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Protein Kinase C: Structural and Spatial Regulation by Phosphorylation, Cofactors, and Macromolecular Interactions

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Contents

Alexandra Newton received her Ph.D. degree in Chemistry from Stanford University in 1986 working on membrane biochemistry. She then spent 2 years doing postdoctoral research in Daniel E. Koshland's laboratory at the University of California, Berkeley, where she was first introduced to protein kinase C. She was on the faculty in the Chemistry Department at Indiana University from 1988 to 1994, before joining the Department of Pharmacology at the University of California, San Diego, where she is now Full Professor. Her research investigates the biology and chemistry of protein kinase C and its upstream kinase, the phosphoinositide-dependent kinase, PDK-1. More information is available at: http://cancer.ucsd.edu/newtonlab/.

I. Introduction

The discovery in the early 1980s that protein kinase C is 'the receptor' for the tumor-promoting phorbol esters catapulted this enzyme to the forefront of research in signal transduction.[1] The pharmacological properties of phorbol esters, present in the milky sap exuded from the plant croton tiglium, had been appreciated over the millennia, but their molecular target had remained elusive until this discovery.[2] The subsequent cloning of protein kinase C in the mid-1980s unveiled a family of kinases whose unifying feature was the presence of a cysteine-rich motif, the C1 domain, amino terminal to the kinase domain.[3] Although we now know that not all protein kinase C isozymes bind phorbol esters and, conversely, that not all phorbol ester receptors are protein kinase C molecules,[4] an avalanche of studies in the 1980s on the effects of phorbol esters on cells pointed to a central role of protein kinase C in cell signaling.[5]

Protein kinase C isozymes transduce the myriad of signals that promote phospholipid hydrolysis.[6] The dogma has been that generation of the lipid second messenger, diacylglycerol, results in the recruitment of most protein kinase C isozymes from the cytosol, where they are maintained in an inactive conformation, to the membrane, where they become allosterically activated.[7] Diacylglycerol is the natural agonist for the C1 domain. This translocation turns out to be only one aspect of the regulation of protein kinase C. In addition to allosteric activation by binding second messengers, protein kinase C isozymes must first be processed by a series of phosphorylation events that render the enzyme catalytically competent.[8,9] A third mechanism is also central to the biological function of protein kinase C: scaffold proteins poise the enzyme at specific intracellular locations.[10] Thus, the intrinsic function of protein kinase C isozymes is regulated by three mechanisms: (1) phosphorylation, which 'primes' the en-

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zyme for catalysis, (2) cofactor binding, which allosterically activates the enzyme, and (3) interaction with targeting proteins that position protein kinase C near its regulators and substrates. This article focuses on how these three molecular mechanisms regulate protein kinase C function.

II. Structure and Function

A. Family Members

The mammalian protein kinase C family comprises 10 isozymes grouped into 3 classes: conventional (α, γ, and the alternatively spliced βI and βII), novel (δ, ε, η/L, θ), and atypical (ζ, ι/λ) PKC isozymes (Figure 1).[6,11,12] In addition, PKC μ and ν are considered by some to constitute a fourth class and by others to comprise a distinct family called protein kinase D. All members have in common a conserved kinase core carboxyl terminal to a regulatory moiety. This regulatory moiety contains two key functionalities: an autoinhibitory sequence (pseudosubstrate) and one or two membrane-targeting modules (C1 and C2 domains and, in the case of protein kinase D, PH domain). The activity of conventional protein kinase C isozymes is stimulated by diacylglycerol, Ca^{2+}, and phosphatidylserine, that of novel isozymes by diacylglycerol and phosphatidylserine, and that of atypical protein kinase C's by phosphatidylserine.

B. Domain Structure

Protein kinase C is a multimodule protein (Figure 1) that is under acute conformational regulation. Both phosphorylation and binding of cofactors induce long-range conformational changes that regulate interdomain interactions, of which the most central is the binding of the autoinhibitory pseudosubstrate sequence to the substrate-binding cavity. A proteolytically labile 'hinge' connects the regulatory moiety to the kinase domain. Cleavage at this hinge releases the kinase domain which, freed from the autoinhibition imposed by the regulatory domain, is constitutively active. It is noteworthy that purification of this cleaved kinase domain (termed protein kinase M for Mg^{2+}, the only cofactor required for activity) from old, but not fresh, bovine brain led to the discovery of protein kinase C.[13]

1. Pseudosubstrate

Kemp and co-workers originally identified a stretch of amino acids in the regulatory region of protein kinase C that had the classic hallmark of a 'pseudosubstrate': the sequence resembled that of a substrate except an Ala occupied the phosphoacceptor position (see Table 1).[14] They found that peptides based on this sequence were effective competitive inhibitors of protein kinase C, and peptides modified to have a Ser at the putative phosphoacceptor position were relatively good substrates of the enzyme. Further evidence that this sequence served an autoinhibitory role came from Makowske and Rosen, who showed that an antibody generated against the pseudosubstrate sequence was able to activate protein kinase C in the absence of cofactors, presumably by withdrawing the pseudosubstrate from the active site.[15] These findings supported the hypothesis that the pseudosubstrate occupied the substrate-binding cavity of protein kinase C, thus maintaining the enzyme in an inactive conformation.

Extensive biochemical studies in the past decade have firmly established that protein kinase C is allosterically regulated by its pseudosubstrate. Using proteases as conformational probes, it was established that activation of protein kinase C is accompanied by release of the pseudosubstrate sequence from the kinase core.[16] Specifically, the pseudosubstrate of protein kinase C is resistant to proteolysis when the enzyme is inactive but highly susceptible to proteolysis at a specific Arg in the pseudosubstrate sequence when the enzyme is active. This unmasking of the pseudosubstrate occurs independently of how protein kinase C is activated: whether by binding its cofactors or by anomalous mechanisms such as by binding cofactor-independent substrates.[17]

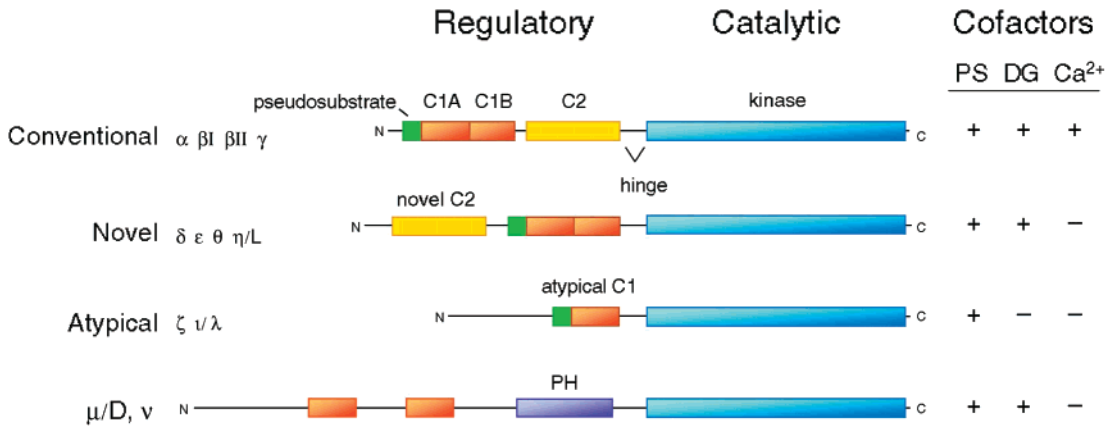

Figure 1. Schematic of primary structures of protein kinase C family members showing domain composition and activators. The N-terminal moiety contains the regulatory modules: the pseudosubstrate (green); the C1 A and C1B domains which bind phosphatidylserine and, for all but atypical protein kinase C's, diacylglycerol/phorbol esters (orange); the C2 domain which binds anionic lipids and, for conventional protein kinase C's, Ca^{2+} (yellow); and the PH domain which binds phosphoinositides (purple). The C-terminal moiety contains the kinase domain (cyan). The requirements for the classical cofactors for protein kinase C subclasses are shown on the right: PS, phosphatidylserine; DG, diacylglycerol; Ca^{2+}. (Adapted from ref 19.)

