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HYPOTHESIS

Hypothesis: Unifying model of domain architecture for conventional and novel protein kinase C isozymes

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Abstract

Protein kinase C (PKC) family members are multi-domain proteins whose function is exquisitely tuned by interdomain interactions that control the spatiotemporal dynamics of their signaling. Despite extensive mechanistic studies on this family of enzymes, no structure of a full-length enzyme that includes all domains has been solved. Here, we take into account the biochemical mechanisms that control autoinhibition, the properties of each individual domain, and previous structural studies to propose a unifying model for the general architecture of PKC family members. This model shows how the C2 domains of conventional and novel PKC isozymes, which have different topologies and different positions in the primary structure, can occupy the same position in the tertiary structure of the kinase. This common architecture of conventional and novel PKC isozymes provides a framework for understanding how disease-associated mutations impair PKC function.

KEYWORDS

C1 domain, C2 domain, protein kinase C

1 | INTRODUCTION

Protein kinase C (PKC) is a multi-module Ser/Thr protein kinase that transduces the abundance of signals resulting in phospholipid hydrolysis.¹ Binding of diacylglycerol (DG) allosterically and reversibly activates these enzymes to effect their major role in maintaining cellular homeostasis. This allosteric regulation is precisely controlled by multiple mechanisms that ensure that the enzyme is only active for specific times at specific locations in response to specific signals. Deregulation of any of these mechanisms results in pathophysiological, with loss-of-function

associated with cancer and enhanced function associated with degenerative disease.² Maintaining autoinhibition is critical to the function of PKC.

DG-sensing PKC isozymes fall into one of two classes: conventional PKC isozymes (cPKC: α , β , γ) have a low-affinity DG-sensing C1B domain and require a Ca^{2+} sensor (C2 domain) for activation, whereas novel PKC isozymes (nPKC: δ , ϵ , θ , η) have a high-affinity DG-sensing C1B domain and are not regulated by Ca^{2+} . Both subtypes share a common architecture of an N-terminal regulatory moiety containing allosteric sensors that constrain the catalytic activity of a C-terminal kinase domain (Figure 1a). This is achieved by an autoinhibitory pseudosubstrate segment that occupies the substrate-binding cavity in the inactive conformation. The pseudosubstrate is immediately followed by tandem C1 domains (C1A and C1B) which serve as DG sensors, although only the C1B binds DG in

Abbreviations: cPKC, conventional protein kinase C; DG, diacylglycerol; nPKC, novel protein kinase C; PIP_2 , phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; SAXS, small angle X-ray scattering.

