REVIEW ARTICLE

Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm

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Phosphorylation plays a central role in regulating the activation and signalling lifetime of protein kinases A, B (also known as Akt) and C. These kinases share three conserved phosphorylation motifs: the activation loop segment, the turn motif and the hydrophobic motif. This review focuses on how phosphorylation at each of these sites regulates the maturation, signalling and down-regulation of PKC as a paradigm for how these sites control the function of the ABC kinases.

Key words: phosphoinositide 3-kinase, phosphoinositide-dependent kinase-1 (PDK-1), phosphorylation motif, protein kinase A (PKA), protein kinase B (PKB)/Akt, protein kinase C (PKC).

INTRODUCTION

Almost half a century ago Krebs, Graves and Fischer reported that the first discovered protein kinase, phosphorylase *b* kinase, was, itself, activated by phosphorylation [1]. Two decades later, Fischer and colleagues showed that the kinase that phosphorylates phosphorylase kinase, later named protein kinase A (PKA), was also phosphorylated [2]. Reversible control by phosphorylation/dephosphorylation is now firmly established as a fundamental mechanism for regulating the function of most of the 500 or so members of the mammalian protein kinase superfamily. Elucidation of the structures of diverse members of this superfamily in the past decade has unveiled the molecular mechanisms for many of these phosphorylations [3,4]. One of the best understood, and most general, is phosphorylation at the 'activation loop', a segment near the entrance to the active site of both serine/threonine and tyrosine kinases that, for many kinases, must be phosphorylated for catalytic competence; indeed, it is this residue that was identified by Fischer and coworkers in their studies on PKA in the late 1970s [2].

Protein kinases A, B (also known as Akt) and C are three major cellular players whose biological function depends on a series of ordered phosphorylations, including activation loop phosphorylation. For protein kinase B (PKB)/Akt and protein kinase C (PKC), and possibly PKA, this cascade of phosphorylations is triggered by phosphoinositide-dependent kinase-1 (PDK-1) [5,6]. PDK-1 plays a pivotal role in cell signalling by providing the activating phosphorylation for an abundance of serine/threonine protein kinases. Although some of the mechanistic details of how PDK-1 regulates its diverse substrates are unique to the specific substrate, the underlying molecular mechanism is the same. Extensive biochemical and cellular studies in the past decade have provided a unifying framework of the controls served by phosphorylation in the life cycle of one PDK-1 substrate, PKC, from its synthesis to its life centre stage as a key signal transducer, and subsequently to its degradation. This review focuses on the life cycle of PKC and the key role played by phosphorylation as a paradigm for control of ABC kinase function by phosphorylation.

ARCHITECTURE OF ABC KINASES

Protein kinases A, B/Akt and C have in common a conserved kinase core whose function is regulated allosterically by a corresponding regulatory moiety (Figure 1). In the case of PKA, the regulatory moiety is a separate polypeptide, whereas the regulatory determinants are on the same polypeptide as the kinase domain for protein kinases B/Akt and C. The regulatory domain serves two key functions: (i) it targets the kinases to the appropriate cellular location; and (ii) it regulates kinase activity by serving as an autoinhibitory module. In the case of PKA, targeting is achieved by interaction of the regulatory subunit with A-kinase anchoring proteins ('AKAPs') [7,8]. In the case of PKB and PKC, primary targeting is achieved by membrane-targeting modules in the regulatory moiety [9,10], with fine-tuning of location achieved, at least for PKC, by additional protein–protein interactions [11,12]. Autoinhibition is achieved by sterically blocking the active site: the inhibitory module is a pseudosubstrate sequence in kinases A and C (shown in Figure 1 by the green rectangles), and the pleckstrin homology (PH) domain in PKB. Binding of cofactors [cAMP for PKA, diacylglycerol for PKC, and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) for PKB] relieves autoinhibition through conformational changes that unmask the active site. There are three mammalian catalytic subunits of PKA (Cα, Cβ and Cγ) and four different regulatory subunits (R1α, R1β, RIIα and RIIβ) [13], three isoenzymes of PKB/Akt that share the same domain composition (PKB α/Akt 1, PKB β/Akt 2 and PKB γ/Akt 3 [9]) and 10 PKC family members (see the next section).

PKC family members

PKC comprises a family of isoenzymes that are grouped into three subclasses on the basis of the domain composition of the

Abbreviations used: PDK-1, phosphoinositide-dependent kinase-1; PH, pleckstrin homology; PI 3-kinase, phosphoinositide 3-kinase; PIF, PDK-1-interacting fragment; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKA/PKB/PKC, protein kinases A, B and C respectively; PRK, PKC-related kinase.

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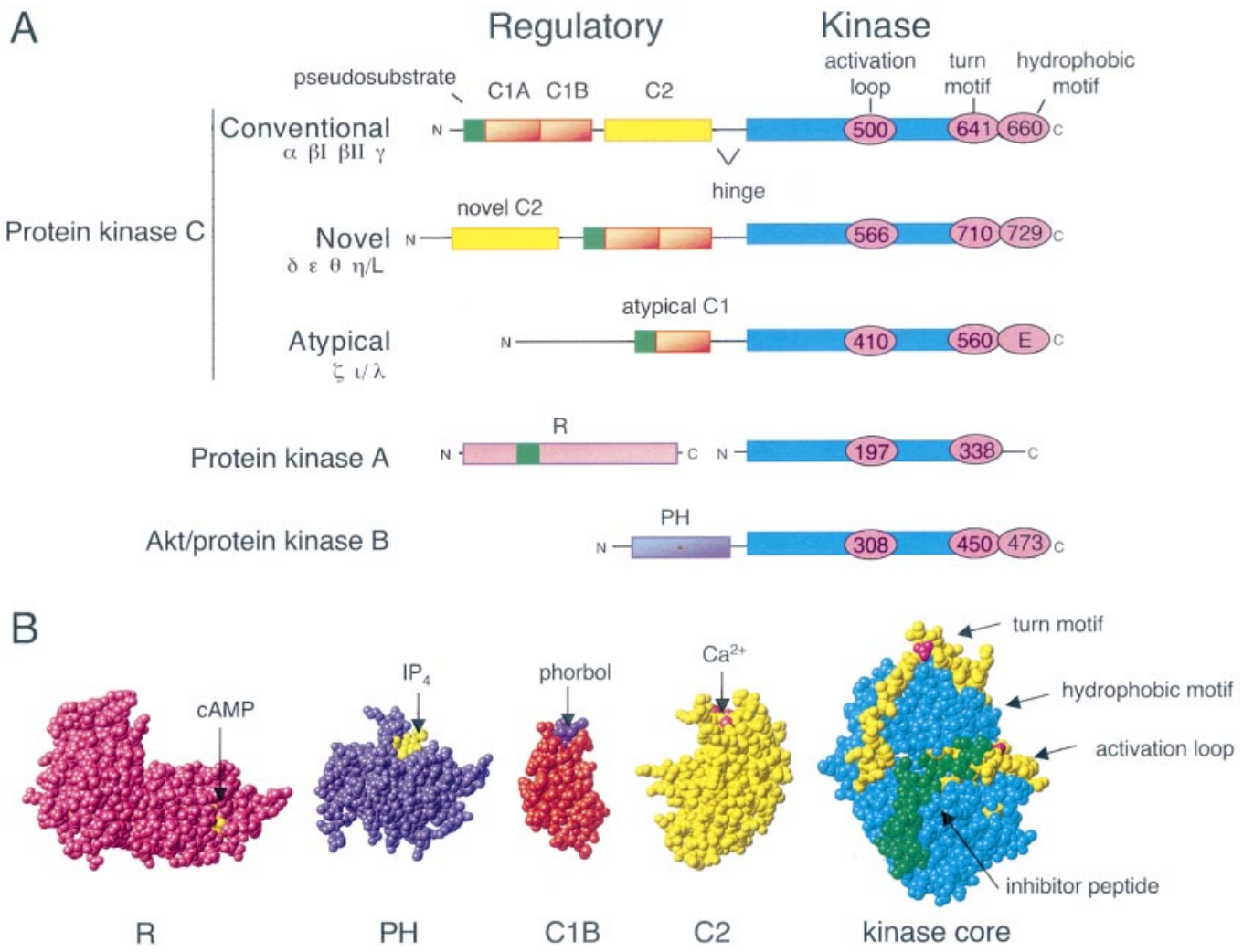

