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Dynamics and Membrane Interactions of Protein Kinase C

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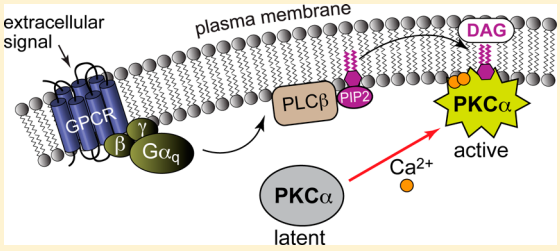
ABSTRACT: Protein kinase C (PKC) is a family of Ser/Thr kinases that regulate a multitude of cellular processes through participation in the phosphoinositide signaling pathway. Significant research efforts have been directed at understanding the structure, function, and regulatory modes of the enzyme since its discovery and identification as the first receptor for tumor-promoting phorbol esters. The activation of PKC involves a transition from the cytosolic autoinhibited latent form to the membrane-associated active form. The membrane recruitment step is accompanied by the conformational rearrangement of the enzyme, which relieves autoinhibitory interactions and thereby allows PKC to phosphorylate its targets. The multidomain structure and intrinsic flexibility of PKC present remarkable challenges and opportunities for the biophysical and structural biology studies of this class of enzymes and their interactions with membranes, the major focus of this Current Topic. I will highlight the recent advances in the field, outline the current challenges, and identify areas where biophysics and structural biology approaches can provide insight into the isoenzyme-specific regulation of PKC activity.



SCOPE

The discovery of PKC[1,2] and its subsequent identification as the receptor for tumor-promoting phorbol esters[3] have spurred the development of a vibrant research field that combines biochemical, genetic, and biophysical approaches. The hallmark of PKC activation is its translocation to cellular endomembranes that occurs in response to second messengers such as Ca^{2+}, diacylglycerol (DAG), and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]. This Current Topic focuses exclusively on the biophysical studies of the PKC isoenzymes and their interactions with membranes. The term "dynamics" is used in two different contexts. "Structural dynamics" refers to the changes in the tertiary structure of the enzyme that accompanies the membrane association step. "Protein dynamics" refers to the motions within the individual domains of PKC that occur on multiple time scales. The intrinsically dynamic nature of PKC is essential for its ability to interact with lipid ligands during the activation process.

INTRODUCTION

PKC is a family of multimodular ~80 kDa Ser/Thr kinases that regulate cell growth, differentiation, apoptosis, and motility.[4−6] All members of the PKC family consist of the N-terminal regulatory and C-terminal catalytic domains, connected by a proteolytically sensitive hinge region (Figure 1). Four novel PKC isoenzymes (nPKCs; ε, δ, θ, and η) are activated by DAG alone, whereas the four conventional PKC isoenzymes (cPKCs; α, βI, βII, and γ) require Ca^{2+} in addition to DAG. The atypical PKCs (aPKCs; ζ and ι/λ) do not bind either second messenger.

A general overview of the conventional PKC signaling pathway is shown in Figure 2A. G protein-mediated activation of phospholipase Cβ (PLCβ) results in the hydrolysis of lipids bearing the PtdIns(4,5)P_2 headgroup. Two second messengers are generated as a result of this reaction, DAG and inositol 1,4,5-trisphosphate (IP_3). The latter induces the release of Ca^{2+} from the endoplasmic reticulum. The recruitment of PKCs to endomembranes in response to DAG/Ca^{2+} relieves the autoinhibitory interaction within the enzyme, thereby activating its kinase function. The regulation of aPKC activity involves protein−protein interactions (reviewed in refs 7 and 8), but overall, their activation mechanism is not well understood.

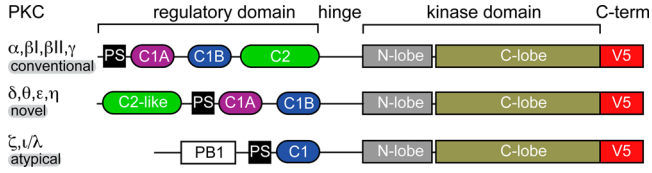


Figure 1. Multimodular structure of PKC isoenzymes. C1 and C2 are conserved region-1 and conserved region-2 domains, respectively. PS is a pseudosubstrate region. PB1 is a Phox and Bem1 domain. The most variable PKC regions are the N-terminal regulatory and C-terminal V5 domains.

Altered levels of expression or activity of DAG-dependent PKC isoenzymes have been implicated in a large number of human diseases such as cardiac disease, cancer, diabetes, and mood disorders.[9−12] PKCα, the most predominant isoenzyme in mouse,[13] rabbit,[14] and human[15] hearts, has been identified as a major regulator of heart contractility,[15,16] platelet aggregation in thrombosis,[17−19] and virtually every development stage of atherosclerotic disease.[20,21] Upregulation of PKCα has been

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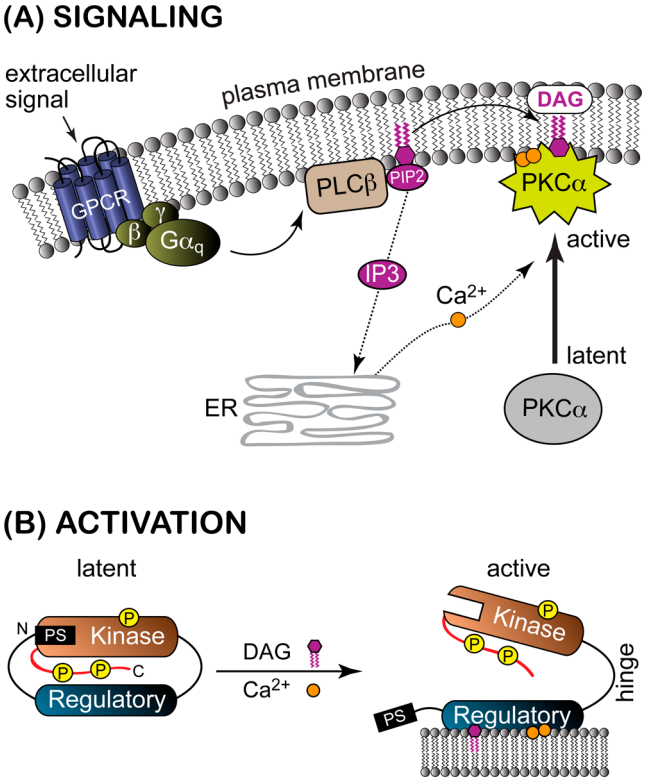


Figure 2. Signaling pathway (A) and activation (B) of PKCα. The abbreviations are given in the text. The C-terminal V5 domain is colored red. DAG-dependent PKCs are constitutively phosphorylated as part of the maturation process, with one site on the kinase activation loop and two sites on the V5 domain.

demonstrated in breast cancer cells,[22,23] melanoma cells,[24] and tissue samples of urinary bladder carcinomas[25] and malignant gliomas.[26] The opposing roles of PKCδ and PKCε in ischemic disease and reperfusion injury[27,28] demonstrated the need for isoenzyme-selective approaches to achieve downregulation[29,30] and upregulation[31−33] of the respective PKC isoenzymes.

Given the differential and sometimes opposing roles of PKC in human disease, there is a need to modulate their function in an isoenzyme-selective manner. One of the means of achieving that is to make use of the differences within the most variable regions, such as the N-terminal regulatory and C-terminal V5 domains. This would also require atomic-level information about PKC in both latent and activated states. However, the structural studies of the PKC family, especially at the atomic level, have not kept pace with the biochemical and genetic advances in the field. This is not due to a lack of effort but rather due to the challenging properties of this class of enzymes, such as their intrinsically dynamic and amphiphilic nature.

These two features are essential for enzyme function, as illustrated in the activation scheme of Figure 2B. The latent cytosolic form, which is autoinhibited by the N-terminal pseudosubstrate region,[34−37] is thought to be compact. The autoinhibitory interaction is released upon interaction of the N-terminal regulatory domain with endomembranes. The N-terminal domain consists of individual domains, each being independently folded and having a specialized function. C1 domains interact with membrane-embedded DAG, while the C2 domain of cPKCs interacts with phosphatidylserine (PtdSer) and PtdIns(4,5)P_2 in a Ca^{2+}-dependent manner. The C2-like domain in nPKCs is not a membrane-binding module. The intrinsic affinities of the C1 and C2 domains for their respective ligands determine the activation threshold of the parent PKC isoenzymes and contribute to the selectivity of the PKC response. It should be noted that PKC activation could be accomplished not only by DAG/Ca^{2+}-driven membrane recruitment but also by the proteolysis of the hinge region and destabilization of the latent form through interference with intramolecular interactions. One example of the latter is the activation of PKC by the pseudo-RACK1 peptide reported by Mochly-Rosen's group.[38]

The focus of this work is the biophysical and structural biology studies of conventional and novel PKC isoenzymes and their interactions with membranes. This Current Topic follows the structural hierarchy of PKC by starting with the full-length enzymes and then narrowing the scope to individual domains. For the latter, I will use several examples from the work of my laboratory.

