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Protein kinase C: a paradigm for regulation of protein function by two membrane-targeting modules

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1. Introduction

Membranes orchestrate a vast repertoire of enzyme functions. Evidence in the past few years has clearly established that their role in cell function extends far beyond merely serving as a stage for the intricate ballet danced by amphitropic proteins as they associate and disassociate from the membrane. Rather, membranes dictate the localization and function of these proteins by specific mechanisms.

Prime examples of enzymes whose activities are regulated by reversible binding to the membrane abound in signal transduction. Here, the communication of signals from the cell surface to intracellular locations depends acutely on properly timed recruitment of specific proteins to the plasma membrane. The importance of membrane targeting is underscored by experiments showing that disruption of membrane targeting stops particular signaling pathways. For example, mutants of the kinase, src, that are not myristoylated do not translocate to membranes and do not transduce src-mediated cell transformation [1]. Similarly, mutants of the small molecular weight G protein, ras, that are not farnesylated do not translocate to membranes and do not transduce ras-mediated cell functions [2,3]. As a third example, the cellular function of the ras exchange factor, SOS, is impaired by removal of its PH (pleckstrin homology) domain, a mutation that does not alter the protein's catalytic activity [4]. Thus, controlled membrane translocation of specific proteins is central to signal transduction.

2. Two membrane-targeting motifs as a mechanism to achieve high-affinity membrane binding

Membrane targeting is mediated by a variety of determinants on the protein: via specific lipid binding domains such as PH, C1 or C2 domains, or through the use of lipid modifications such as isoprenylation or acylation. High-affinity binding is achieved through the use of two membrane-targeting motifs, each binding with low affinity to membranes but with the binding of both domains providing a high-affinity anchorage. When the affinity of one of the motifs is sensitive to stimulus-dependent changes in membrane composition (e.g. lipid hydrolysis) or protein structure (e.g. phosphorylation, acylation), the membrane interaction of the protein is regulated reversibly. This point is illustrated with the myristoyl-electrostatic switch which reversibly regulates the membrane interaction of the src family of kinases and MARCKS [5]. An amino-terminal myristic acid and an adjacent stretch of basic residues comprise the two membrane-targeting modules for these proteins. Elegant studies with src or peptides derived from the protein have revealed that the apparent membrane affinity of the myristate alone is on the order of 10^4 M^{-1} and that of the basic stretch alone on the order of 10^3 M^{-1}; each motif alone is too weak to target src to membranes, but the combination of the two motifs results in an apparent membrane affinity of 10^7 M^{-1}, providing more than sufficient energy to recruit src to membranes. Reversibility is achieved by phosphorylation at the amino terminus, altering the electrostatic potential of the basic domain. For those proteins that take advantage of a myristoyl and palmitoyl group to achieve a high-affinity membrane interaction, reversibility can be achieved by depalmitoylation [6].

Other proteins use a combination of protein:protein and protein:lipid interactions for membrane targeting: rhodopsin kinase, for example, is recruited to membranes by a specific interaction with the light-activated conformation of rhodopsin and by a carboxyl-terminal farnesyl group [7]. Once again, each interaction alone is not sufficient to recruit the enzyme to membranes, but together, a high-affinity membrane interaction is achieved. In this case, reversibility is achieved by the conformation of the substrate: only the light-activated rhodopsin provides the determinants for the receptor:kinase interaction. The related β adrenergic receptor kinase

uses a slightly different approach: membrane targeting is mediated by a PH domain and by interaction with another membrane-interacting protein, $G_{\beta\gamma}$. Both interactions are needed for this kinase to access and phosphorylate its transmembrane substrate, the β adrenergic receptor [8]; reversibility is achieved by sequestration of $G_{\beta\gamma}$.

3. Protein kinase C: the archetypal lipid-regulated enzyme

Members of the protein kinase family of enzymes transduce the myriad of signals that promote phospholipid hydrolysis [9,10]. Generation of diacylglycerol in the membrane drives protein kinase C from the cytosol to the membrane; interaction with this lipid and with the aminophospholipid, phosphatidylserine, results in a conformational change that promotes catalysis [11].