Figure 1 Domain composition of ABC protein kinase family members

(**A**) Primary structure, showing the three classes of PKC isoenzymes, PKA and PKB/Akt. All kinases have a conserved kinase core (coloured cyan) and C-terminal extension that contains two conserved phosphorylation sites: the turn motif and hydrophobic motif (shown by pink circles; note that, in atypical PKCs, a glutamate residue occupies the phosphoacceptor position and in PKA, the sequence ends one residue before the phosphoacceptor position). The kinases vary in the nature of the regulatory moiety. PKC isoenzymes differ primarily in the composition of membrane-targeting modules in the regulatory moiety. All isoenzymes have an autoinhibitory pseudosubstrate sequence (shown in green) that is N-terminal to the C1 domain (coloured orange); the C1 domain is a tandem repeat for conventional and novel PKCs, and functions as a diacylglycerol sensor. Atypical PKCs have an impaired C1 domain that does not respond to diacylglycerol/phorbol esters. Conventional PKCs have a C2 domain (shown in yellow) that serves as a Ca^{2+}-regulated phospholipid-binding module; the C2 domain in novel PKCs binds neither Ca^{2+} nor membrane phospholipids. The regulatory subunit of PKA binds cAMP and is a separate polypeptide; the regulatory moiety of PKB/Akt has a membrane-targeting module (a PH domain) that binds PIP_3. (**B**) Space-filling models of (from left to right) the R1α subunit of PKA (salmon pink) with bound cAMP (shown in yellow) [121], the PH domain of Btk (purple) with bound inositol 1,3,4,5-tetrakisphosphate (IP_4) (yellow) [122], the C1B domain of PKC δ (orange/red) with bound phorbol (purple) [30], the C2 domain of PKC βII (yellow) with bound Ca^{2+} (pink) [123], and the kinase subunit of phosphorylated PKA with bound inhibitor PKI [39] corresponding to the pseudosubstrate (in green), and the activation loop sequence and C-terminal sequence shown in yellow with the phosphate on the turn motif and activation loop coloured pink.

regulatory moiety (Figure 1) [14]. This composition, in turn, dictates the cofactor-dependence of the isoenzymes [15]. The two basic modules are the C1 and C2 domain, and each comes either in a form that binds ligand or in a form that is lacking determinants that allow ligand binding [16,17]. The C1 domain is the diacylglycerol sensor and the C2 domain is the Ca^{2+} sensor. Conventional PKCs contain functional C1 and C2 domains and respond to diacylglycerol and Ca^{2+} signals. Novel PKCs contain a functional C1 domain, but not a non-ligand-binding C2 domain: these isoenzymes respond to diacylglycerol, but not Ca^{2+} signals. Atypical PKCs contain a non-ligand-binding C1 domain and no C2 domain and, as a consequence, respond to neither diacylglycerol nor Ca^{2+}.

There are 10 mammalian PKCs: four conventional isoenzymes (α, βII and the alternatively spliced βI, which differs only in the last 43 residues, and γ), four novel PKCs (δ, ϵ, η/L and θ), and two atypical PKCs (ζ and ι/λ; note that an alternative transcript exists for ζ that only encodes the catalytic domain and is referred to as PKM ζ [18]) [14,19]. PKCs are also found in lower eukaryotes. *Caenorhabditis elegans* has four PKCs that correspond to conventional (PKC2), novel (PKC1 and TPA1) and atypical (PKC3) PKCs [20–23]. *Saccharomyces cerevisiae* has only one PKC, Pkc1 [21]; this contains apparently non-ligand-binding C1 and C2 domains, and responds to neither diacylglycerol nor Ca^{2+} [24]. In addition, Pkc1 contains two motifs, termed HR-1, which are found in a close 'cousin' of PKC,

protein kinase N ('PKN')/PKC-related kinase (PRK), [25,26], and which confer binding to RhoA GTPase [27–29].

Membrane-targeting modules

The C1 domain is present in all PKC isoenzymes (Figure 1). It is a small globular structure (approx. 8 kDa) that has a binding site for diacylglycerol formed by two pulled-apart β sheets [30,31]. Phorbol esters, non-hydrolysable analogues of the endogenous ligand, bind to this same site. Conventional and novel PKCs have two C1 domains, but the preponderance of evidence suggests only one engages ligand *in vivo*. For some isoenzymes, such as PKC δ, it is the C1B domain that is primarily responsible for binding diacylglycerol [32]. For others, such as PKC α, the C1A and C1B domains have equivalent roles in targeting PKC to membranes [33]. The ligand-binding pocket of the C1 domain is impaired in atypical PKCs, and these isoenzymes do not respond to either diacylglycerol or phorbol esters [34]. The C1 domain also specifically binds phosphatidylserine [35]. It is important to note that the C1 domain is found in a number of non-kinase molecules, and so PKCs are not unique in responding to phorbol esters/diacylglycerol [36].