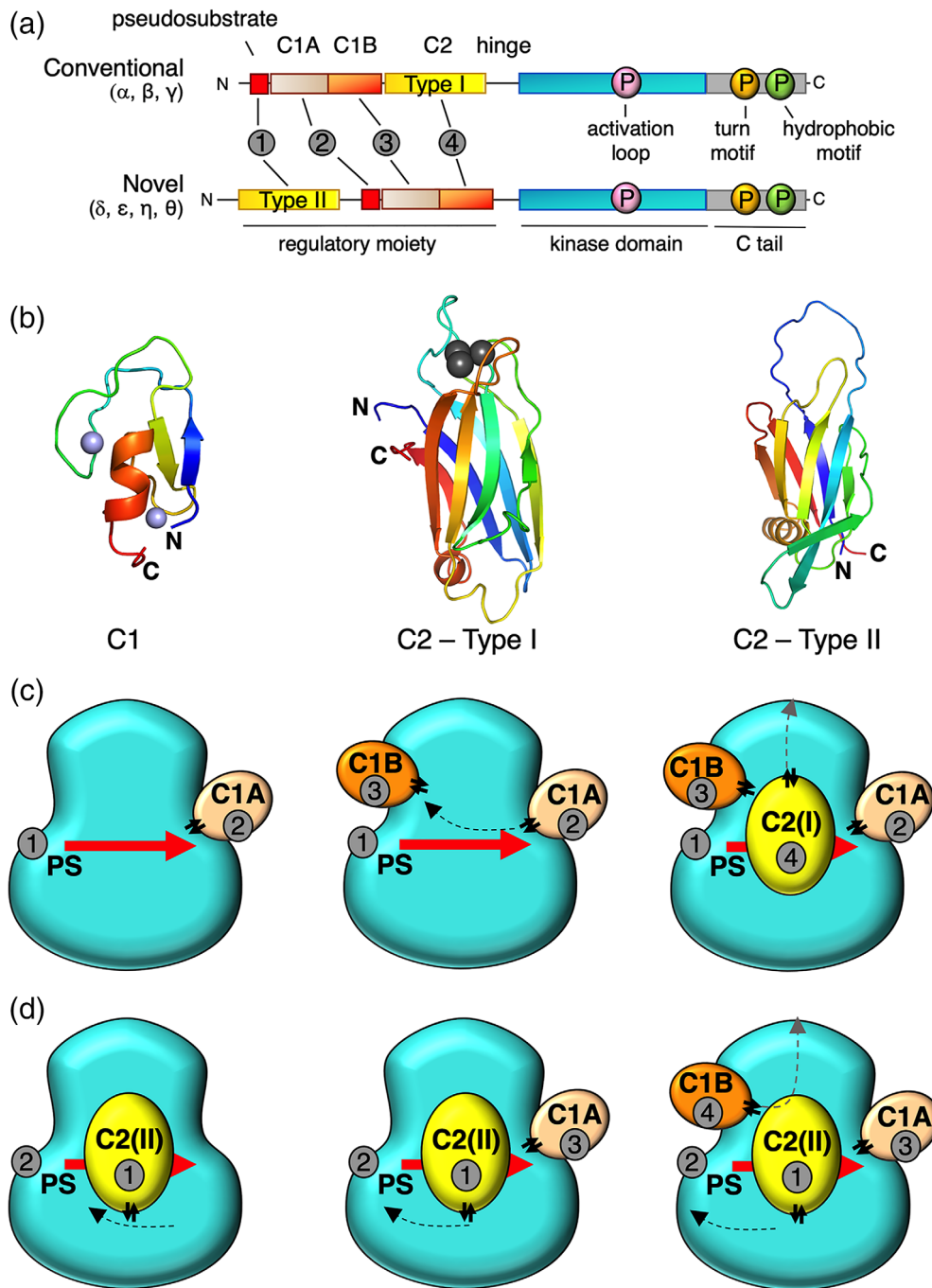


FIGURE 1 Proposed architecture for conventional and novel PKC isozymes. (a) Primary structure and domain composition of conventional and novel PKC isozymes showing pseudosubstrate (PS, red), C1A domain (sand), C1B domain (orange), C2 domain (yellow), kinase (cyan), and C-tail (grey). Three processing phosphorylations indicated in magenta, orange, and green for the activation loop, turn motif, and hydrophobic motif, respectively. Order of domains indicated by grey circles. (b) Structure of C1 domain (PKC δ , PDB ID code 2YUU) and two types of C2 domain: Type I C2 (PKC β , PDB ID code 1A25) in conventional PKC isozymes and Type II C2 (PKC θ , PDB ID code 2ENJ) in novel PKC isozymes highlighting different topology but same overall architecture. Notably, the N and C termini are on opposite ends of each of the two types of C2. Zn²⁺ ions (C1 domains) in purple and Ca²⁺ ions (Type I C2 domain) in grey. (c) Proposed positions of each domain on the kinase module for conventional PKC isozymes and novel PKC isozymes (d) showing common tertiary structure. Domains are numbered in the order they appear in the primary structure in (a). Double arrows indicate positions of the “plugs”

the context of the “mature” protein.³ cPKC isozymes have a Ca²⁺-sensing C2 domain, which targets these PKC isozymes to the plasma membrane via a recognition motif for

phosphatidylinositol-4,5-bisphosphate (PIP₂), a lipid localized to the plasma membrane. The nPKC isozymes also have a C2 domain but, curiously, it has a different

topology,⁴ lacks residues that coordinate Ca^{2+} , and precedes the pseudosubstrate-C1A-C1B segment. The kinase domain is followed by a C-Tail that is a hallmark regulatory region of AGC kinases and serves as docking site for regulatory proteins.⁵ Understanding the mechanism of autoinhibition of PKC would open avenues for allosteric modulators to treat deregulated signaling in disease.

