Chapter 29
The Role of C2 Domains in PKC Signaling

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Abstract More than two decades ago, the discovery of the first C2 domain in conventional Protein Kinase Cs (cPKCs) and of its role as a calcium-binding motif began to shed light on the activation mechanism of this family of Serine/Threonine kinases which are involved in several critical signal transduction pathways. In this chapter, we review the current knowledge of the structure and the function of the different C2 domains in PKCs. The C2 domain of cPKCs is a calcium sensor and its calcium-dependent binding to phospholipids is crucial for kinase activation. While the functional role of the cPKC C2 domain is better understood, phylogenetic analysis revealed that the novel C2 domain is more ancient and related to the C2 domain in the fungal PKC family, while the cPKC C2 domain is first associated with PKC in metazoans. The C2 domain of novel PKCs (nPKCs) does not contain a calcium-binding motif but still plays a critical role in nPKCs activation by regulating C1-C2 domain interactions and consequently C2 domain-mediated inhibition in both the nPKCs of the epsilon family and the nPKCs of the delta family. Moreover, the C2 domain of the nPKCs of the delta family was shown to recognize phosphotyrosines in a novel mode different from the ones observed for the Src Homology 2 (SH2) and the phosphotyrosine binding domains (PTB). By binding to phosphotyrosines, the C2 domain regulates the activation of this subclass of PKCs. The C2 domain was also shown to be involved in protein-protein interactions and binding to the receptor for activated C-kinase (RACKs) thus contributing to the subcellular localization of PKCs. In summary, the C2 domain is a critical player that can sense the activated signaling pathway in response to external stimuli to specifically regulate the different conventional and novel PKC isoforms.

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Introduction

Protein kinase Cs (PKCs) are a family of lipid activated Serine/Threonine kinases which are involved in several critical signal transduction pathways including cell division, differentiation, migration, apoptosis and synaptic plasticity underlying learning and memory formation [1–7]. There are four known families of PKC isoforms in vertebrates: the conventional or Ca^{2+}-activated PKCs (cPKCs) family which includes PKC α, β1, β2 and γ, the novel or Ca^{2+}-independent PKCs (nPKCs) of the epsilon family which includes PKC ε and η also referred to as novel type I, the nPKCs or Ca^{2+}-independent PKCs of the delta family which includes PKC δ and θ also referred to as novel type II and the atypical family (aPKCs) which includes PKC ζ and ι (Fig. 29.1; [2, 8]). All isoforms have a catalytic domain located at the C-terminal and a regulatory domain located at the N-terminal. In the inactive form of the kinase, the pseudosubstrate (*P* in Fig. 29.1) located in the regulatory domain is lodged in the active site located in the catalytic domain and blocks it sterically keeping the enzyme inactive. In order for PKCs to become active, a conformational change is required to move the pseudosubstrate away from the active site and allow binding of the substrate [9, 10]. Conventional PKCs contain two tandem C1 domains which can bind to diacylglycerol (DAG)/Phorbol esters in the regulatory region and a C2 domain which mediates calcium-dependent binding to the membrane lipid phosphatidylserine (PS) and to phosphoinositide-4,5-bisphosphate [PIP2] [11–13]. Novel PKCs also contain two C1 domains that coordinate binding to DAG/Phorbol esters and a C2 domain but their C2 domain is located N-terminal to the C1 domains and lacks the critical aspartic acid residues required for coordinating Ca^{2+} ions in cPKCs [14]. In the nPKCs of the delta family, the C2 domain can also bind phosphotyrosines [15]. Atypical PKCs have one C1 domain which is said to be atypical because it cannot bind DAG/Phorbol esters and do not have a C2 domain but rather a PB1 domain in the regulatory region which mediates protein-protein interactions [16–18]. In *Aplysia californica*, our model system to study memory formation, there are three nervous system specific PKC isoforms one from each major class, namely the conventional PKC Apl I, the novel PKC Apl II which is homologous to the nPKCs of the epsilon family in vertebrates and the atypical PKC Apl III [2, 19, 20]. The present chapter will focus on the role of the C2 domains in PKC signaling. Therefore, the activation mechanisms of atypical PKCs will not be discussed in this chapter.