The C2 domain is present in conventional and novel PKCs [37,38]. In conventional PKCs, this 12 kDa domain serves as a membrane-targeting module that binds anionic phospholipids in a Ca^{2+}-dependent manner. Novel C2 domains lack key residues involved in Ca^{2+} binding and, as a consequence, novel C2 domains bind neither Ca^{2+} nor phospholipids. Like the C1 domain, the C2 domain is found in a number of signalling molecules other than the PKCs [37,38].

PKC is maintained in an inactive conformation by binding of the pseudosubstrate sequence to the substrate-binding cavity (see the structure of PKA with bound inhibitor peptide in Figure 1B). Generation of diacylglycerol and Ca^{2+} recruits PKC to the membrane by engaging the C1 and C2 domains on the membrane. This membrane interaction provides the energy to release the pseudosubstrate from the substrate-binding cavity, allowing substrate binding and phosphorylation [10,15]. However, before such allosteric regulation can occur, PKC must be processed by phosphorylation (see below).

Kinase core

The kinase domains of PKA, PKB/Akt and PKC are highly conserved, with slightly more than 40 % overall sequence identity. The architecture of the kinase domain was first unveiled with the elucidation of the crystal structure of PKA in 1991 [39]. Over 30 protein kinase structures have now been solved, and they all show the same fold: a bilobal structure with an N-terminal lobe that is primarily composed of β sheet, and a C-terminal lobe that is primarily α helix [4,40]. The ATP- and substrate-binding site is located in a cleft between the two lobes. The structure of the kinase domain of PKB/Akt has recently been elucidated [41], but that of the kinase domain of PKC has remained refractory to crystallization, and so we are still dependent on molecular modelling for visualization of this structure [42]. One key feature shared by all three kinases is their key phosphorylation sites, detailed in the next section.

PHOSPHORYLATION IN THE KINASE CORE

The ABC kinases share three conserved phosphorylation motifs that critically regulate their function (Figure 1, pink circles, and Figure 2). These sites serve as phosphorylation-regulated switches to control both intra- and inter-molecular interactions. For all three kinases, phosphorylation is a prerequisite step to allow substrate phosphorylation. Without these priming phosphorylations, the kinases are catalytically inactive.

The activation loop site

As mentioned in the Introduction, phosphorylation at a segment near the entrance to the active site provides an important regulatory mechanism throughout the protein kinase superfamily [4,40,43]. The ABC kinases have a threonine residue in their activation loop segment that must be phosphorylated before the kinases can phosphorylate substrates (Figure 1). This residue corresponds to Thr^{500} in the conventional PKC βII, Thr^{197} in PKA and Thr^{308} in PKB 1/Akt α. The PKA structure revealed that phosphate at this position forms key contacts with an arginine residue adjacent to the base catalyst in the active site (Asp^{166} in PKA), aligning the activation loop segment to allow substrate binding [39,40]. Kinetic studies showed a dramatic increase in phosphoryl transfer rate following activation loop phosphorylation [44]. Mutation of the critical threonine residue to a neutral, non-phosphorylatable residue inhibits the activity of all three kinases, whereas mutation to an aspartate residue in the case of PKA and PKB/Akt, or to a glutamate residue in the case of PKC, results in the formation of a catalytically competent kinase [45–48]. In PKA, this site can be modified by autophosphorylation [45]. In the case of PKC and PKB, this reaction depends on an upstream kinase. The kinase that fulfils this role for PKB and PKC, and a number of other AGC kinases, is PDK-1.

PDK-1: the activation loop kinase

The purification [49,50] and subsequent cloning [51,52] of the activation segment kinase for PKB/Akt provided a major advance in cell signalling, because it soon became apparent that this novel kinase, named PDK-1, provided the priming phosphorylation for many other key members of the AGC protein kinase superfamily (Figure 2). In particular, PDK-1 was quickly shown to be the upstream kinase for conventional [53], novel [54,55] and atypical [54,56] PKC family members. PDK-1 was also rapidly established as the upstream kinase for p70S6 kinase [57,58].

Attesting to its central role in signalling, targeted deletion of PDK-1 in mice is lethal to embryos [59]. Studies with embryonic stem cells deficient in PDK-1 confirm that this kinase is required for the function of PKB/Akt and p70S6 kinase [60]. It is also required for PKC function: levels of PKC isoenzymes are dramatically decreased in stem cells deficient in PDK-1 [61], consistent with their instability in the non-phosphorylated form [62,63]. Interestingly, PKA is processed by phosphorylation in PDK-1-deficient stem cells, suggesting that, although PDK-1 recognizes the activation segment site of PKA effectively *in vitro* [64], PDK-1 is not required for its phosphorylation *in vivo* [60]. Biochemical inhibition of PDK-1 [65] and the use of antisense oligonucleotides against PDK-1 [66] support the results of these genetic deletion studies, showing a requirement for PDK-1 in the function of PKB/Akt. Studies in *C. elegans* have shown that PDK-1 in this simple organism is both required and sufficient to transduce signals from phosphoinositide 3-kinase (PI 3-kinase) to Akt [67]. Deletion of this gene abolishes signalling by Akt, and expression of a constitutively active form of PDK-1 bypasses the requirement for functional PI 3-kinase. Similarly, studies in *Drosophila* have shown that the PDK-1 from *Drosophila* (dPDK-1) activates dAkt-1, and that deletion of dPDK-1 is lethal to embryos [68].

Mammalian PDK-1 is unusual in the kinase superfamily in that it does not appear to belong to a family of isoenzymes.

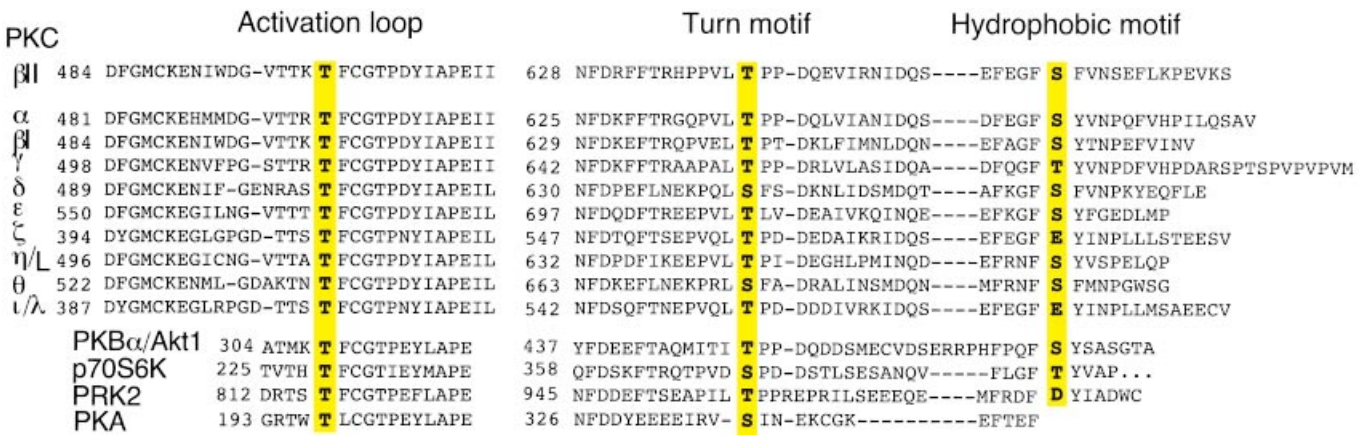

Figure 2 Alignment of the activation segment, turn motif and hydrophobic motif phosphorylation sequences for the PKC isoenzymes, PKBα/Akt1, p70S6 kinase, PRK2 and PKAα

