Asp or Glu and these enzymes can in principle interact with PDK1 as soon as they are expressed in a cell.[105] However, it is possible that the interaction of related PKC 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[108] or PKCζ with hPar3 and hPar6[120] might induce a conformational change in these enzymes that controls their interaction with PDK1.

IX. Phosphatidylinositol 3-Phosphatase PTEN

As discussed above, the production of the lipid second messenger PtdIns(3,4,5)P_3 by growth factor-stimulated PI 3-kinases plays a key role in regulating survival and insulin-signaling pathways in mammalian cells. Only one phosphatidylinositol 3-phosphatase has been clearly demonstrated to convert PtdIns(3,4,5)P_3 back to PtdIns(4,5)P_2 in vivo. This phosphatase, PTEN, thus plays a key role in regulating cellular levels of PtdIns(3,4,5)P_3. PTEN was first identified as a tumor-suppressor phosphatase that was mutated in several types of sporadic human tumors.[121,122] The role of PTEN as a tumor suppressor has been confirmed by studies of PTEN in heterozygote knockout mice which develop a range of tumor types[28,123,124] and the identification of an association between germline PTEN mutations and Cowden disease, a hyperproliferative disorder that includes an increased risk of malignancy.[125,126] Mutation of PTEN has now been shown to occur in a wide range of tumor types but at particularly high frequency in glioblastomas and endometrial tumors.[127,128] Although PTEN was first postulated to function as a tyrosine phosphatase,[121,122,129,130] the most significant breakthrough in understanding the mechanism of tumor suppression by PTEN has been the demonstration that PTEN can dephosphorylate the lipid second messenger PtdIns(3,4,5)P_3 and hence switch off signaling events that are mediated by the PI 3-kinase signaling pathway.[29,131,132]

X. PTEN Protein

PTEN is a 403 amino acid protein, the N-terminus of which contains a phosphatase catalytic domain (residues 7−185) with sequence and structural homology to the family of protein tyrosine phosphatases, although it possesses a wider and deeper cleft in the active site.[133] This is consistent with its activity against PtdIns(3,4,5)P_3, as the phosphorylated inositol headgroup is considerably more bulky and larger than phosphotyrosine. Adjacent to the phosphatase catalytic domain, PTEN also contains a C2 domain (residues 186−351) that has been demonstrated to bind to lipid vesicles in vitro.[133] Partial or complete truncation of the C2 domain completely inactivates the PTEN 3-phosphoinositide phosphatase activity, indicating that this domain may play an important structural role in stabilizing the PTEN catalytic domain.[133−136] Additionally, point mutations that inhibit the lipid binding activity of the C2 domain have been designed that do not affect the 3-phosphoinositide phosphatase activity of PTEN against soluble substrates in vitro.[133] In cells, these mutations greatly diminish the functioning of PTEN in a number of cellular assays.[133] This suggests that the C2 domain of PTEN is required in vivo for this enzyme to interact with cellular membranes and hence to bring PTEN into contact with its phosphoinositide substrates.[133] However, Georgescu et al. recently demonstrated that artificially targeting these PTEN C2 domain mutants to the plasma membrane of cells does not fully restore their function,[137] indicating that the C2 domain may also be required to correctly orient the active site of PTEN so that it can optimally interact with PtdIns(3,4,5)P_3 at the membrane of cells.[137]

The extreme C-terminus of PTEN has been shown to bind to PDZ domains in several proteins, including MAGUK proteins (MAGI1,2,3[133−136,138,139] and hDLG[140]), the MAST205 kinase,[140] and the multi-PDZ adaptor MUPP1,[141] although, the functional significance of these interactions has not been definitively established. It is possible that these protein−protein interactions might bring PTEN into close proximity with specific cellular pools of PtdIns(3,4,5)P_3 substrate and/or potential regulators of PTEN activity that could include protein kinases, protein phosphatases, or even proteases.[135,136,142] The C-terminus of PTEN beyond the C2 domain also contains a number of potential phosphorylation sites, some of which have been reported to be phosphorylated by casein kinase-2 in vitro. Mutation of these sites to Ala appears to reduce the stability of PTEN and also to increase the ability of PTEN to inhibit cell-cycle progression.[135,143]