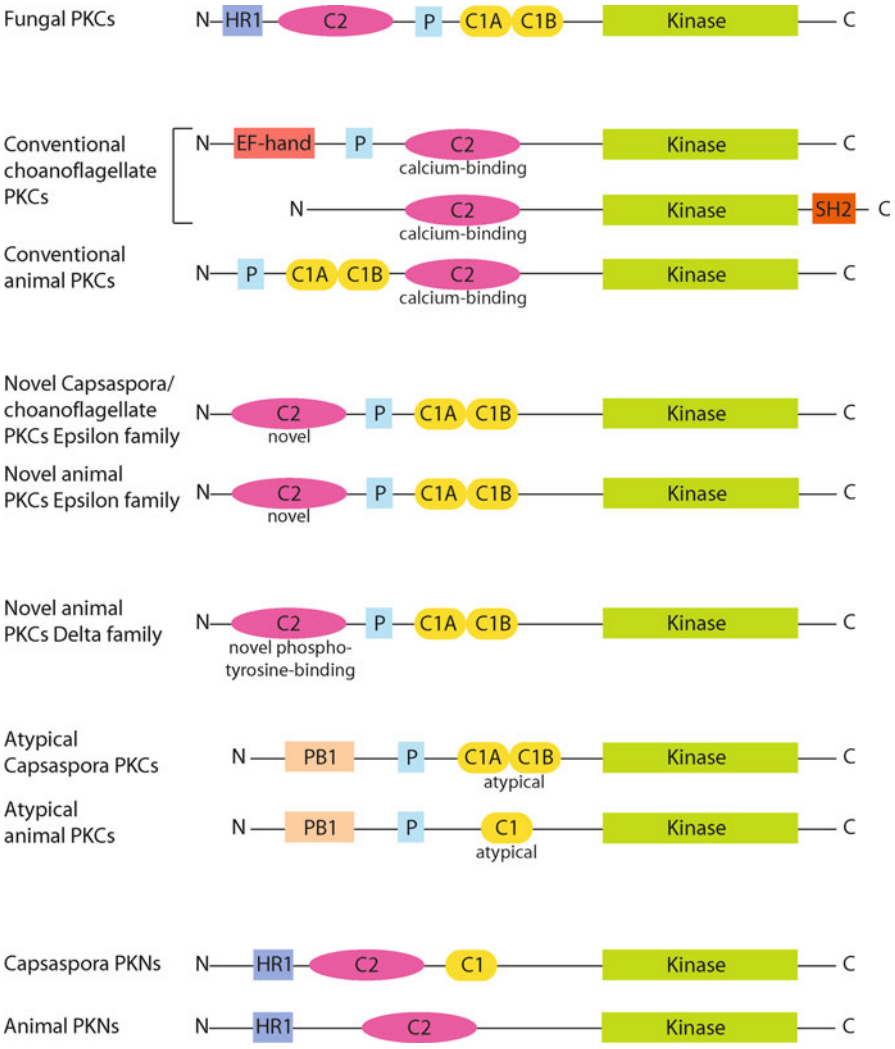

Fig. 29.1 Structure of PKC isoforms. Representative structures from each family are shown. Note that the C2 domains of nPKCs of the epsilon family and nPKCs of the delta family are located N-terminal to the C1 domains and lack the critical aspartic acid residues critical for coordinating Ca^{2+} in cPKCs. The C2 domain of nPKCs of the delta family can also bind to phosphotyrosines. Atypical PKCs don't contain a C2 domain but a PB1 domain that mediates protein-protein interactions. HR1 domains in yeast PKCs and PKNs bind to small GTP-binding proteins such as Ras and Rho. EF hands are calcium-binding domains related to the domains in calmodulin. SH2 (Src homology 2) domains are phosphotyrosine binding domains first defined in Src. *P*, pseudosubstrate

Evolution of C2 Domains

C2 domains are ancient and are found throughout eukaryotes in a wide variety of proteins [21]. In trying to understand the role of C2 domains in PKCs, it would be helpful to know whether the C2 domains in novel type I, novel type II, and conventional PKCs are directly related to each other and how the connection between the C2 domains and PKC evolved. We have previously used bioinformatics to probe the origins of the PKC family and found that (1) the four families of PKCs were already well established by the bilaterian ancestor; (2) The catalytic domains of PKCs and the related kinase protein kinase N (PKN)s are equally similar to fungal PKCs, and thus in early metazoans, the family first diverged into PKCs and PKNs; (3) atypical PKCs diverged from both conventional and novel PKCs before conventional, type I and type II PKCs diverged [2]. Since this study, the genomes of a number of primitive metazoans or species closely related to metazoans, including the choanoflagellates, Salpingoeca and Monosiga brevicollis as well as Capsaspora owczarzaki have been sequenced. We probed these organisms using Blast searches with the C2 domains from type I, type II and conventional PKCs as well as the fungal and PKN-C2 domains and examined proteins that contained C2 domains linked to a catalytic domain with strong homology to PKCs. We then used these C2 domains, as well as an assortment of other metazoan C2 domains from PKCs and PKNs as well as C2 domains from fungal PKCs to probe the evolutionary relationship of C2 domains. We used two additional well-conserved C2 domain families as reference points, the C2a domain from rabphilin, and the C2 domain from the ras GTPase-activating protein 3. These were chosen as the closest C2 domains to the novel and conventional C2 domains observed in blast searches. This analysis (Fig. 29.2) shows that C2 domains of fungal PKCs, PKNs, type I and type II PKCs form a well supported family of related C2 domains that presumably descended from a common ancestor. In contrast, the conventional PKC-C2 domain is first found associated with the catalytic domain of PKCs in choanoflagellates and is not directly related to the C2 domains in novel PKCs.

In Capsaspora owczarzaki, an organism that diverged from metazoans soon after the split between metazoans and fungi [22], there are three proteins with a catalytic domain most similar to PKCs/PKNs. One (gb/EFW44540) has the structure of a novel PKC (C2 domain N-terminal to tandem C1 domains), and the C2 domain of this protein segregates in the analysis with type I novel PKCs or epsilon-like novel PKCs (Figs. 29.1 and 29.2). Additionally, a protein is found (gb/EFW40430.1) with a PB1 domain and a kinase domain segregating with atypical PKCS (Fig. 29.1 and data not shown); notably this protein retains tandem C1 domains predicted to bind diacylglycerol, similar to yeast and animal PKCs, unlike the single atypical C1 domain found in all metazoan atypical PKCs. The third kinase has both the HR1 domains associated with mammalian PKNs and a C1 domain associated with PKCs (gb/EFW43140). The C2 domain in this protein segregates with the PKN C2 domains (Figs. 29.1 and 29.2), and thus we assume that this protein represents the PKN ancestor that had not yet completely lost its C1 domains.