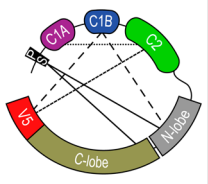
1. STRUCTURAL DYNAMICS OF PKC

The structural dynamics of PKC involves a conformational transition between the latent and activated states. The latent conformation is stabilized by intramolecular interactions. Identifying the structural basis of these interactions has been challenging because of the multidomain structure and the associated flexibility of PKCs, the requirement for sample homogeneity with respect to the phosphorylation state, and the sensitivity of the hinge region to proteolysis. This section summarizes the biophysical studies of latent and activated conformations of full-length PKC.

1.1. Latent PKC. In the latent form, the N-terminal pseudosubstrate (PS) region inhibits the active site, which is located between the two lobes of the catalytic domain. The PS sequence has an Ala instead of the phospho-acceptor Ser/Thr site, thereby achieving an autoinhibitory effect. The structural information about this interaction is currently unavailable for any PKC isoenzyme. Three other intramolecular interactions have been identified, all of which involve the membrane-binding modules of PKC.

1.1.1. "C1B Clamp". In 2011, a partial crystal structure of PKCβII from *Rattus norvegicus* was determined at 4 Å resolution [Protein Data Bank (PDB) entry 3PFQ].[39] One noteworthy feature of the structure is the C1B clamp formed by the three domains that are nonadjacent in the amino acid sequence: C1B, the N-terminal lobe of the kinase, and V5 (Table 1). It was proposed that this intramolecular interaction is inhibitory in nature because (i) the "clamping" of C1B onto

Table 1. Intramolecular Interactions in PKC Isoenzymes

Isoenzyme/ Ref.	Interacting domains	Methods	Schematic view of intramolecular interactions
βII[39]	C1B, V5, and N-terminal lobe of kinase	X-ray crystallography, mutagenesis, and phorbol-ester stimulated membrane translocation	
α[40,41]	C1A and C2	Computational modeling, mutagenesis, in-vitro membrane binding by SPR, DAG-stimulated cellular membrane translocation	
α[42]	C2 and V5	Mutagenesis, FRET, and DAG-stimulated cellular membrane translocation	

PS denotes the pseudosubstrate region responsible for autoinhibition.

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the conserved Phe629 of V5 prevents this residue from interacting with the adenine of ATP and (ii) residues 619−621 of V5 occlude the DAG-binding site of C1B. Another feature of the structure is that the C2 domain makes no intramolecular contacts but is involved in the interactions with the catalytic domain of the neighboring PKC molecules. Understanding how C2 contributes to the stabilization of the latent form at the atomic level will require further studies (*vide infra*). Three PKCβII regions: the pseudosubstrate, C1A domain, and the hinge region between the regulatory and kinase domains could not be identified in the electron density maps and, as a result, are not present in the structure. Overall, the PKCβII structure was interpreted as that of an "intermediate" (i.e., neither latent nor activated) state on the catalytic pathway.

1.1.2. C1 and C2. Using SPR, Slater et al.[41] detected a nanomolar affinity interaction between the chip-immobilized C1 domain consisting of tandem C1A−C1B domains (see Figure 1) and full-length PKCα. This interaction was dependent on the presence of PKC activator tetradecanoylphorbol acetate, a phorbol ester. The interaction partner of C1 was identified as the C2 domain by using the C1−C2 construct of PKCα. In addition, extrinsic addition of the C1 domain was shown to activate PKCα in a concentration- and phorbol ester-dependent manner. Overall, the data were consistent with C1 and C2 domains being engaged in intramolecular interactions that maintain PKC in the latent form.

The question of C1−C2 interactions was also explored by Stahelin et al.[40] Computational docking procedures were applied to the crystal structure of the C2 domain and the homology model of C1A to obtain information about the interdomain interface. From the analysis of the best-scoring docked structures, three putative interactions were identified between the *C1A* and C2 domains: *Asp55*−Arg252, *Arg42*−Glu282, and *Phe27*−Phe255. Disruption of the electrostatic interactions via mutagenesis resulted in the increase in the affinity of PKC for DAG-containing membranes, indicating that C1A is less conformationally restricted and therefore has a more accessible DAG-binding site. Simultaneous charge reversal resulted in PKC variants having membrane affinity comparable to that of the wild-type protein. The data support a direct interaction or "tethering" between the C1A and C2 domains, which is released by the phosphatidylserine component of the plasma membrane when it interacts with C2.

1.1.3. C2 and V5. The existence of intramolecular interactions between the C2 and the C-terminal V5 domains[42] (Table 1) was hypothesized on the basis of the observation that V5 binds the Lys-rich region of Syndecan-4, a known activator of PKCα. The C2 domain has a conserved positively charged region termed the "Lys-rich cluster" (LRC) that by analogy might be involved in intramolecular interactions with V5. Indeed, mutations of two V5 phosphorylation sites, Thr638 and S657, and the acidic residues of the V5 hydrophobic motif resulted in the increase in DAG sensitivity, as reported by the DAG-stimulated translocation of PKCα variants to the plasma membrane.[42] The DAG sensitivity of PKCα variants correlated with the conformational change that was probed using Förster resonance energy transfer (FRET) between the N- and C-termini in the ECFP−PKCα−EYFP fusion protein. Reintroduction of the putative C2−V5 interaction by simultaneous charge reversal of the LRC of C2 and the hydrophobic motif of V5 resulted in membrane translocation behavior similar to that of the wild-type protein. In aggregate, the data support the existence of intramolecular interaction between the C2 domain and the C-terminal V5 region that contributes to the stabilization of the latent form. The functional interplay between the C2 and V5 domains is also evident in PKCβII, where the phosphorylation of Ser660 of the hydrophobic motif increases the affinity of the enzyme for Ca^{2+} and PtdSer.[43]

1.1.4. Summary. In addition to the autoinhibition of the catalytic domain by the pseudosubstrate region, several intramolecular interactions have been identified in the conventional PKC isoenzymes that contribute to the stabilization of the latent form. There is also indirect experimental evidence of the existence of the C1−C2 interactions in novel isoenzymes[41] and a mention of the C2−V5 interaction in PKCε.[44] To assemble the latent form of PKC in its entirety, more detailed information at the atomic level is needed. Given the complexity of the enzyme and its dynamic nature, structural biology approaches that target "pairwise" interactions between the individual domains are viable routes. The validity of the structural findings can then be tested using mutagenesis and functional assays in the full-length systems.

1.2. Activated PKC. The canonical activation route involves the association of PKC with membranes (Figure 2A). To date, the only source of information about the tertiary structure of lipid-associated PKC is the electron miscroscopy study of PKCδ and its regulatory domain.[45] The two-dimensional crystals of both protein species were grown on a lipid monolayer comprising the phosphatidylserine component and DAG with molar concentrations of 45 and 5%, respectively. The three-dimensional (3D) image reconstruction revealed the overall shape of the proteins and allowed the tentative assignment of the domains engaged in the interactions with membranes (Figure 3). According to the assignment, a C1 domain, most likely the C1A domain, is a primary contact of PKCδ with the lipid monolayer. The interactions of the C2 domain with lipids are minor, as expected for a novel PKC isoenzyme. The small protrusion in the structure that contacts the C2 domain was assigned to the C-terminal V5 domain, by comparison with the 3D reconstruction of the regulatory domain alone. The potential role of V5 as a membrane interaction module is discussed in the section on individual domains.

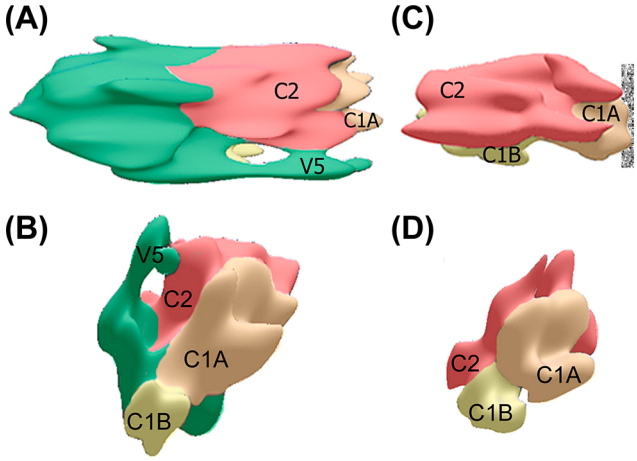

Figure 3. 3D reconstructions of PKCδ (A and B) and its regulatory domain RDδ (C and D) on lipid monolayers, showing the proposed assignments of individual domains (adapted from ref 45). Panels A and C represent the views from the membrane surface, which is shown with a textured bar. The catalytic domain is colored green.

