REVIEW ARTICLE

The extended protein kinase C superfamily

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Members of the mammalian protein kinase C (PKC) superfamily play key regulatory roles in a multitude of cellular processes, ranging from control of fundamental cell autonomous activities, such as proliferation, to more organismal functions, such as memory. However, understanding of mammalian PKC signalling systems is complicated by the large number of family members. Significant progress has been made through studies based on comparative analysis, which have defined a number of regulatory elements in PKCs which confer specific location and activation signals to each isotype. Further studies on simple organisms have shown that PKC signalling paradigms are conserved through evolution from yeast to humans, underscoring the importance of this family in cellular signalling and giving novel insights into PKC function in complex mammalian systems.

INTRODUCTION

Although some 20 years after the discovery of phosphorylase kinase, protein kinase C (PKC) was one of the very first protein kinases to be identified. It was first defined as a histone kinase activity from rat brain which could be activated by limited proteolysis [1]. This was followed by the discovery that this new kinase could also be activated by phosphatidylserine (PS) and diacylglycerol (DAG) in a Ca^{2+}-dependent manner and also by tumour-promoting phorbol esters such as PMA (synonym TPA) (reviewed in [2]). It soon became apparent from chromatographic purifications of PKC that this activity was composed of at least three distinct species, which were designated the α, β and γ isotypes [3]. After 22 years, exhaustive genetic screening has defined a superfamily of mammalian PKC isotypes that currently comprises some twelve distinct genes.

The number of PKC isotypes in existence in some ways represents the greatest challenge to the understanding of PKC function. PKCs have a multitude of cellular substrates and are involved in a bewildering array of biological processes. Against such a broad canvas it is hard to pick out and define physiologically relevant functions and to assign them to individual PKC isotypes. The difficulties of satisfactorily determining the individual contributions of each of the PKC isotypes to any particular process are such that few such studies have been accomplished. The broadly overlapping substrate specificities of the PKC isotypes *in vitro* has led to suggestions of redundancy of function in the PKC superfamily. Although this may be true, it is an intellectually unsatisfying explanation for the diversity of mammalian PKCs. Several recent studies have suggested that specificity within the PKC superfamily is more likely to be effected through regulatory inputs, with PKC isotypes responding to different activation and localization signals.

The wealth of DNA sequence information now available has made possible the comparison of PKCs from a wide range of organisms. This has allowed us to refine our understanding of the domain structure of PKCs and has given valuable insight into the many regulatory elements present in these proteins. Additionally, it has allowed comparison of the mammalian PKC signalling systems with those of simpler organisms where more complete studies have been achieved.

The present review focuses on the information that has been obtained from the comparative analysis of the PKC superfamily and discusses how the potential parallels between PKC signalling in simple organisms may help to elucidate the more complex mammalian signalling pathways.

PKC ISOTYPES

The first PKCs to be identified and cloned were the α, β and γ isotypes, which were initially isolated from brain cDNA libraries [4,5]. This tissue has proven to be a rich source of PKC isotypes, and low-stringency screening of brain cDNA libraries with probes derived from the α, β and γ isotypes yielded three additional PKCs, the δ, ϵ and ζ isotypes [6]. Further low-stringency screens of other tissue cDNA libraries has delivered PKCη [7], PKCθ [8], PKCι (of which PKCλ is the mouse homologue) [9] and, most recently, the PKC-related kinases (PRKs) [10,11]. The mammalian PKC isotypes have been grouped into smaller subfamilies on the basis of their enzymic properties. The best understood and most studied of these groups is the conventional PKCs (cPKCs), which comprise the α, βI, βII and γ isotypes (the PKCβ gene is alternatively spliced to produce two gene products which differ only in their extreme C-terminal ends [12]). These PKC isotypes are activated by PS in a Ca^{2+}-dependent manner; they also bind DAG, which both increases the specificity of the enzyme for PS and also shifts the affinity for Ca^{2+} into the physiological range [13]. The cPKCs are targets of the tumour-promoting phorbol ester PMA, which

Abbreviations used: DAG, *sn*-1,2-diacylglycerol; HR1, homology region-1; GEFs, GDP/GTP exchange factors; GAPs, GTPase-activating proteins; PI, phosphatidylinositide, PKC, protein kinase C; PKD, protein kinase D; cPKC, conventional PKC; aPKC, atypical PKC; nPKC, novel PKC; PRK, PKC-related kinase; PS, phosphatidylserine; RACK, receptor for activated C kinase; V5 region, variable region-5; MAPK mitogen-activated protein kinase; SRF, serum response factor; MEK, MAPK kinase; MEKK, MEK kinase; PMA is a synonym of TPA.

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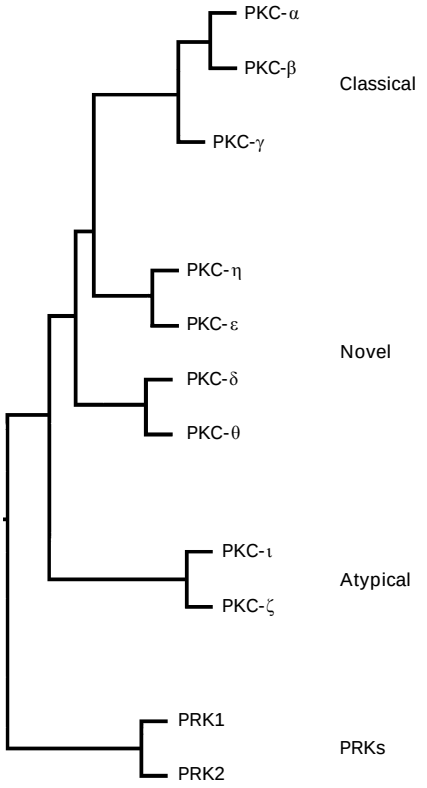


Figure 1 Dendrogram based on a sequence comparison of the PKC superfamily

Protein sequences of the fully-cloned members of the human PKC superfamily were compared using Clustal V software with PAM 250 residue tables. PRK3 is not included as only partial sequence information is available. This Figure is modified from that published in [150] and is reproduced with permission from Elsevier Science.

activates these enzymes by eliminating the requirement for DAG and decreasing the concentration of Ca^{2+} needed for activation [14].

The novel PKCs (nPKCs) consist of the ϵ, η, δ and θ isotypes. These kinases are Ca^{2+}-insensitive, but are still activated by DAG or phorbol esters in the presence of PS (see, for example, [15]). The atypical PKCs (aPKCs), ι and ζ, comprise a third category. Like the nPKCs, these protein kinases are Ca^{2+}-insensitive, nor do they respond to PMA/DAG [16]. Finally, the recently discovered PRKs define a fourth grouping consisting of at least three members, PRKs 1–3. PRK1 (PKN) was isolated in PCR-based and low-stringency screening, simultaneously by this laboratory [10] and by Ono and co-workers [11]. Like the aPKCs, PRKs are insensitive to Ca^{2+}, DAG and phorbol esters [17,18]. However, PRK1 has been shown to bind to the activated RhoA GTPase, which leads to a 4-fold activation of the kinase *in vitro* [19,20]. Recently it has been shown that the other fully cloned member of the PRK subfamily, PRK2, is also capable of binding RhoA [21,22], suggesting that this is a general property of this group.

The categorization of PKC isotypes based on their enzymic properties is strengthened by comparing the relatedness of these enzymes at the protein-sequence level. Sequence comparisons place PKC isotypes into five subgroups (Figure 1). These groupings are identical with those produced by the enzymic criteria described above, with the only exception being the splitting of the nPKCs into two pairs of very closely related kinases, namely δ and θ, and ϵ and η.