Several lines of evidence support the role of PTEN as a phosphoinositide phosphatase that functions to antagonize Class I PI 3-kinase function. First, purified PTEN protein acts as a phosphoinositide phosphatase in vitro, specifically dephosphorylating the 3 position of the inositol ring of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2.[29,132] Second, when overexpressed in cells, PTEN causes dramatic reductions in the levels of both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2.[29,131,132,144] PTEN is also able to dephosphorylate PtdIns(3)P and

the soluble inositol phosphate Ins(1,3,4,5)P_4 (the headgroup of PtdIns(3,4,5)P_3) in vitro. However, experiments investigating the substrate specificity of PTEN in vitro and in cells strongly suggest that the major physiological substrates of PTEN are PtdIns-(3,4,5)P_3 and possibly PtdIns(3,4)P_2 (Bennett, D.; McConnachie, G.; Pass, I.; Leslie, N.; Downes, P. Unpublished results). Further evidence that PTEN functionally antagonizes PI 3-kinase comes from the finding that several of the effects of PTEN expression in cells are mimicked by the PI 3-kinase inhibitors wortmannin and LY294002 as well as by the over-expression of dominant negative mutants of PI 3-kinase.[25,26,29,131,145] Together these data provide convincing evidence that the PtdIns(3,4,5)P_3 and perhaps PtdIns (3,4)P_2 are the physiological substrates for PTEN.

The levels of PtdIns(3,4,5)P_3 have been analyzed in several mammalian PTEN null cell lines. In all of these cells, the concentration of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 were found to be greatly elevated compared to control cells that express PTEN.[131,144,146] Furthermore, one of the downstream targets of PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2, PKB has been shown to be almost maximally activated in unstimulated cells that lack PTEN.[29,131] Much attention has focused on whether the activation of PKB could account for the increased tumorogenic properties of cells lacking PTEN as PKB activity is known to inhibit apoptosis and promote cell survival. It should be noted that it remains to be seen whether most or all cell types that lack PTEN or express mutant forms of PTEN exhibit elevated PtdIns(3,4,5)P_3 levels and PKB activity or whether in some cells other phosphoinositide phosphatases would be able to compensate for the loss of PTEN. Potentially this could have some significance in determining the types of tumors in which PTEN deletion would occur.

As discussed previously, there is now significant evidence that mammalian PKB transduces proliferative and survival signals through phosphorylation of multiple substrates, many of which may not yet have been characterized. Recent work has focused on the role that phosphorylation of the forkhead transcription factors by PKB may play in mediating proliferation and survival.[147−149] Nakamura and colleagues[60] elegantly demonstrated that in cells lacking PTEN, the forkhead transcription factors are aberrantly localized to the cytoplasm due to their phosphorylation by PKB and therefore cannot regulate the transcription of key genes that promote apoptosis and cell-cycle arrest that include the cyclin-dependent kinase inhibitor $p27^{KIP1}$. Evidence for the elevation of p27 levels by PTEN is very strong[26,60,146,150,151] since PTEN-induced stabilization of the p27 protein has also been described, possibly through down-regulation of the levels of the E3 ubiquitin ligase component SKP2.[150]

The pro-apoptotic Bcl-2 family member, BAD, has also been shown to be a substrate for PKB, and its phosphorylation at Ser136 by PKB (Table 1) promotes cell survival.[152] Consistent with this notion, BAD has been shown to be hyperphosphorylated in PTEN null cells.[146] Another established substrate of PKB is GSK3 and its phosphorylation and inhibition by PKB not only is required for stimulation of glycogen and protein synthesis but has also been shown to promote survival of various neuronal cells.[153−155] Furthermore, increased phosphorylation of GSK3 has been demonstrated in PTEN null cells.[156] PKB also phosphorylates and activates endothelial nitric oxide synthase, thereby promoting angiogenesis (formation of new blood supply).[74,157−160] Hypoxia also leads to increased angiogenesis by stimulating PI 3-kinase, PKB, and a transcription factor called HIF (hypoxia-inducible factor-1). HIF1 activates the transcription of many genes mediating the hypoxia response such as VEGF (vascular endothelial growth factor) which stimulates angiogenesis. Consistent with this idea, cells lacking PTEN have been shown to possess elevated VEGF levels.[161−163] The phosphorylation state of other PKB substrates discussed in Table 1, such as caspase 9,[164] Raf,[75] BRCA1,[165] and IκB kinases,[166,167] have not been investigated in PTEN-deficient cell lines to our knowledge.