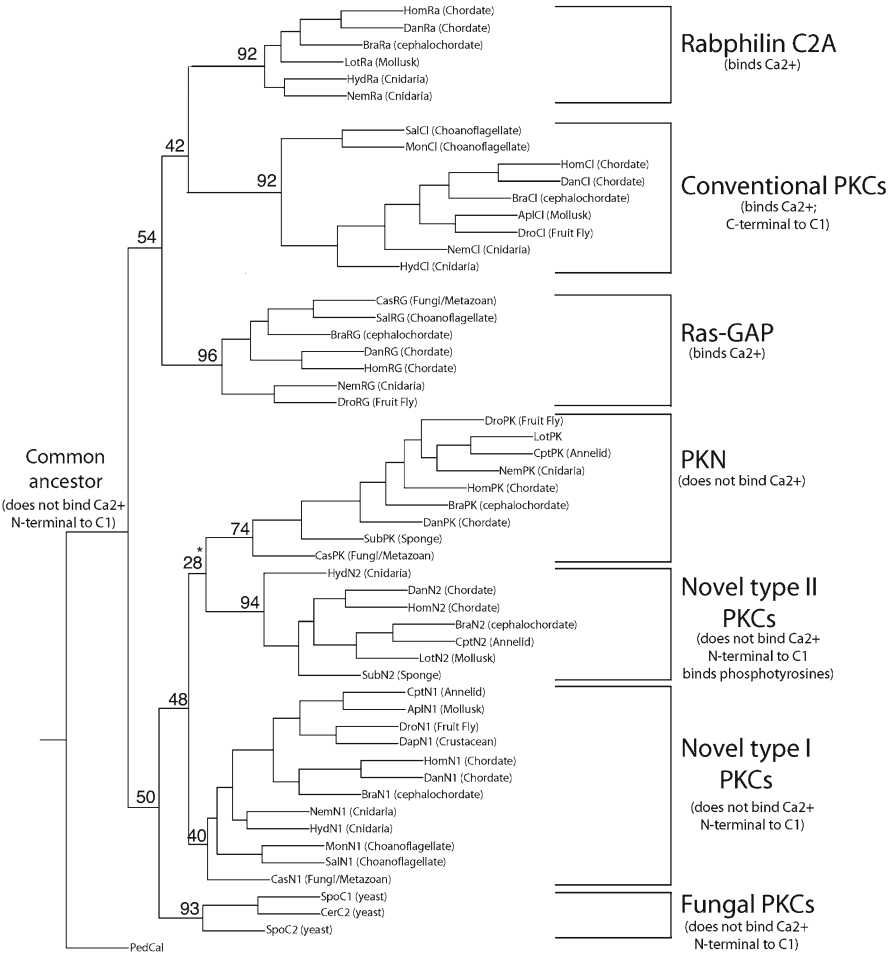

Fig. 29.2 Evolution of C2 domains. Sequences were obtained either from the NCBI site, http://blast.ncbi.nlm.nih.gov/Blast.cgi, or the JGI Genome site, http://genome.jgi-psf.org. Sequences were aligned with Clustal-W, 300 replicates were generated with the Phylip program Seqboot and then the Phylip program ProtDist was used with the Jones-Taylor-Thornton model to generate a Distance Matrix. The Phylip program Neighbor was then used to generate trees from each repetition, the program Consense to generate the consensus tree and Drawgram to make the final tree shown. Confidence numbers are given for critical nodes and represent the percentage of total trees that gave the tree shown. For more information on the workings of these programs, please see http://evolution.genetics.washington.edu/phylip/phylipweb.html. *Abbreviations* for species are: *Apl* Aplysia (Mollusk), *Bra* Brachiostoma (Lancelet, cephalochordate), *Cas* Capsaspora (Fungi/Metazoan), *Cer* S. Cervisiae (yeast), *Cpt* Capitella (Annelid), *Dan* Danio (Zebrafish; Chordate), *Dap* Daphnia (Crustacean), *Dro* Drosophila melanogaster (Fruit Fly), *Hom* Homo (Human; Chordate), *Hyd* (Hydra; Cnidaria), *Lot* Lottia (Mollusk), *Mon* Monosiga Brevicollis (Choanoflagellate), *Nem* Nemostella (Sea Anemone; Cnidaria), *Sal* Salpingoeca (Choanoflagellate), *Spo* S. Pombe (yeast), *Sub* Suberites domoncula (Sponge). The number of trees that matched the consensus tree is shown. *Other possible trees with close, but lower scores; Fungal PKCs with PKNs (20%); Type I and Type II PKCs (15%)

The choanoflagellate Salpingoeca also contains a protein with the structure of a novel PKC whose C2 domain segregates with novel type I PKCs (gb/EGD78676; Fig. 29.1). Unlike Capsaspora, it contains a kinase, EGD75514, with a C2 domain that segregates with conventional PKCs (Fig. 29.2) connected to a catalytic domain that also segregates with conventional PKCs (data not shown). However, this kinase does not contain C1 domains, but instead EF hands at the N-terminal. There is an additional protein, EGD77978 that also has a C2 domain attached to a kinase domain most similar to PKCs, although this C2 domain and kinase domain was difficult to assign to a particular group and is not included in the analysis in Fig. 29.2. This protein has a Src homology (SH2) domain at the carboxy-terminal of the kinase domain (Fig. 29.1). Similar kinases are found in the other choanoflagellate Monosiga brevicollis. We did not find a C2 domain that segregated with atypical PKCs, PKNs or type II novel PKCs in choanoflagellates.

In the Phylip analysis, the C2 domain of type II novel PKCs segregate with the PKN C2 domains, although this was not a strong association (Fig. 29.2). While it is conceivable that novel type I PKCs diverged from PKNs after the divergence of PKCs and PKNs, the previous analysis of kinase domains gave the opposite result [2]. The first appearance of two novel PKCs that are clearly related to the delta novel PKCs or type II nPKCs is in sponge and nematostella, a cnidarian. It is possible, and perhaps likely, that the divergence occurred earlier but in those genomes examined, type II PKCs were lost in the species whose genome has been sequenced. Thus, while this analysis does not determine at what point type I and type II novel PKCs diverged (either before conventional PKCs diverged or afterwards), it seems clear that the C2 domains of both of these proteins, and of PKN are ancestral, while the C2 domain of conventional PKCs became joined with the kinase domain in early metazoans. Thus, the C2 domain N-terminal to the C1 domain arrangement in novel PKCs is the ancestral arrangement, while the conventional PKCs represent a newer evolutionary event.