PKC BUILDING BLOCKS

Closer examination of protein-sequence alignments between PKC isotypes reveals the presence of blocks of homology between family members (Figure 2). In all cases these conserved regions have been shown to define protein domains (or motifs) which confer a specific localization and/or activation input to the isotype. In this way, the functional significance of these groupings becomes more obvious. The distribution of these regulatory protein modules between isotypes also allows a more precise categorization of PKC isotypes and demonstrates that these groupings represent PKC subfamilies.

The C1 domain

The cPKCs and nPKCs contain a C1 domain that is defined by the presence of two repeated zinc-finger motifs, C1a and C1b [23]. Each motif has a conserved pattern of cysteine and histidine residues ($H\text{-}X_{12}\text{-}C\text{-}X_2\text{-}C\text{-}X_{13/14}\text{-}C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_2\text{-}C\text{-}X_7\text{-}C$) that is responsible for the co-ordination of two Zn^{2+} ions [24,25]. NMR analysis of the second motif from the PKCα C1 domain has shown that it folds into a structure distinct from other known zinc-finger domains [26]. Mutational and deletion analysis has provided evidence that the C1 domain is the binding site for phorbol ester [27,28], and this has been confirmed by the solution of the crystal structure of the second zinc-finger motif from PKCδ complexed with PMA [29]. Binding studies have shown that DAG competes with PMA for binding to PKC and the two molecules are therefore assumed to interact with PKC at the same site [30]. The C1 domain is absent from the PRKs, which are not responsive to phorbol esters. The aPKCs are also unresponsive to phorbol esters, but do contain a single zinc-finger motif. Mutational studies have shown that the two zinc fingers in a C1 domain are not equivalent. Mutation of a highly conserved proline residue in the C1a motif of PKCδ had little

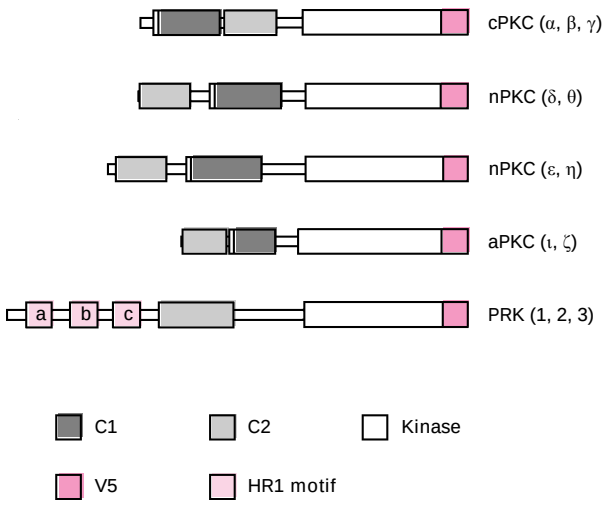


Figure 2 Domain structure of the PKC subfamilies

The Figure shows a comparison of the protein architecture of the various subgroups of the PKC superfamily (adapted from [150] with permission from Elsevier Science). The C1 domain of the aPKCs is smaller than that of the cPKCs and nPKCs as it contains only one copy of the zinc-finger motif.

Table 1 Mammalian C1 domain-containing proteins

The Table summarizes all of the mammalian proteins (in addition to the PKCs) containing consensus C1 domains which have been isolated to date. In many cases these proteins have been shown to bind PMA and/or DAG with a concomitant activation of protein function. Abbreviation: n.d., not done.

Protein	Zn fingers	PMA binding	Comments	Reference
DAG kinase	2	No	C1 not required for DAG binding or catalysis	[32]
Chimaerin	1	Yes	PMA stimulates GAP activity against Rac	[33]
PKD	2	Yes	Activated by PMA and DAG	[34]
Munc-13	1	n.d.	Mammalian homologue of unc-13; synaptosomal	[35]
Raf	1	No	C1 binds to Ras prenyl group	[36]
Ksr	1	n.d.	Raf-related kinase	[37]
Vav	1	Yes	GEF for Ras	[38]
Stac	1	n.d.	Novel SH3-containing protein from brain	[39]

effect on responsiveness to phorbol ester, whereas mutation of the equivalent amino acid residue in C1b caused a 125-fold decrease in phorbol-ester-binding affinity [31]. Interestingly, the single zinc-finger motif of the aPKCs shows much greater homology to the C1a motif than to the C1b. The function of the single zinc finger in the aPKCs is unknown. C1 domains occur in several mammalian proteins other than PKC (Table 1). In some proteins, such as DAG kinase, the domain binds to neither PMA nor DAG [32]. Other proteins have been shown to bind to, and be activated by, phorbol esters. Protein kinase D (PKD) is a human phorbol-ester- and DAG-stimulated protein kinase [34]. The mouse isotype has been termed PKCμ, and it has been suggested that it is a member of the aPKC subfamily [40]. However, examination of the catalytic domain of PKD shows that it is related to the CaMKII-like protein kinases and, indeed, both its substrate specificity [34] and its sensitivity to inhibitors [41] are unlike those of the PKC family. The chimaerin family are GTPase-activating proteins (GAPs) for the small GTPase Rac [42]. Their cellular function seems to be involved with the regulation of the actin cytoskeleton by Rac (and perhaps Cdc42) as they stimulate the formation of lamellipodia and filopodia in a Rac-dependent fashion [43]. The GAP activity against Rac has been shown to be stimulated by PMA treatment *in vitro* [33]. Unc-13 is a *Caenorhabditis elegans* neural-specific protein which binds to both PMA and DAG [44]. Defects in this protein lead to severe disruptions in neurotransmitter release. Three related mammalian homologues have been identified, of which the best studied is Munc13-1. This mammalian Unc-13 has been shown to interact with two synaptic-vesicle proteins, namely Doc2 [35] and syntaxin [45], suggesting a role for this protein in synaptic-vesicle exocytosis. Doc itself contains two C2 domains (see below) and is implicated in Ca^{2+}-dependent exocytosis [46].

The identification of PMA-responsive, non-PKC proteins in mammalian cells raises the need for caution in the interpretation of studies of PKC function that rely solely on the use of phorbol esters as an investigative tool. This is particularly pertinent for the chimaerin and Munc families which are involved in cellular processes which are also potentially regulated by PKCs.

The C2 domain

The C2 domain is found in the cPKCs immediately C-terminal to the C1 domain. Like the C1 domain, the C2 domain has been found to be present in many other proteins, including the synaptotagmins, rabphilin-3A, phospholipases and GAPs [47]. As many of these proteins bind phospholipid in a Ca^{2+}-dependent manner, it has been assumed that the C2 domain confers Ca^{2+}/PS binding to the cPKCs. Recently it has been shown that the isolated C2 domain from PKCβ does indeed bind to phospholipid vesicles in a Ca^{2+}-dependent fashion [48]. Synaptotagmin contains two copies of the C2 domain (C2A and C2B), and it has been shown that the C2A domain confers Ca^{2+}-dependent PS binding to the protein [49]. The three-dimensional structure of the C2A domain of synaptotagmin I has recently been solved in the presence and absence of Ca^{2+}. The C2 polypeptide assumes a compact β-sandwich fold formed by two four-stranded antiparallel β-sheets with Ca^{2+} bound in a cup-shaped depression [50]. Further information has been obtained by NMR-spectroscopic studies on PKCβ, which have shown that two Ca^{2+} ions are co-ordinated by five conserved aspartate residues [48]. Determination of the X-ray crystal structure of the PLC δ1 C2 domain has suggested that Ca^{2+} binding triggers a conformational change which opens a cleft to allow binding of a phospholipid headgroup [51].