The key role of the pseudosubstrate as an autoinhibitory segment that binds the substrate-binding cavity of the kinase domain was identified by Kemp and colleagues over three decades ago.⁶ Although this segment is necessary for the autoinhibition of all PKC family members, numerous studies in the ensuing years have revealed it is not sufficient.^{7–10} Rather, all domains in the regulatory module participate in a network of interactions to maintain autoinhibition. Of these, the C1A domain is the most critical: the binding affinity of the isolated regulatory module (see Figure 1a) for the isolated kinase domain is reduced by three orders of magnitude upon deletion of the C1A domain, but only about 20-fold upon deletion of the C1B or C2 domains.¹¹ Thus, whereas all modules participate in a network of interactions to maintain autoinhibition, the pseudosubstrate-C1A is the dominant autoinhibitory module. But what is this network of interactions?

Unraveling the structure of full-length PKC and demonstrating how these domains communicate with the kinase core has been challenging, likely because these molecules are intrinsically highly dynamic. Whereas the structures of each of the domains of PKC from multiple family members have been solved, a full-length structure has remained elusive. A partial crystal structure of PKC β II has been reported; however, there was insufficient electron density to resolve all the domains.¹² Previous SAXS for two conventional (PKC α and β II) and of two novel PKC isozymes (δ and η) revealed similar scattering curves, with a radius of gyration of 33–34 Å calculated for both classes of isozymes.¹³ *Ab initio* calculations of molecular envelopes showed similar globular shapes, suggesting a conserved tertiary structure of both types of PKC. This raised the intriguing question of how the same 3D architecture could be obtained given the different topologies of the C2 domains and their different order in the primary sequence of conventional versus novel PKC isozymes. Reasoning that contact residues at the regulatory domain:kinase interface would be conserved amongst isozymes, Leonard and colleagues recently proposed potential interaction surfaces of each domain with the kinase.¹³ However, a model of the architecture for full-length PKC was not proposed.

Here we considered the extensive mechanistic information on PKC, the existing structural data, and the properties of individual domains to guide molecular

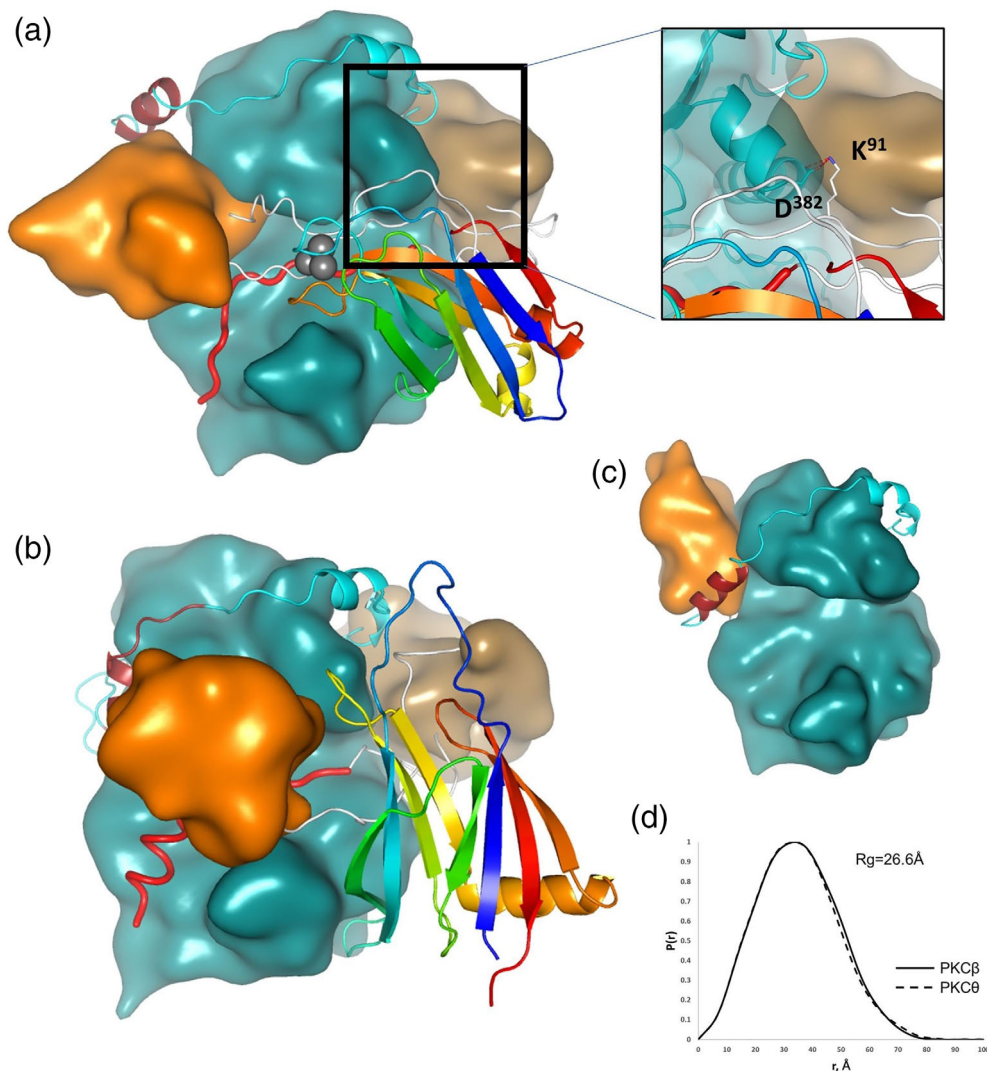
docking of each of the domains on kinase domain. We paid particular attention to the “plug in” property of each regulatory module: the N and C termini are on the same face of the domain, in close proximity (Figure 1b). Therefore, they can be “plugged” into specific sockets of the structure.^{14,15} The proximity of the N and C termini is a well-studied phenomenon and is an important feature of protein domain evolution.^{16,17} This property immediately suggested that both types of C2 domain could occupy the same position on the kinase domain but their “plugs” would be on opposite sides. This would provide an explanation for why they connect to different modules in the primary structure (hinge leading to kinase domain for cPKC and pseudosubstrate for nPKC; Figure 1a).