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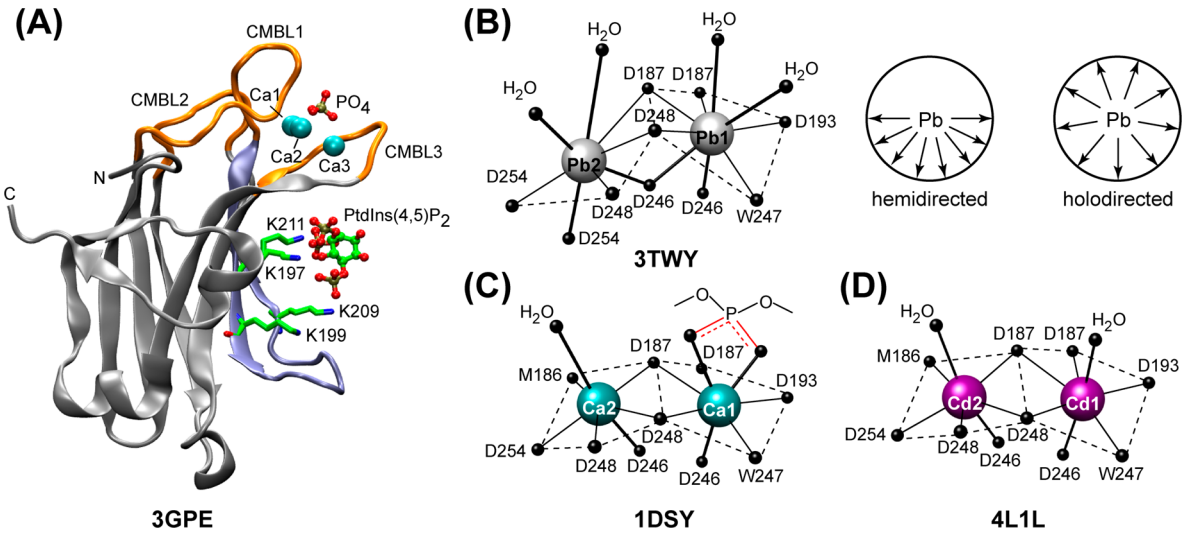

Figure 4. (A) Crystal structure of the C2 domain from PKCα complexed to Ca^{2+} and PtdIns(4,5)P_2.[60] CMBL and LRC regions are colored orange and blue, respectively. Adapted from ref 61: coordination geometries of (B) Pb^{2+}, (C) Ca^{2+}, and (D) Cd^{2+} ions bound to the C2 domain from PKCα. The ligands are the side-chain oxygens of aspartates, with the exception of W247 and M186, where it is the carbonyl oxygen. The coordination sphere of Pb2 is hemidirected: all eight ligands are located in one coordination hemisphere that is facing the viewer. The top axial ligands of Ca1 in PDB entry 1DSY are the phosphoryl oxygens of the short-chain PtdSer analogue.

1.2.1. Evidence of PKC Dimerization. The affinity of individual PKC domains to one another can also manifest itself in intermolecular interactions, provided that the interaction surfaces are solvent-accessible. The latter can be achieved in either fully or partially activated states of PKC. The homodimerization of conventional PKC isoenzymes was first demonstrated using cross-linking with sulfhydryl-selective reagent bis(maleimido)hexane.[46] Slater et al.[41] inferred dimerization from the concentration dependence of PKCα activity in the presence of enzyme activators. Recently, the homodimerization behavior of activated PKCα was characterized in detail using FRET between the donor and acceptor fluorophores that were placed on different PKCα molecules.[47] Using deletion and truncation PKCα variants, it was demonstrated that dimer formation is mediated by the interactions between the respective regulatory domains, as well as the regulatory and catalytic domains. For the latter, the C-terminal V5 domain is crucial in stabilizing the dimer interface: its deletion or mutation of Cys619 to Ala abolished dimerization.

1.2.2. Rearrangement of PKC Tertiary Structure Probed by TIRFM and FRET. The spatial rearrangement of the PKC upon maturation and encounter with membrane-embedded activators were recently explored in two separate studies that made elegant use of FRET[48] and single-molecule total internal reflection fluorescence microscopy (TIRFM).[49] Both studies used conventional PKC isoenzymes that have a Ca^{2+}-responsive C2 domain.

In single-molecule TIRFM experiments, the population-weighted diffusion coefficient of fluorophore-tagged PKCα and its regulatory domain(s) on supported lipid bilayers reported on the protein−membrane interactions. The initial diffusion step for the isolated C2 domain and the full-length PKCα was identical, indicating that C2−membrane interaction is the first step of the activation sequence, which is in agreement with previous functional studies.[50,51] However, the diffusion of full-length PKCα on lipid bilayers exponentially slowed with time, compared to the constant diffusion coefficient measured for the isolated C2. By using different truncation variants of the regulatory domain, the authors identified C1A as the entity involved in the membrane interactions in the absence of DAG. The incorporation of DAG into lipid bilayers resulted in reduction of the diffusion coefficient of both full-length PKCα and the C1A−C1B−C2 form. Frictional drag of both C1A and C1B was needed to quantitatively account for the changes in the diffusion coefficient, suggesting that both domains interact with DAG.

The authors proposed a model in which the Ca^{2+}-driven binding of the C2 domain to PtdSer- or PtdSer/PtdIns(4,5)P_2-containing membranes results in the formation of a "pre-DAG intermediate". In this intermediate, C1A is involved in ligand-independent interactions with membranes, thereby priming itself and C1B for binding to DAG. The interaction of C1B with DAG can then trigger the release of the pseudosubstrate region from the active site of the kinase.

To probe the conformational rearrangement of PKCs during maturation, activation, and downregulation in live cells, Newton's laboratory designed a set of Kinameleon cPKC constructs that had CFP and YFP placed at the N- and C-termini of PKCβII.[48] FRET changes in live cells were monitored under both unstimulated and stimulated conditions, with phorbol 12,13-dibutyrate (PDBu) as an agonist. The pattern of FRET ratios characteristic of different conformational states of PKCβII was established. Short stimulation by PDBu resulted in an increase in the FRET ratios compared to those of the latent form of PKCβII, consistent with the pattern observed previously for PKCδ.[52] Mutations that impair the kinase function of PKCβII and prolonged exposure of wild-type species to PDBu, known to cause PKC dephosphorylation and subsequent degradation, resulted in decreases in FRET ratios. These data correlate well with the kinetics of agonist-stimulated membrane translocation, measured by membrane-to-protein FRET. The kinetics reflects the accessibility of the C1 DAG/phorbol ester-binding site in different PKC states. Wild-type PKCβII and PKCα translocate to membranes slower than their kinase-deficient mutants and the C1A−C1B domain, indicating that the C1 ligand-binding site(s) is masked by the intramolecular interactions in the latent state. Toggling the DAG

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affinities of C1A and C1B by mutating a key residue[53] allowed the evaluation of their relative function roles. Both C1A and C1B contribute to the agonist-driven membrane binding, with C1B making a predominant contribution.

1.2.3. Summary. According to the current activation model that integrates the results of biophysical and biochemical studies of conventional PKC isoenzymes, the Ca^{2+}-dependent interaction of the C2 domain with PtdSer-containing membranes is the first step in the activation sequence. This interaction facilitates the intramolecular rearrangement of PKCs that makes the C1A and C1B ligand-binding sites accessible to membrane-embedded DAG. One such mechanism could be the DAG-independent insertion of the C1A domain into the membranes. The engagement of one or more PKC domains with membranes reduces the dimensionality of the search for DAG and thereby increases the effective affinity of C1B and C1A for DAG.

The functional and structural autonomy of individual PKC domains has been instrumental in dissecting the behavior of full-length enzymes in vitro and in live cells. This property also makes these domains amenable to structural biology studies in which residue-specific information can be readily obtained. The insights into PKC dynamics and membrane interactions obtained from this "divide-and-conquer" approach are discussed in the next section.

2. INDIVIDUAL DOMAINS OF PKC

2.1. C2 Domains. C2 (conserved region 2) domains are independently folded structural and functional modules comprising ~140 amino acids.[54,55] The C2-like domains of novel PKC isoenzymes do not bind Ca^{2+} or membranes but rather serve as protein−protein interaction modules.[56,57] In conventional PKC isoenzymes, the C2 domains target their parent enzymes to the inner leaflet of the PtdSer-containing plasma membrane in response to an increase in cytosolic Ca^{2+} concentration. The functional elements of Ca^{2+}-responsive C2 domains are the Ca^{2+}- and membrane-binding loops (CMBLs) and the LRC, a $\beta3-\beta4$ hairpin region bearing four lysine residues (Figure 4A). The latter are involved in interactions with PtdSer and/or PtdIns(4,5)P_2.[58,59] Three aspects of Ca^{2+}-dependent C2 domains are discussed here: binding to metal ions, dynamics, and membrane interactions.