The protein kinase C enzymes epitomize the use of two membrane-targeting modules to provide sensitive, specific, and reversible regulation of protein function. These two membrane-targeting modules are the C1 domain, which binds diacylglycerol, and the C2 domain which binds acidic lipids [12,13]. High-affinity membrane binding is achieved by having both domains tethered to the membrane, and reversibility is achieved upon metabolism of diacylglycerol, the C1 domain ligand.

4. Domain structure of protein kinase C

There are 11 mammalian isoforms of protein kinase C that have been identified to date and they all share in common an amino-terminal membrane-targeting (regulatory) moiety (ranging from 20–70 kDa) linked to a carboxyl-terminal kinase domain (approximately 45 kDa) (Fig. 1). The kinase domain, which has high similarity to that of protein kinase A, comprises two regions: the ATP binding lobe (C3) and the substrate binding lobe (C4). It is maintained in an inactive state by an autoinhibitory sequence in the regulatory moiety, the pseudosubstrate, that sterically blocks the active site. Binding of cofactors to the regulatory moiety releases the pseudosubstrate from the active site, thus activating protein kinase C [14].

The isozymes have been subdivided into classes based on their cofactor dependence, which in turn is dictated by the modules present in the regulatory moiety. Conventional protein kinase Cs are regulated by Ca^{2+}, diacylglycerol, and phosphatidylserine and contain two C1 domains and one C2 domain; novel protein kinase Cs are regulated by diacylglycerol and phosphatidylserine and contain two C1 domains and a modified C2 domain; atypical protein kinase Cs are sensitive to phosphatidylserine with additional ligands unknown, and contain one atypical C1 domain; and the more recently cloned protein kinase C μ (human; murine homologue protein kinase D) is

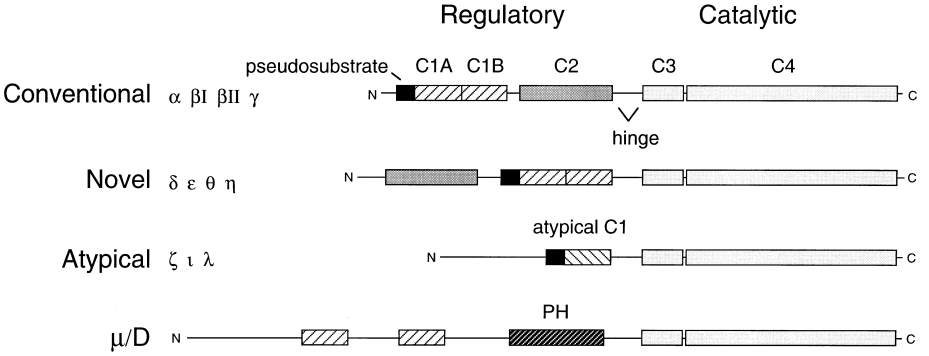

Fig. 1. Schematic representation of protein kinase C's domain composition, showing membrane-targeting modules in amino-terminal regulatory half and kinase domain in carboxyl-terminal half. Membrane-targeting modules are: C1 domain which binds diacylglycerol or phorbol esters in all but the atypical protein kinase Cs (hatched box); C2 domain which binds anionic lipids and, in conventional protein kinase Cs, Ca^{2+} (dark grey box); PH domain which binds phosphoinositides (dark hatched box). The kinase domain (light grey boxes) comprises the ATP binding lobe (C3) and substrate binding lobe (C4). Subclasses and members of each isozyme subclass are listed at the left.

sensitive to both phosphatidylserine and diacylglycerol and contains, in addition to two C1 domains, a PH domain which may confer sensitivity to phosphoinositides [15,16]. Thus, the overall feature of the protein kinase Cs is membrane-targeting modules linked to a kinase domain. With the possible exception of the atypical protein kinase Cs, whose mechanism of regulation has not been established, membrane targeting involves two membrane-targeting modules.

The regulatory moiety is linked to the kinase domain by a flexible 'hinge' that becomes proteolytically labile when protein kinase C is bound to membranes [17–19]. Proteolysis at the hinge region generates two functional moieties: the regulatory half which binds lipids, phorbol esters, and Ca^{2+} [20], and the kinase domain (protein kinase M) which is relieved of inhibitory constraints and is constitutively active [21].

