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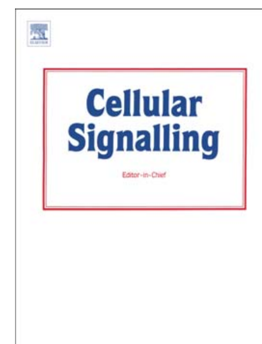

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Regulation of Protein Kinase C function by phosphorylation on conserved and non-conserved sites

(Topical Review article)

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Abstract

Protein Kinase C (PKC) is a family of serine/threonine kinases whose function is influenced by phosphorylation. In particular, three conserved phosphorylation sites known as the activation-loop, the turn-motif and the hydrophobic-motif play important roles in controlling the catalytic activity, stability and intracellular localisation of the enzyme. Prevailing models of PKC phosphorylation suggest that phosphorylation of these sites occurs shortly following synthesis and that these modifications are required for the processing of newly-transcribed PKC to the mature (but still inactive) form; phosphorylation is therefore a priming event that enables catalytic activation in response to lipid second messengers. However, many studies have also demonstrated inducible phosphorylation of PKC isoforms at these sites following stimulation, highlighting that our understanding of PKC phosphorylation and its impact on enzymatic function is incomplete. Furthermore, inducible phosphorylation at these sites is often interpreted as catalytic activation, which could be misleading for some isoforms. Recent studies that include systems-wide phosphoproteomic profiling of cells has revealed a host of additional (and in many cases non-conserved) phosphorylation sites on PKC family members that influence their function. Many of these may in fact be more suitable than previously described sites as surrogate markers of catalytic activation. Here we discuss the role of phosphorylation in controlling PKC function and outline our current understanding of the mechanisms that regulate these phosphorylation sites.

Keywords: PKC, phosphorylation, signalling

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1.1 Introduction

Protein Kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases that play key roles in many of the signalling pathways that control cellular growth, proliferation, differentiation and cell death [1,2]. To date, nine different PKC isoforms that are transcribed from separate genes have been cloned and characterised. Expression of PKCs is widely varied with different cell types expressing different family members. PKCs preferentially phosphorylate substrates that contain a serine/threonine in the context of adjacent amino acids, with different isoforms preferring particular amino acids that surround the phosphorylation site [3,4]. All PKC isoforms share a similar overall structure in that they consist of an N-terminal regulatory domain that is coupled to a highly conserved C-terminal kinase domain (Figure 1). The N-terminal regulatory domain primarily serves two functions. Firstly, it contains one or more modules (C1, C2, PB1 or phosphatidylserine-binding domains) that, when engaged by lipid second messengers or other interacting proteins, tether the enzyme to various locations in the cell such as the plasma membrane. Secondly, the regulatory domain negatively regulates enzymatic activity; located at the N-terminus is a peptide sequence that closely resembles a PKC substrate site, except that the serine/threonine residue is replaced by an alanine (Figure 1). This pseudosubstrate (PS) sequence binds to the substrate-binding cavity in the C-terminus and blocks catalytic activity. Removal of this pseudosubstrate sequence from the kinase domain occurs when lipid second messengers bind to the regulatory domain, resulting in substrate binding and phosphorylation [5].

Situated on the C-terminal kinase domain is the ATP-binding site (C3 domain) and the substrate-binding site (C4 domain) (Figure 1). Interspaced between the N-terminal and C-terminal domains is a non-conserved ‘hinge’ domain that is also

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known as the V3 region (Figure 1). When PKC is active (i.e. bound to lipid second messengers), the hinge domain may become labile to proteolytic enzymes such as Ca^{2+}-dependent calpains. Calpain-mediated cleavage of PKC at the hinge domain produces an isolated kinase domain fragment that is also known as PKM [6,7]. The hinge domains of certain PKCs are also susceptible to cleavage by caspases during apoptosis [8,9], with roles for cleaved PKC isoforms, particularly the kinase domain fragment of PKC δ, in this process [10,11]. The kinase domain fragments that are produced following calpain/caspase-mediated cleavage are generally considered to be constitutively active in the absence of any lipid second messengers, provided that these domains are phosphorylated at a key site known as the activation-loop [12]. As will be discussed in more detail in Section 1.2.1, PKC δ is an exception to this rule however since the cleaved kinase domain of this enzyme can function independently of activation-loop phosphorylation [13].

The nine PKC isoforms are classified into three subgroups, based on the presence or absence of functional membrane-binding modules in their respective N-terminal regulatory domains. These are known as the conventional PKCs (cPKCs; comprised of PKC α, β_I/β_{II} and γ isoforms), the novel PKCs (nPKCs; comprised of δ, ε, η and θ) and the atypical PKCs (aPKCs; comprised of PKC ζ and ι). The structural features and mechanism of activation of these isoforms has been reviewed elsewhere [1,2,14]. Spatial and temporal control of PKC signalling is also influenced via interaction with adaptor/scaffolding proteins that anchor the PKCs to various intracellular locations in the cell. A multitude of adaptor proteins that influence PKC function have been characterised, including A-Kinase Anchoring Proteins (AKAPs) [15,16], Receptors for Activated C Kinases (RACKs) [17,18] and 14-3-3 proteins [19,20]. Termination of PKC signalling is best described for cPKCs and nPKCs.

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These isoforms translocate back to the cytosol following the removal of DAG from the plasma membrane. Removal of DAG is achieved in a number of ways, including downregulation and/or degradation cell surface receptors and conversion of DAG to phosphatidic acid by DAG kinase [21]. Unlike DAG, phorbol esters are not readily metabolised in the cell, which results in persistent PKC signalling. PKC signalling may also be terminated by downregulation of the enzyme from the cell, a process that is often promoted by dephosphorylation of the enzyme at a number of sites (see Section 1.3).

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1.2 Phosphorylation of Protein Kinase C

While it had been demonstrated that PKC family members underwent a post-translational modification shortly after synthesis that was later shown to involve phosphorylation [22], it was not until studies involving mutational analysis of predicted phosphorylation sites [23,24] and mass spectrometry [25,26] that three key phosphorylation sites were identified on the C-terminus that were important for PKC function. These sites are known as the activation-loop (A-loop), the turn-motif (TM) and the hydrophobic-motif (HM). While all three sites are conserved in cPKCs and nPKCs, the aPKCs do not contain a HM residue but instead possess a negatively charged glutamic acid, perhaps mimicking a constitutively phosphorylated state (Figure 1). Furthermore, many of these phosphorylation sites are also conserved on other kinases termed ABC kinases (representing PKA, PKB, PKC and other kinase families such as p70 and p90 ribosomal S6 kinases). Initial investigations that characterised these sites on PKCs probably assumed that similar pathways or mechanisms regulate all isoforms. Recent findings have demonstrated however that this is not always the case, with notable differences reported in PKC isoforms in terms of the regulatory inputs that influence these phosphorylation sites and the impact they have on isoform-specific function. Here we summarise the role of these phosphorylation sites in terms of modulating PKC function.