Although the classical C2 domain is missing from the Ca^{2+}-independent PKCs, close inspection of these enzymes reveals that regions of homology originally termed V_0 in aPKCs and nPKCs, and HR2 in PRKs, are related to the C2 domain [47,52]. In these C2-like domains, one or more of the conserved aspartate residues required for Ca^{2+} binding are missing. It is unclear whether these domains are capable of binding to phospholipids, although activation of these PKCs by phospholipid has been demonstrated *in vitro* (for example, for PRK1 see [53]). It is possible that the general function of these C2-like domains is to make protein–protein interactions. The C2A and C2B domains of synaptotagmin have been shown to bind to syntaxin and clathrin AP-2 respectively [54]. Similarly, a class of proteins has been defined which interacts with the C2 domains of PKCs, i.e. the receptors for activated PKCs (RACKs) [55]. RACKs are not PKC substrates, but instead seem to be important in targetting active PKCs to specific membrane compartments. At present only two RACKs have been fully characterized, namely annexin I [56] and RACK1, a novel protein showing some homology to the β-subunit of heterotrimeric G-proteins [57]. The RACK1-binding site has been mapped to a short region of the PKCβ C2 domain [58]. Peptides derived from this region block the translocation of PKCβ to the plasma membrane in response to PMA [58]. Interestingly, peptides designed to the analogous region of the PKCϵ C2-like domain are also effective in blocking its translocation to the plasma membrane in response to stimuli [59], suggesting a general role for PKC C2 and C2-like domains in mediating protein–protein interactions which target PKCs to cellular compartments. Recent studies have shown that the V_0/C2 domain of PKCδ is a binding site for the growth associated protein GAP-43 [60], providing evidence for the idea that the function of the C2 domain in Ca^{2+}-unresponsive PKCs may be to make protein–protein interactions. GAP-43 does not fit the description of a RACK on two counts: (i) it is a well-characterized substrate of PKC and (ii) the association with PKCδ does not depend on the activation state of the kinase [60]. However, it seems likely that the interaction serves to impose a level of isotype specificity on the phosphorylation reaction by co-localizing the substrate with a specific PKC. Interestingly, although PKCδ does not bind Ca^{2+}, its association with GAP-43 is Ca^{2+}-dependent, adding a further level of complexity to the regulation of this phosphorylation. Clearly a model in which specific RACKs (or even PKC substrates, such as GAP-43) target individual PKC isotypes to distinct cellular compartments is an attractive one

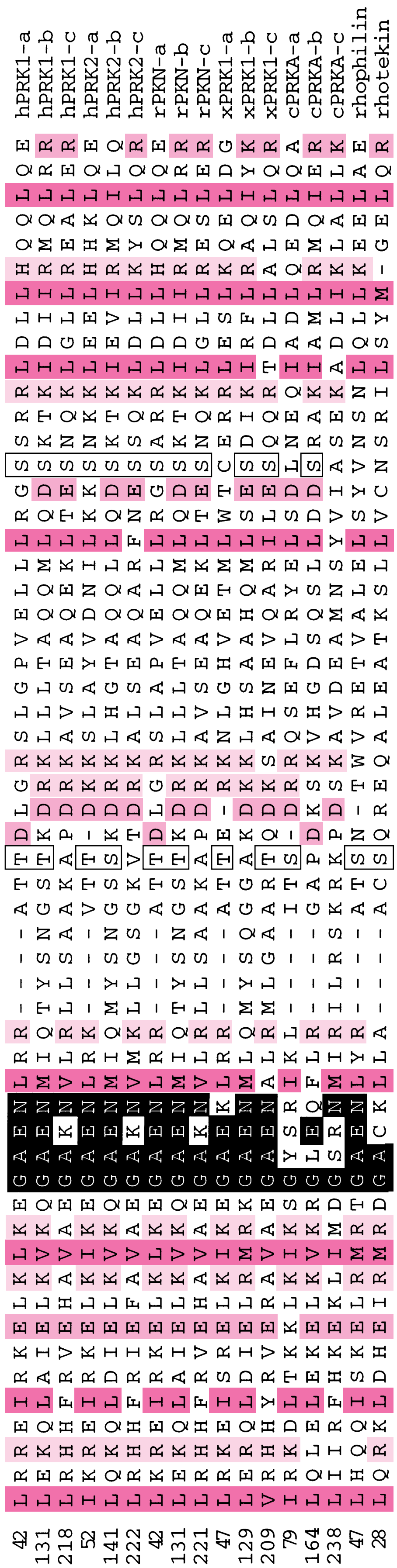


Figure 3 Sequence conservation in the HR1 motif

The sequences of the HR1 motifs from the known HR1-domain-containing proteins from higher organisms are shown aligned. The PRKs from human (h), rat (r), *Xenopus laevis* (x) and *C. elegans* (c) contain three copies of the HR1 motif, whereas only one copy is present in the mammalian proteins rhotekin and rhophilin. Pink colouring indicates conserved basic (pale pink), acidic (medium pink) or hydrophobic (dark pink) residues. Conserved Ser/Thr residues (potential phosphorylation sites) are boxed. Positions in the motif showing preference for a specific residue are shown as white letters on black.

that would go some way towards untangling the PKC signalling network. The recent description of the coatomer protein β-COP as a RACK specific for PKCϵ [61] provides the required selectivity. Ultimately, the validity of this hypothesis rests upon the identification of further isotype-specific protein-binding partners for PKC C2 domains.

The HR1 domain

The HR1 domain, initially identified as a region of homology between PRK1 and PRK2, is composed of three repeats of an approx. 55-amino-acid motif [10]. A single copy of this repeat is found in two other RhoA-binding proteins, rhophilin [20] and rhotekin [62]. Protein alignments of known HR1 repeats from various sources reveals several conserved features (Figure 3). The motif seems to consist of two blocks of homology with a short variable length of linking sequence. The first half of the repeat consists of a block of basic amino acids which have a predicted α-helical structure. This is terminated by a short, partially conserved sequence: Gly-Ala-Glu-Asn. The second half of the motif shows some homology to a leucine zipper in its primary sequence, although the presence of helix-breaking glycine and proline residues mean that it is unlikely to fold as a leucine zipper *in vivo*. The HR1 motif represents the single point of homology between PRKs, rhophilin and rhotekin. This suggests that this sequence defines a Rho-interacting module, and this has indeed been shown to be the case. The first HR1 repeat in PRK1 (HR1a) binds to the active GTP–RhoA complex, but not to the inactive GDP-bound form [63]. The second HR1 motif (HR1b) also binds RhoA, but this binding is weaker and occurs with either the activated (GTP-bound) or inactive (GDP-bound) forms of RhoA ([64]. Further binding analysis has suggested that the HR1a and HR1b motifs make distinct contacts on the RhoA protein and probably both contribute to binding *in vivo* [64]. We have recently shown that PRK1 is targetted to the endosomal compartment by another member of the Rho GTPase family, RhoB. Localization studies have shown that the wild-type RhoB (of which only a fraction is in the GTP-bound state) is as efficient at targetting PRK1 to endosomes as a constitutively GTP-bound mutant RhoB [65]. This suggests that a weak interaction between PRK1 HR1b and RhoB is sufficient to confer binding *in vivo* and that the HR1 domain can act as a localization signal as well as controlling kinase activation.

The HR1c repeat does not bind Rho [64], and its function is unknown. Small protein motifs are often stacked together to form more stable domains [66]. It may be that the three HR1 motifs in PRKs assemble into a higher-order structure that allows further points of contact with Rho. In this respect it is important to note that the published binding studies have been performed with recombinant RhoA which lacks the C-terminal prenylation modification found in the authentic molecule. It is possible that further contact points are made with the modified Rho that may require the complete HR1 domain (or indeed other PKC domains). Alternatively the HR1c motif may confer binding to some other protein, perhaps another member of the Rho GTPase family.