4.1. C1 domain

All protein kinase C isozymes contain one (C1) or two (C1A and C1B) globular C1 domains. The C1 module is present also in a number of unrelated proteins such as diacylglycerol kinase, raf, and *n*-chimaerin [22]. The module has two structural variants: the typical C1 domain which binds phorbol esters/diacylglycerol (Fig. 2A) and the atypical C1 domain which does not bind these ligands. Most protein kinase C family members are responsive to phorbol esters/diacylglycerol, the exception being the atypical protein kinase Cs (ζ, ι, λ) which each contain a single atypical C1 domain.

4.1.1. The C1 domain coordinates two Zn^{2+} atoms

Cloning of the protein kinase Cs in the mid 1980s revealed a conserved Cys-rich motif that is a hallmark of C1 domains [23]. Extended X-ray absorp-

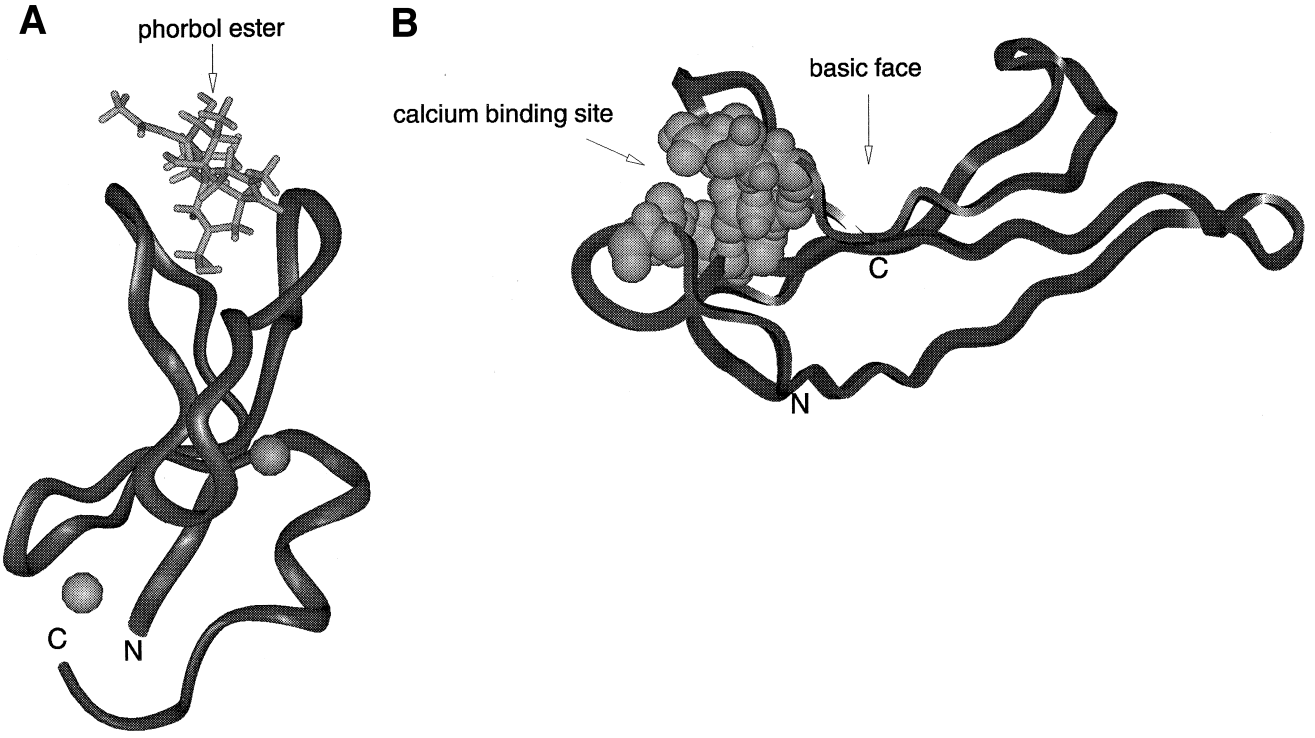

Fig. 2. Ribbon diagram showing the fold of the C1 and C2 domains. A: Ribbon diagram showing the X-ray crystallographic structure of residues 231–280 in the C1B domain of protein kinase C δ with bound phorbol-13-acetate based on the coordinates of Zhang et al. [26]. The arrow indicates the position of the phorbol ester; the grey balls represent the two zinc atoms. B: Ribbon diagram showing a modeled structure of residues 183–257 of the C2 domain of protein kinase C β [45] based on the X-ray crystal structure of first C2 domain of synaptotagmin solved by Sutton et al. [46]. The five aspartates that line the Ca^{2+} binding site, indicated with an arrow, are shown in space-filling representation. The basic face beta sheet behind the Ca^{2+} site is indicated.

tion fine structure (EXAFS) [24] and atomic absorption measurements [25] later established that conventional protein kinase Cs coordinate four Zn^{2+} atoms, corresponding to two per C1 domain. Structural studies have now revealed why the enzyme coordinates Zn^{2+}: the two metal binding sites play a key role in maintaining the fold of the domain by bringing together residues that are far removed in the primary sequence. One metal binding site is near the bottom of the globular domain and involves residues at the N and C termini of the domain, and the second site is in the middle of the molecule [26–28].

4.1.2. Ligand binding alters the surface properties, and not conformation, of the C1 domain