1.2.1 Activation-loop phosphorylation

For the vast majority of PKC isoforms, phosphorylation of the A-loop is crucial for catalysis, since mutation of this residue to a non-phosphorylatable residue abolishes activity (Table 1). In many cases, substitution of the A-loop threonine residue with a negatively charged residue (to mimic phosphorylation) restores catalytic activity. The

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molecular basis of A-loop phosphorylation in controlling catalytic activity has been revealed with the publication of the crystal structures of the kinase domains of PKC θ, PKC ι and PKC β_{II}: phosphorylation at the A-loop makes important contributions in stabilising the active conformation of these kinases by forming ionic contacts with positively-charged residues in the vicinity of the kinase domain [27-29].

While A-loop phosphorylation is required for catalytic activity of most PKC isoforms, a notable exception is PKC δ (Table 1). Mutation of the A-loop residue of PKC δ has no effect on in-vitro catalytic activity [30,31,13]. Instead, phosphorylation of this residue on PKC δ fine-tunes the specificity of the enzyme towards some of its substrates [13,32]. Molecular modelling and mutational analysis have demonstrated that in the absence of A-loop phosphorylation, PKC δ catalytic activity is stabilised by a pair of phenylalanine residues and an additional 20 residues that are located N-terminal to the kinase domain [13]. Interestingly, this pair of phenylalanine residues is not conserved in any other PKC isoform and therefore provides a molecular basis as to why PKC δ can function in the absence of A-loop phosphorylation. The cleaved PKC kinase domain fragments that are formed via calpain/caspase-mediated cleavage generally require A-loop phosphorylation for catalytic activity [12]. PKC δ again appears to be an exception to this rule in that this cleaved kinase domain fragment can function independently of A-loop phosphorylation to modulate the apoptotic cascade [13].

Convincing evidence has demonstrated that A-loop phosphorylation of PKC isoforms is catalysed by the enzyme Phosphoinositide-Dependent Protein Kinase-1 (PDK-1). PDK-1 has been shown to phosphorylate cPKC, nPKC and aPKCs both in vitro and when over-expressed in different cell types [33-36]. Most importantly perhaps is that the intracellular levels and/or A-loop phosphorylation of PKCs is

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reduced in cells that are deficient in PDK-1. For example, embryonic stem (ES) cells that lack PDK-1 have vastly reduced levels of PKC α, β_{II}, γ and ε, demonstrating that phosphorylation by PDK-1 plays an essential role in stabilising these PKCs [37,38]. In the same studies, the total levels of PKC ζ were reported to be relatively stable in ES cells lacking PDK-1 and phosphorylation at the A-loop was abolished [37,38]. In T lymphocytes, Ghosh and colleagues have demonstrated that knockdown of PDK-1 expression inhibits A-loop phosphorylation of PKC θ [39]; this defect is recapitulated in PDK-1-null T cells [40].

PDK-1 is known as a master kinase that phosphorylates the A-loop of many protein kinase families in addition to PKC, such as PKB/Akt [41]. PDK-1 contains a PH domain at its C terminus that binds with high affinity to the lipid products of PI3-kinase (phosphatidylinositol 3,4-diphosphate and phosphatidylinositol 3,4,5-triphosphate). The PH domain of PDK-1 is not required for the A-loop phosphorylation of PKC isoforms, however, since phosphorylation at this site occurs independently of the PI3-kinase pathway [42]. Furthermore, PDK-1 knock-in cells in which the PH domain is disrupted display relatively normal levels of PKC A-loop phosphorylation [38,43]. Instead, PDK-1 catalyses A-loop phosphorylation of PKCs by docking on residues that surround the HM site [44,45,38] (see Section 1.1.4).

While numerous studies have demonstrated that PDK-1 is the A-loop kinase of PKCs in a variety of cell types, more recent studies have also indicated that PKC A-loop phosphorylation may occur independently of this master kinase. For example, although PKC δ expression is reduced in ES cells that lack PDK-1, phosphorylation at the A-loop is detectable on the residual pool of PKC δ [44,38]. The reduced levels of PKC δ in PDK-1-null ES cells are surprising given that this isoform can function independently of A-loop phosphorylation [30,31,13] and instead may be accounted by

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the lower mRNA levels (~50%) that have been reported for this isoform in these PDK-1-null cells [37]. Cantrell and colleagues have also recently shown that deletion of PDK-1 in thymocytes does not abolish PKC δ A-loop phosphorylation [132], while the enzyme has also been reported to autophosphorylate its own A-loop in cardiomyocytes via a mechanism that is dependent on prior tyrosine phosphorylation of the enzyme at the hinge domain by Src kinases [46,32]. These studies imply that autophosphorylation or indeed a kinase other than PDK-1 may catalyse phosphorylation at this site on PKC δ. Similarly, although PDK-1 has been shown to be required for A-loop phosphorylation of PKC θ in T cells [39,40], this isoform is capable of autophosphorylating at this residue following expression in bacteria [47]. Kinases other than PDK-1 have been implicated in PKC A-loop phosphorylation. For example, it has been reported that human biliverdin reductase (hBVR) may phosphorylate the A-loop of PKC β_{II} [48], while 5'-AMP-activated protein kinase (AMPK) has recently been shown to directly phosphorylate the A-loop threonine of PKC ζ during hypoxia [49]. It is clear therefore that PDK-1 and/or alternative pathways may influence PKC A-loop phosphorylation and highlights that our understanding of the mechanisms that catalyse phosphorylation at this site is incomplete and warrants further investigation.

1.2.2 Turn motif phosphorylation

The turn motif (TM) is located outside the C4 kinase domain on a region of the enzyme known as the C-terminal tail and this site is conserved in all PKC isoforms (Figure 1). Crystallisation studies and molecular modelling of the kinase domains of PKC β_{II}, PKC ζ and PKC ι has demonstrated that the phosphate on the TM makes important contacts with other residues on the enzyme to help stabilise the kinase core

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of the enzyme [28,29,50]. While phosphorylation at the TM influences enzyme stability, differences have been reported as to the importance of this site for catalytic activity (Table 1). For example, phosphorylation at the TM is required for PKC β_I and PKC β_{II} activity [51,52], while it is dispensable for PKC α, PKC θ and PKC ι activity [53,31,50]. In contrast, conflicting results have been reported as to importance of TM phosphorylation for PKC δ activity [54,55] and PKC ζ activity [56,50]. The simplest explanation is that individual PKCs may differ in their requirement for TM phosphorylation for catalytic activity, perhaps due to structural differences between the individual isoforms, but this does not explain the differences reported for TM phosphorylation for PKC δ and PKC ζ. An alternative explanation is the possibility that compensatory phosphorylation(s) may have taken place on neighbouring serine/threonine residues of the TM mutants, as reported for PKC β_{II} [52]. Nevertheless, while mutation of the TM site on PKC α was reported to have no effect on catalytic activity, the mutant was susceptible to thermal instability, oxidation, proteolysis and dephosphorylation at other residues [53], which strongly suggests that phosphorylation at this site plays a role in stabilisation of the enzyme rather than directly controlling catalytic activity. TM phosphorylation may also influence function beyond stability and catalytic activity for certain PKC isoforms. As an example, Shaw and colleagues have shown that although mutation of the TM site on PKC θ has no effect on catalytic activity, this mutant demonstrates an enhanced ability to activate the NF-κB pathway in T lymphocytes [31].