The pseudosubstrate site

Juxtaposed to the N-terminus of the C1 domains of the cPKCs, aPKCs and nPKCs is a sequence which retains the hallmarks of a PKC phosphorylation site, but has an alanine at the predicted serine/threonine phosphorylation site [67]. Mutation of this region confers effector-independent activity on the mutant protein [68]. This pseudosubstrate site interacts with the catalytic

domain and is responsible for the intramolecular suppression of catalytic activity prior to effector binding. For PRKs, which lack the C1 domain, studies using the yeast two-hybrid system have defined an interaction between the catalytic domain of PRK1 and the HR1a motif in the regulatory domain. This sequence contains a consensus phosphorylation site for PKCs centred around Ile[46], and synthetic peptides in this region have been shown to be inhibitors of PRK1 activity *in vitro*. It has therefore been proposed that the HR1a motif acts as a pseudosubstrate site to inhibit kinase function [69]. This is an attractive proposal, as it suggests a model whereby Rho binding to PRK could release the pseudosubstrate site from the catalytic domain and lead to activation of the kinase. Interestingly, it has been shown that the activation of PRK1 is accompanied by autophosphorylation within the HR1a motif at Thr[64], although the contribution of this phosphorylation to activity is unknown [70].

The V5 region

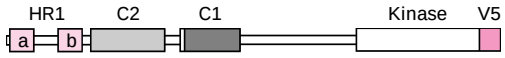
Although too short (approx. 50 amino acids) to be considered a protein domain, the V5 region of PKC plays an important regulatory role in kinase function. The initial assignment of the V5 region was based upon the deterioration in sequence conservation between PKC α, β and γ towards the end of the kinase domain [5]. The subsequent demonstration that the PKCβ gene produced two alternatively spliced transcripts specifically covering this region, provides an objective boundary for this C-terminal V5 region. In itself, this PKCβ alternative splicing and its conservation in other species, imply that this region confers specific properties. In the case of PKCβI and PKCβII there is very good evidence that the V5 domain plays a critical role in localization. Studies in the human U937 monocytic cell line have shown that PKCβI is localized to the microtubules, whereas PKCβII is localized in part to secretory granules [71]. As the only difference between these two proteins is the V5 region, it would seem that this acts as a differential localization signal in these isotypes. Presumably, the V5 region makes a protein–protein interaction, although the protein targets of the PKCβI and βII isotypes in U937 cell have not been identified. Differential localization of PKCβI and βII has also been observed in the MOLT-4 T-lymphoblastoid cell line. Here it has been shown that PKCβII, but not βI, translocates to actin microfilaments upon PMA treatment of cells [72]. In *in vitro* binding studies, PKCβII was seen to associate directly with F-actin; however, it did not bind to polymerized actin, suggesting that translocation to actin microfilaments *in vivo* may be mediated by association with some other cytoskeletal component.

The V5 regions of PKCs also play keys roles with respect to their phosphorylation. This is most clearly elucidated for the cPKCs, where two V5 region phosphorylation sites have been identified, at least one of which is the target of an upstream, non-PKC kinase. Phosphorylation at these sites has been shown to control both the net 'on' rate for PKCα phosphorylation at subsequent regulatory sites [73] and also the rate of PKCα and β dephosphorylation [73–75]. The basis for these effects seems to be in part due to the interaction of the phosphorylated V5 region with the kinase domain itself, inducing a closed, stable conformation. A precedent for this is provided by the equivalent C-terminal region of protein kinase A, which makes extensive contacts, wrapping back around the lower and upper lobes of the kinase domain [76]. Indirect evidence that this region of PKCα is important for stability/activity derives from a deletion analysis of the mammalian protein expressed in *Saccharomyces cerevisiae*, where removal of just the C-terminal 26 amino acids destroys activity [77]. It is noteworthy that the most C-terminal phosphorylation site in the V5 regions of PKCs (the so-called 'FSY' site) is replaced by a glutamic acid residue or an aspartic acid residue in the aPKCs and the PRKs, respectively. In PKCα the equivalent mutation (S657D) partially restores wild-type PKCα properties [73]. Thus it appears that for aPKCs and PRKs, the requirement for an upstream kinase to act on this hydrophobic site is by-passed.

Pkc1: AN ARCHETYPAL PKC?

PKCs have been isolated from a wide-range of eukaryotic sources. Perhaps the simplest of these is the budding yeast *S. cerevisiae*, demonstrating both the age and fundamental importance of this protein kinase. The yeast PKC (Pkc1) was originally identified by low-stringency screens of yeast genomic libraries with mammalian PKCs [78] and has subsequently been re-isolated as a product of a genetic screen for resistance to the PKC inhibitor staurosporine [79]. With the sequencing of the *S. cerevisiae* genome now complete [80], it is clear that Pkc1 represents the sole PKC in this organism. At 132 kDa, Pkc1 is a much larger protein than any of the mammalian PKCs. Examination of its protein sequence shows that this is due to an extended regulatory domain. In the mammalian PKC superfamily the various regulatory modules are differentially distributed between the different isotypes. However, in *S. cerevisiae*, all of these elements are present together in one enzyme, Pkc1 (Figure 4), suggesting that this protein kinase represents an archetypal PKC.

Two copies of the HR1 motif are present at the N-terminus of the kinase [47]. Sequence comparison with the PRKs indicates that these are most closely related to the HR1a and HR1b motifs which are responsible for the binding of RhoA GTPase. Like the PRKs, Pkc1 has been shown to be a downstream effector of Rho GTPase, both genetically and biochemically. Dominant active forms of Pkc1 suppress some the phenotypes of mutations in Rho1, the *S. cerevisiae* Rho GTPase [81], and Rho1 directly activates purified Pkc1 *in vitro* [82]. The site of interaction between Rho1 and Pkc1 has been examined by yeast two-hybrid analysis. Use of Pkc1 truncation mutants has defined a binding site for Rho1 within the C1 domain of the kinase, but not in the first 337 residues of Pkc1 that contain the HR1 and C2 domains



	HR1a	HR1b	C2	C1	Kinase	V5
PKCα	–	–	12	27	49	17
PKCβI (βII)	–	–	13	25	50	13 (21)
PKCγ	–	–	12	25	45	21
PKCδ	–	–	11	26	47	9
PKCθ	–	–	14	26	45	26
PKCε	–	–	13	23	47	21
PKCη	–	–	14	24	45	23
PKCι	–	–	12	–	42	17
PKCζ	–	–	11	–	39	21
PRK1	24	19	11	–	50	26
PRK2	31	15	13	–	51	32

Figure 4 Protein architecture of Pkc1

The structure of Pkc1 comprises an HR1 domain with two copies of the HR1 motif (residues 6–61 and 121–177), a C2 domain (195–369), a C1 domain (415–544) [preceded by the pseudosubstrate site (narrow white box)], the kinase domain (818–1104) and a V5 region (1105–1151). A key to the colourings of these domains is given in Figure 2. The percentage similarities of the respective domains to their counterparts in the mammalian PKC isotypes are shown in tabular form below the structure.

[81]. This is a somewhat surprising result given that the PRKs lack a C1 domain, whereas this domain is present in PKC isotypes which do not interact with Rho GTPases. We have shown that another yeast PKC, the *S. pombe* enzyme Pck2, does interact with Rho GTPase through its HR1 domain (L. Sayers and P. J. Parker, unpublished work). It would seem that Rho1 may make two interactions with Pkc1: an HR1 interaction and an interaction with the C1 domain. Many small GTPases, including Rho1 [83], are modified by the addition of a prenyl group at the C-terminus. It has been recently shown that the small GTPase Ras interacts through its prenyl modification with the C1 domain of the Raf kinase [36]. This interaction appears to stabilize the specific protein–protein interaction between Ras and the Ras-binding domain of Raf. It seems possible that a similar situation exists for the Rho1–Pkc1 interaction, although clearly further work is required to clarify this matter.