2.1.1. Divalent Metal Ions. Binding of Ca^{2+} to C2 domains of conventional PKC isoenzymes is a prerequisite for the association of these domains with membranes. Inspection of the available C2 crystal structures reveals either di- or tripartite Ca^{2+} sites. Our solution nuclear magnetic resonance (NMR) data indicate that at moderate Ca^{2+} concentrations and in the absence of membranes, two Ca^{2+} sites of the C2 domain from PKCα (C2α) are populated.[62] The presence of PtdSer-containing membranes increases the Ca^{2+} affinity of C2 domains and results in binding of an additional Ca^{2+} ion to the protein−membrane complex.[63]

It has been hypothesized that the functional role of Ca^{2+} is to alter the electrostatic potential of C2 and thereby allow the domain to interact with anionic lipids.[64] Recent work from our laboratory suggests that the chemical identity of a metal ion has profound consequences for the protein−membrane interactions.[61,62] All three non-native divalent metal ions that we tested, Pb^{2+}, Cd^{2+}, and Cu^{2+}, bind to C2 with high affinity. However, only Pb^{2+} is able to act as a functional substituent of Ca^{2+} by promoting protein−membrane interactions. This is despite drastic differences in metal coordination: Pb^{2+} at position 2 adopts hemidirected coordination geometry, where all eight ligands are located in one coordination hemisphere, compared to Ca^{2+} that has a uniform spatial ligand distribution (Figure 4B,C). The ability of Pb^{2+} to act as a functional substituent of Ca^{2+}, relevant to the mechanism of Pb(II) toxicity, has been demonstrated in full-length PKCs.[65,66]

In contrast to Pb^{2+}, the coordination geometry of C2-bound Cd^{2+} is identical to that of Ca^{2+} (Figure 4D), yet Cd^{2+} is unable to facilitate protein−membrane interactions. These data support the idea of specific interactions between protein-bound metal ions and anionic lipids and indicate that altering the electrostatic potential of C2α is not sufficient to drive membrane association. Indeed, it was shown that charge reversal mutations in the negatively charged loop region of the apo C2 domain from PKCβII did not promote its Ca^{2+}-independent membrane recruitment.[67] In the crystal structures of C2, Ca^{2+} coordinates the phosphoryl oxygen(s) of the short-chain PtdSer analogue (see Figure 4B). In aggregate, these data suggest that the functional "competency" of divalent metal ions depends on their ability to expand the coordination sphere in the all-oxygen environment presented by the protein and anionic lipids, rather than their coordination geometry in the membrane-free state of C2 domains.

2.1.2. Dynamics. Ca^{2+} binding stabilizes conventional C2 domains, resulting in a 30−38 °C increase in the melting temperature.[68] A comparison of the apo and metal ion-complexed C2α structures[61,62,69] indicates that the average conformation of the protein backbone does not significantly change upon metal ion binding. One notable difference between the apo and metal ion-complexed structures is the conformation of the metal-coordinating side chains and elevated *B* factors for the CMBLs in apo C2α. This observation prompted us to investigate the backbone dynamics of C2α in different states of metal ligation (apo, C2α·Pb, and C2α·Ca_2) using NMR relaxation techniques (K. Morales and T. Igumenova, manuscript in preparation).

We found that CMBL1, CMBL3, and LRC in apo C2α have elevated dynamics on the subnanosecond time scale. The subnanosecond dynamics of C2α is not significantly affected by its interactions with Pb^{2+} or Ca^{2+}. In addition, CMBL1 and CMBL3 in apo C2α undergo a chemical exchange process that occurs on the microsecond time scale. Binding of a single Pb^{2+} is sufficient to attenuate this process, indicating a significant reduction in conformational flexibility. In the Ca^{2+}-complexed state of C2α, we detected a chemical exchange process that involves the N- and C-terminal regions, particularly a short α-helix H3 close to the C-terminus.

Molecular dynamics simulations conducted on the C2 domain from PKCβ[70] (βI and βII are the splice variants that differ only in the V5 domain) revealed that CMBL1, CMBL3, and the C-terminal α-helix H3 undergo significant fluctuations during the 1 ns trajectory. While these fluctuations are present in the Ca^{2+}-bound C2 structures, removal of Ca^{2+} increases their amplitude. The average position of CMBL1 is similar for the apo and Ca^{2+}-complexed protein; however, CMBL3 moves away from CMBL1 in the apo C2 presumably because of the electrostatic repulsion.

In summary, conventional C2 domains show a range of dynamic behavior on multiple time scales. Ca^{2+} binding alters the conformational exchange behavior of the loop regions and the termini. The conformational flexibility of loop regions appears to be a shared theme among the lipid-binding modules of PKC (*vide infra*).

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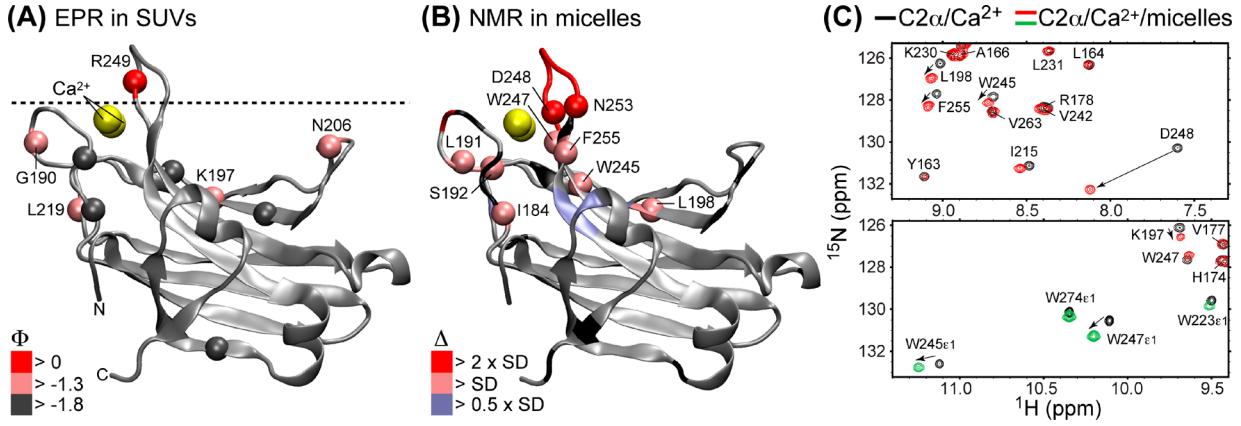

Figure 5. Probing the C2−membrane interactions with EPR and NMR. (A) Adapted from ref [74]. Depth parameters Φ are mapped onto the structure of the Ca^{2+}-complexed C2α (PDB entry 1DSY[69]). The spheres correspond to the backbone N−H groups, to facilitate a comparison with the NMR data. The dashed line represents the bilayer phosphate plane. (B) CSP analysis of C2α in mixed DPS/DPC micelles. The N-H groups are color-coded according to the deviation SD from the mean CSP value. Loop residues that enter an intermediate exchange regime upon micelle binding (N189, R249, T250, T251, and R252) are colored red, with no sphere representation. Prolines and residues whose N-H groups are not spectrally resolved are colored black. The CSP values were calculated as described previously[75] using $^{15}N-^{1}H$ HSQC spectra, two expansions of which are given in panel C. The data in panel C illustrate the changes in chemical shifts experienced by C2α upon interactions with mixed micelles; the cross-peaks of Nε1-Hε1 groups of the Trp side chains in the micelle-complexed form are colored green.

2.1.3. Membrane Interactions. High-affinity Ca^{2+}-dependent interaction of C2 domains with PtdSer[71] and PtdIns(4,5)P_2 is responsible for targeting their parent enzymes to the plasma membrane. According to the stopped-flow fluorescence study of C2α, hydrophobic and electrostatic interactions play a role in membrane recruitment, through their contributions to the "on" and "off" rates, respectively.[72]

The first structural information about the geometry of interactions of C2 with membranes was obtained by electron paramagnetic resonance (EPR) spectroscopy.[73] Small unilamellar vesicles (SUVs) composed of either POPC and POPS, or a lipid mixture designed to mimic the inner leaflet of the plasma membrane, were used as membrane mimics. Single-Cys variants of C2α were covalently modified with MTSL, a functional group bearing a stable nitroxide radical. Power saturation EPR measurements were conducted to determine the site-specific membrane depth parameters Φ, which were subsequently used to dock the crystal structure of the Ca^{2+}-complexed C2α onto the membrane. A model that emerged from the EPR restraints has the CMBL3 protruding into the headgroup region, while the CMBL1 and Ca^{2+} ions are at the headgroup−water interface (Figure 5A). The β3−β4 segment of LRC is positioned almost parallel to the membrane surface, because of the interactions between the LRC residues and the PtdSer headgroups.

It is instructive to relate this model to the results of NMR experiments that we conducted in a mixed micelle system comprising 1,2-dihexanoyl-*sn*-glycero-3-[phospho-L-serine] (DPS) and *n*-dodecylphosphocholine (DPC). The advantage of NMR is that the stable-isotope enrichment has no effect on the protein structure or its functional properties. Interaction of C2α with micelles changes the electronic environment of the NMR-active nuclei, resulting in the changes in their chemical shifts. The C2−micelle interaction is dependent on the presence of DPS and Ca^{2+}, indicating that mixed micelles are a good membrane mimic for this protein system.

An overlay of the $^{15}N-^{1}H$ HSQC spectra of Ca^{2+}-complexed C2α in the absence and presence of mixed DPS/DPC micelles illustrates the chemical shift perturbation (CSP) of the backbone N-H and the Trp side-chain Nε1-Hε1 groups due to binding (Figure 5B,C). For example, the chemical shifts of Trp245 and Trp247, two highly conserved residues that are essential for membrane association[50] and control the "on" rate of the process,[72] are significantly perturbed, suggesting a direct interaction with the micellar environment. Mapping the CSP onto the 3D structure of the Ca^{2+}-complexed C2 domain reveals that the primary membrane interaction site is CMBL3, with CMBL1 being less involved. The LRC-harboring face of C2α experiences mild CSPs (colored blue in Figure 5B), which is consistent with EPR data and further supports the near-parallel orientation of the domain with respect to the surface of the membrane mimetic. These and other types of experimental data can be further used as ambiguous restraints to dock C2 domains to membrane mimetics[76] and obtain the atomic-level information about the geometry of C2−membrane interactions.