NMR and X-ray studies have established that the C1 domain folds into a globular beta sheet-rich structure [26,27], with two pulled-apart beta sheets providing the ligand binding pocket [26]. Fig. 2A shows the structure of the C1B domain of protein kinase C δ with bound phorbol acetate solved by Hurley and coworkers [26]. A striking finding from structural studies was that the conformation of the C1B domain of protein kinase C δ was not significantly different with or without bound phorbol acetate [26]. Rather, the surface hydrophobicity of the domain was markedly increased. Specifically, in the absence of phorbol binding, the top half of the C1 domain is relatively hydrophilic because of the water-lined groove formed by the pulled-apart beta sheets. Once phorbol binds this groove, the domain is 'capped' so that the top third provides a contiguous hydrophobic surface. This structural finding beautifully accounts for biochemical data (see below) showing that phorbol esters recruit protein kinase Cs to membranes by acting as a hydrophobic anchor. Solution NMR studies have recently confirmed that the ligand-binding half of the C1 domain contains residues that interact with lipid based on changes in their NMR signals in the presence of lipid micelles [28].

4.1.3. Are the C1A and C1B domains functionally equivalent?

The stoichiometry of phorbol ester binding to protein kinase C is one ligand per protein kinase C [29–32], suggesting that the protein's function is regulated by only one C1 domain. Studies on GST-constructs of isolated C1A and C1B domains reveal that the isolated domains bind phorbol esters with similar affinities when derived from protein kinase C γ [33], but only the C1B was shown to bind phorbol esters tightly when derived from protein kinase C δ [34]. For protein kinase C δ, the two domains are not equivalent in the context of the full-length protein: mutation of the C1A domain had little effect on the phorbol ester-dependent translocation of protein kinase C δ in NIH 3T3 cells, whereas mutation of the C1B domain decreased translocation significantly [35]. Thus, at least for the δ isozyme, the non-equivalence of the C1A and C1B domains could account for why only the latter is relevant in the phorbol ester-binding function of protein kinase C in vivo. For isozymes such as protein kinase C γ, it is possible that the orientation of the domains associated with the full-length protein is such that only one can access the membrane-bound ligand.

Stubbs and coworkers have found that a fluorescent phorbol ester analogue, sapinotoxin-D, recruits protein kinase C to membranes with biphasic kinetics and have suggested that these kinetics reflect binding of the probe to a low-affinity and high-affinity phorbol binding site [36]. Curiously, such biphasic kinetics are not observed in the membrane interaction of protein kinase C that is mediated by phorbol myristate acetate or phorbol dibutyrate [30,37,38].