The HR1 domain is followed by a C2 like domain and then a C1 domain with two zinc fingers. Sequence comparison suggests the existence of a second C2-like domain between the C1 domain and the catalytic region [78], although the sequence similarity is too weak to be proven conclusively [47]. Pkc1 therefore has the potential to respond to all of the physiological activators of the mammalian PKCs. This question has been addressed to some extent by *in vitro* studies of the enzymic properties of the purified protein. From these studies it is clear that Pkc1 does not respond to Ca^{2+} [84], and this is not surprising, given that only one of the four aspartic acid residues required for Ca^{2+} binding are present in the Pkc1 C2 domain [47]. A similar lack of responsiveness to Ca^{2+} has been noted for the *S. pombe* Pck1 [85] and PKCs from the filamentous fungi *Trichoderma reesei* and *Aspergillus niger* [86]. Purified Pkc1 is not activated by combinations of PS, DAG or PMA *in vitro* [84]. However, recent studies have shown that Pkc1 is potently stimulated by PS in the presence of activated Rho1 [82]. Thus it would seem that dual inputs from the C2 domain (PS) and the HR1 and/or C1 domains (Rho1) are required for activation. The possible contribution of DAG to Pkc1 activation is unclear. Addition of DAG to Pkc1 incubated with PS and Rho1 produces no further activation of kinase activity [82]. Unfortunately, the effect of DAG and Rho1 on Pkc1 activity over a range of concentrations of PS has not been published. There is some indirect evidence for a role of DAG in Pkc1 regulation *in vivo*. Mutation of conserved cysteine residues in the Pkc1 C1 domain has been shown to mimic some of phenotypes of Pkc1 deletion, although this could be due to an inhibition of Rho1 binding [87]. More compelling are studies with the *S. cerevisiae* cyclin-dependent kinase cdc28. Genetic evidence has shown that cdc28 activation in late G_1 phase leads to a stimulation of DAG production, probably through the activation of a phosphatidylcholine-dependent phospholipase C and that this DAG production parallels Pck1 activation. Although strictly correlative, these data suggest a possible role for DAG in the regulation of Pkc1 *in vivo* [88]. No doubt further studies will resolve this question and provide the biochemical base upon which to establish links to the genetic analysis.

EXTENDING THE FAMILY: NEMATODE PKCS

More clues to the development of the PKC superfamily come from the study of the nematode worm *C. elegans*. This extremely simple organism contains only approx. 1000 somatic cells and yet has a primitive neural system and exhibits a range of behavioural responses to its environment. Treatment with PMA causes uncoordinated movement and growth arrest [89]. Screening for mutant worms resistant to PMA led to the identification of the *tpa-1* gene (*tpa* referring to TPA, a synonym of PMA) [90]. The *tpa-1* gene product encodes a protein (TPA1A) with substantial homology with the novel PKCs, especially to the PKCδ and θ grouping [90]. It has been shown recently that a second product of the *tpa-1* gene, TPA1B, is produced by alternative splicing. Interestingly, the effect of this splice is to remove very neatly the N-terminal V_0/C2-like domain while leaving the C1 domain intact [91]. Mutations in the *tpa-1* gene lead to a reversal of both the growth-inhibitory and locomotory effects of PMA, suggesting that TPA1 is responsible for the observable phenotype of phorbol ester on the organism [90]. A second *C. elegans* PKC gene, *pck-1*, has been isolated through screening of cDNA libraries with a PKCβ probe [92]. PKC1B is most related to the second grouping of novel PKCs, ϵ and η. Its expression in *C. elegans* is restricted to a subset of sensory neurons, suggesting parallels with the distribution of PKCs ϵ and η in mammals [92]. As with *tpa-1*, the *pkc-1* gene is also alternatively spliced to produce a second PKC, PKC1A. This isotype differs only in the presence of an additional 56 amino acids at the N-terminus [93]. The PKC1B isotype shows differential expression during development with a peak of protein in the early larval stages [92]. It would seem likely that this isotype is required for post-embryonic development of the neurosensory system.

The *pkc-2* (*kin-11*) gene was identified through the *C. elegans* genome sequencing project. The predicted product of this gene shows high homology with the cPKCs, and its structure suggests that it will have similar enzymic properties. It has a C2 domain which contains the invariant residues required to confer Ca^{2+} binding and also a well-conserved C1 domain. Recent isolation and cloning of the PKC2 cDNAs has revealed a complex pattern of alternative splicing, generating variation at both ends of the protein [94]. The *pkc-2* gene gives rise to two 3′-splice variants, PKC2A and 2B, which differ in their C-termini. This has an obvious parallel with the splice variants of cPKCβ and, indeed, comparison of the protein sequences reveals that the length and relative position of the alternatively spliced exon is the same for PKC2 and PKCβ. These findings suggest that PKCβ and PKC2 share a common ancestor, perhaps the original cPKC [94], and that this alternative splicing of the C-terminus has been conserved from nematodes to man. Indeed, closer examination of the protein sequences suggests that PKC2A corresponds to PKCβI and PKC2B to βII. Three splice variants have been isolated at the 5′-end of the PKC2 cDNA that encode alternative N-terminal extensions of 50 (exons 1A plus 1A′), 13 (exon 1B) and 15 (exon 1C) residues [94]. This gives a potential six isotypes of PKC2, although it is not known whether all combinations are expressed. The kinase activity of recombinant PKC2 is stimulated by Ca^{2+} [94] and may correspond to the Ca^{2+}- and DAG/PS-dependent PKC-like kinase activity isolated from *C. elegans* tissue extracts [95]. The observed Ca^{2+}-dependence demonstrates that the enzyme is a cPKC both by structural and enzymic criteria. The level of expression of the protein kinase rises dramatically upon hatching and then remains relatively constant throughout adult life, although, as with PKC1, there appears to be stage-specific regulation of the expression of splice variants. As with the other nematode PKCs, the cellular role of PKC2 is at yet unknown. Clues to its function may come from its localization; immunodetection of the endogenous protein shows that the distribution of PKC2 in adult *C. elegans* is restricted largely to a subset of neurons and to somatic cells in the gonad [94].

The sequencing of the *C. elegans* genome is now 75% complete, with well over 5000 genes identified so far. Analysis of these sequences predicts the presence of five PKC-related proteins, three of which being the products of the *tpa-1*, *pkc-1* and *pkc-2*

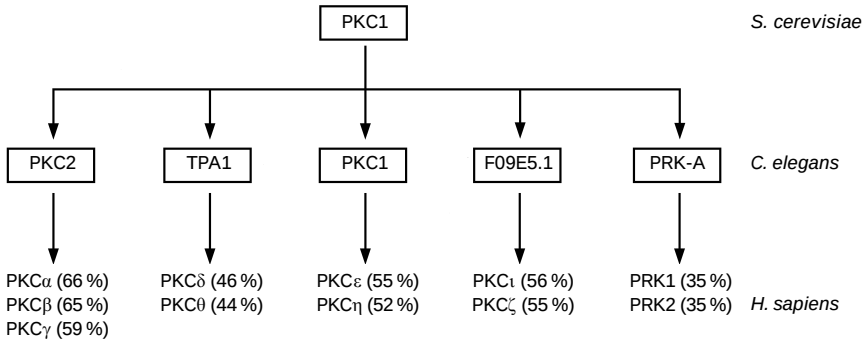


Figure 5 Expansion of the PKC superfamily

Five PKC genes have been identified in the nematode worm *C. elegans*. Each of these corresponds to a specific subgroup of the mammalian PKC superfamily, both by overall similarity and protein architecture. The diagram shows the percentage similarities between each of the *C. elegans* PKCs and their corresponding mammalian isotypes.

genes. As the mammalian PKC superfamily appears split into five groups of related enzymes, so a single *C. elegans* PKC seems to correspond to each of these groups (Figure 5). The predicted *F09E1.1* gene product is highly homologous with the aPKCs and shares the same domain structure, whereas the PRK-A kinase (H. Mellor and P. J. Parker, unpublished work), encoded by the *F46F6.2* gene, contains a highly conserved HR1 domain (containing all three HR1 motifs) and appears to be the nematode equivalent of the PRK subfamily. While other PKCs may be identified within the remaining 25 % of the *C. elegans* genome remaining to be sequenced, it seems clear that expansion of the PKC family occurred early in metazoan evolution to create five main PKC subfamilies. It also seems likely that subsequent gene duplication has lead to the appearance of multiple members of these subfamilies in mammals. Whilst the first expansion would appear to have involved gene rearrangement, the products of the second expansion have retained their modular architecture and are presumably products of gene duplication.