2.1.4. C2 and PtdIns(4,5)P_2. Another lipid that is essential for targeting conventional isoenzymes to plasma membranes is PtdIns(4,5)P_2, whose effective molar concentration in the membrane is ∼1%. PtdIns(4,5)P_2 contributes to PKC−membrane interactions by increasing the residence time of the protein at the membrane.[59] Mutagenesis studies,[59,77,78] followed by the determination of the crystal structure of C2α complexed to Ca^{2+}, 1,2-diayl-*sn*-glycero-3-(phosphoinositol-4,5-bisphosphate), and 1,2-dihexanoyl-*sn*-glycero-3-(phospho-L-serine),[60] demonstrated that C2α interacts with the headgroup of PtdIns(4,5)P_2 via the LRC (see Figure 4A). The amine groups of three lysine residues of the LRC, K209, K211, and K197, are engaged in electrostatic interactions with the phosphate groups of PtdIns(4,5)P_2. In addition, the side chains of N153 and Y195 form hydrogen bonds with one of the phosphate groups. It was shown using site-directed EPR methods that PtdIns(4,5)P_2 alters the membrane docking geometry of C2α.[74] Through interactions with LRC, the bulky headgroup of PtdIns(4,5)P_2 tilts the long axis of C2α by 40 ± 10° toward the bilayer normal, resulting in a more "vertical" domain orientation compared to that shown in panels A and B of Figure 5.

The interaction of C2 domains with PtdIns(4,5)P_2 in conventional PKC isoenzymes is enhanced by Ca^{2+}.[58,77]

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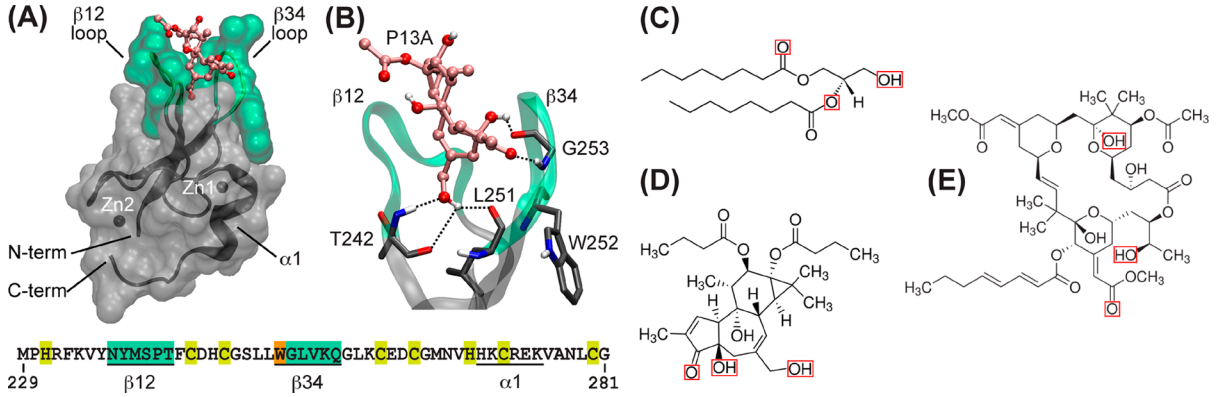

Figure 6. C1 domains and their ligands (adapted from ref 75). (A) Primary and tertiary structure of C1Bδ complexed to phorbol-13 acetate, P13A (PDB entry 1PTR[85]). The loop regions β12 (residues 237−242) and β34 (residues 252−257) are colored green; α1 is a short α-helix comprising residues 270−275. W252 and Zn^{2+}-coordinating residues are colored orange and yellow, respectively, in the primary structure. (B) Expansion of the C1Bδ ligand-binding site showing hydrogen bonds between P13A and the backbone atoms of C1Bδ. The side chain of W252 is not involved in direct interactions with the ligand. (C−E) Chemical structures of representative C1 ligands: (C) 1,2-dioctanoyl-*sn*-glycerol, (D) phorbol 12,13-dibutyrate, and (E) bryostatin-1. Red boxes mark the chemical groups that according to the molecular modeling studies[90−92] are involved in hydrogen bonding interactions with the protein.

Similarly, the presence of PtdIns(4,5)P_2 reduces the Ca^{2+} concentration required for membrane binding and thereby increases the Ca^{2+} sensitivity of PKCs.[78] To quantitatively characterize the interplay between the metal ions and PtdIns(4,5)P_2, we made use of a single high-affinity Pb^{2+}-binding site of C2α and generated three species that differ in the state of metal ligation: apo C2α, C2α·Pb, and C2α·Ca_2.[79] Using NMR-detected binding experiments with a short-chain PtdIns(4,5)P_2, we established that metal ions make an individual and comparable contribution to the energetics of binding of PtdIns(4,5)P_2 to C2α. Specifically, the affinity of C2α for PtdIns(4,5)P_2 increases 4−6-fold with progressive saturation of metal ion-binding sites. In a complementary NMR-detected binding experiment, we determined that the affinity of the ternary C2α·Pb·[C4−PtdIns(4,5)P_2] complex to the second Pb^{2+} ion is 8-fold higher than that of the binary C2α·Pb complex. The synergistic action of these two ligands occurs through modulation of the electrostatic potential of C2α and involves no significant structural changes.

The synergistic effect of the C2 ligands invites a question about whether the recruitment of Ca^{2+}-dependent C2 domains to membranes is target [PtdSer/PtdIns(4,5)P_2]-activated or messenger (Ca^{2+})-activated.[80] Current evidence suggests the dominant role of target lipids in the membrane recruitment process. While the affinity of isolated C2 domains for Ca^{2+} is too low to respond to physiological Ca^{2+} concentrations, it increases significantly in the vicinity of target anionic lipids, PtdSer and PtdIns(4,5)P_2. Target lipids can then recruit partially metal-bound species of C2 to the membranes, where acquiring an additional metal ion further stabilizes the complex.

2.1.5. Summary and Outlook. Originally considered to be nonspecific electrostatic switches, conventional C2 domains have emerged as dynamic protein modules that interact with membranes in a nuanced and complex manner. Structural and biophysical studies of Ca^{2+}-dependent C2 domains have greatly advanced our understanding of metal ion-dependent protein−lipid interactions. One question that still remains unanswered is how the binding of divalent metal ions to C2 initiates the activation sequence of conventional PKC isoenzymes.

Another area of interest is the geometry of multivalent interactions between the full-length regulatory domains and membranes. Given the short length of linker regions between C1 and C2, the orientation and position of the domains in the membrane will be interdependent and influenced by the membrane composition and chemical identity of lipid ligands. Last but not least, novel PKC isoenzymes rely mostly on C1 domains for membrane recruitment. Do their C2 domains play an indirect role in facilitating membrane binding, or do they simply function as intra- and intermolecular interaction modules?

2.2. C1 Domains. C1 (conserved region 1) domains are independently folded Zn^{2+} fingers of 50 amino acids. Their function is to target parent PKCs to diacylglycerol (DAG)-containing endomembranes. The affinity of PKCs for DAG has important implications for the cellular localization of PKC isoenzymes and selectivity of their response.[81] The domains occur in tandem, C1A and C1B, and either precede the C2 domain in conventional isoenzymes or follow the C2-like domain in novel isoenzymes (Figure 1). The sequence identity of C1A and C1B domains is typically 36−40% within a given PKC isoenzyme. Eight residues that coordinate two structural Zn^{2+} ions are strictly conserved across all C1 domains.[82] Several 3D structures of C1 domains from PKC isoenzymes have been determined using NMR[83,84] and X-ray crystallography.[85−87] Only C1B from PKCδ proved to be crystallizable when complexed to the following ligands: a water-soluble phorbol ester, phorbol-13 acetate;[85] and general anesthetics, methoxymethylcyclopropane and cyclopropylmethanol that bind to the surface of the domain outside of the canonical DAG pocket.[86,87] There are currently no structures of C1 complexed to its native PKC agonist, diacylglycerol.