The schematic in Figure 1c illustrates the rationale for a general docking of the domains for conventional PKC isozymes. The pseudosubstrate peptide binds into the active site of the kinase with its C-terminus directed toward the C-helix. The linker between the P+1 residue and the C1A domain is only 9 residues long; therefore, we reasoned that it may be positioned at the C-helix surface of the kinase. Positioning of the C1B domain was the major challenge as the proposed position of C1A is incompatible with the previously published structure of PKC β II.¹² As the linker between C1A and C1B is only 15 residues long, it is impossible to circumvent the N-lobe of the kinase and connect it to the N-terminus of C1B that was positioned behind the C-terminal tail. The latter contains an important regulatory NFD-helix that was shown to be in a direct contact with the C1B domain of PKC β II.¹² If we consider that the N and C termini of the C1A domain are in close proximity, it would be logical to suggest that the C1A-C1B linker must be antiparallel to the pseudosubstrate peptide. Following this logic, we positioned the C1B domain near the N-terminus of the pseudosubstrate, next to the NFD-helix in the C-terminal tail, albeit at its “front” side, opposite to the position proposed earlier¹² (Figure 2, compare A and C). To position the C2 domain we followed our biochemically validated model that suggests binding of the C2 over the pseudosubstrate region covering the active site of the kinase.¹⁸ As the N-terminus of the C2 must be close to the C-terminus of C1B (6 residue linker), we concluded that the C2 “plug” must be facing the N-lobe of the kinase, as shown in Figure 1c. This position would satisfy not only the constraints for the connection between the C1B and C2 domains, but would easily accommodate a long linker between the C2 and the N-terminus of the kinase domain.

With this domain positioning in mind, we discovered that the same logic applied to the novel PKC isozymes results in a structure with the same overall geometry (Figure 1d). As the C2 in this case precedes the pseudosubstrate and the “plug” of the C2 has to be reversed with respect to the conventional C2 domains, its

FIGURE 2 Conserved architecture of conventional and novel PKC isozymes.

(a) Proposed structure of PKC β II highlighting positions of the C1B (orange), NFD helix (burgundy) in the kinase domain (cyan), C1A domain (sand), and C2 domain (ribbon diagram colored from blue (N terminus) to red (C terminus) to indicate topology; Ca²⁺ ions in grey). Linkers connecting domains are shown in white with the pseudosubstrate (red) positioned in the active site. Inset: Interaction between Lys91 (C1A-C1B linker) and Asp382 (kinase) shown. (b) Proposed structure of PKC θ , colored as in (a) showing inverted C2-domain topology yet same overall structure. (c) Previously reported structure of PKC β II¹² (pdb:3PFQ) showing C1B domain (orange) positioned on top of NFD helix (burgundy). (d) Pair distance distribution function $P(r)$ computed from the proposed models showing similar shapes and radii of gyration of conventional PKC β and novel PKC θ



C-terminus should face the C-lobe of the kinase and, thus, can be easily connected the N-terminus of the pseudosubstrate. Positioning of the latter and the two C1 domains can be the same as in the previous case with the only difference that the C-terminus of the C1B will lead toward the N-terminus of the kinase domain instead of the C2 domain.