Table 1. Optimal Peptide Sequence and Pseudosubstrate Sequence of Protein Kinase C Isozymes[a]

	Position												
isozyme	−7	−6	−5	−4	−3	−2	−1	**0**	+1	+2	+3	+4	+5
PKC α													
optimal	R	R	R	R	R	K	G	**S**	F	R	R	K	A
pseudo.	N	R	F	A	R	K	G	**A**	L	R	Q	K	N
PKC βI	F	K	L	K	R	K	G	**S**	F	K	K	F	A
	V	R	F	A	R	K	G	**A**	L	R	Q	K	N
PKC βII	Y	K	L	K	R	K	G	**S**	F	K	K	K	A
	V	R	F	A	R	K	G	**A**	L	R	Q	K	N
PKC γ	R	R	R	R	R	K	G	**S**	F	K	R	K	A
	P	L	F	C	R	K	G	**A**	L	R	Q	K	V
PKC δ	A	R	R	K	R	K	G	**S**	F	F	Y	G	G
	P	T	M	N	R	R	G	**A**	I	K	Q	A	K
PKC ϵ	Y	Y	X	K	R	K	M	**S**	F	F	E	F	F
	R	P	R	K	R	Q	G	**A**	V	R	R	R	V
PKC η	A	R	R	R	R	R	R	**S**	F	R	R	X	R
	F	T	R	K	R	Q	R	**A**	M	R	R	R	V
PKC ζ	R	R	F	K	R	Q	G	**S**	F	F	Y	F	F
	K	S	I	Y	R	R	G	**A**	R	R	W	R	K
PKC μ	A	A	L	V	R	Q	M	**S**	V	A	F	F	F

[a] Optimal peptide sequences were identified by Cantley and co-workers using an oriented peptide library in which the P-3 position was fixed as Arg and the phosphoacceptor postion fixed as Ser (note the screen did not include Trp, Cys, Ser, or Thr).[35] For each isozyme, the optimal sequence identified by the screen is indicated as well as the pseudosubstrate sequence of the isozyme. The residue at the phospho-acceptor position (P0) is shown in bold. Note that no pseudosubstrate sequence has been identified for protein kinase C μ. The selectivity for a specific residue at a particular position is typically 2-fold, except for the P-5 position in protein kinase C μ which has a 6-fold selectivity for Leu. (Adapted from ref 35.)

The question then arises, how do cofactors cause release of the pseudosubstrate? Recent studies of energetics suggest that engaging of the membrane-targeting modules on the membrane provides the energy to release the pseudosubstrate from the kinase core.[18]

2. Membrane-Targeting Modules

A large number of signaling enzymes take advantage of two membrane-targeting modules to reversibly regulate their spatial distribution.[19,20] In many cases, each module binds membranes with low affinity, with tight membrane binding achieved when both domains are engaged on the membrane. When the affinity of one module for membranes is dependent on stimulus-dependent changes in membrane composition (e.g., generation of lipid second messengers) or protein structure (e.g., phosphorylation), the interaction with the membrane is reversibly regulated. Protein kinase C has served as a paradigm for the reversible regulation of membrane location by the concerted action of two membrane-targeting modules.

The role of these modules is not just to regulate the membrane binding and hence pseudosubstrate location of protein kinase C. Rather, studies with chimeric proteins have revealed that the nature of the regulatory module can affect both the substrate specificity and biological function of the full-length protein.[21,22] Furthermore, the isolated regulatory modules influence cellular function: for example, microinjection of the C1B domain in xenopus oocytes

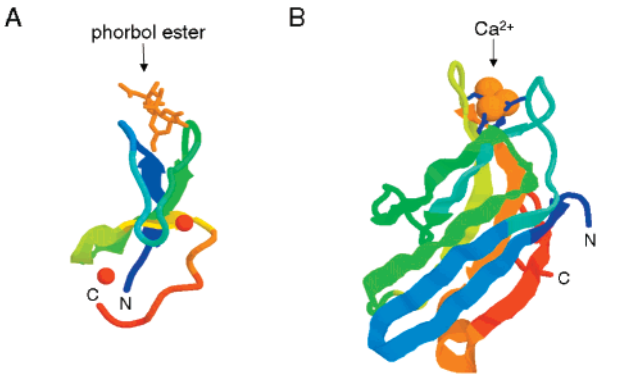

Figure 2. Structures of membrane-targeting modules of protein kinase C. (A) Ribbon diagram showing the X-ray crystallographic structure of residues 231−280 in the C1B domain of protein kinase C δ bound with phorbol-13-acetate determined by Hurley and co-workers.[26] Phorbol acetate is shown in orange; the red spheres represent the two zinc atoms. (B) Ribbon diagram showing the X-ray crystallographic structure of residues 157−289 of the C2 domain of protein kinase C β from the structure solved by Sprang and co-workers.[31] The five aspartates that line the Ca^{2+} binding site, indicated with an arrow, are shown in blue in stick representation, and the three Ca^{2+} ions are represented by orange spheres. Both structures are colored to indicate the connectivity of the strands, from blue at the amino terminus to red at the carboxyl terminus.

promotes meiotic maturation[23] and the regulatory moiety of protein kinase C modulates phospholipase D activity.[24] Thus, the membrane-targeting modules regulate not only the membrane location and equilibrium of the pseudosubstrate in and out of the active site, but additional functional parameters that involve protein:protein interactions.

a. C1 Domain. The C1 domain is a Cys-rich region of approximately 50 residues that is present in all protein kinase C isozymes. In conventional and novel protein kinase C's it is present as a tandem repeat, named C1A and C1B.[25] Atypical protein kinase C's contain a single copy of the domain, termed atypical because it does not bind phorbol esters. It is noteworthy that there is a growing family of proteins unrelated to protein kinase C that contain either typical or atypical C1 domains.[4]

Determination of the crystal[26] and NMR[27] structures of the C1B domain revealed a globular domain, with two-pulled apart beta sheets forming the ligand binding pocket (Figure 2A). Two Zn^{2+} atoms are coordinated by His and Cys residues at opposite ends of the primary sequence, helping to stabilize the domain. In atypical C1 domains, one face of the ligand-binding pocket is compromised and the module cannot bind phorbol esters or diacylglycerol.[28]