Recently, Zhang and Aravind [23] performed sequence-structure analysis of the C2 domain combined with phylogenetic analysis to infer the ancestral functions and subsequent diversification of C2 domains during eukaryotic evolution. They identified several novel versions of the C2 domain and their analysis shows that all families of C2 domains, except for PKC-C2 domains, lack the calcium-binding signature [23]. In agreement with our findings, they suggest that the common ancestor of all C2 domains probably did not bind calcium [23].

C2 Domain Structure

The C2 domain comprises about 130 residues and was first identified as the second of four conserved domains in the mammalian calcium-activated PKCs (Fig. 29.3; [26, 27]). The notion that this domain could act as a calcium-binding motif came from the observation that cPKCs which contained a C2 domain were regulated by calcium whereas nPKCs which were originally thought to lack a C2 domain, were

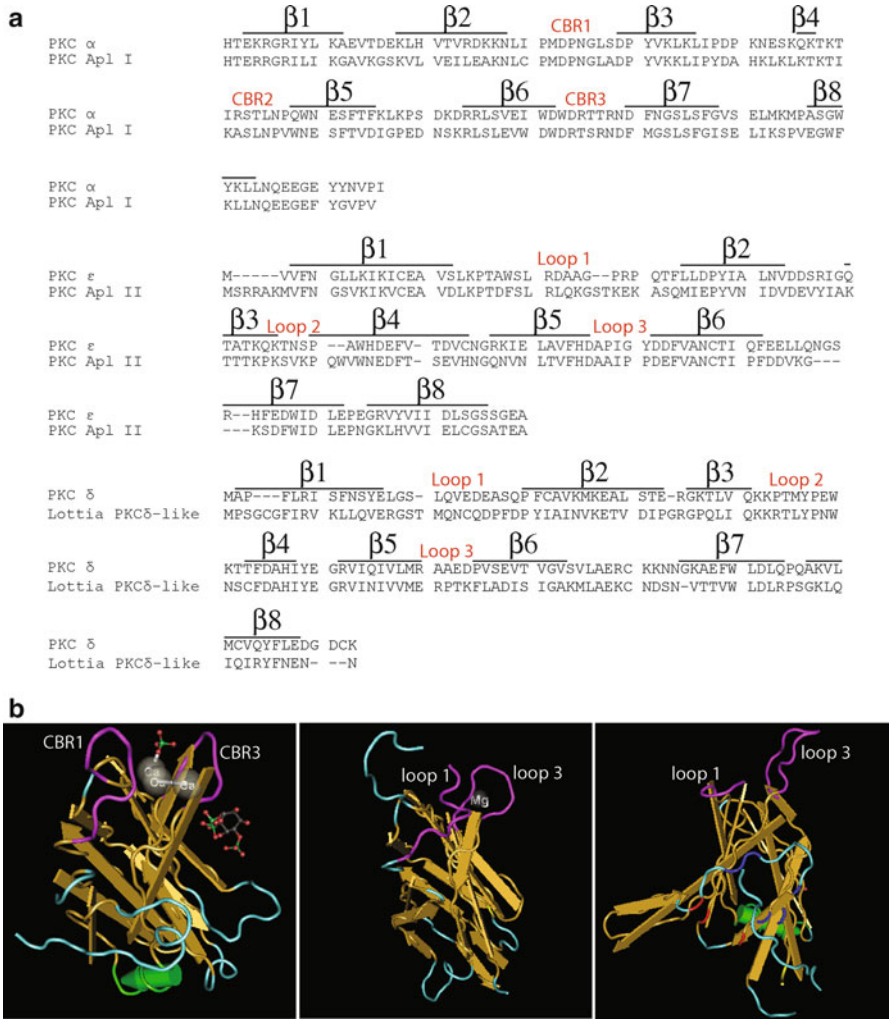

Fig. 29.3 Sequence alignment of the C2 domains and a representation of the overall structure of the C2 domains in PKCs. (**a**) Comparison of the C2 domains from vertebrate (*Mus musculus*) and invertebrate (*Aplysia californica*) conventional and novel PKCs of the epsilon family as well as from vertebrate (*Mus musculus*) and invertebrate (*Lottia*) novel PKCs of the delta family. The sequence of the conventional Ca^{2+}-dependent PKCα was aligned with that of PKC Apl I, the novel Ca^{2+}-independent type I PKCε with that of PKC Apl II and the novel Ca^{2+}-independent type II PKCδ with that of the Lottia PKCδ-like protein. *CBR*, calcium-binding region. (**b**) Three-dimensional representation of the structure of the different C2 domains based on the C2 domain of PKCα (*left panel*, [24]), the C2 domain of PKCε (*middle panel*, [25]) and the C2 domain of PKCδ (*right panel*, [15]). The structures were generated using CN3D 4.1 (produced by the National Center for Biotechnology Information; http://www.ncbi.nlm.nih.org). CBR1 and CBR3 are highlighted in *purple* in PKCα-C2 domain (*left panel*). *Loop 1* and *loop 3* are highlighted in *purple* in PKCε (*middle panel*) and PKCδ (*right panel*) C2 domains. In PKCδ-C2 domain (*right panel*), the residues that interact with the phosphates are highlighted in *red* and the residues that make contact with the peptide are highlighted in *dark blue* [15]

calcium-independent [26, 27]. We later reported that nPKCs do contain a phylogenetically conserved C2 domain that doesn't bind calcium [14, 19, 28]. The topology of the β-strands that form the C2 domains in conventional and novel PKCs is different [8]. The conventional C2 domains have a type I or a synaptotagmin-like topology whereas the novel C2 domains have a type II or phospholipase C (PLC)-like topology, consistent with their lack of common ancestry (see above). Topology I and topology II have a similar structure but differ in the orientation of the eight β-strands that form the C2 domain.