The TM site on PKC β_{II} was initially described as an autophosphorylation site, as it was observed that expression of a kinase-inactive PKC β_{II} mutant did not incorporate phosphate on its TM site [57]. Lack of phosphorylation in kinase-inactive PKC mutants however has recently been attributed to the fact that mutation of the

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critical lysine in the ATP-binding site of these proteins results in increased susceptibility to phosphatases when expressed in cells, rather than defects in the ability of these mutants to autophosphorylate [58]. More recent evidence has demonstrated that the mammalian Target of Rapamycin (mTOR) pathway regulates TM phosphorylation of some, but not all, PKCs [59-61]. mTOR is a serine/threonine kinase that associates with other proteins to form multi-protein complexes. One of these complexes, mTORC1, is sensitive to the drug rapamycin while the other complex, mTORC2, is insensitive to acute rapamycin treatment [62]. mTORC2 has been implicated in TM phosphorylation of cPKCs (α, β_I, β_{II}, γ) and PKC ε in murine embryonic fibroblasts (MEFs) [59,60]. In MEFs that lack critical components of the mTORC2 complex, the levels of expression of cPKCs and PKC ε are vastly reduced and TM phosphorylation is also absent on the residual pool of PKC α (A-loop and HM phosphorylation are also absent). The effect of mTORC2 on these PKCs appears to be indirect. Nonetheless, the instability of these PKCs in cells that lack mTORC2-specific components is reminiscent of the reduced levels of expression of PKCs that have been reported in PDK-1-null ES cells [37]. Whether this lower expression of PKCs in mTORC2-deficient MEFs is due to absent TM phosphorylation or is also a consequence of lack of phosphorylation at the A-loop/HM sites is unknown at present. In line with this, it is well known that lack of phosphate at one site on PKC such as the HM increases its sensitivity to phosphatases, which results in dephosphorylation of other sites on the enzyme, leading to protein instability [63-65]. Since mTORC2 has also been implicated in HM phosphorylation of PKCs (see Section 1.2.3), it is possible that this instability is also due to lack of phosphorylation at the HM and/or the A-loop. It appears that mTORC2 does not regulate all PKC isoforms in MEFs however, since the levels of TM phosphorylation of PKC δ/θ or

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levels of expression of PKC δ, θ, η, ζ and ι are unaffected in cells that lack mTORC2-specific components [59,60]. This implies that other signalling pathways or autophosphorylation may regulate these isoforms at this site. Indeed, the TM of PKC δ has been reported to be an autophosphorylation site [54,66]. While deletion of mTORC2-specific components in MEFs appears to have no effect on TM phosphorylation or expression of PKC θ, this pathway regulates TM phosphorylation of PKC θ in T lymphocytes [61]. A role for the mTORC2 pathway in PKC θ phosphorylation in T cells but not in MEFs highlight the fact that regulatory inputs that influence PKC function are likely not just to be isoform but also cell type specific.

The phosphorylation state of the TM site also serves as a switch to regulate the binding of chaperone proteins to help protect PKCs from degradation. For example, Heat-shock protein-70 and keratins bind PKCs that have become dephosphorylated at the TM. This interaction stabilises PKC and facilitates re-phosphorylation at this site and thus helps to preserve the function of these enzymes [67,68].

1.2.3 Hydrophobic-motif phosphorylation

The HM phosphorylation site is conserved in cPKCs and nPKCs, but is absent in aPKCs (Figure 1). Molecular modelling studies have shown that the negatively charged phosphate at the HM site on PKC β_{II} and PKC θ forms hydrogen bonds with an invariant glycine residue and thus serves to stabilise the enzyme [27,29]. While this phosphorylation site is not conserved in aPKCs, the negatively charged glutamic acid residue at the HM site on PKC ι performs a similar function [28]. The sequence surrounding the HM also serves as a docking site for the A-loop kinase PDK-1 [44,45,38].

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Like the TM site, mutation of the HM site to a non-phosphorylatable residue in cPKCs and nPKCs has been reported to have varying effects on the catalytic activity and function of the enzyme (Table 1). For example, two studies differed with regard to the effect of HM phosphorylation on PKC α activity [63,65]. Both reports concluded however that phosphorylation at this site promotes the formation of a 'closed' conformation that protects the enzyme from inactivation by phosphatases. Mutation of the HM site on PKCβ_{II} has no effect on catalytic activity but phosphorylation at this position is required for enzyme stability and the function of a signalling-competent enzyme [69]. Curiously, mutation of the HM site on PKC δ increases catalytic activity [70], while mutation of the HM site on PKC θ reduces activity 5-fold [31]. These studies again highlight the different roles that these conserved phosphorylation sites have on PKC function.

While cPKCs were initially reported to autophosphorylate at both the TM and HM [57], the mTORC2 complex has also been implicated in HM phosphorylation of PKC α in MEFs [71,72,59], while mTORC2 also influences PKC θ phosphorylation at this site in T lymphocytes [61]. Interestingly, while the levels of expression of PKC δ are unaffected following deletion of mTORC2 components in MEFs [59,60], phosphorylation of PKC δ (and PKC ε[*]) at the HM motif is sensitive to rapamycin treatment in human embryonic kidney cells, which therefore implicates the mTORC1 complex in phosphorylation of these isoforms [73,66]. It is clear therefore that mTORC1, mTORC2 and possibly other pathways impact on the HM of different PKCs and contribute to their function in diverse ways.

[*] As the rapamycin-insensitive mTORC2 pathway influences PKC ε stability [59], it is interesting that PKC ε phosphorylation is also sensitive to rapamycin, which therefore implicates both mTORC pathways in PKC ε function.

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Similar to the TM site, the phosphorylation state of the HM is also regulated by chaperone proteins that include the Hsp family [74]. In this case, Hsp-90 and Cdc37 perform a dual role in regulating PKCs. Hsp-90 and Cdc37 first facilitate the phosphorylation of PKCs at the HM by binding to newly synthesised cPKCs and nPKCs during their life cycle (aPKC phosphorylation is not regulated by these chaperones as this subclass of PKCs do not contain a HM site) and, secondly, these chaperones help stabilise the steady-state level of all subclasses of the PKC family.

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1.3 Dephosphorylation of PKC

PKC signalling is terminated by the metabolism of DAG, which results in the translocation of cPKCs/nPKCs back to the cytoplasm. It has also been reported that dephosphorylation of PKCs can occur at sites such as the A-loop and HM in response to stimuli such as Tumor Necrosis Factor-α, which is likely to be a key step in turning off the signalling capacity of these kinases [75]. Prolonged PKC signalling that is promoted by phorbol esters or sustained DAG signalling results in downregulation of PKCs via the ubiquitin/proteasome system [76,77] and studies have shown that dephosphorylation of PKCs at the A-loop, TM and HM sites occurs prior to downregulation [78]. Dephosphorylation of PKCs in cells was initially shown to be sensitive to okadaic acid, which implicated the PP2a-like phosphatases in this process [79,80,75]. More recent studies have identified an additional family of okadaic acid-insensitive phosphatases called PHLPPs that specifically dephosphorylate the HM (but not the TM) of PKCs upon prolonged phorbol ester treatment [81]. It should be noted however that while dephosphorylation serves as an important switch to turn off the activity of these enzymes, dephosphorylation does not necessarily predispose the enzyme to degradation [79]. Consistent with this, it has been reported that downregulation of PKC α can occur through a ubiquitin/proteasome pathway that does not involve dephosphorylation of the enzyme [82].