Elucidation of the structure of the C1B domain of protein kinase C δ in the presence and absence of bound phorbol acetate unveiled a surprisingly simple and effective mechanism of how ligand binding recruits the C1 domain to membranes.[26] Binding of ligand does not result in any significant conformational change in the domain. Rather, ligand binding dramatically alters the surface properties of the module to create a hydrophobic surface for membrane interaction. Specifically, ligand caps the hydrophilic ligand-binding pocket so that the top third of the C1

domain presents a contiguous hydrophobic surface. Thus, membrane targeting is achieved simply by ligand altering the surface properties of the module, in the absence of conformational changes.

b. C2 Domain. The C2 domain is an independent membrane-targeting module found not only in conventional and novel protein kinase C's, but also in a large number of proteins unrelated to protein kinase C.[29] As with the C1 domain, there is a second messenger-regulated and a second messenger-independent variant of the C2 domain: the C2 domain in conventional protein kinase C's binds membranes in a Ca^{2+}-dependent manner; however, novel C2 domains do not bind Ca^{2+}.

The C2 domain is a β-strand-rich, globular domain with loops formed by sequences at the opposite ends of the primary structure coming together to form a pocket[30] (Figure 2B). There are two topologically distinct variants of the C2 domain which differ in the connectivity of the strands: in the protein kinase C's, the type I topology exists for domains that follow the C1 domain (i.e., conventional isozymes) and the type II topology exists for domains that precede the C1 domain (i.e., novel isozymes). In Ca^{2+}-responsive C2 domains, this pocket is lined by multiple aspartic acid residues that coordinate 2−3 Ca^{2+} ions (3 in the case of the C2 domain of protein kinase C β isozymes[31]). In novel C2 domains, key aspartic acid residues are missing and the domain does not bind Ca^{2+}.[32] A variety of studies have narrowed the membrane-binding site of the C2 domain to the Ca^{2+} binding region, suggesting that the module approaches the membrane in a 'jaws first' orientation (reviewed in ref 20).

For conventional protein kinase C's, coordination of Ca^{2+} in the C2 domain causes a dramatic increase in the affinity of protein kinase C for anionic membranes. This membrane interaction shows little selectivity for phospholipid headgroup, beyond the requirement for negative charge. The Ca^{2+} binding site has a high negative electrostatic potential that is effectively neutralized upon Ca^{2+} binding. However, this charge neutralization is not sufficient to effect membrane binding since (1) mutation of acidic residues does not promote membrane binding[33] and (2) novel C2 domains which are lacking key aspartic acid residues do not bind anionic membranes constitutively. Rather, the actual coordination of Ca^{2+} is required for membrane binding, suggesting that Ca^{2+} acts as a bridge between the C2 domain and phospholipid headgroups. Consistent with this, elucidation of the crystal structure of the C2 domain of protein kinase C α in the presence of phosphatidylserine revealed that the phosphoryl group of the lipid headgroup contributed to the coordination of the bound Ca^{2+}.[34]

While the conventional C2 domains clearly function as a Ca^{2+}-regulated membrane anchor, the function of novel C2 domains, like that of atypical C1 domains, remains elusive. An attractive possibility is that these ligand-independent modules control the spatial distribution of protein kinase C via protein:protein interactions.

3. Catalytic Domain

The catalytic domain of protein kinase C has approximately 40% amino acid identity with protein kinase A,[17] differing primarily in its carboxyl-terminal sequence. The enzyme phosphorylates Ser or Thr residues, typically in the context of a basic sequence, and with a maximal catalytic rate of on the order of 10 reactions per second. Screening of an oriented peptide library has generated a series of 'optimal' substrate sequences for each isozyme (Table 1). However, selectivity for specific residues at specific positions is modest (typically 2-fold preference for a specific residue at a particular position) compared with kinases such as protein kinase A that have much stricter requirements for substrate binding.[35] As an example of the 'loose' preference for substrates, protein kinase C can, unlike protein kinase A, phosphorylate both the D- and L-stereoisomers of a number of alcohols.[36] This promiscuity in in vitro assays suggests that factors beyond primary sequence must be the critical determinants in targeting substrates in vivo.

The crystal structure of protein kinase C has remained refractory to elucidation; however, molecular modeling based on the solved structure of protein kinase A[37] has been useful in identifying surface-exposed residues.[17] As expected, residues that maintain the fold of the kinase domain or are important in catalysis (see papers by Taylor and Johnson in this issue) are conserved, with differences in residues found primarily on the surface of the protein.

The sequence of protein kinase A terminates with a Phe that tucks into the protein (see alignment in Figure 3). All protein kinase C isozymes have this Phe which is immediately followed by a conserved phosphorylation site named the hydrophobic phosphorylation motif (see below). This phosphorylation motif is then followed by a short sequence unique to each isozyme. This carboxyl-terminal sequence is critical to the regulation of protein kinase C: it forms the docking site for the upstream kinase, PDK-1, and, as such, is essential for the function of the enzyme. Constructs lacking this sequence are insoluble and nonfunctional.[38] This sequence also critically regulates the subcellular distribution of protein kinase C. Fields and co-workers found that the carboxyl-terminal 13 residues dictate the nuclear translocation and biological function of protein kinase C βII, with elegant experiments showing that these 13 residues are sufficient to confer protein kinase C βII functionality to a chimera of protein kinase C α and βII.[39]

III. Regulation

Protein kinase C is under acute structural and spatial regulation: its phosphorylation state, conformation, and subcellular location must be precisely defined for its physiological function. Thus, the kinase must be processed by phosphorylation, have its pseudosubstrate exposed, and be localized at the correct intracellular location for unimpaired signaling. Perturbation at any of these points of regulation disrupts signaling by the kinase.