4.1.4. Atypical C1 domains have a compromised ligand-binding site

Extensive studies from numerous groups have failed to detect phorbol ester binding, or effects of C1 ligands on enzyme activity, for the atypical protein kinase Cs [39,40]. The molecular basis for this difference was unveiled when the structure of an atypical C1 domain, that of Raf, was elucidated. Atypical C1 domains do not have consensus sequences present in the typical C1 domains that form a hydrophobic face of the binding site in typical C1 domains, thus compromising the structure of the binding site in atypical C1 domains [41]. Whether atypical C1 domains mediate separate functions, unrelated to diacylglycerol/phorbol ester binding, remains to be determined.

4.2. C2 domain

The C2 domain is present in conventional protein kinase Cs and, in a modified form, in novel protein kinase Cs [12,42,43]. This module is also present in a number of unrelated proteins that share the property of binding membranes in a Ca^{2+}-dependent manner. Although usually present in amphitropic proteins such as cytosolic phospholipase A2, phospholipase C γ, and other proteins that reversibly bind membranes, a C2 domain is also present in transmembrane proteins such as synaptotagmin where its membrane interaction may be important in close apposition of membranes preceding fusion. A recent comprehensive review has aligned 65 C2 domains from diverse protein families [44].

4.2.1. Structure of the C2 domain

Fig. 2B shows a modeled structure of the C2 domain of protein kinase C β [45] based on the crystal structure of the first C2 domain of synaptotagmin [46]. NMR and X-ray crystal structures of the C2 domains from synaptotagmin and phospholipase C reveal an eight-stranded anti-parallel beta sandwich, with loops formed by opposite ends of the sequence coming together to form an aspartate-lined mouth [46–48]. On the back face of this mouth are bulky hydrophobic residues (the sequence WDWD is particularly noteworthy in the protein kinase C C2 domains, with the aspartates pointing into the Ca^{2+} binding site and the tryptophans pointing out from the back face). Behind the 'jaws' of this domain is a highly basic surface that is present in most C2 domains. The C2 domain has two topological folds that differ only in that the first strand of one topology corresponds to the eighth strand of the second topology as a result of different positions for the amino and carboxyl termini [44].

Sequence analysis of the C2 domain of protein kinase C ε reveals that consensus residues that maintain the C2 domain fold are present, however key aspartates in the Ca^{2+} binding site are mainly absent [12]. This presents the intriguing possibility that the C2 domain of novel protein kinase Cs is already structured to bind anionic lipids; the C2 domain of conventional protein kinase Cs may require Ca^{2+} to structure the domain for lipid binding.

4.2.2. The aspartate-lined mouth coordinates at least two Ca^{2+} ions

Biophysical and structural studies are consistent with two, and possibly more, metal ions binding to the Ca^{2+} site in the C2 domain. Crystallographic studies revealed two Sm^{2+} ions [48] and two Ca^{2+} or three La^{3+} [49] bound to the C2 domain of phospholipase C δ, and NMR studies showed two Ca^{2+} ions bound to the C2 domain of synaptotagmin [47]. Equilibrium binding measurements by Falke and coworkers recently established that the C2 domain of cytosolic phospholipase A2 also binds two Ca^{2+} ions [50]. Thus, the novel architecture for a Ca^{2+} binding site found in the C2 domain appears to be engineered for binding more than one metal ion, in contrast to other structures such as the EF-hand where a single metal is coordinated in the Ca^{2+} binding pocket.

4.2.3. Ca^{2+} is not an electrostatic switch in regulating the function of the C2 domain

The surface potential around the Ca^{2+} binding site is fairly electronegative as a result of the five aspartates involved in Ca^{2+} coordination. Based on this, Rizo and coworkers proposed that Ca^{2+} may act as an electrostatic switch in regulating the function of the C2 domain: binding of this cation would essentially neutralize this region of the C2 domain, thus promoting interaction with anionic membranes [51]. Recent mutagenesis studies have revealed that this is not, surprisingly, the mechanism by which Ca^{2+} regulates the C2 domain. Specifically, neutralization of the electronegative potential around the Ca^{2+} site of protein kinase C βII, by mutation of two aspartates to arginine, did not mimic the effect of Ca^{2+} in recruiting protein kinase C to membranes. Rather, this mutation dramatically reduced protein kinase C's affinity for Ca^{2+}, which was still essential for binding to anionic lipids [45]. Thus, Ca^{2+} does not recruit C2 domain-containing proteins to membranes by providing an electrostatic switch.