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1.4 PKC phosphorylation: constitutive or inducible?

Phosphorylation of PKCs at the A-loop, TM and HM sites have been described as maturational or priming events that are required for the processing of these enzymes to signalling-competent (but still inactive) forms. This theme developed because it was demonstrated that PKCs (predominantly cPKCs) are phosphorylated at these sites shortly after synthesis [83,42] and are often highly phosphorylated at these sites in many types of cells grown in culture, even under quiescent conditions [26,66,84]. In the absence of phosphorylation at one or more of these sites, catalytic activation of these isoforms is impaired or enzyme stability is compromised. A model has therefore been proposed for PKC phosphorylation [14,85] in which (a) the primary translation product is phosphorylated at the A-loop by PDK-1 at the plasma membrane shortly after synthesis (b) PDK-1 disassociates from PKC, which is then followed by phosphorylation at the TM and HM sites via mTORC1/mTORC2-dependent pathways or autophosphorylation (c) these phosphorylations allow PKC to adopt a stable and 'closed' enzyme conformation in which the pseudosubstrate occupies the substrate-binding site and the enzyme predominantly localises to the cytosol. The enzyme is now in an inactive but signalling-competent state that is ready to be activated by lipid second messengers such as DAG. Phosphorylation is therefore constitutive and is a priming event that enables catalytic activation in response to cellular signalling (Figure 2).

While this model undoubtedly holds true for PKCs in many types of cells, it is now clear that PKCs can also exist in non/hypo-phosphorylated forms, with cellular stimulation resulting in inducible phosphorylation at some or all of these sites. For example, inducible phosphorylation has been reported for cPKCs (at all three sites) [86-88], nPKCs (at all three sites) [89-91,61] and aPKCs (PKC ζ on its A-loop site)

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[92,49] following stimulation. These findings suggest that an additional model should be made that describes inducible phosphorylation of PKCs during their life cycle (Figure 2). This new model poses new and interesting questions that remain to be addressed however. One outstanding question is: how is PKC stability and/or conformation maintained in cells in the absence of phosphorylation at these sites? This is an intriguing question, since PKCs are often unstable in cells when phosphorylation is lacking at one or more of these sites, as demonstrated in PDK-1 or mTORC2-deficient cells. Possible stabilising factors include chaperones such as the Hsp family, keratins and Cdc37. Newton and colleagues have demonstrated however that Hsp-70 only associates with 'mature' (i.e. phosphorylated) PKC that has undergone dephosphorylation at the TM, and that this chaperone does not associate with the newly synthesised PKCs that have yet to undergo these regulatory phosphorylations [67]. Other candidates include Hsp-90 and Cdc37, which bind to newly synthesised PKCs and facilitate the phosphorylation of these isoforms at the HM [74]. Another possible candidate is the Centrosome and Golgi localised PKN-associated protein (CG-NAP), a member of the AKAP family (also known as AKAP-450). CG-NAP/AKAP-450 binds immature, non-phosphorylated PKC ε following synthesis and serves as a scaffolding protein to regulate the phosphorylation process [93]. AKAPs have been shown to facilitate phosphorylation of PKCs that follow the constitutive phosphorylation model. Whether these proteins function to anchor and stabilise non-phosphorylated PKCs in cells until such time that cellular stimulation recruits PKCs to membranes and induces phosphorylation requires further work however. Other key questions that remains to be addressed include how other putative activation-loop kinases (e.g. AMPK or hBVR) fit into the models that

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describe PKC phosphorylation, and 'where-when-how' they interact with individual PKCs during their life cycle.

The observation that cellular stimulation may induce PKC phosphorylation at the A-loop, TM or HM has often resulted in phosphorylation-specific antibodies to these sites being used as 'activation markers' of these enzymes, which could be misleading for some isoforms. For example, phosphorylation of PKC α or β_{II} at the HM does not necessarily correlate with catalytic activation, but instead is more indicative of a stable or 'closed' enzyme conformation [65,69], while A-loop phosphorylation of PKC δ does not reflect catalytic activity at all [13]. In addition, A-loop phosphorylation of other isoforms may not always be reliable markers to monitor catalytic activation. As an example, Newton and colleagues have shown that mature cPKCs that are isolated from cells are quantitatively phosphorylated at the TM and HM sites, while only ~ 50% of this species retains phosphate at the A-loop [26]. Although the reasons for this dephosphorylation at the A-loop are unclear, re-phosphorylation of this species by PDK-1 at the A-loop does not increase catalytic activity, because phosphorylation of the A-loop site is dispensable once catalytic competence has been achieved for these PKCs (i.e. once TM/HM phosphorylation has taken place and the 'closed' enzyme conformation has occurred) [35]. A key question that remains therefore is whether the inducible phosphorylation of PKCs that has been observed at these sites reflect true activation events that act as switches to increase the catalytic activity of these proteins, or whether they just reflect an enhancement of the processing of PKC to more competent/mature states. Until such questions become clearer, we suggest that caution should be used when using phosphorylation-specific antibodies to these sites to assess PKC activation, a point that has also been highlighted by other researchers [94]. Other assays that should be used in parallel

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include intracellular translocation assays, immune-complex kinase assays or phosphorylation of downstream substrates. As will be discussed in the Section 1.5, recent studies including phosphoproteomic analysis of PKCs has revealed additional and sometimes non-conserved phosphorylation sites on the enzymes. Some of these sites may be more suitable markers of catalytic activation.

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1.5 New phosphorylation sites on PKC isoforms

Research into PKC-dependent signalling pathways over the past two decades has revealed additional phosphorylation sites on PKC family members that are regulated in response to cellular events such as receptor stimulation by growth factors or the cell cycle. While some of these sites are conserved on PKCs, other phosphorylation sites have been shown to be specific to a particular family member and thus are likely to contribute to the regulation of individual isoforms. Indeed, those phosphorylation sites that are both unique to particular isoforms and sites of autophosphorylation can be used as surrogate markers to monitor catalytic activity. Although autophosphorylation sites have been mapped on individual PKCs (PKC α - [95]; PKC δ - [96]; PKC θ - [97]) and antibodies to these sites have been raised and used as surrogate markers to monitor the catalytic activation of these isoforms, such reagents are not yet commercially available.