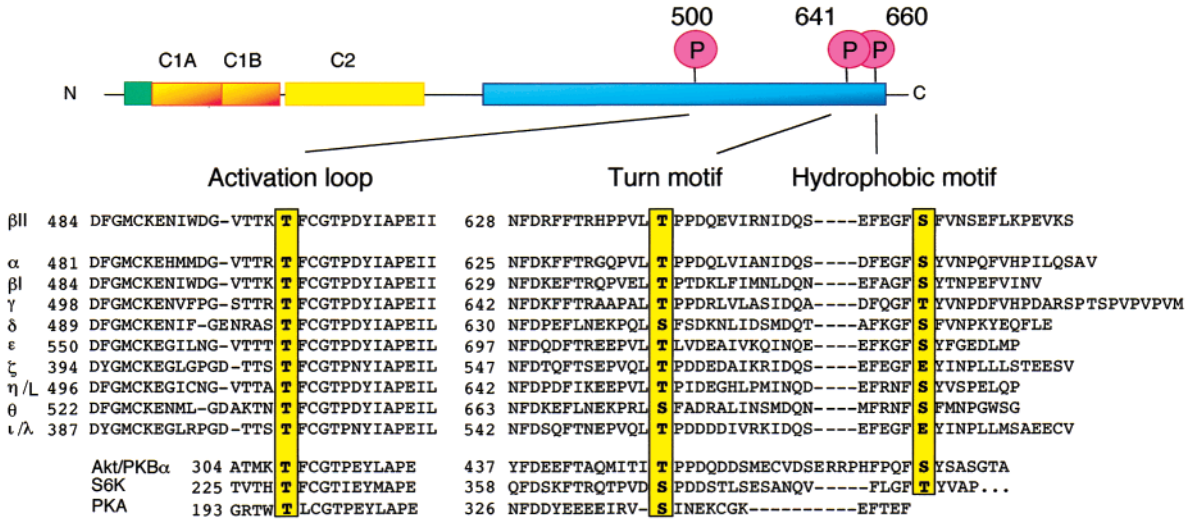

Figure 3. Alignment of activation loop, turn motif, and hydrophobic motif phosphorylation sites in protein kinase C isozymes, Akt, p70S6 kinase, and protein kinase A. The schematic of protein kinase C on top shows the relative positions of the phosphorylation sites, with numbering referring to that of protein kinase C βII. Sequences used in the alignment are as follows: human protein kinase C α, ϵ, ζ, η/L, θ and ι/λ; rat protein kinase C γ and δ;[120] protein kinase C βI and βII;[121] murine Akt α;[122] rat S6 kinase;[123] murine protein kinase A.[124] Amino acid residue numbers are indicated to the left of the sequences.

A. Phosphorylation Is Required for Catalytic Competence

1. Protein Kinase C Is Processed by Three Ordered Phosphorylations

Protein kinase C isozymes are processed by a series of ordered phosphorylations that are required for the enzyme to gain catalytic competence and correct intracellular localization.[40,41] It is this phosphorylated species that has been extensively studied over the past two decades, and it is this species that transduces signals. Unphosphorylated species accumulate in the detergent-insoluble fraction of cells and are not competent to signal.

The identification in 1998 of the phosphoinositide-dependent kinase, PDK-1, as the upstream kinase for protein kinase C isozymes swept protein kinase C signaling into a new era.[42−44] PDK-1 was originally discovered as the upstream kinase for protein kinase C's close cousin, Akt/protein kinase B.[45] The sequence similarity of the activation loop of Akt to that of the protein kinase C isozymes made it an excellent candidate for the elusive upstream kinase for protein kinase C, and experiments rapidly verified this for both conventional[44] and atypical[42,43] protein kinase C's. While it had been appreciated since the late 1980s that the enzyme was processed by phosphorylation,[46] the mechanisms of these phosphorylations are only now being unveiled.[9,47]

Figure 3 shows the three phosphorylation positions, conserved not only among protein kinase C family members, but also among other AGC family members. The first phosphorylation occurs on a loop near the entrance to the catalytic site, the activation loop; the second appears to be, by comparison to the protein kinase A structure, at the apex of a turn and has been named the turn motif; the third is flanked by hydrophobic residues and is referred to as the hydrophobic site.

The phosphorylations described below are conserved among the protein kinase C isozymes and are required for the function of the enzymes. Additional phosphorylations on both Ser/Thr and Tyr residues modulate the function of mature protein kinase C (e.g., refs 48 and 49).

a. Activation Loop Phosphorylation and PDK-1. The first and rate-limiting step in the processing of protein kinase C is phosphorylation on the activation loop by PDK-1. PDK-1 plays a pivotal role in signaling by turning on the catalytic function of diverse members of the AGC family of kinases.[50] These kinases require activation loop phosphorylation to gain catalytic competence, such that the activation loop phosphorylation site functions as a switch. Structural and biochemical studies of diverse members of the protein kinase superfamily, including both Ser/Thr kinases and tyrosine kinases, have unveiled the molecular mechanisms of this switch (e.g., see the paper by Johnson in this issue). Phosphate at this conserved segment plays two roles: it correctly positions residues for catalysis, and it unmasks the entrance to the substrate binding cavity).[51]

Mutational and biochemical analysis of conventional protein kinase C's has established that negative charge at the phosphorylatable residue of the activation loop is the first and required step in the maturation of protein kinase C. Mutation of this residue in protein kinase C α (T497) or protein kinase C βII (T500) to a neutral, nonphosphorylatable residue results in expression of a dephosphorylated protein that accumulates in the detergent-insoluble fraction of cells.[52,53] (Note that some compensating phosphorylation at adjacent Thr in the activation loop is possible:[53] the triple mutant (e.g., T497A/T498A/T500A in protein kinase C βII) precludes this and results in completely dephosphorylated protein.) This accumulation of unphosphorylated enzyme does not

result from increased phosphatase sensitivity of the activation loop mutant: pulse chase experiments have established that nonphosphorylatable activation loop mutants of protein kinase C do not become phosphorylated at the C-terminal sites (Dutil, E. M.; Gao, T.; Newton, A. C. Manuscript in preparation). Thus, phosphorylation at T500 is prerequisite for the maturation of the enzyme. Substitution of Thr with Glu at the activation loop results in the generation of fully functional, mature enzyme, suggesting that Glu is an appropriate phosphate mimic at this position.[52,53]

Although phosphorylation at the activation loop is required for the maturation of protein kinase C, once the enzyme is phosphorylated at the C-terminal sites, the activation loop phosphate is dispensable.[40] Thus, the activation loop phosphorylation is required to initiate the C-terminal processing, but once this event is completed, phosphate on the activation loop site is not essential for activity. In fact, mass spectrometric analyses of the mature protein kinase C in overexpression systems or in bovine tissue suggests that only one-half the protein kinase C molecules are phosphorylated at the activation loop.[40]

It is noteworthy that the processing of protein kinase C δ by phosphorylation differs somewhat from that of other isozymes.[54] This isozyme has a Glu positioned five residues preceding the phosphorylatable Thr (see Figure 3; Glu 500) which seems to partially fulfill the role of activation loop phosphorylation.[55] This function of Glu 500 likely accounts for modest activity of protein kinase C δ when expressed in bacteria,[56] contrasting with the inactivity of other isozymes expressed in bacteria.

b. Turn Motif. Phosphorylation at the activation loop triggers the rapid phosphorylation of a motif in a Pro-rich domain. This site corresponds to Ser338 in protein kinase A (see the paper by Taylor in this issue) which is positioned at the apex of a turn on the upper lobe of the kinase domain. Molecular modeling suggests that this site occupies a similar position in protein kinase C, hence the name 'turn motif'. Several lines of evidence suggest that this site is modified by autophosphorylation. First, kinase-inactive constructs of protein kinase C expressed in COS cells do not become phosphorylated at this position. This lack of phosphorylation does not result from the requirement for phosphorylation at the activation loop because kinase-inactive mutants that have Glu at the activation loop phosphorylation site are also not processed by phosphorylation.[57] Second, the enzyme effectively autophosphorylates at neighboring positions in vitro.[58] Curiously, this site is modified by a heterologous kinase in protein kinase C's close cousin, Akt.[59,60]