How, then, does Ca^{2+} regulate the affinity of the C2 domain for membranes? Structural studies in the presence or absence of Ca^{2+} do not reveal any global structural changes in the domain [47,49]. Rather, localized re-arrangements of basic residues on the loops of the Ca^{2+} mouth may promote membrane interactions. As discussed below, several lines of evi-

dence are consistent with the loops of the Ca^{2+} site providing the C2 domain's membrane contacts.

4.2.4. Membrane-interacting determinants are localized near the Ca^{2+} binding site

Mutagenesis of the C2 domain of protein kinase C βII has established that the only region of the molecule that interacts significantly with the membrane is localized to the actual Ca^{2+} binding site. Specifically, the highly charged beta sheet behind the Ca^{2+} site (indicated in Fig. 2B) is not positioned near the membrane because dramatic reduction in charge of this beta sheet, by substituting four Lys with Ala, has no effect on protein kinase C's membrane affinity [45]. Furthermore, a previously proposed phosphatidylserine binding motif located at the opposite end of the Ca^{2+} site does not contribute to protein kinase C's membrane interaction; mutation of the two carboxyl-terminal basic residues in the proposed motif of FXFXLKXXXKXR, had no effect on protein kinase C's membrane affinity [52].

Based on the crystal structure of phospholipase C δ, Hurley and coworkers proposed that the C2 domain must approach the membrane 'jaws' first [48] since this was the only positioning of the protein that would also allow access of the active site to membrane lipids. Biochemical data also support this 'jaws' first orientation: stopped flow kinetic measurements are consistent with Ca^{2+} dissociating rapidly from soluble C2 domain and considerably more slowly from membrane-bound C2 domain, suggesting that Ca^{2+} is masked in the membrane-bound complex [50]. By a completely separate approach, Mosior and Epand estimated that the Ca^{2+} binding site involved in membrane binding lies approximately 0.3 nm from the plane of the membrane [53], consistent with relatively close membrane apposition of the Ca^{2+} site.

Taken together, the mutagenesis of the C2 domain, structural information, and kinetic measurements are consistent with a model in which the C2 domain binds membranes via residues that are on, or near, the loops that comprise the Ca^{2+} site. Whether Ca^{2+} serves as a bridge in the lipid interaction is unknown. However, it is worth mentioning that novel protein kinase Cs do not require Ca^{2+} for membrane binding and, since their C2 domain is missing critical aspartates in the Ca^{2+} binding groove of the C2 domain [12], the lipid interaction is not mediated by a Ca^{2+} bridge for these protein kinase Cs.

4.2.5. Lipid specificity of C2 domains

The Ca^{2+}-dependent binding of protein kinase C to membranes has been clearly established to require anionic lipids [54,55]. In the absence of diacylglycerol, Ca^{2+} increases the affinity for anionic phospholipids with no headgroup requirements other than the presence of negative charge [56]. Presumably this Ca^{2+}-dependent binding in the absence of diacylglycerol is mediated by the C2 domain. The isolated C2 domain of synaptotagmin also has a preference for anionic lipids [57]. However, the requirement for anionic lipids is not a general feature of C2 domains. Cytosolic phospholipase A2 binds phosphatidylcholine vesicles independently of phosphatidylserine content [58], and a recent analysis of its isolated C2 domain showed that it bound phosphatidylcholine vesicles an order of magnitude more tightly than phosphatidylserine vesicles [59].