Technological advances in mass spectrometry and phosphoproteomics have revolutionised the cell-signalling field and have also resulted in the identification of new phosphorylation sites on PKC family members. Some of these sites appear to be constitutively phosphorylated whilst others are dynamically regulated. The advantage of phosphoproteomics is that it can quantify global changes in site-specific phosphorylation levels of thousands of proteins in cells in an unbiased, systems-wide manner. For example, this technology has been used to (a) identify proteins in cells whose phosphorylation state is modulated via receptor stimulation [98-101], (b) identify proteins whose phosphorylation state is regulated by the cell cycle [102-104] and (c) indentify proteins whose phosphorylation state is perturbed as a consequence of drug/inhibitor treatment [105,106]. As phosphoproteomics studies and indeed smaller, conventional studies continue to identify new phosphorylation sites on PKC

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family members, the next major challenge will be to characterise the impact of these phosphorylations on PKC function. This will hopefully be facilitated by the production of commercially available phosphorylation-specific antibodies to these sites. Here we have used literature searches and phosphorylation site database searches* to summarise these additional phosphorylation sites on PKCs that have been reported from both conventional and phosphoproteomic studies. In particular, we focus on those phosphorylation sites on PKCs that are inducible or regulated in cells (Figure 3).

*Database searches included Phosida (www.phosida.com), Phosphosite (www.phosphosite.org), phosphoELM (http://phospho.elm.eu.org), Human Protein Reference Database (www.hprd.org) and dbPTM (http://dbptm.mbc.nctu.edu.tw)

PKC α: PKC α undergoes phosphorylation at Thr-250 (numbering of amino acids from human sequences are used throughout this manuscript) located in the C2 domain in response to phorbol esters that mimics DAG signalling [95] and also in response to cells adhering to fibronectin [107] (Figure 3). Thr-250 is an autophosphorylation site and hence the phosphorylation status of this isoform at this site has been used as a marker of PKC α catalytic activity [95]. This site is unique to cPKC isoforms (PKC α, $\beta_I/_{II}$, γ). Phosphoproteomic profiling of HeLa cells has revealed that PKC α phosphorylation also occurs at Thr-209 in the C2 domain and at Ser-319 in the hinge domain of the protein during mitosis [102; Phosida database] (Figure 3), while increased phosphorylation at Ser-319 on PKC α was also detected in a phosphoproteomic study of mouse macrophages stimulated with lipopolysaccharide (LPS) [100]. Thr-209 is conserved on other cPKC isoforms while Ser-319 is unique to

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PKC α. The impact of Thr-209/Ser-319 phosphorylation on PKC α function is unknown at present. PKC α has also been reported to undergo phosphorylation at Tyr-658 in mast cells in response to FcεR1 stimulation [108] (Figure 3). Tyr-658 is located directly after the HM site and is conserved on some (PKC β_I, PKC γ, PKC η, PKC ε, PKC ζ, PKC ι) but not all (PKC β_{II}, PKC δ, PKC θ) isoforms. Tyr-658 phosphorylation is catalysed by the tyrosine kinase Syk and phosphorylation at this position provides a docking site for the recruitment of the Grb-2/Sos complex, thus contributing to the activation of the Ras/extracellular signal-related kinase pathway in these cells [108].

PKC β_I/β_{II}: PKC $\beta_{I/II}$ has also been reported to autophosphorylate at the site that is equivalent to Thr-250 of PKC α in the C2 domain [unpublished data from ref. 95] (Figure 3). PKC β_I has also been shown to undergo phosphorylation on Tyr-662, the site located directly after the HM site, in mast cells in response to FcεR1 stimulation [108] (Figure 3). This Tyr is not present in the corresponding position of PKC β_{II}.

PKC γ: To the best of our knowledge, no additional phosphorylation sites on PKC γ that are dynamically regulated in cells have been described to date.

PKC δ: PKC δ has been reported to undergo phosphorylation on a variety of sites in response to diverse stimuli. For example, phosphorylation of PKC δ has been reported on Thr-141, Tyr-155 (both located either side of the pseudosubstrate sequence), Thr-295, Ser-299, Ser-304, Tyr-313, Tyr-334 (all located in the hinge domain) and Tyr-514, Tyr-567 (both located in the kinase domain) (Figure 3). Many of these sites are

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not conserved on other PKCs, which strongly suggests that these sites regulate unique functions of the enzyme. Other residues have also been reported as phosphorylation sites on PKC δ, but cellular regulation has not been demonstrated at these sites to the best of our knowledge. The large number of phosphorylation sites that have been identified on PKC δ likely reflects the multitude of signalling cascades that this kinase is involved in, such as apoptosis and proliferation [109,110]. While PKC δ plays an important role in apoptosis, its role in this process is dictated by cell type, stimulus and intracellular localisation [10,11,111]. This is probably best exemplified in glioma cells, where PKC δ positively influences etoposide-mediated apoptosis, whereas it plays a protective role in TRAIL-induced apoptosis in the same cells [112]. In many cases, the generation of a kinase domain fragment of PKC δ via caspase-3-mediated cleavage at the hinge domain is necessary for PKC δ to modulate the apoptotic cascade and it has been demonstrated that phosphorylation of different residues on the enzyme regulates this process. For example, the apoptotic stimulus TRAIL induces phosphorylation of PKC δ at Tyr-155 in glioma cells and phosphorylation at this site is necessary for translocation of the enzyme to the endoplasmic recticulum and for the cleavage of PKC δ to the kinase domain fragment. Phosphorylation at Tyr-155 is therefore an important step in regulating the ability of this enzyme to act as an anti-apoptotic kinase in this pathway [112]. PKC δ is also phosphorylated on Tyr-313 and Tyr-334 in the hinge domain in response to apoptotic stimuli such as TRAIL or cisplatin [113]. These sites are located on either side of the caspase-3 cleavage site in the hinge domain on PKC δ (Figure 3). Phosphorylation at Tyr-334, rather than at Tyr-313, is required for the cleavage of PKC δ to the kinase domain fragment in TRAIL/cisplatin-treated cells [113]. In contrast, phosphorylation at Tyr-313 is required for the cleavage of PKC δ and apoptosis in response to oxidative stress

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(H_2O_2) in neuronal cells [114]. Notably, oxidative stress also results in phosphorylation of Tyr-334 (hinge domain) and Tyr-514 (located in the kinase domain) [114,115], although the role of these sites in the H_2O_2-mediated apoptotic cascade has yet to be determined. Evidence indicates that phosphorylation at Tyr-313/Tyr-334 is mediated by Src family kinases. However, phosphorylation of PKC δ at Tyr-313/Tyr-334 by Src kinases is not always associated with cleavage by caspase-3 at the hinge domain. Ceramide-induced apoptosis in HeLa cells is also regulated in a PKC δ-dependent manner and results in phosphorylation at Tyr-313/Tyr-334. While phosphorylation of PKC δ at Tyr-313/Tyr-334 is required for ceramide-induced apoptosis, cleavage of PKC δ to the kinase domain fragment does not occur in response to this stimulus [116]. These studies highlight the fact that the signals that activate PKC δ and regulate its ability to modulate apoptosis is cell type and stimulus specific.