Negative charge at the turn motif is necessary and sufficient for the function of mature protein kinase C. First, selective dephosphorylation of mature protein kinase C at the activation loop and the hydrophobic motif results in no change in the catalytic activity of the enzyme in in vitro assays. Thus, once the enzyme has matured, phosphate at the turn motif is all that is needed for the catalytic function of the enzyme. Dephosphorylation at this position abolishes activity.[40] Biochemical studies indicate that phosphate at this position locks protein kinase C in a catalytically competent, thermally stable, and phosphatase-resistant conformation.[61,62] Second, constructs of protein kinase C βI with Ala in place of the phosphorylatable Thr of the turn motif accumulate as completely dephosphorylated species in the detergent-insoluble fraction of cells.[62,63] Note that other Ser/Thr in proximity of the turn motif can undergo compensating phosphorylations, so that multiple mutations are required to abolish turn-motif phosphorylation in protein kinase C βII;[62] such compensating phosphorylations could account for why mutation of the turn motif in protein kinase C α (Thr 638) and δ (Ser 643), although impairing catalysis significantly, did not abolish activity.[61,64]

In addition to its role in protein kinase C maturation and stability, the turn motif may serve as a docking site for protein:protein interactions. In particular, the sequence surrounding this motif conforms to a 14−3−3 protein-binding site,[65] presenting the intriguing possibility that phosphorylation at this position may regulate protein:protein interactions.

c. Hydrophobic Motif. Following phosphorylation of the turn motif, protein kinase C isozymes rapidly autophosphorylate at the hydrophobic motif, with an intramolecular mechanism shown for protein kinase C βII.[57] Note that protein kinase C δ could be an exception to this autophosphorylation mechanism: in addition to the anomalous activation loop regulation described above, studies by Parker and co-workers suggest that the hydrophobic site may be regulated by an upstream kinase, possibly in complex with protein kinase C ζ.[66] The hydrophobic phosphorylation site is the least conserved of the three processing sites, with atypical protein kinase C's having a phosphate mimic, Glu, at the phospho-acceptor position. It is also the one site whose phosphorylation is not essential for function. Mutation of the Ser at this position to Ala in protein kinase C α or βII results in significant production of mature, detergent-soluble, activatable enzyme. However, biochemical analyses reveal that such mutants have decreased thermal stability and increased phosphatase sensitivity.[67−69] Thus, phosphate at this position influences the subcellular location and stability of protein kinase C but in a manner that is not critical for function.

The hydrophobic site has been proposed to be modified by its own upstream kinase, tentatively referred to as PDK-2.[45] This proposal was based on studies with Akt. For this kinase, the activation loop phosphorylation catalyzed by PDK-1 depends on engaging the PH domain of Akt on membranes by binding 3′-phosphoinositides, an interaction that unmasks the activation loop phosphorylation site. Once phosphorylated at the activation loop, Akt rapidly incorporates phosphate at its hydrophobic motif. Because the first, rate-limiting step is phosphoinositide-dependent, the phosphorylation of the hydrophobic site of Akt appears to be phosphoinositide-dependent, hence the proposal that it is mediated by a putative PDK-2. Despite heroic purification and cloning efforts, such a kinase has remained refractory to identification. Could autophos-

phorylation, as occurs for conventional protein kinase C's, regulate the hydrophobic site in other kinases? Recent studies show that phosphorylation of the hydrophobic site of Akt also depends on the intrinsic catalytic activity of this kinase and that, in addition, this kinase effectively autophosphorylates at the hydrophobic site in vitro.[60] This would suggest that PDK-1-mediated phosphorylation of the activation loop followed by autophosphorylation at the hydrophobic site is a general regulatory mechanism.

The hydrophobic site has a second role in addition to stabilizing the structure of protein kinase C: the sequence surrounding the hydrophobic phosphorylation motif forms a docking site for PDK-1.[70] This site is exposed for PDK-1 binding in the unphosphorylated form of protein kinase C but becomes masked in the phosphorylated (and inactive) conformation.[71] PDK-1 actually has a higher affinity for hydrophobic phosphorylation sequences with a negative charge at the phospho-acceptor position, and such peptides effectively displace PDK-1 from protein kinase C, promoting autophosphorylation of the liberated carboxyl terminus.[71] The ability of peptides to displace PDK-1 may account for the proposal by Alessi and co-workers that a peptide based on the carboxyl terminus of the protein kinase C-related kinase, PRK-1, which they termed PIF (for PDK-1 Interacting Fragment), converts PDK-1 into a PDK-2 kinase.[72] Rather than causing this unprecedented change in substrate specificity, PIF may be promoting the hydrophobic site autophosphorylation of Akt by disengaging PDK-1 and rendering the carboxyl terminus accessible to the active site for autophosphorylation.

2. Cellular Control of Protein Kinase C Phosphorylation

It is clear that the phosphorylation of the activation loop catalyzed by PDK-1 is a required step in signaling by protein kinase C. This begs the following question: what are the cellular inputs that control this event? Now that PDK-1 has been identified as the upstream kinase, significant effort is being directed into understanding what regulates its intrinsic activity. This kinase has high basal activity in vitro, and mounting evidence suggests that the enzyme is constitutively active in cells. Nonetheless, substrate phosphorylation by PDK-1 is under exquisite control in vivo. A major factor in this specificity is achieved by the substrate itself, which must be in the correct conformation and location in order to be phosphorylated by PDK-1.[50] In the case of protein kinase C, pulse chase experiments have revealed that newly synthesized protein kinase C localizes to the membrane where it adopts a conformation in which the pseudosubstrate is out of the active site, thus exposing the activation loop phosphorylation site.[73] This conformation is essential to target protein kinase C for phosphorylation by PDK-1.

A major challenge lies in determining whether this processing by protein kinase C is constitutive or whether additional inputs regulate its phosphorylation by PDK-1. PDK-1 itself is regulated by multiple phosphorylations[74] (in addition to autophosphorylation on its own activation loop)[75] and has a PH domain which binds 3-phosphoinositides,[76] suggesting at least two potential regulatory mechanisms. However, the finding that >90% of protein kinase C is in the 'mature' form under most published conditions suggests that any regulation of PDK-1 is masked under conditions used to study protein kinase C. The possibility that external signals may, in fact, regulate the PDK-1 step in protein kinase C processing is suggested by a recent report showing that the phosphorylation of protein kinase C δ is dependent on phosphatidylinositide-3-kinase lipid products in HEK293 cells grown in suspension but not in adherent cells.[77] The current explosion of research into PDK-1 is likely to shed light on its regulation soon.

Mounting evidence points to a role for phosphatases in controlling the phosphorylation state of protein kinase C. In particular, prolonged activation following phorbol ester treatment results in complete dephosphorylation of protein kinase C coupled to translocation to a detergent-insoluble cell fraction. In addition, serum starvation results in selective dephosphorylation of the hydrophobic site, suggesting that dephosphorylation rather than the constitutive phosphorylation provides the regulation of this site[66,78] (Edwards, A. E. Newton, A. C. Manuscript in preparation). Similarly, passage of quiescent fibroblasts triggers the selective dephosphorylation of the hydrophobic site in protein kinase C ϵ by a rapamycin-sensitive mechanism.[79] Parker and co-workers noted that the phosphorylation state of the hydrophobic site of another novel protein kinase C, the δ isozyme, is also rapamycin-sensitive.[66] Whether this reflects the involvement of mTOR kinase activity or, as proposed recently, phosphatase activity regulated by mTOR,[80] remains to be resolved.