Calcium-Binding C2 Domain

The first C2 domain structure to be elucidated was that of PKCβ [29, 30]. The crystal structure of the C2 domain complexed with Ca^{2+} and o-phospho-L-serine has been determined to 2.7 Å resolution. The C2 domain of PKCβ is an antiparallel β sandwich similar in structure to the first C2 domain of synaptotagmin I with which it shares 28% amino-acid sequence identity. Each of the two sheets is composed of four antiparallel β strands. The Ca^{2+}-binding sites are located between the polypeptide loops that connect β2 to β3 and β6 to β7 and have been designated calcium-binding regions (CBRs; Fig. 29.3) I and III, respectively [31]. Three Ca^{2+}-binding subsites are arranged linearly within a broad trough lined by aspartate residues in loops β2–β3 and β6–β7 and a phosphoserine molecule binds to a lysine-rich cluster in C2. The authors suggested by model building that the C1 domain could provide carboxylate and carbonyl ligands for two of the three calcium sites and that Ca^{2+}-mediated interactions between the C1 and the C2 domains could contribute to enzyme activation as well as to the creation of a positively charged PS-binding site. Verdaguer and colleagues [32] later elucidated the three-dimensional structure of the Ca^{2+}-bound PKCα-C2 domain in the absence or presence of 1,2-dicaproyl-*sn*-phosphatidyl-L-serine (DCPS) by X-ray crystallography at 2.4 and 2.6 Å resolution respectively. The structure of PKCα-C2 domain retains most of the features found in the C2 domain of PKCβ including the organization of the calcium-binding region. However, only two calcium ions were found to bind to PKCα-C2 domain. The location corresponding to the third PKCβ-C2 calcium-binding subsite is occupied by a water molecule in PKCα-C2. In this study, a different model was suggested to explain the interaction of cPKCs with membranes. In this model, one calcium ion directly mediates the PS-specific recognition while the CBR3 might penetrate into the phospholipids bilayer [32]. Ochoa and colleagues [33] later determined the structure of PKCα-C2 domain crystallized in the presence of Ca^{2+} with either 1,2-diacetyl-sn-phosphatidyl-L-serine (DAPS) or 1,2-dicaproyl-sn-phosphatidic acid (DCPA) at 1.9 and 2.0 Å respectively. The structures showed the presence of an additional binding site for anionic phospholipids. The additional site was not located inside the calcium-binding pocket but in the vicinity of the conserved lysine-rich cluster in the concave surface of the C2 domain. In the DCPA complex, the absence of the serine headgroup and of its specific interactions seems to reduce the affinity

for the binding site inside the calcium pocket. Furthermore, in the DAPS-C2 domain complex, a third Ca^{2+}, which binds an extra phosphate group, was identified in the CBRs. In the complex with DCPA, the third Ca^{2+} might be present with partial occupancy. The third calcium ion is likely to bind with extremely low affinity in solution but might become trapped physiologically in a ternary C2 domain–membrane complex similar to what has been proposed to happen in PKCβ [34]. Finally, Guerrero-Valero and co-workers [24] determined the 3D structures of the ternary and quaternary complexes of the C2 domain of PKCα, crystallized in presence of Ca^{2+} and 1,2-diayl-*sn*-glycero-3-[phosphoinositol-4,5-bisphosphate] [PtdIns(4,5)P2] or Ca^{2+}, PtdIns(4,5)P2 and 1,2-dihexanoyl-*sn*-glycero-3-[phospho-L-serine] (PtdSer). They showed that PtdSer binds specifically to the calcium-binding region, whereas PtdIns(4,5)P2 occupies the concave surface of strands β3 and β4 allowing for the domain to be anchored to the membrane by two points. They further show that Tyr-195 and Trp-245 directly interact with the phosphate group of the inositol ring and mutating those residues impairs the ability of PKCα to localize to the plasma membrane [24].

Calcium-Independent C2 Domain

Ochoa and colleagues have determined the crystal structures of PKCε-C2 domain crystallized both in the absence and in the presence of the two acidic phospholipids, 1,2-dicaproyl-sn-phosphatidyl-L-serine (DCPS) and DCPA at 2.1, 1.7 and 2.8 Å resolution, respectively [25]. PKCε-C2 domain structure is an eight-stranded, antiparallel, β-sandwich with a type II topology similar to that of the C2 domains from PLC and from novel PKCδ (Fig. 29.3). Site-directed mutagenesis experiments and structural changes in the PKCε-C2 domain from crystals with DCPS or DCPA indicated that loops joining strands β1-β2 and β5-β6 participate in the binding to anionic membranes (Fig. 29.3; [25]). The pocket situated between loops 1 and 3 in the C2 domain of PKCε presents major differences with the corresponding pocket, the Ca^{2+}-binding pocket, defined by the CBRs in conventional PKCs. Ca^{2+} ions bind to the Ca^{2+} binding C2-domain in conventional PKCs mainly through the carboxylate groups from five conserved aspartate residues (187, 193, 246, 248 and 254 in PKCα) situated in CBR1 and CBR3. Three of the five conserved Ca^{2+} binding aspartate residues, 187, 246 and 248 in PKCα, are replaced by residues Phe36, His85 and Ala87, respectively, in PKCε. The two aspartate residues still present in PKCε, Asp39 (from loop 1) and Asp93 (from loop 3), appear to play only a structural role [25]. Furthermore, Asp86 and Asp92 in the β5-β6 loop help coordinate a magnesium (Mg^{2+}) ion one directly and one indirectly. A model for binding of PKCε-C2 domain to membranes was proposed by Ochoa and colleagues who stipulated that loop 1 would remain mostly on the surface of the membrane while loop 3 would insert into the membrane.