We then proceeded to dock each domain on the structure of PKC. We used the ClusPro protein docking server¹⁹ with spatial restrictions to achieve positions close to the proposed models (the restrained residues had to be within 15Å proximity). For the docking of the C1A of PKC β II, the restriction was applied to Phe43, a residue that is highly conserved in all C1A domains but not in C1B domains and was suggested previously to be at a protein-protein binding interface.¹³ For the C1B docking in PKC β II, Asp116 was restricted to a close proximity to the NFD helix. This residue is highly conserved in all C1B domains but not in C1A domains, and was also previously identified as a conserved C1B-specific residue

that may mediate protein:protein interactions.¹³ The C2 domain docking was based on our previous model with the correction to the “plug” orientation.¹⁸ Flexible linkers between the pseudosubstrate, the C1 domains, and the C2 domain were generated using UCSF Chimera²⁰ and Modeller.²¹ The model for PKC θ was created using the PKC β II model as a template using PKC θ known domain structures.

2 | DISCUSSION

Here, we present a unifying model for both conventional and novel PKC isozymes that accounts for the inverted topology and order of the Type I versus Type II C2 domains. This model is consistent with existing biochemical data (Table 1)^{11–13,18,22} and defines new interfaces that can now be verified experimentally. This conserved architecture not only underscores the role of each domain in tethering the pseudosubstrate in the kinase domain, but

TABLE 1 Comparison of existing biochemical data with proposed model predictions

Domain	Residue	Isozyme(s)	Disrupted autoinhibition?	Prediction with new model
C1A	R42	α	Yes ¹¹	Interaction with E655 in C-Tail
	F43	α , β II, δ , η , θ	Yes ^{11,13}	Interaction with F656, F659, or F661 in C-Tail
	T54	β II	No ¹³	No predicted interactions
	D55	α , β II	Yes ^{11,13}	Interaction with R76, domain unfolding
	F72	α	Yes ¹¹	Interaction with F84, domain unfolding
C1B	T108	β II	No ¹³	No predicted interactions
	L125	β II	No ^{12*}	Disrupt phorbol-binding pocket
C2	K236	β II	No ²²	No predicted interactions
	R238	β II	No ²²	No predicted interactions
Kinase	L358	α , β II, δ , η , θ	Yes ^{12,13}	Could interact with Y422 or M145
	L367	β II	Yes ¹²	L367D could unfold N-lobe due to DE(D) negative patch
	D382	β II	Yes ¹⁸	Interaction with K91 (between C1A and C1B)
	Y422	β II	Yes ¹²	Could interact with L358/in nucleotide binding region
	Y430	β II	Yes ¹²	Could pi-stack with H140 in C1B
C-Tail	F629	α , β II, δ , η , θ	Yes ^{12,13} /No ¹⁸	No predicted interactions
	F633	α , β II, δ , η , θ	Yes ^{12,13}	Could pi-stack with F114 in C1B
	E655	β II	Yes ¹⁸	Interaction with R42 in C1A

Note: Disruption of autoinhibition upon mutation of the indicated residues in conventional or novel PKC isozymes was determined experimentally either by assessing increased membrane translocation^{11–13,18,22} or increased basal activity¹⁸ in cells, or increased cofactor-independent activity of pure protein *in vitro*.²² Mutated residues were examined in the new structural model and predictions were made about inter- and intradomain interactions that may affect autoinhibition. Residues are numbered according to PKC β II.

*Phorbol-induced membrane translocation impaired.

also draws attention to linker regions as playing key roles in maintaining the autoinhibited conformation.

The estimated radius of gyration for our models of both conventional and novel PKC isozymes estimated by CRYSOLO and GNOM programs from ATSAS 2.8 package²³ is the same (Figure 2d), consistent with experimental observations.¹³ At 27 Å, it is slightly smaller than the reported 33–34 Å.¹³ The maximum dimension of our models (around 80 Å) is also smaller than the experimentally measured 110–115 Å¹³; this difference can be explained by the absence of the large linker between the C2 and the kinase domain in PKC β II and between C1B and the kinase domain in PKC θ in our models.