Sequences shown are for human PKC isoenzymes α, ε, ζ, η/L, θ and ι/λ and rat PKC γ and δ [124], rat PKC βI and βII [125], murine PKB α/Akt1 [126], rat p70S6 kinase [127] and murine PKA α [128]. Amino acid residue numbers are indicated to the left of the sequences. Reprinted with permission from [15]. © (2001) American Chemical Society.

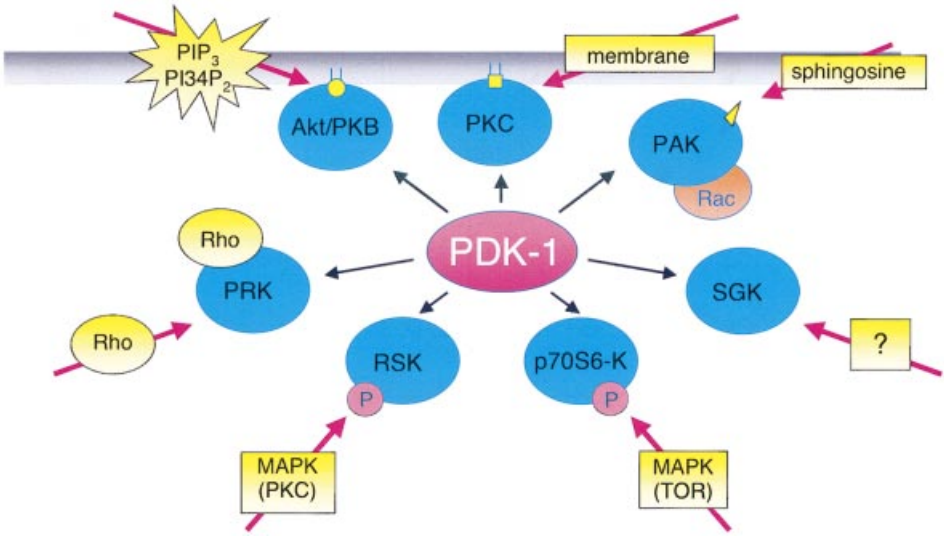

Figure 3 PDK-1 plays a pivotal role in cell signalling by phosphorylating the activation segment of diverse kinases

Specificity is dictated by the substrate: priming events of the substrate (indicated in yellow) provide a conformational switch to allow phosphorylation by PDK-1. Examples of this conformational switch are phosphoinositide binding to PBK/Akt, membrane binding of newly-synthesized PKC, binding of sphingosine to Rac-bound PAK, phosphorylation of p70S6 kinase and p90RSK by MAPK (and possibly other pathways), and binding of Rho to PRK. Reprinted from Cell **103**, A. Toker and A. Newton, "Cellular signalling: pivoting around PDK-1", pp. 185–188, © (2000), with permission from Elsevier Science.

Rather, a single gene product fulfils the function of PDK-1. Most compelling is the finding with stem cells deficient in PDK-1, showing that Akt phosphorylation at the activation segment is abolished [60]. Moreover, analysis of the human genome does not reveal a significantly related gene. Curiously, a novel kinase was described recently in *C. elegans*, which has modest similarity to the kinase domain of PDK-1 (32% identity and 56% similarity to *C. elegans* conventional PDK-1), but which lacks a PH domain; this kinase phosphorylated PKB/Akt when transfected into mammalian cells, and was named PIAK (phospholipid-independent PKB/Akt kinase) [69]. In mammals, however, PDK-1 appears to be unique. Whether the pleiotropic effects mediated by PDK-1 are regulated by association of the kinase with multiple (regulatory) binding partners is an unexplored question.

How is signalling specificity maintained when one upstream kinase dictates the function of so many different downstream kinases? Nature has designed an efficient mechanism: the substrate dictates when it needs to be phosphorylated by PDK-1 (Figure 3). That is, PDK-1 substrates need to be at the right location and in the right conformation before they can be phosphorylated by PDK-1, whose intrinsic activity appears to be constitutive. For example, PKB/Akt becomes a substrate for PDK-1 once its PH domain engages PIP_3 on the membrane, exposing the PDK-1 phosphorylation site. (It is unfortunate that

the naming of PDK-1 reflects the phosphoinositide-dependence for phosphorylation of this first-known substrate [49]. Phosphorylation of other substrates, such as conventional PKCs, is not phosphoinositide-dependent [70].) As discussed below, PKC becomes a substrate for PDK-1 when it is membrane-bound and in an 'open' conformation in which the pseudosubstrate is not occupying the substrate-binding cavity.

For some substrates, the phosphorylation by PDK-1 is a constitutive process involved in the maturation of the enzymes. Conventional PKC family members epitomize this class of substrates. Here, PDK-1 phosphorylates the enzymes to render them catalytically competent. However, once phosphorylated, the enzymes adopt an autoinhibited conformation that is only relieved upon binding of the appropriate second messengers. Thus PDK-1 primes conventional PKC family members, but does not directly activate them. In contrast, the regulation of PKB/Akt is reversed: the unphosphorylated species is maintained in an autoinhibited state. Generation of PIP_3 unmasks the PDK-1 site, allowing phosphorylation. The phosphorylated species is locked in an activated conformation that no longer requires cofactor binding (Figure 3). These mechanistic details are discussed in greater detail below.

The turn motif

The ABC kinases also have a conserved phosphorylation site in a segment of the C-terminus corresponding to Ser^{338} in PKA (Figure 2). In the PKA structure, the phosphorylated residue anchors the C-terminus at the top of the upper lobe of the kinase domain, with the phosphorylated threonine residue at the apex of a tight turn (see Figure 1B). This motif is thus referred to as the 'turn motif', and in most cases the phosphorylated threonine residue is flanked by proline residues (Figure 2). Mutation of this residue in PKA destabilizes the kinase domain [45], probably as a result of losing the anchorage at the top of the kinase domain. Interestingly, mutation of this residue (Thr^{450}) to alanine in PKB/Akt has no affect on the activity of PKB/Akt [71,72]. In the case of PKC βII, mutation of the turn motif residue (Thr^{641}) results in compensating phosphorylation of adjacent residues to produce functional kinase [63]. Mutation of both the turn-motif threonine residue and adjacent compensating sites abolishes kinase activity. Mutation of the turn motif in PKC α was reported to be tolerated, but the question of compensatory phosphorylations was not addressed [62]. Thus structural studies suggest that the phosphate at the turn motif is critical in stabilizing the kinase core by anchoring the C-terminus at the top of the upper lobe of the kinase core: this stabilization role is supported by biochemical studies of PKA and PKC, but, curiously, not PKB/Akt, where other mechanisms (including compensating phosphorylations) may help tether the C-terminus in place.