As discussed in the Introduction, in addition to PKB and PDK1, several other proteins have been shown to possess PH domains that bind specifically to PtdIns(3,4,5)P_3 and/or PtdIns(3,4)P_2. For example, the Bruton tyrosine kinase (Btk) and the related kinase Itk contain a PH domain that binds to PtdIns-(3,4,5)P_3, and they are activated through phosphorylation by a Src -family kinase following interaction with PtdIns(3,4,5)P_3.[5] It has recently been demonstrated that overexpression of PTEN in cells prevents the recruitment of Itk to cell membranes, thereby inhibiting its activation, and also that PTEN has been identified in a genetic screen as a functional antagonist of Btk signaling.[168,169] The activation of the small GTPase Rac by certain agonists is dependent upon PI 3-kinase, apparently due to the recruitment to the plasma membrane and/or activation of Rac GDP/GTP exchange proteins that possess PH domains that interact with PtdIns(3,4,5)P_3. This notion of how Rac is activated agrees well with the observation that PTEN null cells possess a greater proportion of cellular Rac that is associated with GTP and processes that are mediated by activated Rac such the rate at which cells migrate and/or spread is increased.[145,170] Interestingly, the phenomenon of tumor cell invasion, which is related to cell motility, is also dependent on PI 3-kinase and is up-regulated by PTEN deletion.[171−173] Thus, several pathologically significant cellular processes that have been shown to be PI 3-kinase dependent have now been demonstrated to be deregulated in cells lacking PTEN. Other processes known to require PI 3-kinase activity might also give a selective advantage to a tumor cell, for example, the regulation of some steps in translation or the uptake of nutrients such as amino acids and glucose into cells. It will be interesting to see whether these processes are upregulated in tumors that have lost PTEN expression.

The pathways that mediate the activation of Class IA PI 3-kinases by growth factor receptors and the production of PtdIns(3,4,5)P_3 as well as some of the downstream effectors of these pathways are highly

conserved in worms (*C.elegans*), flies (*Drosophila*), and humans. PTEN homologues have been identified in *Drosophila* and *C. elegans,* and genetic analysis of these organisms strongly confirms that PTEN plays a key role in antagonizing signal transduction processes mediated by PI 3-kinase including the activation of PKB.[174−180]

XI. PTEN—A Protein Phosphatase?

It should be noted that human PTEN, in addition to its 3-phosphoinositde phosphatase activity, also possesses significant phosphotyrosyl phosphatase activity.[130] Thus, it cannot be ruled out that some of the cellular effects of PTEN could be mediated by dephosphorylation of protein substrates rather than lipid substrates. Thus far only two protein substrates have been proposed for PTEN, namely, the focal adhesion kinase (FAK) and the adaptor protein Shc, both of which are dephosphorylated by PTEN in vitro and following the overexpression of PTEN in cells.[170,181] However, it is unlikely that PTEN is dephosphorylating these proteins directly as a mutant of PTEN that lacks lipid phosphatase activity but still retains activity toward tyrosine phosphorylated peptides in vitro does not lead to FAK dephosphorylation in cells.[145] In addition, phosphorylation of FAK and Shc on tyrosine residues has been shown to occur downstream of PI 3-kinase, so that overexpression of PTEN in cells would be expected to induce dephosphorylation of FAK and Shc indirectly.[182,183] Other recent data, however, have also implicated the protein phosphatase activity of PTEN in down-regulating cyclin D1 expression in a breast cancer cell line.[151] It will be interesting to see whether other evidence supports this pathway.