The proposed model for the tertiary structure of conventional and novel PKC isozymes differs in several important aspects from the partial crystal structure of PKC β II¹² (Figure 2c) and a re-interpretation of this structure.¹⁸ First, the C1B in our model binds to the NFD-helix at the side proximal to the active site of the kinase, whereas Hurley and colleagues placed it on the back-side positioned to clamp on to Phe629 of the NFD helix. In our model, Phe629 is not predicted to interact with the C1B. Consistent with this lack of interaction, mutation of this residue

in PKC β II was shown to have no detectable effect on the basal activity or activation in cells.¹⁸ Instead, our model positions the C1B to interact with Phe633 in the C-Tail. Indeed mutation of Phe633 has been shown to increase the rate of membrane translocation of PKC β II, consistent with reduced autoinhibition.¹³ Furthermore, Biondi and colleagues found a similar position for the C1A domain of atypical PKC isozymes based on ¹H/²H exchange, which showed that the C1A domain also clamps over the α C helix and hydrophobic motif segment of the C-tail.²⁴ In a re-interpretation of the crystal packing,¹⁸ the C2 domain was proposed to clamp over the pseudosubstrate to stabilize the autoinhibited conformation. In our new model, the C2 domain occupies the same overall position to clamp the pseudosubstrate in place but is rotated. Residues in the C2 domain previously proposed to be at the kinase interface, Lys205 and Lys209,¹⁸ are also predicted to be at the C2: kinase interface in our new model. These basic residues, which are part of a basic PIP₂-sensing face,^{25,26} were proposed to interact with Asp382 in the kinase domain and Glu655 in the C-Tail: mutation of either of these acidic residues to Lys resulted in an accelerated rate of membrane translocation, consistent with unmasking of the regulatory



domains and reduced autoinhibition. Although co-mutation of the “partner” basic residues in the C2 domain to acidic residues (to reverse the predicted ion pairs) reduced the rate of membrane translocation back to that observed for wild-type enzyme, the C2 mutations disabled the plasma membrane “sensor” confounding interpretation. In the current model, Asp382 is predicted to bind a conserved Lys in the linker between the C1A and C1B, and Glu655 is predicted to interact with a conserved Arg in the C1A (Table 1). Our model is also consistent with the conserved residues in conventional and novel PKC isozymes proposed by Lucic et al¹³ as mediating interdomain interactions. For example, Phe43 in the C1A is predicted to interact with the C-Tail and Leu358 in the kinase domain could interact with Met145 or Tyr422.

An important feature of this model is the role of the linker regions in coordinating domain interactions. Lucic et al previously noted that the linker regions are not conserved in sequence but are almost invariant in length and are, most probably, unstructured.¹³ The C1B-C2 linker has previously been shown to contribute to autoinhibition⁹; whereas a GST-fusion of the regulatory moiety of PKC α effectively inhibited the activity of full-length PKC α , mutation of residues in this linker to a stretch of Ala decreased the ability of the regulatory domain to inhibit PKC α . In our model of PKC β II, the C1A-C1B linker crisscrosses over the pseudosubstrate to reinforce its interaction in the substrate binding cavity. Interactions between the conserved Lys91 in the C1A-C1B linker with Asp382 in the kinase domain may provide an additional layer of autoinhibition (Figure 2a, inset). Thus, the pseudosubstrate is secured in the substrate-binding cavity by the C2 domain and by linkers that weave over the bound pseudosubstrate.

In a physiological context, aberrant PKC that is not properly autoinhibited is unstable and shunted to degradation. This property of PKC provides a major mechanism by which cancer-associated mutations in PKC isozymes are loss-of-function²⁷: perturbation of interdomain surfaces that activate the enzyme are paradoxically loss-of-function because the aberrant PKC is degraded by quality control mechanisms.²⁸ Indeed, the pseudosubstrate of PKC β is a functional hotspot for mutations,²⁹ either because mutation of a residue strengthens the affinity for the pseudosubstrate for the kinase (resulting in decreased activity) or because the mutation decreases autoinhibition to promote the degradation of the enzyme and lower steady-state levels.²⁸ Our model accounts for why cancer-associated mutations occur throughout the domain structure of the enzyme and exemplify the exquisite conformational control driven by a coordinated network of domains and linkers.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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