The hydrophobic motif

The third conserved phosphorylation site of the ABC kinases contains a serine or threonine residue flanked by hydrophobic residues, and was first identified in PKC and p70S6 kinase [73–75]. The sequence of PKA ends at the hydrophobic residue (phenylalanine) immediately preceding the phosphoacceptor site (Figure 2). The crystal structure of PKA reveals that this terminal phenylalanine residue is tucked into a hydrophobic pocket on the back side of the active site (Figure 1B) [39]; thus the back side of the kinase core has a binding pocket for the hydrophobic motif. The hydrophobic site binding pocket is also apparent in the recently elucidated structure of the PKB/Akt kinase core [41]. In this latter study of both phosphorylated and unphosphorylated PKB/Akt, the C-terminus was too disordered to enable its structure to be assigned, but phosphorylation-dependent changes in the core of the kinase suggested a model in which phosphorylation of the hydrophobic site orders the C helix, a key contact in the upper lobe of the kinase domain, to align residues more favourably for catalysis [41]. This model was subsequently confirmed following elucidation of the structure of an Akt in which the C-terminus had been replaced with sequences containing a constitutive negative charge at the hydrophobic motif: this study elegantly established that negative charge at the phosphoacceptor position of the hydrophobic motif triggers an intramolecular 'clamp' with the N-terminal lobe of the kinase, which orders the C helix and allows key stabilizing contacts to be made with the activation loop segment [76].

Structural studies linking phosphorylation of the hydrophobic site to kinase core stabilization are well supported by biochemical studies. Mutation of the hydrophobic site to an alanine residue in PKC reveals that phosphorylation of this site is not required for PKC function; rather, it stabilizes the enzyme [77–80].

The hydrophobic site is also important in kinase function because it provides a docking site for PDK-1. A series of studies by Alessi and co-workers provided the first evidence that PDK-1 interacts with high affinity with sequences corresponding to this hydrophobic phosphorylation motif by identifying the C-terminus of the PKC-related kinase PRK2 in a yeast two-hybrid screen for PDK-1-binding partners [81]. This kinase has a hydrophobic motif that has a constitutive negative charge (aspartate) at the phosphoacceptor position. They termed this sequence PIF (for PDK-1-interacting fragment). It has since been shown that the hydrophobic motif is the docking site of PDK-1 for a variety of substrates, including PKC and p70S6 kinase [82,83]. Alessi and co-workers went on to propose that the hydrophobic motif binding pocket, apparent in the PKA structure, is present in PDK-1 but is not occupied, because PDK-1 does not have a C-terminal hydrophobic motif [84]. Rather, it was proposed that PDK-1 is stabilized by docking on to the hydrophobic motif of its substrate kinases. This hypothesis is supported by mutagenesis studies, showing that mutation of key residues in the hydrophobic motif binding pocket reduce the phosphorylation of one substrate, p70S6 kinase, by PDK-1 [85]. Indeed, the recent elucidation of the crystal structure of the kinase domain of PDK-1 delineates the hydrophobic motif binding pocket (termed 'PIF pocket' by the authors), with biochemical studies defining residues that form a potential phosphate-docking site near the PIF pocket [86,87]. Interestingly, the hydrophobic motif of PKB/Akt has a very low affinity for PDK-1, and mounting evidence suggests that mechanisms other than docking to the hydrophobic site may determine the phosphorylation of PKB/Akt by PDK-1 [85]. This docking is, however, central to the regulation of PKC by PDK-1, as discussed below.

THE LIFE CYCLE OF PKC: CONTROL BY PHOSPHORYLATION

Maturation of PKC

Pulse–chase analysis has revealed that newly synthesized PKC associates with a membrane compartment in the cell [70]. Biochemical analysis of this newly synthesized species of enzyme has established that it is in an 'open' conformation, in which the autoinhibitory pseudosubstrate sequence is removed from the substrate-binding cavity [88]. Biophysical analysis suggests that this species of PKC is tethered at the membrane by multiple weak interactions. These comprise low-affinity interaction of the C1 and C2 domain to anionic lipids {each contributing approx. 1 kcal $\cdot$ mol^{-1} (where 1 kcal $\approx$ 4.184 kJ) in binding energy [35]},

and interaction of the basic pseudosubstrate with anionic lipids (contributing approx. 8 kcal · mol^{-1} in binding energy [89]). These three interactions synergize to tether PKC in the open conformation to membranes. This open conformation is critical to the subsequent processing of PKC, because the activation loop sequence is unmasked. This sequence is masked when the pseudosubstrate occupies the substrate-binding cavity [88].

In this 'open' conformation, the unphosphorylated C-terminus of newly synthesized PKC is also exposed, and provides a docking site for PDK-1. Specifically, PDK-1 binds the unphosphorylated hydrophobic motif of newly synthesized PKC, positioning it to phosphorylate the exposed activation loop sequence [83]. PDK-1 phosphorylates the activation loop, and is released from the C-terminal docking site, thus exposing sites on this segment for phosphorylation. The turn motif and hydrophobic motif are then phosphorylated, an event that proceeds via an intramolecular autophosphorylation mechanism in the case of conventional PKCs [90]. PKC ϵ, a novel isoenzyme, also autophosphorylates on the C-terminal sites [55], but evidence has been presented to suggest that the reaction on the hydrophobic motif is catalysed by another kinase for PKC δ [91]. The finding that mutation of the phosphorylatable residue of the turn motif to an alanine prevents autophosphorylation of the hydrophobic motif suggests that phosphorylation of the turn motif precedes that of the hydrophobic motif [63]. PKC undergoes distinct shifts in electrophoretic mobility following phosphorylation of the turn motif (electrophoretic mobility is retarded by the equivalent of 2 kDa) and the hydrophobic motif (also with a retardation of electrophoretic mobility by the equivalent of 2 kDa) [73]; phosphorylation of the activation loop sequence does not affect the electrophoretic mobility of PKC. These shifts in electrophoretic mobility have allowed analysis of the kinetics of processing of PKC by pulse–chase analysis, and reveal a half-time of approx. 10 min for endogenous PKCα in COS cells [70].