Phosphorylation of PKC δ at Tyr-313 by Src family kinases has also been reported in a variety of cell types in response to non-apoptotic stimuli [117-119,46,120,32,121,100]. While some of these studies have shown that phosphorylation at Tyr-313 is required for PKC δ function, one consequence of Tyr-313 phosphorylation is that it regulates the ability of the enzyme to autophosphorylate its own A-loop and thus fine-tune substrate specificity [32,46]. It is also likely that interdependency exists between the A-loop and Tyr-313 sites on PKC δ, as mutation of the A-loop residue to a non-phosphorylatable residue inhibits Tyr-313 phosphorylation [122].

Phosphorylation of PKC δ at Tyr-567 in the kinase domain has also been reported in platelets stimulated with thrombin and appears to partially contribute to PKC δ activation [119]. Finally, a number of autophosphorylation sites have been

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mapped to the pseudosubstrate sequence (Thr-141) and to three sites in the hinge domain (Ser-295/Ser-299/Ser-304) on PKC δ [96,123] (Figure 3). Phosphorylation of PKC δ at Ser-299 serves as a useful marker for catalytic activation of the enzyme [96].

PKC ε: PKC ε has been reported to undergo phosphorylation at a number of sites in the hinge domain that regulates its ability to control the completion of cytokinesis, the final stage in cell separation during cell division [124]. This was elegantly demonstrated by studies from the Parker laboratory, who showed that cytokinesis was dependent on the interaction of PKC ε with 14-3-3β. Furthermore, this interaction required the sequential phosphorylation of PKC ε in the hinge domain at Ser-350 (by p38 MAPK), followed by phosphorylation at Ser-346 (by GSK-3β) and at Ser-368 (by auto-phosphorylation or cPKCs) [124]. These sites in the hinge domain of PKC ε are unique to this isoform (Figure 3). The same laboratory also reported phosphorylation of PKC ε at Ser-346/Ser-368 in cells stimulated with LPS [125]. A phosphoproteomic study of LPS-stimulated mouse macrophages has identified an additional phosphorylation site on PKC ε at Thr-309 in the hinge domain that is dephosphorylated in a time-dependent manner in response to LPS [100] (Figure 3). The consequence of dephosphorylation at this site on PKC ε is unknown at present.

PKC η: A recent phosphoproteomics study of Interferon-gamma treated mouse macrophages has revealed a two-fold decrease in abundance of PKC η over a 24 hour period [99]. Furthermore, this decrease in protein abundance was paralleled by dephosphorylation at Ser-317 (11-fold downregulation) and Tyr-676 (3-fold downregulation). Ser-317 is located in the hinge domain and is not conserved on other

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PKCs, while Tyr-676 is the residue immediately after the HM site and is phosphorylated at the corresponding site on PKCα / PKC β_I by the tyrosine kinase Syk [108] (Figure 3). The reason for this loss in PKC η abundance and dephosphorylation at these sites requires further investigation.

***PKCθ*:** PKC θ is activated downstream of T cell receptor (TCR) signalling in T lymphocytes and plays a key role in the ability of these cells to regulate immune responses [126]. Three sites have been mapped on PKC θ that are phosphorylated following TCR stimulation. These are Tyr-90 (located in the C2-like domain), Thr-219 (located between the tandem C1A/B domains) and Thr-685 (located on the C-terminal tail between the TM and HM sites) (Figure 3). TCR stimulation leads to phosphorylation of PKC θ at Tyr-90 by the tyrosine kinase Lck [127], and in-vitro studies indicate that phosphorylation at this position enhances the affinity of the enzyme for lipid membranes [128]. Thr-219 on PKC θ is also phosphorylated in T lymphocytes in response to TCR stimulation and has been reported to be an autophosphorylation site [97], thereby serving as a useful marker of catalytic activity. While phosphorylation at Thr-219 does not affect the catalytic activity of PKC θ or its ability to bind lipid second messengers, phosphorylation at this site is required for the proper localisation of the enzyme in stimulated T cells [97]. Phosphoproteomics analysis has also revealed that PKC θ undergoes phosphorylation at Thr-685 between the TM and HM sites in response to TCR-stimulation [101]. The impact of Thr-685 phosphorylation on PKC θ function in T cells remains to be addressed.

PKC ζ: To the best of our knowledge, no additional phosphorylation sites on PKC ζ that are dynamically regulated in cells have been described to date.

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PKC ι: Nerve growth factor (NGF) stimulates phosphorylation of PKC ι at Tyr-265 in rat PC12 cells [129]. Phosphorylation at this site is mediated by Src family kinases and appears to be a necessary step for the nuclear translocation of the enzyme. Tyr-256 is located on the C3 domain (ATP binding site). This residue is only conserved on other aPKC family members (PKC ζ and the isoform of PKC ζ that lacks the regulatory domain) (Figure 3). Interestingly, a phosphoproteomics study of HeLa cells has revealed that the phosphorylation status of PKC ι changes dramatically during the cell cycle [104; Phosida database]. Although the precise residue(s) has not been conclusively identified, phosphorylation appears to be localised in a 'hotspot' of serine/threonine residues that are located at the N-terminus of the enzyme and precede the PB1 domain (Thr-3, Ser-7, Ser-8, Thr-9, Ser-11, Thr-13 and Ser-19). Phosphorylation in this domain occurs during mitosis and is downregulated in early S phase. These sites are not conserved on other PKC isoforms (Figure 3). Since PKC ι has been implicated in many types of cancers and is the first PKC to be identified as a human oncogene [130], it will be intriguing to investigate the role of phosphorylation in PKC ι signalling and its effect on cell cycle deregulation/cancer progression.

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1.6 Conclusion

Research over the past couple of decades on PKC isoforms has clearly demonstrated that phosphorylation constitutes one of several regulatory inputs that influence these key transducers of intracellular signalling. While phosphorylation of PKCs on conserved sites such as the A-loop had previously been described as key events that occurred early during their life cycle, we now know that the role of phosphorylation in modulating PKC function is much more complex. This is highlighted by recent findings that show that (a) PKC phosphorylation on sites such as the A-loop may occur much later during their life cycle and predominantly in response to cellular stimulation and (b) that phosphorylation on sites that are unique to individual family members provides isoform specificity and thus licences these kinases to execute specific cellular events such as apoptosis (PKC δ) or cytokinesis (PKC ε). The finding that interdependency exists between phosphorylation sites (e.g. PKC δ at A-loop and Tyr-313 in the hinge domain) or that phosphorylation may occur in a sequential manner (e.g. PKC ε at the hinge domain) underscores this complexity. The characterisation of these recently identified sites on PKCs should go some way in widening our knowledge of how these kinases are regulated and how they contribute to various signalling pathways. In particular, the characterisation of autophosphorylation sites on individual PKCs and the production of commercially available phospho-specific antibodies to these sites will facilitate monitoring of PKC activity in-situ and thus allow direct correlations to be drawn between PKC activity and different disease states.

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Table 1: The effect of mutation of A-loop, TM and HM phosphorylation on the catalytic activity of PKC isoforms is summarised.

Figure 1: Structure of PKC subfamilies. Shown are the conventional, novel and atypical PKC subfamilies and their corresponding domains in the regulatory domain and C-terminal domain. PS = pseudosubstrate site. P:P = Protein:Protein interaction domain.