The features and functional elements of C1 are illustrated using the structure of C1Bδ complexed to phorbol-13 acetate, P13A (Figure 6A). The domain has a treble-clef fold,[88] with two Zn^{2+} coordination sites that are formed by three Cys side chains and one His side chain. The secondary structure elements include a short C-terminal helix and four β-strands. The ligand-binding pocket is formed by two loops between β-strands 1 and 2 (β12 loop) and β-strands 3 and 4 (β34 loop). Phorbol-13 acetate, considered a "partial" PKC activator[89] compared to DAG and hydrophobic phorbol esters, forms

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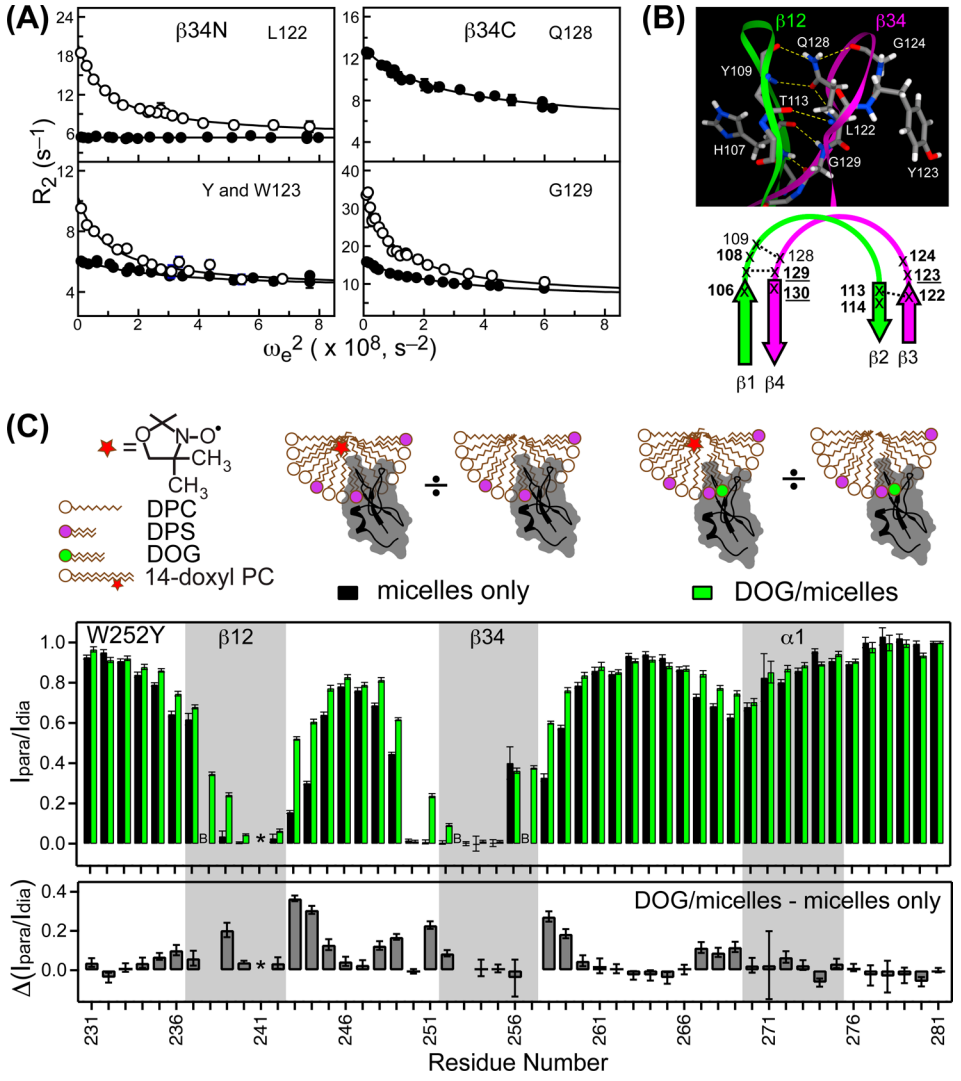

Figure 7. Dynamics of C1 loops and their interaction with mixed micelles. (A) Differences in the microsecond time scale dynamics of wt (●) and Y123W (○) C1Bα, illustrated using relaxation−dispersion curves for the N-H backbone groups of L122, Y123, Q128, and G129. (B) Intra- and interloop hydrogen bonds that stabilize β12 and β34 loops (top) and summary of conformational dynamics of loop hinges (bottom). Residues with quantifiable dispersion in both the wt and the mutant are underlined; residues with quantifiable dispersion in either the wt or the mutant are shown with regular or bold font, respectively (adapted from ref 107). (C) Probing the depth of insertion of C1Bδ into the micellar environment using PRE. The I_{para}/I_{dia} intensity ratios of the N-H resonances of W252Y C1Bδ, complexed to paramagnetic and diamagnetic preparations of mixed DPS/DPC micelles, are plotted as a function of amino acid sequence. Data in the absence and presence of DOG are shown with black and green bars, respectively. Residues whose broadening in the micelle-bound state is unrelated to PRE are labeled with "B". The ratios were normalized to G281, the most C-terminal residue. $\Delta(I_{para}/I_{dia})$ is the difference in I_{para}/I_{dia} ratios between the DOG-bound and micelle-only W252Y mutant (adapted from ref 75).

hydrogen bonds exclusively with the backbone groups of the protein with no specific side-chain contacts (Figure 6B).

C1 domains show considerable promiscuity by binding a wide range of naturally occurring compounds (see, e.g., Figure 6D,E), some of which have therapeutic potential.[93] Some of the most potent C1 ligands are tetracyclic diterpenoids called phorbol esters (PEs).[94] Because of their tumor-promoting activity, PEs have found widespread application as pharmacological and research tools for studying the function of PKCs and their role in carcinogenesis.[11,95] Another class of C1 ligands, bryostatins, has long been known to antagonize the tumor-promoting effects of PEs. Bryostatins have recently risen to renewed prominence because of their effect on reducing the synaptic loss and plaque formation associated with Alzheimer's disease in animal models,[96] and the development of efficient synthetic routes for potent bryostatin analogues.[97−99]

Despite the high level of sequence identity of C1A and C1B domains, their intrinsic affinities for DAG and PE differ, with the notable exception of PKCγ. As demonstrated by the studies in isolated C1 domains,[100−102] C1A has an affinity for DAG higher than that of C1B, and the pattern is reversed for the PEs. Although this pattern generally holds for the full-length enzymes,[103−106] additional factors, such as masking of the DAG-binding site by intramolecular interactions,[48] and "priming" steps, such as ligand-independent partitioning of the C1A domain into the membrane,[49] ultimately determine the individual functional roles of C1A and C1B domains in the cellular context.

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2.2.1. Dynamics of β12−β34 Loop Regions. Inspection of the available NMR structural ensembles of apo C1 domains revealed a significant variability in the conformation of loop regions β12 and β34, suggesting conformational plasticity of the C1 ligand-binding site. We have investigated the dynamics of the C1B domain from PKCα (C1Bα) on multiple time scales using solution NMR techniques.[107] To quantify the subnanosecond dynamics of the backbone N-H groups, we measured three relaxation parameters: the ^{15}N transverse and longitudinal relaxation rate constants and $\{^1H\}-^{15}N$ nuclear Overhauser enhancement. Interpretation of these relaxation parameters using the Lipari−Szabo formalism produced a set of residue-specific order parameters, S_{NH}^2. These parameters describe the spatial restriction of the protein N-H vectors, with 1 corresponding to total rigidity and 0 corresponding to unrestricted motions. The S_{NH}^2 data revealed an overall increase in the flexibility of ligand-binding loops compared to the flexibility of other regions of the protein, with the most dynamic residues being Ser111 of β12 with an S_{NH}^2 of 0.73 and Tyr123 of β34 with an S_{NH}^2 of 0.65.

The C1B residue at position 123 or equivalent has an important functional role: its identity, Trp or Tyr, toggles the affinity of the domain for DAG. As demonstrated by Newton's laboratory,[53] C1 domains having either native or mutated Tyr (Trp) at this position have reduced (increased) affinity for DAG. Using ^{15}N rotating-frame relaxation−dispersion NMR experiments, we characterized the dynamics of the N-H backbone groups in wild-type apo C1Bα and its Y123W variant. We found that loop hinges undergo chemical exchange on the microsecond time scale (Figure 7A).[107] As a result of the Tyr → Trp mutation, the kinetics of the exchange, in the form of the sum of the forward and reverse rate constants, is significantly altered, suggesting that the mutation perturbs the conformational equilibrium of apo C1Bα. The same type of correlation among residue identity, DAG affinity, and chemical exchange behavior was observed in another pair of C1 domains, the Trp-containing wild-type C1Bδ domain and its W252Y variant (M. Stewart and T. Igumenova, manuscript in preparation).

Our NMR and computational results[107] indicate that, for both wild-type (wt) and Y123W C1Bα, the conformation with open ligand-binding loops is likely to represent the major conformer of apoprotein in solution. We speculate that (i) C1 alternates between open- and partially closed loop conformations, with the latter having higher affinity for DAG, and (ii) the Tyr → Trp mutation contributes to DAG affinity via preferential partitioning of Trp in the interfacial membrane region and possibly by shifting the conformational equilibrium of apo C1 toward the partially closed loop state. This is in agreement with the recent results from Falke's group,[92] who conducted extensive molecular dynamics simulations of the C1A and C1B domains from PKCα in lipid bilayers. They observed a broad distribution of distances between the tips of β12 and β34 loops in apo C1 domains, with C1A having on average a wider groove. The average loop tip distance increased in the presence of lipid bilayers, whereas interactions with ligands, especially DAG, resulted in its decrease. In addition, the distribution of distances becomes narrower, suggesting that ligand binding rigidifies C1 domains. The preferential partitioning of the β34 Trp into the hydrophobic environment was later demonstrated by us quantitatively for the wt C1Bδ/W252Y pair (*vide infra*).[75]

In conclusion, C1 domains possess a high degree of conformational plasticity in their ligand-binding loop region. It is plausible that modulation of the binding site geometry through loop dynamics is the property that allows C1 domains to accommodate chemically diverse ligands. Stabilization or preselection of a given conformer by a lipid ligand is likely to contribute to both binding affinity and ligand specificity.