C. elegans is an extremely manipulatable and powerful genetic system. The presence of five representative isotypes of the PKC superfamily in this organism greatly simplifies the study of PKC function. As yet only the *tpa-1* gene has been mutated. It is to be hoped that genetic dissection of the other four genes will allow a functional distinction of PKC subfamilies with relevance for the more complex mammalian system.

PKC SIGNALLING IN YEAST

Genetic studies in yeast have made rapid progress in delineating the PKC signalling pathways in this simple organism. In the interests of clarity, the present review will focus on Pkc1 signalling in *S. cerevisiae* which represents the most complete story, although significant progress has been made in studies of PKC pathways in the fission yeast *S. pombe* (see, for example [96]).

Deletion of Pkc1 in *S. cerevisiae* leads to an arrest of protein synthesis and cell growth at a point in the cell cycle prior to mitosis and after DNA synthesis [78]. Growth can be restored to ΔPkc1 mutants by the addition of osmotic stabilizing agents, such as sorbitol, to the medium, suggesting that the defect involves a weakening of the cell wall. When ΔPkc1 yeasts are returned to a hypotonic medium, budding cells undergo immediate lysis, further suggesting that the lethality of the cell-wall defect is manifest on bud formation [97]. Electron-microscopic studies have shown this to be true, revealing thinned cell walls in ΔPkc1 yeast and holes at the bud tip [98,99]. The Pkc1 pathway has been shown to be stimulated by at least two signals. The first is cellular stress in the form of hypotonic osmotic shock [100] or heat shock [101]. The second is cell-cycle-dependent establishment of polarized growth. Budding yeast exhibit polarized growth in the vegetative state during bud formation (reviewed in [102]). Conditional mutants of Pkc1 arrest with small buds, indicating that Pkc1 is not required for bud establishment but for the subsequent bud emergence [97]. *S. cerevisiae* also exhibits polarized growth under two other conditions. Haploid cells will respond to mating pheromone by undergoing sexual development involving cell cycle arrest and the production of a mating projection (reviewed in [103]). Haploid or diploid cells will also exhibit polarized growth under conditions of nitrogen starvation, where they switch to a pseudohyphal growth mode in order to explore the environment for nutrient sources (reviewed in [102]). Pkc1 signalling pathways have also been shown to be required for polarized growth in both of these conditions; i.e. in response to mating pheromone or nitrogen starvation [104–106], demonstrating a general role for Pkc1 in this process. Polarized growth and the cellular response to hypotonic shock or heat shock all require remodelling of the cell wall, and this commonality between the various activating signals for Pkc1 suggests that the dynamic regulation of the cell wall is the cellular site of Pkc1 action. Dissection of the Pkc1 signalling pathways indicates how this may be achieved.

Genetic studies have led to an understanding of PKC signalling pathways in yeast that is more extensive and, in many ways, more conclusive than our current understanding of signalling in the mammalian PKC signalling networks (Figure 6). As mentioned above, Pkc1 is immediately downstream of the Rho GTPase Rho1 [81]. The activity of Rho1 is governed by the opposing actions of GDP/GTP exchange factors (GEFs) and GTPase-activating proteins (GAPs). The GEFs Rom1 and Rom2 both contain a DH domain which catalyses the exchange of GDP for GTP on Rho1, causing its activation [107]. The GAPs Bem2 [108] and Sac7 [109] have both been shown to catalyse the hydrolysis of GTP on Rho1 *in vitro* and therefore are potential negative regulators of Rho1. Whether both proteins fulfil this role *in vivo* is uncertain, as only Sac7 deletions are capable of suppressing the phenotype of mutations in Rom2 [109]. Further upstream is the yeast Tor2 protein, which is related to the PI 3-kinases. Genetic evidence suggests that Tor2 stimulates Rom2 activity (and hence Pkc1 activity) through the Rom2 PH domain [109]. It is not known whether Tor2 acts directly on Rom2, although an attractive proposal is that stimulation of

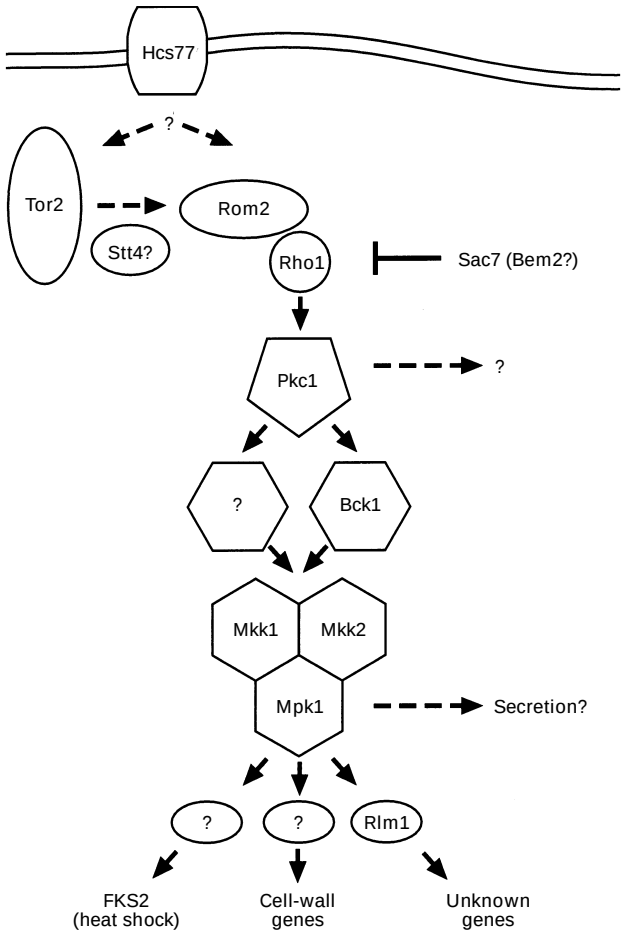


Figure 6 PKC signalling pathways in budding yeast

The diagram summarizes current knowledge of the Pkc1 signalling pathway in *S. cerevisiae*. Direct activations are shown as continuous arrows. Activations that are presumed or which may be indirect are shown as broken arrows.