The crystal structure of PKCη-C2 domain was later elucidated by Littler and colleagues [35]. The structure is similar to that of PKCε-C2 domain except for

differences in the loop regions C-terminal to the two α-helices: the structure of PKCε becomes flexible for several residues immediately C-terminal to α1 following Asp-27, PKCη instead forms a loosely structured but well-defined helical turn [35]. The authors further identified two potentially phosphorylated serine residues contained within helix α1 which might regulate PKCη.

Phosphotyrosine-Binding C2 Domain

Even though PKCε-C2 and PKCδ-C2 domains share the same type II topology and diverged from a common ancestor (see above), they are considered to be representatives of different PKC subclasses. Indeed, they share only a 19% sequence identity with a number of insertions and deletions that correspond to large structural differences observed [25]. These differences include the presence in the PKCδ-C2 domain structure of a helix, between strands β6-β7, and of a protruding β hairpin with a mostly basic sequence that might define an interaction site with anionic membranes unique to PKCδ-like molecules (Fig. 29.3; [36]). Most importantly, the C2 domain of PKCδ directly binds to phosphotyrosine peptides in a sequence specific manner as shown by Benes and colleagues [15] who elucidated the crystal structure of PKCδ-C2 domain bound to a peptide containing phosphotyrosine. Prior to the Benes study, the only signaling domains known to recognize phosphotyrosine were the SH2 and the phosphotyrosine binding domains [PTB] [15, 37–39]. Benes and colleagues showed that PKCδ-C2 domain mediates the association of PKCδ with a transmembrane protein overexpressed in colon cancer, CDCP1. The activity of Src promotes this interaction by phosphorylating key residues on CDCP1, which leads to the formation of a CDCP1-Src-PKCδ complex [15]. Moreover, the crystal structure of PKCδ-C2 domain bound to a substrate peptide revealed a novel mode of phosphotyrosine recognition, different from the ones observed in SH2 and PTB domains. Indeed, the interaction with the tyrosine is maintained by stacking against a histidine residue. Overall, the structure of PKCδ-C2 domain is significantly divergent from other C2 domains only in the region where phosphotyrosine binding occurs which is situated on the opposite side of where calcium and phospholipids bind in cPKCs.

Functions of the Different C2 Domains

Calcium-Dependent Binding to Phospholipids

Conventional PKC-C2 domains clearly function as a Ca^{2+}-regulated membrane anchor. Coordination of calcium in the C2 domain causes a dramatic increase in the affinity of cPKCs to phospholipids [13, 32]. Binding to calcium is thought to be the primary step in kinase activation. First, it transiently recruits the enzyme to

the membrane where its physiological activator, DAG, resides. Second, in conjunction with the C1 domain interacting with DAG, binding of the C2 domain to PS and PIP2 provides the energy to release the autoinhibitory pseudosubstrate sequence from the substrate-binding cavity, allowing substrate phosphorylation [11–13, 24, 32, 40–43]. As such, vertebrate cPKCs as well as the cPKC Apl I from *Aplysia californica* both require calcium in association with DAG for translocation to membranes and activation [44, 45].

It is important to note that while both PS and PIP2 bind to the conventional PKC-C2 domain in a calcium-dependent manner, they bind in different ways. PS binds to the C2 domain through the Ca^{2+}-binding region with calcium acting as a bridge between the protein and the membrane lipid while in the PIP2 binding mode, Ca^{2+} is needed but does not directly mediate the phosphoinositide-domain interaction, since PIP2 binds to the lysine-rich cluster which is more distant [11, 13, 24, 32, 41, 43]. Furthermore, PS and calcium binding seems to be a prerequisite for productive phosphoinositide binding [41]. Indeed, PIP2 alone cannot drive the membrane attachment of the domain but further stabilizes the Ca^{2+}- and PS-dependent membrane binding [41].

Multiple studies have suggested that the C1 domain of cPKCs is masked in the inactive form of the kinase by the C2 domain and is inaccessible to DAG [8, 12]. This phenomenon was first described for cPKCγ, when Oancea and Meyer showed that the isolated C1 domain translocated to cellular membranes with exogenous DAG far more quickly than did the full-length PKCγ, while, in response to Ca^{2+}, the isolated C2 domain translocated to membranes with the same kinetics as the full-length protein [46]. They postulated that the region N-terminal to the C1 domain encompassing the pseudosubstrate acts as a clamp to keep the C1 domain inaccessible to DAG. The clamp is released when the C2 domain binds to membranes. Slater and co-workers later demonstrated that the complete C1 domain of PKCα was able to bind and activate the full-length enzyme in a phorbol ester/DAG-dependent manner in the absence of phospholipids. Furthermore, it was found that the C1 domain bound to a protein fragment containing the C2 domain of PKCα, and they proposed that the activating conformational change in PKCα results from the dissociation of intramolecular interactions between the C1 and C2 domains [47]. Stahelin and co-workers further performed homology modeling and a docking analysis of C1A and C2 domains of PKCα which revealed a highly complementary interface that comprised Asp55-Arg252 and Arg42-Glu282 ion pairs [48]. Mutations of these residues in the predicted C1A-C2 interface showed large effects on *in vitro* membrane binding, enzyme activity, PS selectivity and cellular membrane translocation of PKCα supporting their involvement in interdomain interactions [48]. Finally, Stensman and Larsson [49] showed that acidic residues in the C-terminal tail of PKCα bind basic residues in the C2 domain, and that this interaction maintains the kinase in a closed conformation that masks the C1a domain. The C1a domain is presumably revealed when the basic patch on the C2 domain engages PIP2 in the membrane [49]. This model is strengthened by the evidence that, while mutation of residues in either charged region to Ala residues sensitizes the enzyme to exogenous DAG, swapping the residues in the basic and acidic patches restores DAG insensitivity [49].