4.2.6. The C2 domain mediates functions other than membrane binding

A number of functions in addition to membrane interaction have been ascribed to C2 domains, with many of the determinants mediating these other functions localized to the basic sheet behind the Ca^{2+} site (Fig. 2B). For example, peptides based on sequences in the basic sheet of protein kinase C βII's C2 domain compete with binding to the protein kinase C anchoring protein, RACK 1 [60]. Similarly, specific Lys residues in the basic face of the C2B domain of synaptotagmin (but not protein kinase C α) were shown to mediate binding to inositol polyphosphates [61,62]. Consistent with this, a recently cloned putative IP4 receptor contains two C2 domains [63], and peptides derived from the basic face of the C2B domain effectively bind IP4 [64]. Thus, the C2 domain may form a multi-functional module, with membrane-targeting functions localized to the Ca^{2+} binding loops, and additional functions arising from the basic face or other regions of the domain.

4.3. Pseudosubstrate

All protein kinase Cs, with the possible exception

of protein kinase C μ [65], contain an autoinhibitory pseudosubstrate domain that maintains protein kinase C in an inactive conformation by sterically blocking the active site [66–68]. Activation of protein kinase C is always coupled to removal of this autoinhibitory domain from the active site [67]. Although typically protein kinase C is activated by binding of lipids to the C1 and C2 domains, binding of Arg-rich proteins or peptides to protein kinase C is able to release the pseudosubstrate from the active site, allowing Arg-rich proteins such as protamine sulfate to be phosphorylated in a cofactor-independent manner [69,70].

Mosior and McLaughlin showed that a synthetic peptide based on residues 19–36 of the pseudosubstrate of protein kinase C β (containing six basic and one acidic residues) bound to membranes containing 25 mol % phosphatidylserine with an apparent affinity of 10^4 M^{-1} [71]. They proposed that the pseudosubstrate could thus contribute on the order of 6 kcal mol^{-1} in stabilizing protein kinase C's membrane interaction.

The pseudosubstrate precedes the C1A domain, and is thus far removed in primary sequence from the kinase core (Fig. 1). Thus, ligand binding to the C1 and C2 domains likely results in long-range conformational changes that cause the release of the pseudosubstrate from the active site. Understanding the molecular mechanism of how binding of molecules to the regulatory domains releases the basic pseudosubstrate from the acidic substrate binding cavity may await elucidation of how protein kinase C's domains are interfaced.

4.4. Kinase domain

The kinase domain of protein kinase C has significant similarity to that of the archetypal kinase, protein kinase A. Molecular modeling based on the crystal structure of protein kinase A has identified determinants on the surface of the molecule that are unique to the protein kinase C family [72,73]. In particular, a cluster of acidic residues near the entrance to the active site is conserved in all protein kinase Cs and may serve as an electrostatic gate to maintain the basic pseudosubstrate in the active site [72]. The kinase has a high affinity for basic peptides, with limited sequence specificity [74], suggesting that determinants beyond the active site may dictate substrate specificity in vivo [11]. For example, the increasing identification of protein kinase C anchoring proteins suggests that specificity may be modulated by targeting isozymes to specific subcellular locations via protein:protein interactions [75–78].

4.4.1. The carboxyl terminus of protein kinase C interfaces with the regulatory domain

The kinase domain also modulates protein kinase C's membrane affinity. This domain comprises a kinase core that is similar to that of protein kinase A and includes a carboxyl-terminal extension of approximately 20 amino acids that contains a conserved hydrophobic phosphorylation motif: FXXF(S/T)(F/Y). Recent studies have established that phosphorylation of this motif, which appears to be a required step in the maturation of protein kinase C, increases protein kinase C's membrane affinity. Specifically, mutagenesis of Ser-660 to Ala in protein kinase C βII resulted in a protein with 10-fold reduced affinity for phosphatidylserine-containing membranes compared with protein mutated to Glu at that position, or wild-type protein with phospho-Ser at that position [79].