XII. 5-Phosphatases—SHIP1 and SHIP2

Several cellular lipid phosphatases have been identified that will dephosphorylate PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 in vitro, most of them members of a family of phosphatases that has been termed the inositol polyphosphate 5-phosphatase family.[184,185] These include the synaptojanins,[186,187] Oculocerebrorenal (Lowe) (OCRL) phosphatase,[188] the type II[185,189] and type IV[190] 5-phosphatases, as well as the SH2-containing inositol phosphatase-1 (SHIP1) and SHIP2.[191] However, thus far evidence is lacking that the synaptojanins, OCRL, the type II, and type IV 5-phosphatases antagonize growth factor-stimulated PI3K signaling, and studies indicate that the principle cellular substrate for some of these phosphatases may be PtdIns(4,5)P_2 rather than PtdIns(3,4,5)P_3.[184−186,188] In contrast, there is significant evidence that SHIP1 and SHIP2 function as cellular PtdIns(3,4,5)P_3 phosphatases converting PtdIns(3,4,5)P_3 to another putative second messenger PtdIns(3,4)P_2. These proteins in addition to possessing a phosphatase catalytic domain have an N-terminal SH2 domain (Figure 6). They efficiently dephosphorylate the 5 position of the inositol ring of PtdIns(3,4,5)P_3 and have much weaker activity toward PtdIns(4,5)P_2 in vitro.[144,190,192] Overexpression studies using SHIP-deficient cells suggest that both

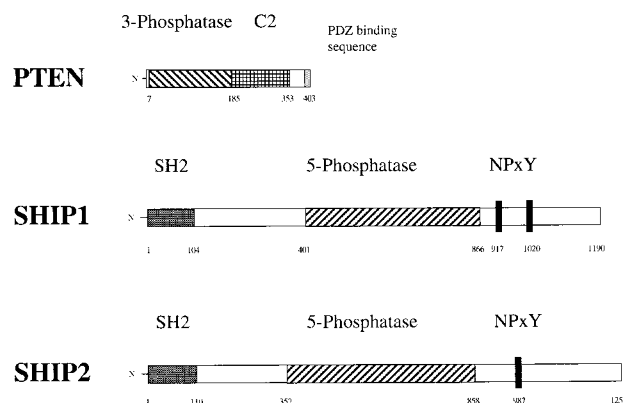

Figure 6. Domain structure of the PtdIns(3,4,5)P3 phosphatases. The domain structures of PTEN, SHIP1, and SHIP2 are shown. The 3-phosphatase domain of PTEN and the 5-phosphatase domain of the SHIP proteins do not share sequence homology. The NPxY sequence in the SHIP proteins can be tyrosine phosphorylated and then forms a binding site for some PTB domains, including that of the adaptor protein Shc.

SHIP1 and SHIP2 proteins are able to reduce cellular levels of PtdIns(3,4,5)P_3 and interestingly also reduce PKB activity.[144,193−195] This latter result has been interpreted to imply that PtdIns(3,4,5)P_3 rather than PtdIns(3,4)P_2 is the major activator of PKB in vivo, although as discussed above PtdIns(3,4)P_2 is as good as PtdIns(3,4,5)P_3 in enabling PDK1 to phosphorylate and activate PKB in vitro. Significantly, cells derived from SHIP1 null mice only possessed elevated levels of PtdIns(3,4,5)P_3 and PKB activity following cytokine stimulation and not in unstimulated cells.[194] The duration of PtdIns(3,4,5)P_3 elevation was also significantly increased in cells lacking SHIP1. It is possible that the PtdIns(3,4,5)P_3 phosphatse activity of the SHIP proteins may be more tightly regulated than that of PTEN due to the high elevation of PtdIns(3,4,5)P_3 and PKB activity in PTEN null cells compared to the SHIP1-deficient cell lines that have been investigated to date. These data, together with the normal development of SHIP1 null mice[194] and of SHIP2 null mice up to birth,[196] suggest that PTEN may play a greater role as a global regulator of PtdIns(3,4,5)P_3 levels. However, it is also possible that through functional redundancy these experiments hide phenotypes that would be observed in mice completely lacking both the SHIP1 and SHIP2 proteins. A recent study demonstrates that SHIP2 is likely to play a major role in regulating insulin sensitivity in animals as mice lacking SHIP2 die shortly after birth from very low glucose levels in the blood. Moreover, even adult mice that are heterozygous for the SHIP2 mutation have markedly increased insulin sensitivity, which is associated with an increased glucose tolerance and enhanced insulin signaling such as recruitment of the GLUT4 glucose transporter to the plasma membrane and increased glycogen synthesis in skeletal muscle.[196] These data strongly indicate that inhibitors of SHIP2 and or perhaps SHIP1 could show promise for the treatment of diabetes.