The rate-limiting step in the maturation of PKC appears to be the release of PDK-1 from PKC to expose the C-terminus to allow autophosphorylation. Consistent with this, disruption of the docking of PDK-1 to the hydrophobic motif increases the phosphorylation of PKC [83]. On the other hand, overexpression of PDK-1 slows the rate of processing, probably by favouring the binding to the C-terminus, thus preventing the autophosphorylation step by masking the C-terminus [70]. The physiological regulators for release of PDK-1 are not well understood. Whether the release depends on the equilibrium binding of PDK-1 to the C-terminus, or whether it is regulated by binding to higher-affinity docking sites such as the C-terminus of PRK, which contains the PIF motif, remains to be determined.

Phosphorylation of the C-terminal sites results in a number of key conformational rearrangements that lock PKC into a more thermally stable, protease- and phosphatase-resistant conformation. Specifically, several lines of evidence suggest that phosphorylation of the C-terminus locks this segment on the upper lobe of the kinase domain, freeing the substrate-binding cavity to bind the pseudosubstrate sequence. First, a comparison with the structure of phosphorylated PKA suggests that the phosphorylated turn motif tethers the C-terminus on the upper lobe of the kinase core, away from the substrate-binding cavity (see the kinase structure in Figure 1) [39]. Secondly, a recent analysis identifying a phospho-hydrophobic site binding pocket suggests that, once phosphorylated, the hydrophobic motif is engaged in this pocket on the back side of the upper lobe (see the kinase structure in Figure 1) [87]. This binding pocket contains two basic residues that are conserved in AGC kinases that have hydrophobic motif phosphorylation sequences (notably PKC and PKB/Akt, but not PKA), which are proposed to interact with the phosphate on the phosphorylated hydrophobic motif. Presumably, anchoring of the C-terminus away from the substrate-binding cavity allows the pseudosubstrate to gain access. Thus PKC adopts the 'closed' conformation, loses its primary membrane anchor and localizes to the cytosol. This species of PKC is referred to as the mature species: it is catalytically competent, but is not active because it is maintained in the autoinhibited state by the pseudosubstrate sequence.

The rate of processing of conventional PKC isoenzymes is the same in the presence or absence of PI 3-kinase inhibitors [70]. Thus the PDK-1 step in the phosphorylation of these PKCs is not phosphoinositide-dependent. This underscores the concept that the phosphoinositide-dependence for the phosphorylation of PKB/Akt results from a requirement of the substrate, PKB/Akt, for phosphoinositides in order to be recognized by PDK-1. PKC provides an example of how it is the conformation of the substrate that dictates whether it will be phosphorylated by PDK-1 or not.

PKC 'in its prime'

It is the fully phosphorylated 'mature' species of PKC that has been reported to translocate to membranes in the avalanche of studies on PKC distribution triggered by the hallmark discovery that PKC translocates to the membrane following phorbol ester treatment of cells [92,93]. Extensive biochemical studies coupled with fluorescent imaging studies in cells provide a fairly detailed understanding of the molecular mechanisms of this translocation [10,94,95].

For conventional and novel PKCs, the bulk of the enzyme in the cell is in this matured form, localized to the cytosol. Isoenzyme-specific binding partners may also localize a fraction of the PKC to specific intracellular sites [11,12]. The bulk enzyme is diffusing in the cell, occasionally colliding with the membrane, but coming off immediately because there are no significant interactions with the membrane. Signals that elevate intracellular Ca^{2+} and diacylglycerol have the following effect on conventional PKCs: Ca^{2+} binds the C2 domain of cytosolic PKC, the immediate consequence being that, upon the next few diffusion-controlled collisions with the membrane, the C2 domain engages on the membrane, thus tethering PKC there [96,97]. This is a low-affinity interaction and is not sufficient to activate PKC. It then diffuses in the plane of the membrane to find its membrane-embedded ligand, diacylglycerol. Binding of diacylglycerol to the C1 domain results in a high-affinity interaction of PKC with the membrane, and the energy of this interaction is used to release the pseudosubstrate from the substrate-binding cavity. In this open conformation, mature PKC binds substrates and effects downstream signalling [15,19,98].

Novel PKCs do not have the advantage of being pre-targeted to membranes by a Ca^{2+}-sensing C2 domain. As a consequence, their rate of translocation to membranes is approximately an order of magnitude lower than that of conventional PKCs, and this probably reflects the decreased probability of encountering a membrane-bound ligand (diacylglycerol) from the solution compared with encountering it from the membrane [97].

Atypical PKCs respond to neither diacylglycerol nor Ca^{2+}, and the only clear regulation of these isoenzymes is their requirement for PDK-1 for the phosphorylation step [54,56]. These isoenzymes differ somewhat from the conventional and novel PKCs in that activation of PI 3-kinase results in a moderate increase in activation loop phosphorylation. That is, these isoenzymes still have a high basal level of activation loop phosphorylation, but it increases 2- or 3-fold following activation of PI 3-kinase. These isoenzymes have a pseudosubstrate sequence that allosterically

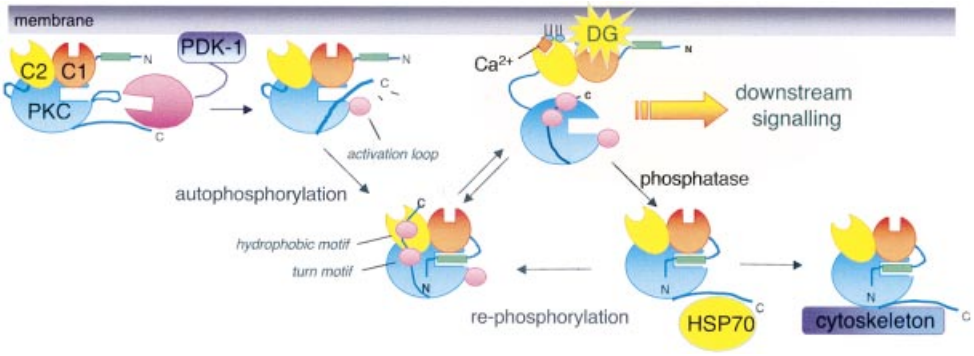

Figure 4 Model showing the life-cycle of PKC, from its biosynthesis to its eventual down-regulation

Newly synthesized PKC associates with the membrane in an open conformation in which the pseudosubstrate (coloured green) is released from the substrate-binding cavity (shown as a rectangular indent in a cyan circle), and in which the C-terminus is exposed to allow PDK-1 to bind (far left). PDK-1 phosphorylates the activation loop segment and is released from its docking site on the C-terminus. The liberated C-terminus accesses the substrate-binding site and is autophosphorylated by an intramolecular mechanism. The phosphorylated enzyme is released into the cytosol (shown in the middle of the diagram), where it is maintained in an inactive conformation by the bound pseudosubstrate. Generation of Ca^{2+} and diacylglycerol targets PKC to the membrane. Engagement of the C1 and C2 domains on the membrane provides the energy to release the pseudosubstrate from the substrate-binding cavity, allowing downstream signalling. In this active conformation, PKC is rapidly dephosphorylated. The molecular chaperone Hsp70 binds the dephosphorylated turn motif and stabilizes PKC, allowing it to become rephosphorylated and to re-enter the pool of signalling-competent PKC. In the absence of Hsp70 binding, or as a result of chronic activation, dephosphorylated PKC accumulates in a detergent-insoluble cell fraction, where it is eventually degraded.

regulates the enzyme, but what the inputs are for this regulation are not as well defined as for conventional and novel PKCs [99].