Figure 2: Prevailing model and new model of PKC phosphorylation. *Prevailing model of PKC phosphorylation*: Shortly after synthesis, PKC is phosphorylated at the A-loop by PDK-1 at the plasma membrane. PDK-1 disassociates from PKC, which is then followed by phosphorylation at the TM and HM sites via mTORC1/mTORC2-dependent pathways or autophosphorylation. These phosphorylations allow PKC to adopt a stable and 'closed' enzyme conformation in which the pseudosubstrate occupies the substrate-binding site and the enzyme predominantly localises to the cytosol. The enzyme is now in an inactive but signalling-competent state that is ready to be activated by lipid second messengers such as DAG. Phosphorylation is therefore constitutive and a priming event that enables catalytic activation in response to cellular signalling. *New model of PKC phosphorylation*: PKC remains in the cytosol in a non/hypo-phosphorylation state after synthesis. The conformation of this protein (i.e. open or closed conformation) is unknown at present. Cellular stimulation results in the recruitment of PKC to the plasma membrane, and this is followed by phosphorylation at one or more sites such as the A-loop. Phosphorylation of these sites on PKC permits the enzyme to catalyse substrate phosphorylation.

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Figure 3: New phosphorylation sites identified on PKC isoforms. Phosphorylation sites that have been described for individual isoforms are shown as circles with the phosphorylated PKC isoform indicated. Numbering according to human PKC amino acid sequences is used throughout. Other PKC isoforms that have a serine, threonine or tyrosine residue in the corresponding position as the phosphorylated residue are also indicated in smaller font below the phosphorylated residue. *indicates that although this site is also present on the indicated isoform, phosphorylation on this isoform has not been reported. Underlined PKC isoforms indicate autophosphorylation sites.

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Isoform	A-loop	TM	HM
PKC α	Required for activity [23]	Not required for activity [53]	Required for activity [63] Not required for activity [65]
PKC β_I	Not reported	Required for activity [51]	Not reported
PKC β_{II}	Required for activity [24]	Required for activity [52]	Not required for activity [69]
PKC γ	Not reported	Not reported	Not reported
PKC δ	Not required for activity [30,31,13]	Required for activity [54] Not required for activity [55]	**Negatively regulates activity [70]**
PKC ε	Not reported	Not reported	Not reported
PKC θ	Required for activity [31]	Not required for activity [31]	Required for activity [31]
PKC η	Not reported	Not reported	Not reported
PKC ζ	Required for activity [33,34]	Required for activity [50] Not required for activity [56]	Not present in PKC ζ
PKC ι	Required for activity [131]	Not reported	Not present in PKC ι

Table 1: The effect of mutation of A-loop, TM and HM phosphorylation on the catalytic activity of PKC isoforms is summarised. The references are indicated in brackets.

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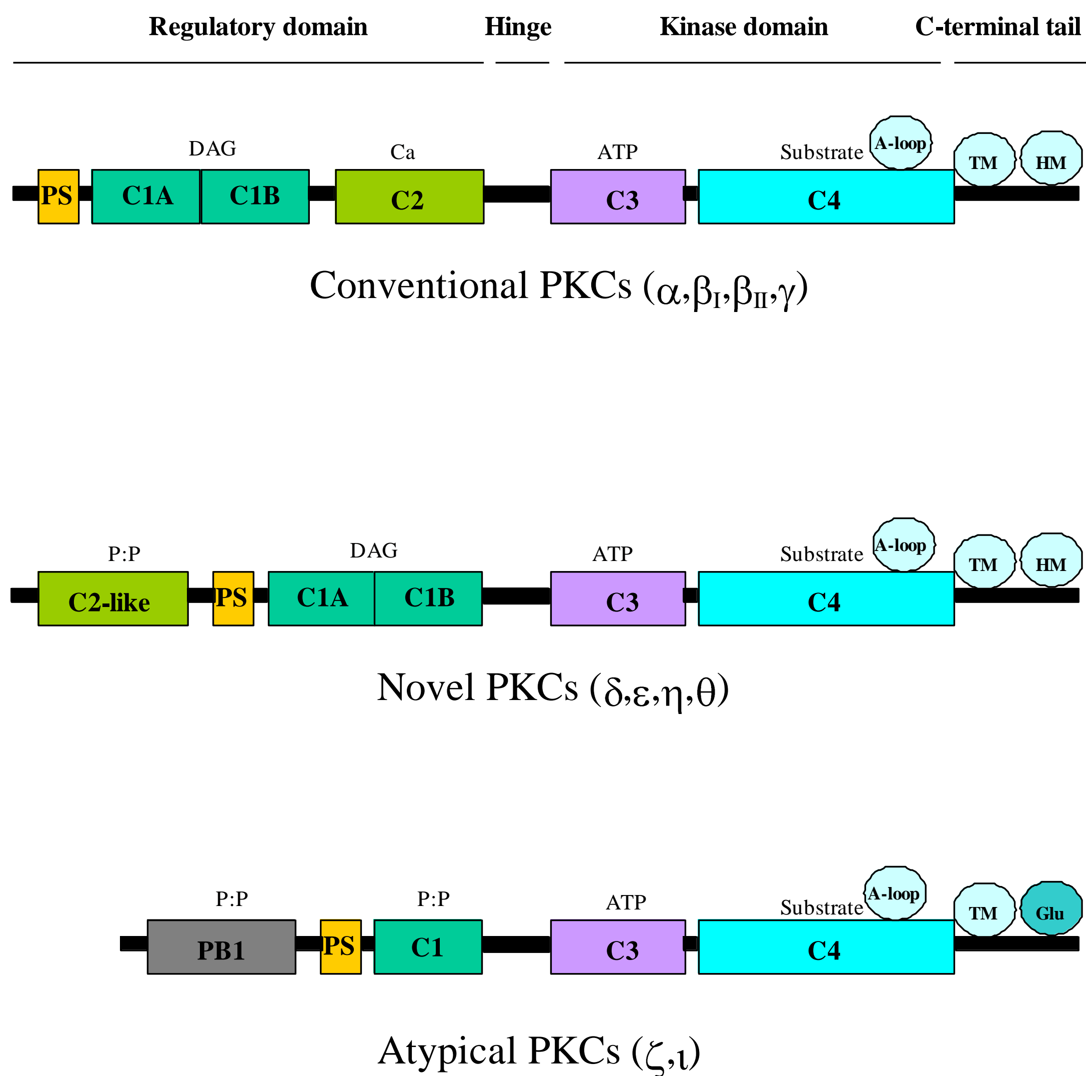