B. Membrane-Binding Modules Allosterically Regulate Protein Kinase C

The hallmark of protein kinase C activation in cells is its translocation to the plasma membrane. The discovery that protein kinase C is 'the receptor' for phorbol esters catalyzed an explosion of studies on the effects of phorbol esters on cells that were linked by one unifying theme: protein kinase C redistribution from the cytosol to the membrane. While initial studies relied on cellular fractionation, recent advances in cellular imaging have allowed elegant capture, in real time, of the ligand-dependent translocation of GFP-tagged constructs of protein kinase C in vivo.[81,82]

Initial studies on protein kinase C focused on its requirement for cofactors for activation, and three classical activators were identified: phosphatidylserine, Ca^{2+}, and diacylglycerol. Later studies linked the requirements for activity to the ability to promote membrane binding. Coupled now with structural information, the extensive biochemical and biophysical studies on protein kinase C have established the central roles of the C1 and C2 domains in orchestrating this spatial redistribution which leads to activation.

1. Phosphatidylserine

The activity of all isozymes of protein kinase C is regulated by phosphatidylserine, an aminophospholipid found exclusively on the cytoplasmic leaflet of membranes, which, typically, comprises approximately 15 mol % of the total lipid.[83] Binding studies revealed that, in the absence of diacylglycerol, protein kinase C displays little selectivity for phosphatidylserine compared with other monovalent anionic lipids. However, the presence of diacylglycerol causes protein kinase C to bind phosphatidylserine-containing surfaces (membranes or detergent:lipid mixed micelles) with an order of magnitude higher affinity than membranes composed of other anionic lipids. Thus, the recognition of phosphatidylserine depends on C1 domain ligands. This stimulation by phosphatidylserine is stereospecific for sn-1,2-phosphatidyl-L-serine. While it had been suggested that this requirement arose from unique membrane-structuring properties of phosphatidylserine optimizing the membrane surface for protein kinase C binding, recent studies with enantiomeric membranes have established that the stereospecific requirement results from molecular determinants on protein kinase C recognizing the shape of the phosphatidylserine molecule.[84]

Initial studies focused on the C2 domain as conferring this sensitivity to phosphatidylserine. However, mutations covering most surfaces of the domain, including a proposed phosphatidylserine binding motif, have no significant effect on the phosphatidylserine selectivity of the full-length protein.[33,85] Rather, studies with isolated domains of protein kinase C βII have recapitulated the phosphatidylserine specificity induced by diacylglycerol in the isolated C1B domain. Specifically, the isolated C1B domain binds to anionic membranes containing 2 mol % phorbol esters with 15-fold higher affinity if the anionic lipid is phosphatidylserine compared with phosphatidylglycerol[18] (Figure 4). Consistent with this finding, it is the C1 domain that is present in all isozymes of protein kinase C. The precise determinants on the C1 domain that recognize phosphatidylserine remain to be determined. In this regard, Cho and co-workers identified an Asp on the surface of the C1A domain of protein kinase C α which they suggest might be involved in phosphatidylserine specificity by impeding the interaction with other anionic lipids.[86]

2. Diacylglycerol and Phorbol Esters

Phorbol esters and diacylglycerol cause a dramatic increase in the affinity of protein kinase C for membranes, serving as 'molecular glue' to recruit protein kinase C to membranes. Both ligands regulate protein kinase C by the same mechanism; however, they can have very different biological effects for two reasons. First, membrane recruitment and activation initiated by diacylglycerol is short-lived because diacylglycerol is rapidly metabolized, in contrast to phorbol esters which are not readily metabolized and result in constitutive activation of protein kinase C. Second, phorbol esters are over 2 orders of magnitude more potent than diacylglycerol, on a molar basis, in recruiting protein kinase C to membranes. The remarkable potency of phorbol esters is illustrated by the finding that 2 mol % phorbol esters (i.e., two phorbol ester molecules per 98 lipid molecules) increases the affinity of protein kinase C for phosphatidylserine-containing membranes by 4 orders of magnitude. This 4-orders of magnitude increase is reproduced in the isolated C1B domain (see Figure 4: full-length protein kinase C binds to anionic membranes with an apparent affinity of 10^2 M^{-1} in the absence of phorbol myristate acetate (PMA) and an apparent affinity of 10^6 M^{-1} in the presence of PMA; similarly, the C1B domain binds anionic lipids with an apparent affinity of 10^1 M^{-1} in the absence of PMA and an apparent affinity of 10^5 M^{-1} in the presence of PMA). This incredible potency of PMA allows it to recruit the C1 domain to membranes in the complete absence of anionic lipids (Figure 4: PMA (2 mol %) causes both full-length

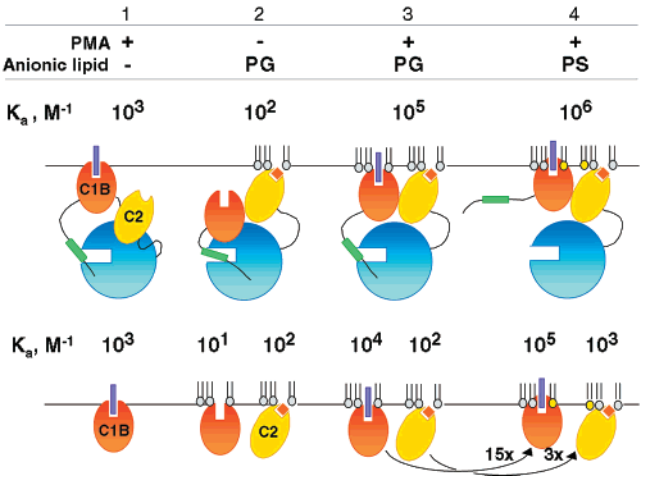

Figure 4. Schematic comparing the approximate binding constants of full-length protein kinase C βII with those of the isolated C1B and C2 domains for membranes of four different compositions. The C1B domain is shown in orange and the C2 domain in yellow either in the context of the full-length protein (upper panel) or alone (lower panel). Shown are the apparent equilibrium constants for binding to (1) neutral membranes containing 2 mol % PMA (note that the C2 domain does not bind to neutral membranes); (2) anionic membranes containing 40 mol % phosphatidylglycerol (PG) and no PMA; (3) anionic membranes containing 40 mol % phosphatidylglycerol (PG) and 2 mol % PMA; and (4) anionic membranes containing 40 mol % phosphatidylserine (PS) and 2 mol % PMA. Bulk neutral lipid is phosphatidylcholine. Binding constants were obtained in the presence of 0.2 mM Ca^{2+}. The *x*-fold increase in affinity of the isolated C1B and C2 domains for binding membranes containing PS instead of PG is indicated below the domains. For simplicity, the C1A domain is not shown and is proposed not to contribute significantly to the membrane interaction for this isozyme. The pseudosubstrate (green rectangle) has a binding affinity of approximately 10^4 M^{-1} for anionic membranes,[96] an interaction that would be relevant to the panel on the far right where the enzyme is active and the pseudosubstrate removed from the active site. The difference in binding energy between engaging of the separate modules (C1 domain, C2 domain, and pseudosubstrate) is on the order of 8 kcal mol^{-1} and is proposed to account for the energy required to release the basic pseudosubstrate from the substrate-binding cavity. Data taken from ref 18. Violet rectangle represents PMA; orange square represents Ca^{2+}; yellow lipid represents phosphatidylserine.

protein kinase C and the C1B domain to bind membranes composed of the neutral lipid phosphatidylcholine with an apparent affinity of 10^3 M^{-1}).