2.2.2. Dynamics of the Structural Zn^{2+} Site. In addition to loop dynamics, we have also detected and characterized the structural dynamics of the Zn(2) coordination sphere in the 50-mer construct of C1Bα.[108] The nature of this dynamic process was Sγ of Cys151, the last Cys residue of the domain, alternating between the Zn^{2+}-bound thiolate and free thiol states. This finding is surprising because structural cysteine-rich Zn^{2+} sites that stabilize protein folds are considered to be unreactive. We determined the structures of the two exchanging conformations of C1B that differ in zinc coordination using NMR methods and demonstrated the chemical reactivity of Cys151 in longer C1B-containing constructs such as C1B−C2. Our data suggest that Cys151 serves as an entry point for the reactive oxygen species that are known to activate PKCα in a process involving Zn^{2+} release.[109]

2.2.3. Interactions with Membrane Mimics and Lipid Ligands. Compared to Ca^{2+}-dependent C2 domains, much less is known about the interactions of C1 with membranes at the atomic level. Extensive biophysical and biochemical studies of full-length PKC isoenzymes have provided valuable information about the properties of their respective C1 domains; this is especially relevant to novel PKC isoenzymes, where C1 is responsible for the majority of membrane interactions in the activated form. It was demonstrated using vesicle sedimentation, enzyme activity assays, and SPR methods that PKCα and PKCδ have high specificity for PtdSer,[106,110] while for PKCε, the specificity is low and other anionic lipids, such as POPG, promote membrane association.[110] In all of the referenced studies, mutagenesis was used to assess the role of individual PKC residues in membrane binding and the associated activation of the enzyme. Stopped-flow experiments with fluorescence-based detection provided insight into the kinetics of the membrane binding process for the Trp-containing variant of C1B from PKCβII. The data led to a model that incorporates an initial membrane preassociation step, which subsequently facilitates the search for a membrane-embedded DAG.[111]

The extent of interactions of PKC with membranes was characterized using a series of lipid monolayer penetration experiments, during which the surface pressure change of a lipid monolayer is measured upon injection of a protein solution. The conventional PKC isoenzymes, PKCα and PKCγ,[101] were found to have membrane penetration powers higher than those of novel isoenzymes, PKCε[110] and PKCδ.[106] The same type of experiment conducted on the isolated C1 and C2 domains revealed that the extent of C1A−C1B interactions with monolayer lipids is comparable with that of the full-length PKCα and is larger than that of the isolated C2 domain.[112] This observation points to the pivotal role of the C1 domain in driving the PKC−membrane interactions.

We used paramagnetic relaxation enhancement (PRE) to probe the depth of insertion of the C1Bδ domain into membrane-mimicking micelles at the level of individual residues.[75] A paramagnetic agent, the doxyl-labeled lipid, was incorporated into the mixed DPS/DPC micelles either in the absence or in the presence of short-chain diacylglycerol, 1,2-

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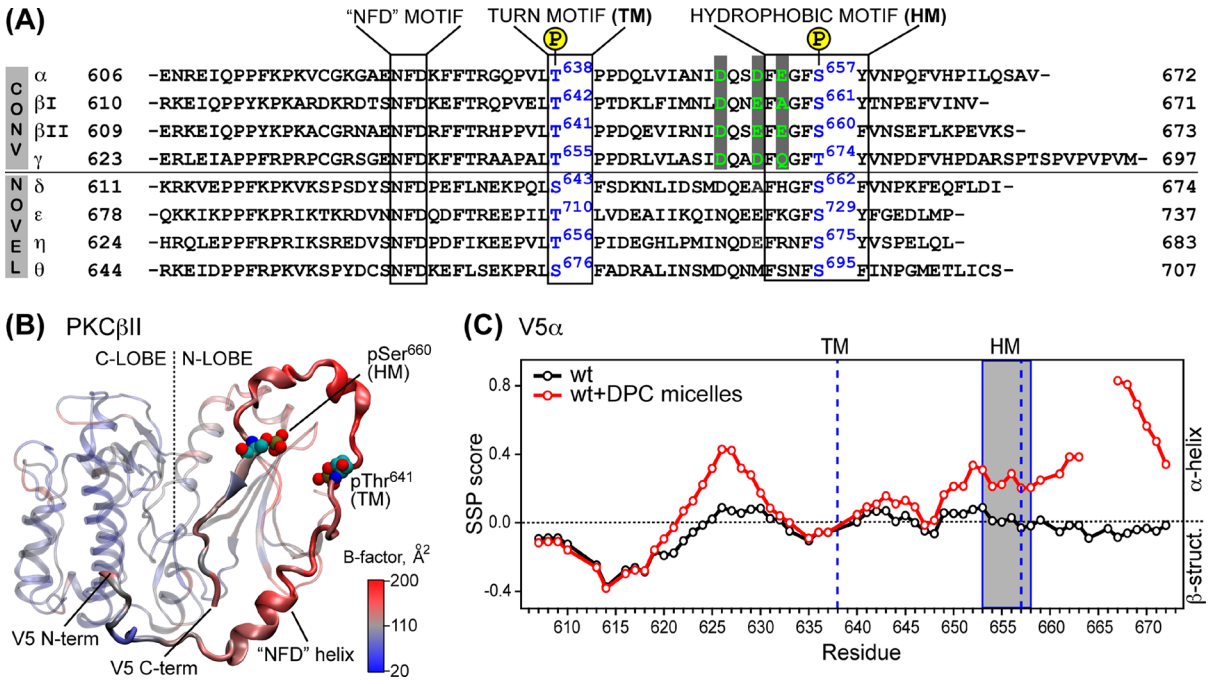

Figure 8. Properties of the V5 domain. (A) Alignment of the V5 primary structures of PKC isoenzymes from *Mus musculus*. The conserved motifs are boxed. Acidic residues implicated in the intramolecular interactions with the C2 domain are colored green. (B) Catalytic domain (residues 339−679) taken from the partial crystal structure of PKCβII, PDB entry 3PFQ.[39] The *B* factors of backbone Cα atoms are mapped onto the structure as a color gradient. The N/C-lobes of the kinase domain and V5 are shown with transparent and opaque representations, respectively. (C) SSP scores calculated using NMR chemical shift data for the free (black) and micelle-bound (red) V5α. Upon binding to DPC micelles, V5α acquires a partial α-helical structure, most notably in the NFD region and the most C-terminal residues (adapted from ref 133).

dioctanoyl-*sn*-glycerol (DOG). The principle behind PRE-based experiments is that the cross-peaks of protein residues penetrating into the hydrophobic core of the micelles will be broadened, because of the efficient relaxation caused by an unpaired electron. The dependence of peak intensity ratios obtained for the paramagnetic and diamagnetic preparations of micelles indicates that both β12 and β34 loops undergo significant insertion into the micelle core (Figure 7C, top graph). Addition of DOG resulted in a change in C1Bδ−micelle interaction geometry, as evidenced by the differences in the (I_{para}/I_{dia}) ratios (Figure 7C, bottom graph). On the basis of the CSP analysis of C1Bδ bound to DPC-only and DPC/DPS micelles, we concluded that the C-terminal helix α1 is likely involved in the interactions with the PtdSer headgroup. Using NMR-detected binding experiments, we demonstrated that (i) the presence of PtdSer enhances the affinity of C1Bδ for micelles 2-fold and (ii) the DAG-sensitizing Trp252 (position equivalent to position 123 in conventional PKC isoenzymes) enhances the affinity of the domain for micelles in the absence of ligand. Our data are consistent with the models of Newton[111] and Falke[49] that include initial ligand-independent partitioning of C1 domains into the lipid bilayer and point to the pivotal role of an aromatic residue at position 123/252 in facilitating this process. The NMR-based restrains, such as CSP data and PRE ratios, can be used as ambiguous restraints to drive the protein−micelle docking and obtain the geometry of protein−micelle interactions, as described by Dancea et al.[76]

2.2.4. Summary and Outlook. C1 domains are highly dynamic hydrophobic membrane-binding domains that can interact with chemically diverse ligands, in addition to their endogenous lipid activator, DAG. High-resolution structures of ligand-complexed C1 domains in the presence of suitable membrane mimetics are needed to fully understand the molecular basis of ligand affinity and specificity. With respect to membrane interactions, much of what was said in the context of future C2 domain studies applies here: the geometry of protein−membrane interactions and the influence of linker regions on these interactions are the topics that require further investigation.

One of the most important questions in the field is how the binding of divalent metal ions initiates the activation sequence of conventional PKC isoenzymes. As described in section 1.1, C1A has been implicated in intramolecular autoinhibitory interactions with C2. What is the structural basis of these interactions, and how does Ca^{2+} binding interfere with them to make the lipid-binding sites on C1 and C2 domains accessible?