FIGURE 1

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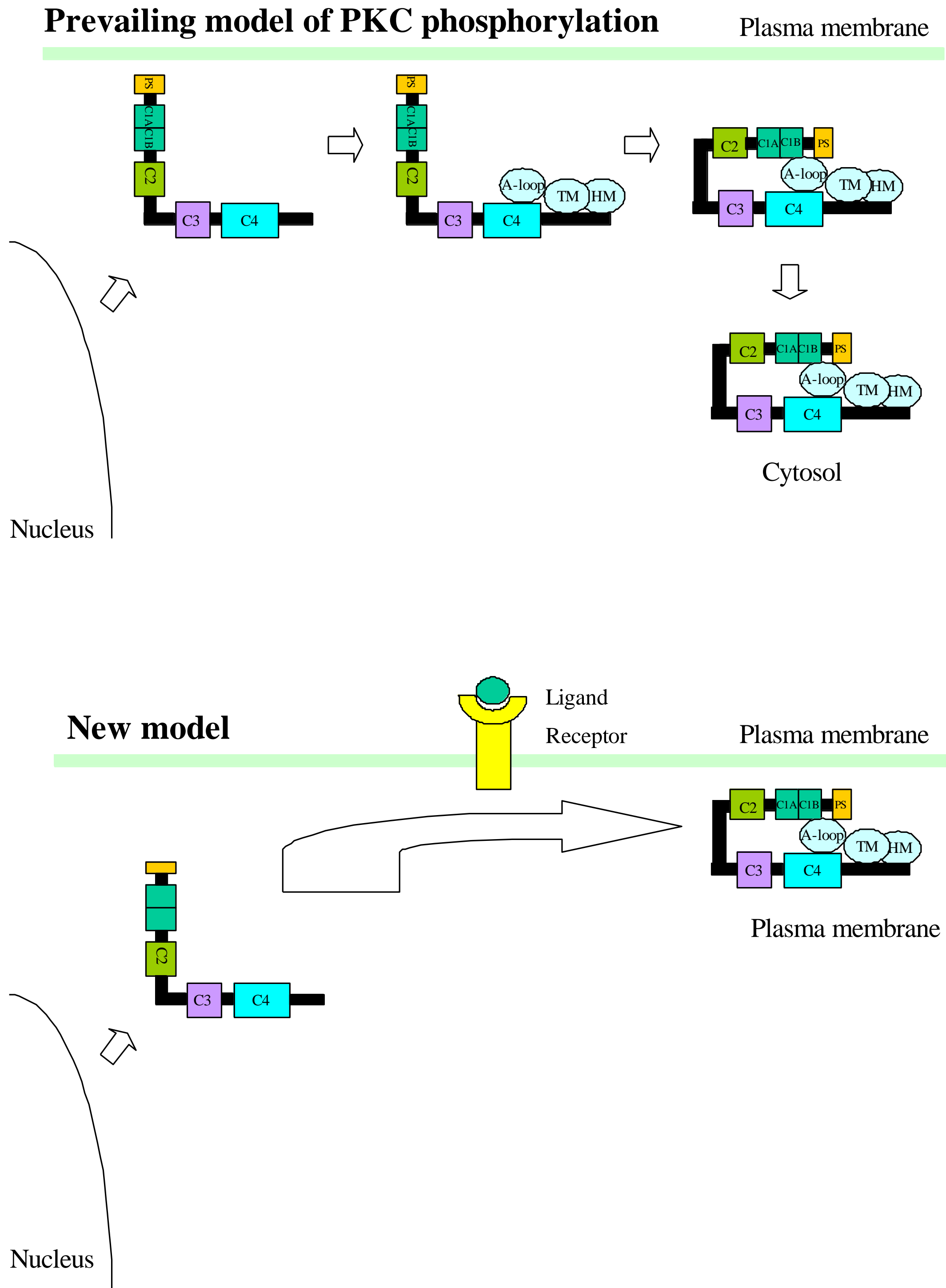

FIGURE 2

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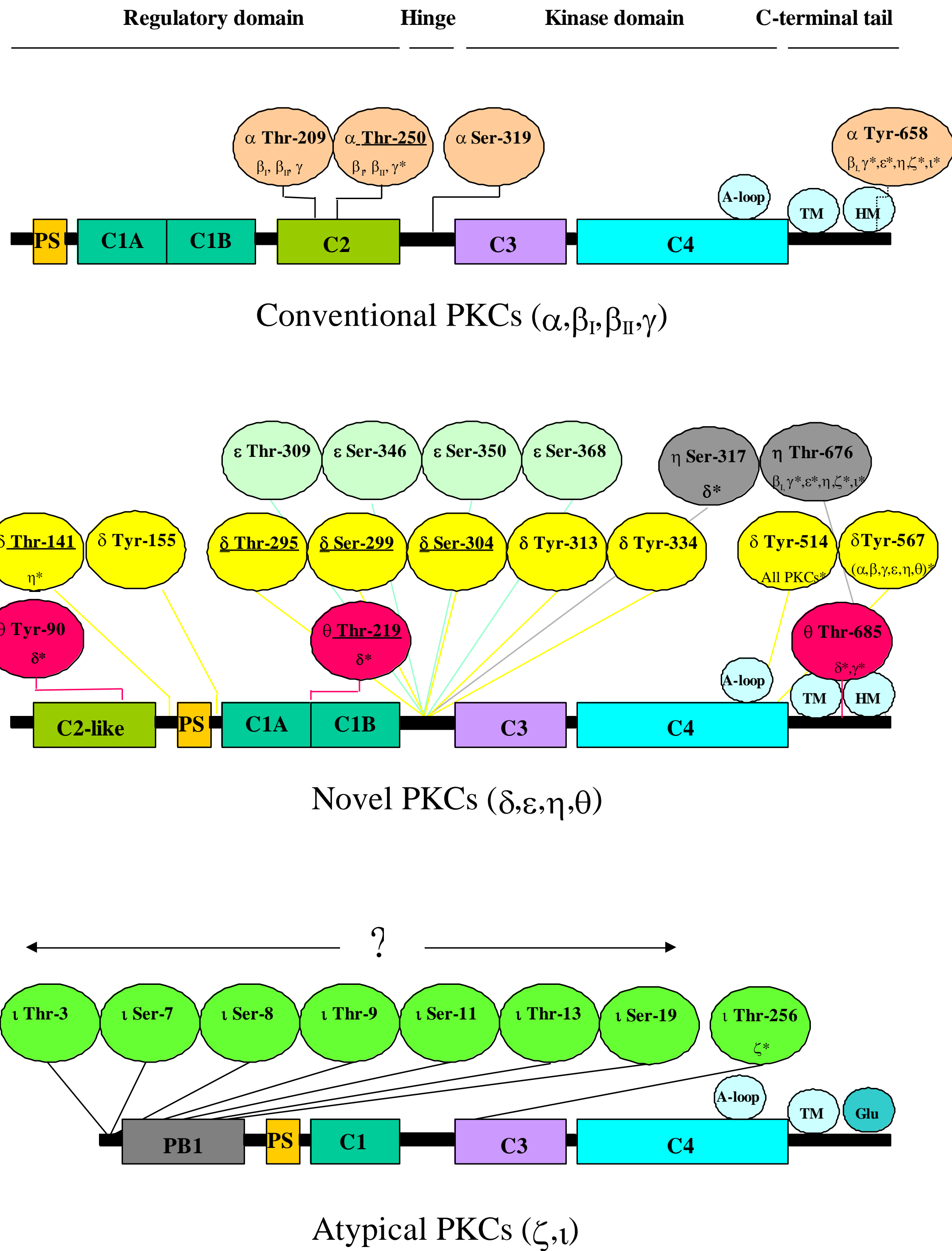

FIGURE 3