Structural analyses have elegantly revealed the mechanism of this 'molecular glue': as mentioned above, occupancy of the ligand binding cavity in the C1 domain results in presentation of a contiguous hydrophobic surface.[26] Thus, C1 domain ligands recruit protein kinase C by altering the surface properties of the domain to favor membrane penetration.

Most isozymes of protein kinase C contain two C1 domains, the C1A and C1B, yet biochemical studies generally reveal a stoichiometry of one phorbol ester bound per protein kinase C molecule.[87−90] However, recent studies with a fluorescent analogue of phorbol ester suggest that two ligands bind one protein kinase C α[91]). Blumberg and co-workers have shown that, in the case of protein kinase C δ, it is the C1B domain that mediates in vivo functionality: mutation of the C1A domain to impair phorbol ester binding had little effect on the phorbol ester-dependent translocation of this isozyme in NIH 3T3 cells, whereas mutation of the C1B impaired translocation.[92] In contrast, both domains play equivalent roles in causing protein kinase C α translocation.[93] Studies with isolated C1A and C1B domains have also provided contrasting results but consistent with in vivo results: only the C1B domain binds phorbol esters in the case of protein kinase C δ;[94] however, both the C1A and C1B bind phorbol esters in vitro in the case of protein kinase C γ.[95] Thus, phorbol esters may regulate protein kinase C by either C1 domain (or both simultaneously) for some isozymes (e.g., protein kinase C α) but only through the C1B domain in other isozymes (e.g., protein kinase C δ).

3. Calcium Ion

The conventional protein kinase C's stand out in their unique regulation by Ca^{2+}: this cation facilitates translocation by increasing the affinity of the C2 domain for anionic lipids. It should be noted that increased intracellular Ca^{2+} is not essential for protein kinase C translocation and activation: if sufficiently tight binding is achieved by the C1 domain, the C2 domain does not need to be fully engaged on the membrane for activation. Thus, phorbol ester stimulation can cause translocation of conventional protein kinase C isozymes in the absence of changes in intracellular Ca^{2+}.

One of the earliest observations about protein kinase C was that diacylglycerol and Ca^{2+} 'synergize' in activating the enzyme. That is, the presence of one ligand dramatically decreased the amount of the second ligand required to activate the enzyme. This synergism does not result from allosteric interactions between the C1 and C2 domains. Rather, each ligand independently causes protein kinase C's affinity for anionic lipids to increase. If one dissects out the contribution of anionic lipids, there is no synergism. Thus, varying the Ca^{2+} concentration by 5 orders of magnitude has no effect on the affinity of protein kinase C for neutral membranes containing phorbol esters,[90] an interaction mediated only by the C1 domain.

An attractive regulatory mechanism for conventional protein kinase C's is that elevated intracellular Ca^{2+} recruits protein kinase C to membranes by the C2 domain and, by the reduction in dimensionality, dramatically increases the probability of finding diacylglycerol and engaging the C1 domain. Thus, engaging the C2 domain increases the efficiency of the search for diacylglycerol. Consistent with this, Oancea and Meyer proposed that the C2 domain docks the membrane first, based on their studies with protein kinase C α: they found that the kinetics of translocation triggered by engaging the C2 domain (i.e., by increasing intracellular Ca^{2+}) is faster than that triggered by engaging the C1 domain (i.e., by addition of phorbol esters).[82]

4. Model for Membrane Interaction

Figure 5 presents the current working model for the cofactor regulation of mature (i.e., phosphorylated) protein kinase C. In the inactive conformation (i.e., in the absence of second messengers), protein kinase C resides in the cytosol with its pseudosubstrate bound to the active site and sterically blocking substrate binding. Studies with model membranes reveal that the C1 and C2 domains function as independent membrane-targeting modules and can independently recruit protein kinase C to membranes. The isolated C1 and C2 domains can be recruited to membranes by a relatively weak interaction, with binding constants on the order of 10^2−10^5 M^{-1}, depending on the concentrations of diacylglycerol/phorbol esters, anionic lipids, and Ca^{2+}. The binding energy provided by tethering a single domain is insufficient to result in significant release of the pseudosubstrate. However, the tethering of both domains results in a binding constant $\geq 10^6$ M^{-1}. This binding constant is less than that of the sum of the binding constants for the isolated C1 and C2 domains and pseudosubstrate sequence,[96] suggesting that the difference (roughly estimated to be on the order of 8 kcal mol^{-1}) could be the energy required to release the pseudosubstrate from the substrate-binding cavity.[18]

C. Anchoring Proteins Are Spatial Regulators of Protein Kinase C

The biological function of protein kinase C depends on its correct spatial distribution. This localization is orchestrated by a battery of binding partners for protein kinase C that serve the role of anchoring or scaffold proteins.[10,97] These proteins position protein kinase C isozymes near their substrates, near regulators of activity such as phosphatases and kinases, or in specific intracellular compartments.

The essential role of anchoring/scaffold proteins in regulating protein kinase C is epitomized in the Drosophila phototransductive system. Here, the scaffold protein ina D coordinates a number of proteins involved in phototransduction through a series of PDZ domains, each specific for a particular protein. Abolishing its interaction with any one of these proteins, including PKC, results in mislocalization of the relevant signaling protein and disrupts signaling.[98]