The molecular chaperone Hsp70 prolongs the signalling lifetime of PKC

The active species of PKC is highly sensitive to dephosphorylation: biochemical studies have shown that membrane-bound PKC is two orders of magnitude more sensitive to dephosphorylation than is soluble PKC [100]. In fact, prolonged activation by treatment of cells with phorbol esters results in dephosphorylation and down-regulation of PKC [101]. The dephosphorylated species localizes to the detergent-insoluble fraction of cells, where it is eventually proteolysed. Studies with PKC α suggest the involvement of caveolin-dependent targeting to endosomes [102]. Limited evidence suggests that proteolysis may be through a ubiquitin-mediated pathway [103,104]. Treatment with phorbol esters is frequently used to 'down-regulate' PKC in experiments aimed at addressing the involvement of PKC in cell signalling [105]. Atypical PKCs are resistant to this phorbol-ester-mediated down-regulation.

Although the phosphorylation of conventional PKCs is constitutive, their dephosphorylation may be under agonist control [101]. Both phorbol esters and ligands such as tumour necrosis factor α ('TNFα') result in PKC inactivation and dephosphorylation [106–108]. Furthermore, serum selectively promotes the dephosphorylation of the activation loop segment in conventional PKCs, thus uncoupling the phosphorylation of the activation loop from that of the C-terminal sites [70]. The hydrophobic site of PKC ϵ has also been reported to be selectively dephosphorylated by a rapamycin-sensitive phosphatase [109].

How does the cell maintain signalling by PKC when the active species is so sensitive to down-regulation? Nature has, once again, devised a mechanism to protect PKC. Specifically, recent studies have shown that the dephosphorylated turn motif provides a specific binding site for the molecular chaperone, Hsp70 [110]. This dephosphorylation-dependent binding of Hsp70 stabilizes PKC and allows it to become rephosphorylated and cycle back into the pool of functional PKC. Disruption of the interaction of Hsp70 with PKC results in accumulation of dephosphorylated PKC in the detergent-insoluble fraction of cells.

A model showing the life cycle of PKC, from its biosynthesis to its eventual down-regulation, is depicted in Figure 4.

PKC AS A PARADIGM FOR REGULATION OF THE ABC KINASES BY PHOSPHORYLATION

The ABC kinases are all regulated by two co-ordinated mechanisms: (i) phosphorylation, required to structure the enzymes for catalysis; and (ii) second messengers, required to allosterically regulate the activity of the enzymes. For PKA and PKC, phosphorylation occurs first and is, with the possible exception of atypical PKCs, constitutive (Figure 5, right-hand panel). Thus cells maintain a pool of processed PKA and PKC ready to respond to their respective second messengers. Binding of these second messengers 'unmasks' the substrate-binding site by dissociation of the R subunit (PKA) or the pseudosubstrate sequence (PKC), allowing substrate binding and phosphorylation.

In the case of PKB/Akt, allosteric regulation precedes phosphorylation (Figure 5, left-hand panel). For this kinase, generation of PIP_3 engages the PH domain on the membrane and triggers the second step: phosphorylation by PDK-1. The dependence on PIP_3 for the phosphorylation of PKB/Akt probably arises for two reasons: first, by analogy with PKC, engaging the PH domain on the membrane probably unmasks the activation loop segment and allows PDK-1 to access it. Secondly, co-localization of both PDK-1 and PKB/Akt on the membrane via their respective PH domains increases the probability of interaction and phosphorylation. This co-localization is significant in the case of PKB/Akt because, unlike PKC, PDK-1 does not appear to dock with significant affinity on the C-terminus of PKB/Akt.

A third regulatory mechanism epitomized by PKC is the role of molecular chaperones in sustaining the signalling lifetime of the ABC kinases. Specifically, dephosphorylation of the turn motif of PKA, PKB/Akt and PKC promotes the binding of Hsp70, an interaction that, in the case of PKC, allows the kinase to be recycled back into the signalling pool of PKC [110].

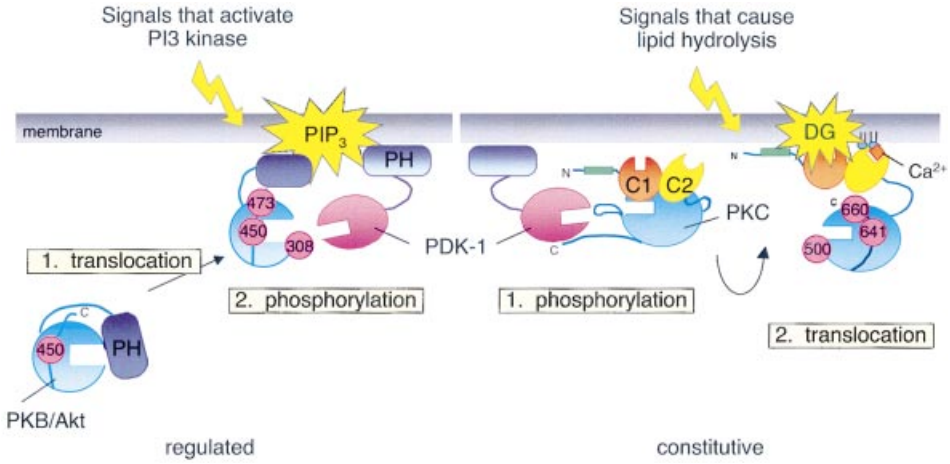

Figure 5 Model showing that the PDK-1 step is constitutive in the case of PKC, but is regulated in the case of PKB/Akt

PKB/Akt and PKC are both under the co-ordinate regulation of second messenger/allosteric mechanisms and phosphorylation triggered by PDK-1. However, the order of these events is reversed. In the case of PKB/Akt, generation of PIP_3 targets PKB/Akt to the membrane, an event which allows PDK-1 phosphorylation. Thus this phosphorylation is acutely regulated by PI 3-kinase. In the case of PKC, PDK-1 phosphorylation is constitutive because newly synthesized PKC is targeted to the membrane in a conformation that promotes PDK-1 binding and phosphorylation; this phosphorylated species is released into the cytosol and subsequent generation of diacylglycerol and Ca^{2+} recruit PKC to the membrane, allowing activation by allosteric mechanisms.