2.3. Pseudosubstrate Variable-1 Region and C-Terminal Variable-5 Domain. *2.3.1. Pseudosubstrate Region.* The pseudosubstrate (PS) region, sometimes termed Variable-1 (V1), is located at the N-terminus in the conventional PKCs and between the C2-like (PB1) and C1 domains in novel (atypical) PKCs (Figure 1). The PS region is a short amino acid sequence that is similar to PKC substrates, except that an Ala replaces the Ser or Thr phospho-acceptor sites; it also contains four to six positively charged amino acids. Mosior and McLaughlin[113] demonstrated that the PS-derived peptide from PKCβI/βII binds to LUVs containing anionic lipids, and that the binding curve is sigmoidal with respect to the molar fraction of the anionic lipid. While the energetics of binding would not be sufficient to release the PS from the autoinhibitory interaction, PS could be involved in the stabilization of the activated PKC by anchoring it to the membrane, in addition to the lipid ligand-dependent interactions of C1 and C2. This is further supported by the observation that the V1−C1−C2−GFP construct from PKCγ is

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partially prelocalized to the plasma membrane in unstimulated live cells, whereas the C1−C2−GFP construct is uniformly distributed in the cytoplasm.[114]

2.3.2. C-Terminal Variable-5 (V5) Domain. V5 is the most C-terminal domain of PKCs comprising 60−80 amino acids that varies in both length and amino acid composition. V5 has three conserved regions: the "NFD" motif, the turn motif (TM), and the hydrophobic motif (HM) (Figure 8A). Current experimental evidence suggests that V5 plays a role in several processes that regulate PKC activity, such as enzyme maturation, Ca^{2+} sensitivity, interactions with scaffolding proteins, and downregulation via interactions with Pin1.

2.3.2.1. Maturation. Conventional and novel PKCs undergo three ordered phosphorylation reactions to reach catalytic maturity. Two phosphorylation sites belong to V5 and are the Ser/Thr residues of the HM and TM. In addition, V5 serves as an interaction site between the newly synthesized PKC and phosphoinositide-dependent kinase, PDK-1, which phosphorylates a conserved Thr residue on the activation loop that is not part of the C-terminal V5 region.[115] Experimental evidence supports the involvement of the mammalian target of rapamycin complex 2 in the phosphorylation of the TM[116,117] and autophosphorylation of the HM.[118]

2.3.2.2. Ca^{2+} Sensitivity. The negatively charged phosphoryl group at the HM is crucial for the proper Ca^{2+} response of conventional PKCs. Replacement of Ser660 of the HM with Ala increases the Ca^{2+} concentration required for full membrane binding and activity of PKCβII by almost an order of magnitude.[43]

2.3.2.3. Interactions with Scaffolding Proteins. PKCα interacts with PICK1, a scaffolding protein implicated in synaptic plasticity, using the last four amino acids of V5, QSAV.[119] PICK1 was later shown to be a substrate of PKCα.[120] V5 also forms part of the PKCβII-binding site for RACK1, a scaffolding protein that is critical for proper subcellular localization and stabilization of the activated PKC.[121]

2.3.2.4. Downregulation of PKC via Interactions with Pin1. A recent addition to the repertoire of cellular mechanisms that control PKC activity was the finding that Pin1, a peptidyl-prolyl isomerase, downregulates PKCα and PKCβI/βII through its action on the TM of the V5 domain.[122] Isomerization of V5 makes the activated (i.e., membrane-bound) enzyme susceptible to dephosphorylation and ubiquitin-mediated degradation.

2.3.2.5. Structural Preferences and Dynamics of V5. In the available crystal structures of catalytic domains of PKC,[123−132] V5 is wrapped around the N-terminal lobe of the kinase domain. The electron density of V5 is not uniformly well-defined, giving rise to "missing" regions in the crystal structures. Together with the elevated values of *B* factors, this indicates a relatively high degree of static or dynamic disorder. The structure of the catalytic domain of PKCβII that has a fully defined V5 domain illustrates the secondary structure elements of V5 (Figure 8B). V5 has two helical segments, one of which is the NFD motif that has been implicated in the autoinhibitory interactions with the C1B domain.[39] The other helical segment is located between the TM and HM. The phosphate group of the TM, pThr641, is engaged in electrostatic interactions with K355, K374, and R415 of the kinase N-lobe. The phosphate group of the HM pSer660 is hydrogen-bonded to the side-chain amide group of Gln411 of the N-lobe.

To determine the conformational preferences and dynamics of the V5 domain in its "free" form, we have prepared and characterized the isolated V5 domain from PKCα (V5α) using NMR spectroscopy and circular dichroism.[133] V5α is intrinsically disordered, with a moderate propensity to form extended β-structures in the N-terminal region and weak propensity to form α-helical structures in the NFD region, and between the TM and HM (Figure 8C). This was shown by calculating the secondary structure propensity (SSP)[134] scores that describe the likelihood of a peptide adopting a helical structure (maximal score of +1) or an extended β-structure (minimal score of −1). Introduction of the phosphorylation-mimicking mutation, T638E/S657E, into V5α did not alter the conformational preferences of the protein but resulted in a change in the population of the "cis-trans" conformer of the TM segment, Thr638-Pro639-Pro640, which has been implicated in Pin1 interactions.[122]

The subnanosecond dynamics of the V5α backbone was quantified using a set of NMR relaxation parameters that we interpreted using reduced[135] spectral density mapping[136] formalism. Regions with weak propensity for α-helical structure formation, such as the region upstream of the NFD motif and the TM−HM segment, show a degree of motional restriction higher than that of the rest of the protein residues.

These structural and dynamical data indicate that the C-terminal V5 domain has a high degree of conformational flexibility, especially in the isolated form that serves as a model of V5 in the immature and activated states of the kinase. The association of V5α with the N-terminal lobe of the kinase domain during the final step of maturation may include both "conformational selection" and "folding upon binding" mechanisms.[137]

2.3.2.6. Interactions with Micelles. Using NMR and CD spectroscopy, we demonstrated that V5α has a propensity to partition into the hydrophobic environment and acquire partial helical structure upon doing so.[133] This was manifested in a significant change in the V5α chemical shifts upon addition of zwitterionic DPC micelles. The SSP scores of micelle-bound V5 indicate a significant increase in the helical structure content, especially at the C-terminus (Figure 8C). The increase in helical content was also evident in the circular dichroism data.

Given our results for the V5α−DPC micelle interactions, we sought to evaluate the amphiphilicity of the C-terminal segments of V5 domains that follow HM. The hydrophobic moment, $\langle\mu_H\rangle$,[138] was calculated with the program *hmoment* of the EMBOSS,[139] assuming a helical structure and using a 10-residue window. For V5α, the μ value of the last 14 amino acids is larger than 0.35, indicating a significant degree of amphiphilicity. Regions with $\langle\mu_H\rangle$ values exceeding 0.35 were present in all PKC isoenzymes except βI. We conclude that the C-terminal segments of V5 have a moderate degree of amphiphilicity. This suggests that V5 may play a role in anchoring immature[140,141] and even activated PKC to the membrane surface.

2.3.2.7. Summary and Outlook. The two distinct features of V5 are its conformational plasticity caused by a large fraction of unstructured regions and moderate amphiphilicity. These biophysical properties of V5 underlie its multifaceted function. Further studies are required to understand the structural basis of interactions of V5 with scaffolding proteins, Pin1, and membranes. Because V5 is the most variable domain among the PKC isoenzymes, atomic-level information about its interactions with binding partners will provide insight into the isoenzyme specificity and inform the design of isoenzyme-selective agents.

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CONCLUDING REMARKS

This Current Topic presents an overview of biophysical studies of dynamics and membrane interactions of PKC. Significant progress has been made in understanding the structural dynamics of full-length PKC isoenzymes. The rearrangement of PKC tertiary structure reveals an intrinsically amphiphilic character of the enzyme, by exposing hydrophobic membrane-interacting regions that were previously sequestered from the solvent in the latent form. Because of the disruption of intramolecular interactions in the activated PKC, individual membrane-binding domains serve as good models for gaining atomic-level information about PKC−membrane interactions.

Despite significant progress, much remains to be learned about these incredibly complex enzymes that regulate signal transduction processes at membrane surfaces. The high-resolution structure of latent PKC remains elusive. Several aspects of the membrane recruitment process, such as the interplay between membrane-binding domains, the effect of membrane composition, and the structural basis of interactions with lipid ligands and adaptor/regulatory proteins, require further investigation. The structural and biophysical studies will be deemed successful when the spatiotemporal activation sequence of PKC isoenzymes is characterized at the level of atomic detail. Having this information will provide the molecular framework for understanding the functional response of PKC isoenzymes to different stimuli and inform the development of ways to modulate PKC activity for therapeutic and research purposes.

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Notes

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ABBREVIATIONS

PKC, protein kinase C; DAG, diacylglycerol; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; MTSL, *S*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)-methylmethanesulfonothioate; DPS, 1,2-dihexanoyl-*sn*-glycero-3-[phospho-L-serine]; DPC, *n*-dodecylphosphocholine; CSP, chemical shift perturbation; LRC, lysine-rich cluster; CMBL, Ca^{2+}- and membrane-binding loop; PtdSer, phosphatidylserine; PDBu, phorbol 12,13-dibutyrate; DOG, 1,2-dioctanoyl-*sn*-glycerol; C2α, C2 domain from PKCα; C1Bα, C1B domain from PKCα; V5α, V5 domain from PKCα.

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