While the above studies are in favor of C1-C2 domain interactions and C2 domain-mediated inhibition, a recent study by Leonard and co-workers [50], who elucidated the crystal structure of PKCβII, suggested the conformation of PKCβII is best described by a single closed auto-inhibited state in which the C2 domain projects away from, and has limited contact with, the rest of the structure [50]. In their model, PKCβII translocates to the membrane upon Ca^{2+} release in the cell, where the calcium-binding regions of its C2 domain mediate bridging to PS, with an adjoining site on the C2 domain binding PIP_2 [50]. Subsequent binding of DAG to the C1A domain results in disengagement of the C1A domain, which in turn forces the removal of the pseudosubstrate from the catalytic cleft. There are a number of possible explanations for the discrepancy between this model (C2 domain as a separate module) and the earlier studies (C2 domain interacting with the C1 domains). First, since the C2 domain in the crystal was implicated in the intermolecular interactions involved in generating the crystal, its placement in the native protein may not be the same as in the crystal. Second, C1-C2 domain interactions may be isozyme specific in cPKCs. Indeed, Johnson and co-workers [51] showed that the C1 and C2 domains of PKCβII were independent membrane-targeting modules, with each, independently of the other, containing determinants for membrane recognition [51]. More crystallization studies of the different PKC isoforms will help to address this issue.

Calcium-Independent Binding to Phospholipids

While structure analysis (see above) revealed binding of the novel C2 domain to phospholipids, several studies reported a low binding affinity or no binding to phospholipids both *in vitro* and *in vivo* [48, 52–55]. As such, Jose Lopez-Andreo and colleagues reported that the C2 domain of PKCε can bind to PA but the amount of PA required to bind to the C2 domain is high compared to that for the Ca^{2+}-dependent binding of the C2 domains of cPKCs to PS [48, 55–58]. Furthermore, Giorgione and co-workers showed that C2 domain deletion in PKCε showed no effect on binding to PS/DAG containing membranes [48, 55]. However, in this study, saturating amounts of DAG were used, and this might have compensated for the lack of the C2 domain. In *Aplysia*, deleting the C2 domain in PKC Apl II did not affect translocation in response to 1,2-dioctanoyl-*sn*-glycero-3-phosphate (DiC8-PA), a cell permeable analog of PA [54].

Whereas C2 domain seems to bind poorly to lipids, Pepio and Sossin have shown that phosphorylation of PKC Apl II-C2 domain greatly increases its binding affinity to phospholipids [59]. However, a PKC Apl II with the C2 phosphomimetic mutation showed less translocation than the wild type protein in Sf9 cells and in isolated sensory neurons from *Aplysia* (Farah, CA and Sossin, WS, in preparation). It rather seemed that phosphorylation of the C2 domain was acting on C2 domain-mediated inhibition and that lipid binding to the C2 domain of PKC Apl II is dissociated from protein translocation in vivo (Farah, CA and Sossin, WS, in preparation).

In agreement with this, mutating isoleucine 89 to an asparagine in loop 3 of the vertebrate PKCε decreases translocation in response to DOG and to DiC8-PA in RBL-2H3 cells but does not affect PKCε activation by PA in an *in vitro* assay [58].

While the contribution of lipid binding to the C2 domain to protein translocation of nPKCs *in vivo* remains unclear, there seems to be a consensus that the novel C2 domain acts as an autoinhibitory module that impedes DAG binding to the C1 domains [12, 48, 53–55, 60]. Indeed, For PKC Apl II, removal of the C2 domain lowered the amount of lipid required to activate the enzyme [52, 61]. Furthermore, Pepio and Sossin showed using mixed micelle assay that the presence of the C2 domain lowers the affinity of the C1 domain to phorbol esters and this inhibition is removed by PA demonstrating that C2 domain-mediated inhibition could be regulated [53]. Farah and colleagues later confirmed that the C2 domain of PKC Apl II interacts with its C1 domain to inhibit DAG binding and that PA activates the kinase by binding to the C1b domain and removing C2 domain-mediated inhibition in live cells [54]. Binding to PA was mediated by Arg-273 in the C1b domain and this residue is conserved in the nPKCε suggesting this mechanism is conserved through evolution. In agreement with this, the C1b domain of PKCε was shown to preferentially bind to PA-containing vesicles [62].

In vertebrates, evidence for C2 domain-mediated inhibition of nPKCs came from a study by Stahelin and coworkers who reported that the deletion of the C2 domain of PKCε induced a faster membrane translocation in HEK293 cells [48]. Furthermore, the C2-deleted construct had a higher affinity (~60%) for PS/DAG membranes and a higher level of activity (<50%) than the PKCε wild type. Moreover, Melowic and colleagues reported that the deletion of the C2 domain of PKCθ greatly enhanced its affinity to PS/DAG-containing membranes. The authors proposed that the C2 domain of PKCθ is involved in keeping the enzyme in an inactive conformation, presumably by interacting with the C1a and C1b domains [60]. For PKCδ, removal of the C2 domain induces a faster response of the protein to C1 ligands [55].

Both PKCε and PKC Apl II require PA production for translocation in cells and it was shown that DAG synergizes with PA for translocation of both proteins [54, 58]. However, the model proposed for translocation of PKCε stipulates that synergism is due to PA binding to the C2 domain [58]. The main justification for this model was the *in vitro* binding of the C2 domain of PKCε to PA and the lack of translocation when residues in the C2 domain responsible for PA binding were mutated. While these mutations decreased the affinity of the C2 domain for PA, they also could have strengthened C1-C2 domain interactions, and this could be the reason for their effect on translocation. For PKC Apl II, it was suggested that PA binds first to the C1b domain to remove C2 domain-mediated inhibition and allow for DAG binding to the C1 domains [54]. One possible model that would encompass the two suggested ones is that PA would bind to the C1 domain first removing C2 domain-mediated inhibition and disengaging the C1 domains. Once C2 domain-mediated inhibition is removed, the C2 domain would bind to PA and assist in protein translocation and activation along with DAG binding to the C1 domains. In favor of this model, Farah and co-workers reported that PKC Apl II R273H, in which C2 domain-mediated inhibition is removed, translocates slightly better in response to 5HT in

isolated sensory neurons than PKC Apl II ΔC2-R273H suggesting a small positive contribution of the C2 domain once C2 domain-mediated inhibition has been removed [54].