Significant research has shown that both SHIP1 and SHIP2 possess SH2 domains and are recruited to growth factor/cytokine receptors following stimu-

lation of cells through the interaction of the SH2 domain with tyrosine-phoshorylated receptors.[144,191,193,197] There is no evidence that this interaction activates the SHIP phosphatatses but may play an important role in bringing the phosphatases to their physiological PtdIns(3,4,5)P_3 substrate at cell membranes. SHIP1 and SHIP2 also become phosphorylated on tyrosine residues following stimulation; however, this does not appear to affect the enzymic activity of SHIP, and the role that this phosphorylation plays is not clear.[144,192,198] While the expression of SHIP1 is largely restricted to haematopoietic cells, SHIP2 appears to be more widely expressed.

Another potentially significant difference between the metabolism of PtdIns(3,4,5)P_3 by 5-phosphatases and by PTEN is the production of PtdIns(3,4)P_2 and PtdIns(4,5)P_2, respectively. Due to the relative differences in the abundance of these lipids, the dephosphorylation of the 3 position of PtdIns(3,4,5)P_3 will have an insignificant effect on the total cellular concentration of the abundant PtdIns(4,5)P_2 whereas dephosphorylation of the 5 position of cellular PtdIns(3,4,5)P_3 will have a very significant effect on the concentration of PtdIns(3,4)P_2. SHIP1 and SHIP2 phosphatases are thus likely to play critical roles in regulating the production and cellular levels of PtdIns(3,4)P_2. There has been a lot of speculation that PtdIns(3,4)P_2 is a key second messenger in its own right[199,200] There has also been considerable debate as to whether PtdIns(3,4)P_2 regulates the same physiological processes as PtdIns(3,4,5)P_3, as many of the PH domains that interact with PtdIns(3,4,5)P_3 also bind to PtdIns(3,4)P_2. Furthermore, recent findings indicate that agonists such as hydrogen peroxide[199] and cross-linking of platelet integrin receptors[200] elevate PtdIns(3,4)P_2 without increasing PtdIns(3,4,5)P_3 through the activation of a PtdIns 3P 4-kinase, also suggestting that PtdIns(3,4)P_2 may be able to regulate physiological processes distinct from those controlled by PtdIns(3,4,5)P_3. The two putative adaptor proteins termed TAPP1 and TAPP2[11] are the first proteins to be identified that interact with PtdIns(3,4)P_2 and not with PtdIns(3,4,5)P_3 or any other phosphoinositide tested and may therefore be key mediators of cellular responses that are regulated specifically by this second messenger.

XIII. Concluding Remarks

The elucidation of the mechanism by which PKB was activated by PDK1 in cells provided the first example of how the second messenger PtdIns(3,4,5)P_3 could activate downstream signaling processes. However, there remains many major unsolved questions for future research to address. A major challenge will be to clarify the mechanism by which PtdIns(3,4,5)P_3 induces the phosphorylation of the hydrophobic motif of PKB and other AGC kinases members. New genetic and pharmacological approaches are also urgently needed to dissect the roles of each individual AGC kinase in mediating agonist-dependent responses because information obtained by overexpression of constitutively active and dominant negative mutants of AGC kinases is not providing physiologically reliable results. Another major question is to define the role(s) that other proteins that bind to PtdIns(3,4,5)P_3 and or PtdIns(3,4)P_2, depicted in Figure 2, play in mediating PI 3-kinase-dependent signaling processes. It is also not clear whether PtdIns(3,4)P_2 regulates a group of cellular responses that are distinct from PtdIns(3,4,5)P_3, and if so, it is necessary to define what these responses are. Growth factors such as EGF, PDGF, IGF1, and insulin all activate PI 3-kinase and PKB and yet frequently regulate distinct physiological responses in the same cell line. A major challenge for future work will be to identify whether these agonists can activate specific components of signal transduction pathways that lie downstream of PI 3-kinase. Finally, we still understand little about the mechanism by which the PTEN and SHIP phosphoinositide phosphatase activity is regulated in vivo. As PTEN plays such a critical role in regulating the cellular levels of PtdIns(3,4,5)P_3, it is very likely that its activity will be subject to tight regulation and understanding these mechanisms will provide important insights into the pathway by which cells regulate the overall levels of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2.

XIV. Acknowledgments

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