Rom2 activity is mediated by the binding of a PI lipid product of Tor2 to the Rom2 PH domain. However, the identity of the presumed lipid product(s) of the Tor2 kinase is as yet unknown. Genetic evidence has also placed another PI lipid kinase upstream of Pkc1; the phosphatidylinositide (PI) 4-kinase, Stt4 [110,111]. Interestingly, both the mammalian homologue of Tor2 [112] and yeast Tor2 (F. Cooke, unpublished work) have been shown to co-purify with an associated, uncharacterized, PI 4-kinase activity. This suggests the possibility that Stt4 may lie in the pathway leading to Tor2-dependent activation of Pkc1, although further work is clearly needed to clarify the biological relevance of the genetic interaction between Stt4 and Pkc1. Looking further upstream, recent studies have identified a candidate cell-surface receptor for the Pkc1 pathway [113]. Hcs77 (also termed Wsc1 [114]) is an integral plasma-membrane protein that has been shown genetically to be upstream of Pkc1 [113]. Deletion mutants of Hcs77 show the ΔPkc1 cell-lysis phenotype and also fail to activate Mpk1 (see below) in response to heat shock. These findings, combined with the cell-surface localization of the protein, have lead to the proposal that Hcs77 is the mechanosensor for the Pkc1 pathway [113]. It will be interesting to see how mutations of Hcs77 interact with the Tor2/Rom2/Rho1 arm of the Pkc1 pathway.

Downstream of Pkc1 is a mitogen-activated protein kinase (MAPK) module of the paradigm MEKK > MEK > MAPK, where MEK is MAPK kinase and MEKK is MEK kinase. Genetic screens for extragenic suppressors of a conditional Pkc1 deletion have identified Bck1 (bypass of C kinase, also termed Slk1 [104]) as a downstream effector of the yeast PKC [115]. Bck1 is upstream of two related MEKs, Mkk1/Mkk2, which are functionally redundant in the Pkc1 signalling pathway [116]. These proteins are in turn upstream of the last member of the MAP kinase module, Mpk1 ([116,117], also termed Slt2 [118]). Deletion of either Bck1, Mkk1/Mkk2 or Mpk1 leads to a cell lysis phenotype similar to ΔPkc1 mutants; however, the magnitude of the phenotype is less severe, only being evident at higher temperatures. This has lead to the proposal that a bifurcation in the pathway occurs at the level of Pkc1, with a second, as-yet-uncharacterized signalling pathway parallel with the Bck1/Mkk/Mpk1 MAP kinase module [116]. Genetic analysis has also suggested that there may be an alternative downstream partner for Pkc1 within the MAP kinase module. The activation of Mpk1 during heat shock is absolutely dependent on Bck1 [101]. However, whereas the activation of Mpk1 that occurs in response to mating pheromone requires Pkc1 and Mkk1/Mkk2, there is only a partial effect of the deletion of Bck1. This has lead to the suggestion of an alternative mechanism for activation of Mkk/Mkk2 by Pck1 under certain conditions [119]. Hopefully, further studies will resolve this issue.

MAP kinase modules are generally seen to regulate transcription-factor activity, and this would seem to be the case with the Pkc1 pathway. Mutation of Pkc1 causes reduced expression of several genes involved in cell-wall synthesis, most notably *FKS1* [subunit of (1–3)-β-glucan synthase], *MNN1* (α-1,3-mannosyltransferase) and *CSD2* (chitin synthase III) in mid-exponential-phase cells [120]. This indicates that at least part of the effects of the Pkc1 pathway on cell-wall integrity are mediated through regulated gene expression. Two transcription factors, namely SBF and Rlm1, have, on the basis of their genetic interactions with the Pkc1 pathway, been proposed as candidates to execute this regulation, Genetic studies of this part of the pathway are particularly difficult. Transcriptional regulation of the genes involved in cell-wall synthesis in yeast appears to be complex and multifactorial. Mutation of any transcription factor involved the expression of cell-wall genes could potentially aggravate the phenotypes of mutations of the Pkc1 pathway and/or display a cell-wall-lysis phenotype. This gives rise to difficulties in addressing the biological relevance of such genetic data to Pkc1 signalling.

SBF is composed of a regulatory subunit (Swi6) and a DNA-binding protein (Swi4, reviewed in [121]). Swi4 controls the expression of several genes involved in cell-wall synthesis at the G_1/S-phase boundary [120], and mutants in Swi4 have temperature-sensitive cell-wall defects [120,122]. It has been shown that Mpk1 can phosphorylate Swi6 and Swi4 *in vitro*, leading to the proposal that Swi4 is downstream of the Pkc1 pathway [122]. However, whereas expression of Swi4 suppresses ΔBck1 and ΔMpk1 mutants [122], overexpression of Pkc1 can suppress mutants in Swi4 [113]. Further, Pkc1 deficient mutants still show Swi4-dependent transcription of cell-wall genes at the G_1/S-phase boundary [120]. Taken together, the data suggest that Swi4 acts in a pathway governing cell-wall integrity that may be parallel with the Pkc1 pathway, rather than co-linear.

Stronger evidence exists for a role for the Rlm1 transcription factor as a downstream effector of the Pkc1 MAP kinase module. This protein was isolated as a suppresser mutant of cells expressing an constitutively active Mkk1 [123]. Rlm1 shows homology with the MADS family of transcription factors which

includes the mammalian serum response factor (SRF, reviewed in [124]). Rlm1 interacts with Mpk1 in a two-hybrid binding assay [123] and is phosphorylated by Mpk1 *in vitro* [125]. Most importantly, expression of Rlm1 reporter constructs is entirely dependent on Mpk1 activity *in vivo* [126]. As yet, the identity of the genes under the control of Rlm1 is unknown. However, they most likely represent only a subset of the genes regulated by the Pkc1 pathway, as deletion of Rlm1 does not lead the cell-lysis phenotype seen with ΔPkc1 mutants [123]. A recent detailed study of the *FKS2* gene has shed light on this. Fks2 is an alternative subunit to Fks1 for the (1–3)-β-glucan synthase. Fks1 is constitutively expressed in cells with some cell-cycle-dependent fluctuations. However, Fks2 expression is normally very low, but is induced under three conditions: exposure to mating pheromone, heat shock or growth on a non-fermentable carbon source [127]. Levin and co-workers have shown that two genetically separate pathways control the expression of the *FKS2* gene, one dependent on calcineurin and the other on the Pkc1 MAP kinase module [128]. Pkc1 signalling controls only one of the three triggers to *FKS2* expression, the induction during heat-shock. The Pkc1-dependent regulatory element in the *FKS2* gene promoter has been mapped and shows no requirement for Rlm1 for activation through Pkc1 [128]. It would seem that at least one other, as-yet-unknown transcription factor acts downstream of Pkc1 to control expression of the *FKS2* gene.

Regulated gene expression is only one of the probable outcomes of Pkc1 signalling pathways in yeast. As mentioned above, the phenotypes of deletions of members of the Pkc1 MAP kinase module are less severe than that of Pkc1-deleted strains, suggesting the existence of other downstream pathways from Pkc1. Additionally, mutations of members of the Pkc1 MAP kinase module have some phenotypes that are hard to explain in terms of regulated gene expression. Polarized growth involves the transport of secretory vesicles along actin cables to the site of cell-wall synthesis. Conditional Mpk1 mutants show disruption of this process, with an accumulation of vesicles in the cytoplasm and a delocalization of cortical actin structures [105]. Clearly other targets of the Pkc1 MAP kinase pathway remain to be identified.

Although still incomplete, our current knowledge of PKC signalling pathways in yeast presents interesting and potentially informative parallels with the mammalian system. Broadly speaking, this information can be used in two ways. The most concrete of these is the use of interactions defined in the yeast Pkc1 pathway to predict possible signalling partners in higher organisms. The majority of the members of the yeast Pkc1 pathway have mammalian homologues, and comparative studies would focus on these proteins as candidate PKC effectors. The interaction between Pkc1 and Rho1 [81] was reported over a year before the discovery of the analogous interaction between PRK1 and RhoA [19,20], illustrating the potential predictive value of yeast studies, albeit with hindsight in this case. On a larger scale, comparisons between yeast and mammals can also be made for entire pathways. Such arguments based upon analogy are more diffuse and may have less predictive power. However, in three cases the parallels are compelling and suggest potential directions for future research in mammalian studies.