Phosphotyrosine Binding

PKCδ can be activated by tyrosine phosphorylation independently of cleavage or production of DAG and this mechanism is specific to this class of PKCs [63, 64]. Different tyrosine residues were shown to be phosphorylated in PKCδ depending on cell stimuli [64–67]. PKCδ can be phosphorylated by various tyrosine kinases and can even associate with tyrosine kinases such as Src, Fyn, Lyn, Abl, PYK2, Lck, and growth factor receptors [64, 65, 67–76]. The mechanism of interaction of PKCδ with tyrosine kinases remained unclear until Benes and co-workers discovered that the PKCδ-C2 domain was a phosphotyrosine-binding domain [15]. Indeed, phosphorylation of PKCδ on tyrosines was facilitated by binding of activated tyrosine kinases to the C2 domain of PKCδ [15]. The effects of tyrosine phosphorylation on PKCδ activity are diverse. Indeed, the catalytic activity of PKCδ was shown to be reduced by tyrosine phosphorylation in *v-ras*-transformed keratinocytes [77], and in epidermal cell treated with epidermal growth factor [69]. However, tyrosine phosphorylation enhanced PKCδ enzymatic activity in various cell lines following stimulation with phorbol esters, growth factors and hormones [65, 68, 78–83]. In particular, phosphorylation of PKCδ at Tyr-311 located in the hinge domain led to, and was critical for, activation of the kinase in response to H_2O_2 in COS-7 cells [64]. Thus, by interacting with phosphotyrosines, PKCδ-C2 domain controls activation of the kinase and allows for cross-talk between two distinct signaling pathways. Preliminary results indicate that PKCθ, which is a member of the same family and shares 70% homology with PKCδ, also contains a phosphotyrosine binding domain and is likely to be regulated by the same mechanism [15].

Other Protein–Protein Interactions

Mochly-Rosen and co-workers discovered that translocation of PKC was associated with binding of each activated PKC isozyme to a corresponding anchoring protein, which they termed RACK, for receptor for activated C-kinase [84–86]. Examples of RACK proteins include RACK1 for PKCβ and β'cop (RACK2), which is a member of the coatomer complex COPI that binds several coatomer proteins and the small G protein ARF, for PKCε [87–92]. Mochly-Rosen and co-workers showed that RACKs bind to a site on PKC, which is only exposed when the enzyme is activated by binding to its cofactors such as DAG and PS. This is consistent with the concept that in the inactive form of the enzyme, the C2 domain interacts with the C1 domain, and the C2 domain is then released after enzyme activation. Such binding brings

PKCs closer to their cellular substrates [93, 94]. The C2 domain was shown to be critical for binding to RACKs and peptides derived from RACK-binding site were shown to act as selective inhibitors of their respective PKC isozymes [85, 95, 96]. RACK binding site was also suggested to be masked in the inactive form of the kinase by intramolecular interactions taking place with a pseudo-RACK site also located in the C2 domain which resembles and mimics a sequence in the RACKs [85, 95, 96]. Peptides derived from the pseudo-RACK site were shown to be selective activators of their respective PKC isozymes.

Schechtman and co-workers demonstrated that mutating Asp-86 to an asparagine in the pseudo-RACK domain of PKCε inhibits protein translocation [97]. They proposed a model in which Asp-86 would be involved in the intramolecular interactions between RACK and pseudo-RACK sites [97]. While this in an attractive idea, it is also possible that this mutation is acting on C1-C2 domain interactions to increase C2 domain-mediated inhibition. Studies with pharmacological peptides derived from C2-RACK and C2-pseudo-RACK domains have allowed tremendous insight into the role of the different PKC isoforms in various pathological conditions and many of those peptides are currently in clinical trials, emphasizing the important role of C2 domain in PKC regulation [95].

One issue with the model of RACK binding is the cellular localization of RACKs. RACK1 is a ribosomal protein [98] that may be more important for PKC regulation of translation [99, 100], while β'cop is involved in retrograde trafficking from Golgi to ER, and may be important for PKC regulation of Golgi trafficking [87, 101]. However, PKC translocation to different subcellular compartments, including the plasma membrane cannot be solely explained by these protein-protein interactions. It is possible that additional RACKs play important roles in PKC translocation. It is also possible that some of the actions of the peptides derived from RACK and pseudo-RACK domains are due to regulation of C1-C2 domain interactions, and not C2 domain-protein interactions.

Conclusions

In this chapter, we have described the multiple functions of the C2 domain in conventional, novel type I and novel type II PKCs. The C2 domain seems to act as a sensor of the intracellular signaling cascade activated in response to external stimuli. When calcium is produced as a second messenger, its binding to the conventional C2 domain along with DAG binding to the C1 domains will activate cPKCs. When PA is produced as a second messenger, its binding to the C1 domains of nPKCs will remove C2 domain-mediated inhibition and allow for binding of DAG to the C1 domains thus activating the kinase. Association of the C2 domain of nPKCs of the delta family with phosphotyrosines specifically regulates the activation of this subclass. Finally, protein-protein interactions involving the C2 domain were shown to be critical for kinase activation and for its subcellular localization in response to external stimuli.

It is worthwhile emphasizing that PKC can transduce signals not only in the plasma membrane but also in other subcellular compartments such as the Golgi, mitochondria and the nucleus in response to different stimuli [55, 102–104]. Interestingly, the magnitude and the duration of PKC signaling at these different compartments seems to be differentially regulated [105] and it will be interesting to determine the contribution of the C2 domain to the differential activation of PKC in those subcellular regions.

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