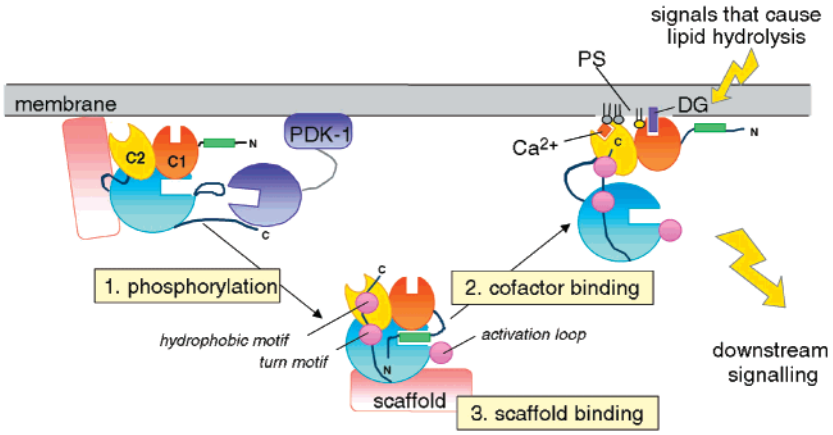

Figure 5. Model summarizing the spatial, structural, and conformational regulation of protein kinase C by (1) phosphorylation, (2) cofactor binding, and (3) interactions with anchoring/scaffold proteins. Newly synthesized protein kinase C associates with the membrane in a conformation that (a) exposes the activation loop for phosphorylation (pseudosubstrate (green rectangle) is exposed) and (b) exposes the carboxyl terminus for binding PDK-1. The first step in the posttranslational modification of protein kinase C is phosphorylation at the activation loop by PDK-1 (pink circle represents phosphate). This phosphorylation correctly aligns residues for catalysis. PDK-1 is released from protein kinase C, triggering the autophosphorylation of the two carboxyl-terminal sites (turn motif and hydrophobic motif). The fully phosphorylated species is then released into the cytosol, where it is maintained in an autoinhibited conformation by the pseudosubstrate (middle panel) and in a stable, phosphatase-resistant conformation by restructuring of the carboxyl-terminal interactions. Generation of diacylglycerol, and, for conventional protein kinase C's, Ca^{2+} mobilization, provides the allosteric switch to activate protein kinase C. This is achieved by engaging the C1 and C2 domains on the membrane (right panel), thus providing the energy to release the pseudosubstrate from the active site, allowing substrate binding and catalysis. In addition to the regulation by phosphorylation and cofactors, anchoring/scaffold proteins (blue oblongs) play a key role in protein kinase C function by positioning specific isozymes at particular intracellular locations.

An abundance of anchoring proteins bind protein kinase C. Some binding proteins regulate multiple protein kinase C isozymes, while others control the distribution of specific isozymes. There are binding proteins for unphosphorylated protein kinase C, for phosphorylated but inactive protein kinase C, and for phosphorylated and activated protein kinase C. For example, the anchoring protein CG-NAP has recently been shown to localize newly synthesized (unphosphorylated) PKC ϵ to the Golgi/centrosome.[99] Members of the AKAP family of scaffold proteins (for A Kinase Anchoring Proteins) position phosphorylated but inactive protein kinase C near relevant substrates.[100] A family of proteins called RACKs (for Receptors for Activated C Kinase) anchor the active conformation of phosphorylated protein kinase C at specific cellular locations.[101] Other proteins named STICKs (for Substrates That Interact with C Kinase) bind inactive, phosphorylated protein kinase C and then release the activated kinase following their phosphorylation.[102]

Anchoring proteins for protein kinase C have diverse functions: some positively regulate signaling, while others negatively regulate it. For example, the interaction of protein kinase C βI with the tyrosine kinase, Btk, positively regulates JNK, leading to transcriptional activation of cytokine genes in mast cells.[103] In the case of protein kinase C ζ, its interaction with the protein PAR 3 activates pathways leading to embryonic polarity and subsequent asymmetric cell division.[104] Conversely, binding to the protein PAR-4 serves to inactivate protein kinase C ζ, leading to apoptosis.[105] Thus, protein:protein interactions play key roles in regulating signaling pathways revolving around protein kinase C.

Protein kinase A contains a specific structural motif[106] in the regulatory subunit that binds AKAPs, and conversely, AKAPS contain a strictly defined sequence that recognizes this structural motif.[107] Thus, the interaction and subsequent biology of protein kinase A:AKAPs can be effectively disrupted using peptides based on the anchoring protein's recognition sequence. In marked contrast, anchoring partners bind protein kinase C by multiple mechanisms. Some proteins bind determinants in the regulatory domain, and others bind determinants in the kinase core. For example, protein kinase C ϵ has an actin-binding motif located between the C1A and C1B domains.[108] Determinants in the C1 domain also regulate a very different interaction: the binding of protein kinase C isozymes to the PH domain of the tyrosine kinase, Btk.[109] By comparison, the anchoring region of protein kinase C ζ for PAR 3 and its mammalian counterpart, ASIP, resides in the kinase domain.[110] Nor are there defined consensus protein kinase C binding sites on anchoring proteins. Rather, the interactions described to date rely on determinants unique to each binding partner. Thus, nature has taken advantage of the unique surfaces of protein kinase C isozymes to tether them to specific intracellular sites. These unique surfaces permit the design of peptides to competitively inhibit specific isozyme:anchor protein interactions.[111]

Mochly-Rosen and co-workers have taken this concept one step further by proposing that peptide agonists, rather than antagonists, can be designed to promote (rather than inhibit) protein kinase C functions regulated by anchoring proteins for activated enzyme, the RACKs.[112] They propose that the motif on protein kinase C that binds its cognate anchoring protein is masked by an intramolecular interaction with another domain of protein kinase C, in a similar way that the pseudosubstrate is masked by the substrate-binding cavity. They have proposed

that this 'masking' domain (which they call a pseudoRACK sequence) comprises a sequence similar to the binding site on the actual anchor protein, so that peptides based on this 'masking' motif will effectively disengage the intramolecular interaction. Disruption of this intramolecular interaction is proposed to activate protein kinase C by the same mechanism that binding to RACKS stabilizes the active conformation of the enzyme. In the case of protein kinase C ϵ, the sequence NDAPIGYD in its C2 domain (also referred to as V1 region) has been identified as a pseudoRACK based on its similarity to the sequence NNVALGYD in the RACK, β'-COP.[113,114] The use of peptides based on this 'masking' motif has, in fact, proved promising in promoting protein kinase C ϵ activation in cardiac myocytes, an event that is involved in protecting hearts from ischemia.[114]

D. Summary

Figure 5 summarizes the molecular mechanisms regulating the biological function of protein kinase C. Unphosphorylated protein kinase C is positioned at the membrane in a conformation that targets it for phosphorylation by PDK-1. Phosphorylation by PDK-1 triggers the intramolecular autophosphorylation at the carboxyl-terminal sites, an event which has two important consequences: (1) protein kinase C adopts its 'mature' conformation and (2) the mature protein kinase C redistributes to the cytosol. This phosphorylated species is catalytically competent but is maintained in an inactive conformation because the pseudosubstrate occupies the substrate-binding cavity. Generation of diacylglycerol and in the case of conventional protein kinase C's elevation of intracellular Ca^{2+} recruits protein kinase C to the membrane by engaging the C1 and C2 domains. The energy provided by the interaction of these domains with the membrane releases the pseudosubstrate, allowing substrate binding and phosphorylation. Anchoring proteins play a key role in poising all species of protein kinase C (i.e., unphosphorylated, phosphorylated but inactive, and phosphorylated and activated) at specific intracellular locations. Disruption of any of these mechanisms impairs signaling.

The past decade has unveiled the molecular mechanisms governing the intrinsic function of protein kinase C. Major challenges ahead are to understand how PDK-1, the upstream kinase of protein kinase C, is regulated and how specific isozymes effect downstream signaling events. To this end, the generation of mouse knock-outs of specific protein kinase C isozymes and of PDK-1,[115−118] the use of novel approaches to inhibiting specific isozymes,[119] and the identification of protein kinase C in lower eukaryotes, where genetic manipulation is readily performed,[11] poise the field to attain this goal.

IV. Abbreviations

AKAP	A kinase anchoring protein
PDK-1	phosphoinositide-dependent kinase-1
PIF	PDK-1 interacting fragment
PKC	protein kinase C
PG	phosphatidylglycerol
PMA	phorbol myristate acetate
PS	phosphatidylserine

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