The most obvious parallel between PKC signalling in yeast and mammals is the regulation of gene expression by the Rho GTPase. In mammalian cells, RhoA induces the expression of a number of immediate early genes in response to serum, including c-fos [129]. The response is mediated by a member of the MADS box family of transcription factors, the SRF (reviewed in [130]). In yeast the Rho1 GTPase also activates a member of the MADS box family of transcription factors, Rlm1, through the Pkc1 MAP kinase pathway. Although currently nothing is known of the signalling pathway to the SRF from RhoA, it is possible that an uncharacterized MAP kinase pathway may be involved. By analogy with yeast, PRKs are an obvious candidate effector for this aspect of RhoA signalling. As yet, only a modest stimulation of SRF activity has been reported in cells overexpressing PRK2 [21]. However, it is possible that these studies require another PRK (1 or 3) or some additional regulatory input to achieve PRK activation.

In mammals, a pathway parallel with that involving Rho and SRF involves the classical MAP kinase cascade (i.e. Raf/MEK/ERK) and the transcription factors of the TCF subfamily of ets-related proteins (reviewed in [130,131]). This pathway can be triggered by phorbol esters and the evidence indicates the involvement of PKC. Thus activated PKC mutants will stimulate the pathway and dominant negative PKC mutants will inhibit, showing that PKC is both necessary and sufficient for the response to phorbol esters. The actual mechanism involved is far from clear, with conflicting evidence as to whether PKC acts directly on c-Raf-1 or not (see [132]. Interestingly, there is evidence that direct cPKC phosphorylation of cRaf-1 contributes to desensitization rather than activation [132]. The control of cRaf-1 through Ras, tyrosine and serine/threonine protein kinases has yet to be crystallized into a coherent story, and this has complicated the elucidation of the PKC input(s). It is clear that certain PKC isotypes can activate MEK independent of cRaf-1 [132]. This has parallels with the yeast Pkc1 pathway where, as mentioned previously, alternative routes to Bck1 can be used by Pkc1 to activate Mkk1/Mkk2 (Figure 6). It may be that the resolution of this signalling junction in yeast will help to clarify PKC signalling to the classical MAP kinase cascade in mammals.

Finally, Bussey has remarked on the potential parallels between Rho action at the polarised bud site in yeast and focal adhesions in mammalian cells [133,134]. Focal adhesions are concentrated aggregates of cytoskeletal proteins and signalling molecules at the plasma membrane which mediate signalling between the cytoskeleton and the extracellular matrix through integrins (reviewed in [135]). Rho action at the bud site and at focal adhesions involves some PKC-independent functions. Thus, in yeast, Rho1 stimulates (1–3)-β-glucan synthase at the bud site directly, independent of Pkc1 [136,137]. In mammalian cells RhoA stimulates the formation of actin stress fibres at the focal adhesion through the actions of two non-PKC proteins, p140mDia [138] and ROK [139]. This has parallels with the localization of actin cables at the yeast bud site. However, it seems that other parallels exists between the yeast bud site and focal adhesions that do involve PKC function. In yeast it has been proposed that a mechanosensor (perhaps Hcs77) triggers a Rho1-dependent Pkc1 response to heat shock or osmotic shock. Mammalian cells also have signalling pathways to respond to mechanical stress which includes the tensile stress exerted by neighbouring cells and the substratum as well as specialized conditions of stress such as the fluid shear stress experience by vascular endothelial cells. Several lines of evidence point to integrins as the mechanosensors involved in these responses (reviewed in [140]). PKCs play an important, if ill-defined, role in integrin signalling (reviewed in, e.g., [141]). PKCα has been shown to be localized to focal adhesions in rat embryo fibroblasts [142], and recently it has been shown that RACK1 binds directly to β-type integrins in a PMA-stimulated fashion, suggesting how PKCs may localize to this compartment [143]. Perhaps the most compelling data is the recent finding that the specific depletion of PKCϵ using antisense oligonucleotides prevents the stimulation of the ERK1/ERK2 MAP kinase pathway caused by shear stress

of human umbilical-vein endothelial cells [144]. This presents the best evidence to date for a direct parallel between PKC-dependent mechanosensor pathways in yeast and mammals.

OTHER GENETIC SYSTEMS

Genetic analysis of PKC pathways in higher organisms can be expected to give additional information to studies in unicellular yeast. Transgenic mouse knockouts have been established for PKCγ [145] and β [146] with associated mild neurological and immune disorders respectively. It is possible that the reason for the fairly benign phenotypes of the single PKC knockouts in mice is that other members of the same PKC subfamilies are able to substitute partially for the deleted gene. In this case, the crossing of single knockout mice to remove an entire PKC subfamily may give more definitive results. As mentioned above, genetic studies in *C. elegans* may represent a complementary way of addressing this problem, as each PKC subfamily is represented by a single isotype. While such studies are still in their early stages, Rubin and co-workers have recently made rapid progress in characterizing the nematode PKCs and it would seem likely that this system will contribute significantly to our understanding of PKC signalling.

Further information may come from other genetic study systems. In *Drosophila* it has been shown that an eye-specific PKC isotype (InaC) interacts with InaD [147], a scaffolding protein for the phototransduction cascade. This interaction has been shown to occur through one of the five PDZ domains in InaD and serves to regulate the subcellular location of InaC to the rhabdomere [148]. Recently PKCα has been shown to associate with a protein of unknown function, PICK1. The interaction is between the PICK1 PDZ domain and a PDZ-binding motif at the C-terminus of PKCα [149], demonstrating once again that paradigms of PKC regulation are preserved across species boundaries.

PERSPECTIVES

Dissecting the complex mesh of mammalian PKC signalling pathways presents several problems. First is the presence of so many PKC isotypes, all with seemingly broadly overlapping substrate specificities. Secondly is the number of potential substrates for these enzymes. The substrate specificity of many protein kinases is governed by the catalytic domain, making the identification of cellular substrates relatively straightforward. However, most proteins contain consensus phosphorylation sites for PKC, and many of these are phosphorylated by PKC *in vitro*. Addressing the biological relevance of these phosphorylations is far from easy. Comparative studies can help to address this problem by providing information about specificity in the PKC signalling pathway. Comparisons of the protein sequences of PKCs from various sources shows that most of the diversity between isotypes is in the regulatory region, and it is here that we can look for specificity in PKC signalling. These comparative studies have defined a number of conserved domains in the regulatory region, and in all cases these have been shown to confer an activation and/or localization input to the enzyme. Each PKC subfamily contains a different arrangement of these regulatory domains, suggesting a method by which isotype specificity may be determined by a unique pattern of localization and activation. The identification of these domains allows us to design experiments to find their binding partners. It also has predictive power; for example, a RhoA-dependent signalling pathway containing a PKC substrate is more likely to involve a PRK than a cPKC. Comparative studies in simple organisms can give further information about specificity in the PKC signalling pathway. The reduced number of PKC isotypes makes the results of genetic studies more conclusive. Thus deletion of one of the 12 PKC isotypes in mice has a mild phenotype, whereas deletion of the sole PKC in *S. cerevisiae* causes growth arrest. Presumably there are the same degree of biologically irrelevant *in vitro* PKC substrates in yeast as there are in mammals. However, the ability to analyse a clear phenotype has made it possible to identify biologically relevant PKC substrates directly and to elucidate the signalling pathway in yeast to much greater detail than has been possible in mammals.

Evidence so far suggests that many of the paradigms of PKC signalling are conserved across species from yeast to mammals. It seems likely that information gained form the study of PKCs and PKC signalling in simple organisms will help to unravel these complex and important mammalian signalling pathways.

Note added in proof (received 27 April 1998)

The cDNA corresponding to the *C. elegans* gene sequence F46F6.2 has recently been characterized by Rubin and co-workers and designated PKC3 [151].

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