Without this interaction with Hsp70, dephosphorylated PKC is targeted for degradation. Thus the turn motif serves as a switch that is central not only for the maturation of these kinases, but as a protection mechanism from the conformational stress of signalling. Indeed, heat-shock proteins are emerging as regulators of a diverse range of protein kinases [111]. Of particular relevance, Hsp90 has been shown recently to protect Akt/PKB from dephosphorylation, hence sustaining the active state of Akt and protecting cells from apoptosis [112]. Hsp90 has also been shown to bind directly and stabilize PDK-1, thus prolonging the signalling lifetime of this master kinase [113].

Phosphorylation step

The processing of PKA by phosphorylation is similar to that of PKC in that the processing is constitutive, and the C-terminal phosphorylation is mediated by autophosphorylation [45]. As discussed above, phosphorylation by PDK-1 triggers the autophosphorylation of the turn motif and hydrophobic motif of PKC. Kinetic analyses have shown that the autophosphorylation of the hydrophobic site of conventional PKCs proceeds by an intramolecular mechanism [90]. Consistent with this, phosphorylation of this site *in vivo* depends on the catalytic competence of PKC [90]. PKA follows the same activation-loop-phosphorylation-triggered, autophosphorylation mechanism. However, unlike PKC, PKA is also able to autophosphorylate at the activation loop sequence [45]. Nonetheless, an upstream kinase may modify this site *in vivo*: a recent study has shown that mutations in PKA that disrupt autophosphorylation at the activation loop segment *in vitro* do not disrupt the processing of PKA *in vivo*, whereas mutations that disrupt phosphorylation by PDK-1 *in vitro* do disrupt the processing of PKA *in vivo* [114]. PKA also differs from PKC in not being regulated by phosphorylation at the hydrophobic motif: the sequence of PKA ends at the phenylalanine residue at the P-1 position of the phosphoacceptor site of this motif (Figure 2).

Similar to PKC, PKB/Akt has all three processing phosphorylation sites: the activation loop segment, the turn motif, and the hydrophobic motif (Figure 2). The mechanisms of phosphorylation at these sites have both differences and similarities to the mechanism of phosphorylation of PKC at these sites. In common is the dependence of both kinases on PDK-1 for phosphorylation of the activation loop sequence. For both kinases, access to this site is controlled by the conformation of the substrate: the pseudosubstrate (PKC) and PH domain (PKB/Akt) need to unmask the activation loop by engagement on the membrane. For both kinases, by analogy with PKA and recent structural information from PKB/Akt, phosphorylation probably positions residues correctly for catalysis and substrate binding. The differences relate to the two C-terminal sites. In contrast with PKC, phosphorylation of the turn motif of PKB/Akt is mediated by a distinct kinase, and does not depend on activation loop phosphorylation or on the intrinsic activity of PKB/Akt [71,72]; nor does phosphorylation of this site affect the activity or function of PKB/Akt in any readily discernible way [71,72].

The mechanism of phosphorylation of the hydrophobic site of PKB/Akt is the subject of controversy [5,6,9]. The phosphoinositide-dependence of phosphorylation of this site has led to the proposal that a separate upstream kinase tentatively named 'PDK-2' catalyses the phosphorylation of this site [49]. To this end, several candidate activities have been proposed, including most recently one that associates with PKC ζ [91,115] and a lipid-raft-associated activity [116]. Early suggestions that the integrin-linked kinase, Ilk, serves this function [117,118] are at odds with this protein lacking key residues required for catalysis, leading to the suggestion that the effects of Ilk on the phosphorylation state of the hydrophobic site *in vivo* may reflect allosteric or scaffolding mechanisms [119]. Thus, despite considerable effort, no kinase that fulfils the role of PDK-2 has been identified.

A second possibility is that, like PKC, the hydrophobic site of PKB/Akt is regulated by autophosphorylation. Evidence in favour of this is the finding that pure PKB/Akt autophosphorylates at this site *in vitro* following phosphorylation by PDK-1 [72]. The phosphorylation at the hydrophobic site, but not the PDK-1 site, depends on the catalytic activity of PKB/Akt. Similarly, the phosphorylation of the hydrophobic site *in vivo* is abolished in kinase-inactive constructs of PKB/Akt, independent

of negative charge at the other phosphorylation positions. It has been argued that the PI 3-kinase-dependent phosphorylation of the hydrophobic site in PDK-1$^{-/-}$ cells, where activation loop phosphorylation is abolished, implies the existence of PDK-2 [9,60]. One could also argue that this supports the autophosphorylation model: engaging the PH domain of PKB/Akt on the membrane unmasks the active site to allow autophosphorylation of the hydrophobic site. The basal activity of AGC kinases that occurs in the absence of activation loop phosphorylation would readily account for this autophosphorylation [44], regardless of whether it occurs by an intramolecular reaction, as it does for PKC, or by an intermolecular reaction, which would be favoured once Akt is concentrated on the plane of the membrane. Thus it is reasonable to hypothesize that, like PKC, PDK-1 phosphorylation of the activation loop sequence triggers the autophosphorylation of the hydrophobic site, an event required to stabilize the signalling-competent conformation of PKB/Akt. Thus the phosphoinositide-dependence of this phosphorylation reflects the phosphoinositide-dependence of binding the PH domain of PKB/Akt to membranes: this binding unmasks the active site to both expose the PDK-1 site and to allow autophosphorylation.

In addition to conformational mechanisms controlling the dephosphorylation of the ABC kinases by unmasking phosphorylation sites, regulation by site-specific phosphatases provides an attractive model for regulating these kinases. Pharmacological manipulation to alter selectively the phosphorylation of the hydrophobic site of PKC and PKB/Akt has been interpreted to imply that a separate kinase phosphorylates this site, but could equally well be accounted for by separate phosphatase regulation of this site [91,101,120]. Much remains to be elucidated regarding the dephosphorylation mechanisms of these kinases.

Conclusion

The phosphorylation of the ABC kinases epitomizes how subtle variations in mechanistic details of the same general theme can add complexity and diversity to cellular regulation. All three kinases are regulated by two co-ordinated steps: phosphorylation and second-messenger binding. The order of these steps varies, but with the same outcome, and there are subtle differences in the mechanisms of the phosphorylations, but, again, with the same functional outcome. With much of the detail of the phosphorylation of the ABC kinases elucidated, the next major challenge is to identify the mechanisms of site-specific dephosphorylation of these key signalling molecules.

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