Whether the carboxyl terminus regulates the C2 domain indirectly by modulating its conformation, or whether it interfaces with the C2 domain providing determinants that could interact with Ca^{2+} or the membrane, awaits elucidation of the structure of protein kinase C. Two additional lines of evidence, however, support the possibility that the carboxyl terminus interfaces with determinants in the C2 domain. First, the Ca^{2+} regulation differs significantly for two isozymes that differ only in the carboxyl-terminal 50 amino acids, the alternatively spliced protein kinase C βII and βI [80]. These protein kinase Cs have identical C2 domains, revealing that determinants in the carboxyl terminus modulate the affinity of the C2 domain for Ca^{2+}. Second, an RNA aptamer that selectively inhibits βII and not βI protein kinase C does not inhibit the cleaved kinase domain of protein kinase C βII even though this is the region of the molecule that is different from protein kinase C βI ([81] and Keranen and Newton, unpublished data). This reveals that the surface the aptamer binds to must include both the carboxyl terminus (to account for the selectivity for βII) and the regulatory region

(because only intact protein kinase C is inhibited). Taken together with the finding that phosphorylation of the carboxyl terminus increases protein kinase C's affinity for C2 domain ligands (Ca^{2+} and anionic lipids), these data are consistent with the carboxyl terminus of protein kinase C interfacing with the C2 domain.

4.4.2. Phosphorylation renders protein kinase C catalytically competent

Protein kinase C is covalently modified by phosphorylation at three conserved positions: a trans-phosphorylation at a site near the entrance to the active site that appears to be required to correctly align residues for catalysis, and two autophosphorylations at the carboxyl terminus that appear to be important to lock protein kinase C in a catalytically competent conformation and to release the mature enzyme into the cytosol [82,83]. These phosphorylations are required to process the mature, lipid-regulated protein kinase C that is the subject of this review. Whether additional phosphorylations modulate the ligand interactions of protein kinase C is unknown, although protein kinase C δ is tyrosine phosphorylated in response to stimulation [84,85]. In addition, all isozymes are tyrosine phosphorylated in response to oxidative stress [86].

5. Lipid regulation of protein kinase C

5.1. Measuring protein kinase C's membrane interaction

The interaction of protein kinase C with lipids is best measured by monitoring the binding of enzyme to lipid surfaces. Because membrane binding does not necessarily result in activation of protein kinase C, relying on the ability of lipids to activate protein kinase C does not always serve as a reliable indicator of whether protein kinase C interacts with these lipids. For example, protein kinase C that is quantitatively bound to Triton X-100 mixed micelles containing 10 mol % phosphatidic acid has no significant activity [87]; similarly, protein kinase C has no detectable activity under conditions where 50% of it is bound to vesicles containing 10 mol % phosphatidic acid [56].

The binding of protein kinase C to lipid bilayers can be directly measured by determining the fraction of enzyme partitioning with sucrose-loaded vesicles after centrifugation to separate free enzyme from vesicle-bound enzyme [88,89]. This method requires only nM amounts of protein kinase C, because protein kinase C in the supernatant or membrane pellet can be detected based on its enzymatic activity [89].

Alternatively, membrane binding can be measured by following the striking change in protein kinase C's proteolytic sensitivity upon membrane binding. Specifically, the hinge separating the regulatory and catalytic moieties of protein kinase C becomes two orders of magnitude more sensitive to proteolysis when protein kinase C is bound to anionic membranes [80,90]. The advantage of the latter is that it does not involve separation of bound from free enzyme, thus the enzyme's equilibrium distribution between the membrane and solution may not be significantly perturbed by the detection method. A second advantage of this method is that it allows examination of the interaction of protein kinase C with Triton X-100 mixed micelles, which cannot be centrifuged or chromatographically separated from free enzyme.

Energy transfer from tryptophans on protein kinase C to dansyl-labeled phosphatidylethanolamine has also been used to detect protein kinase C's membrane interaction [91]. Although a sensitive technique, the negative charge of the labeled headgroup participates in recruiting protein kinase C to membranes.

5.2. Lipid structure, rather than membrane structure, is the critical determinant in protein kinase C's recognition of phosphatidylserine

Protein kinase C is maximally activated when bound either to bilayers or Triton X-100 mixed micelles containing phosphatidylserine and diacylglycerol (Fig. 3). Thus, a bilayer structure is not required for protein kinase C's activity. Rather, the critical determinant in activating protein kinase C is the presence of specific lipid structures: *sn*-1,2-phosphatidylserine-L-serine and *sn*-1,2-diacylglycerol [56,92,93].

Why is the activation of protein kinase C so dependent on these two lipids? The presence of a spe-

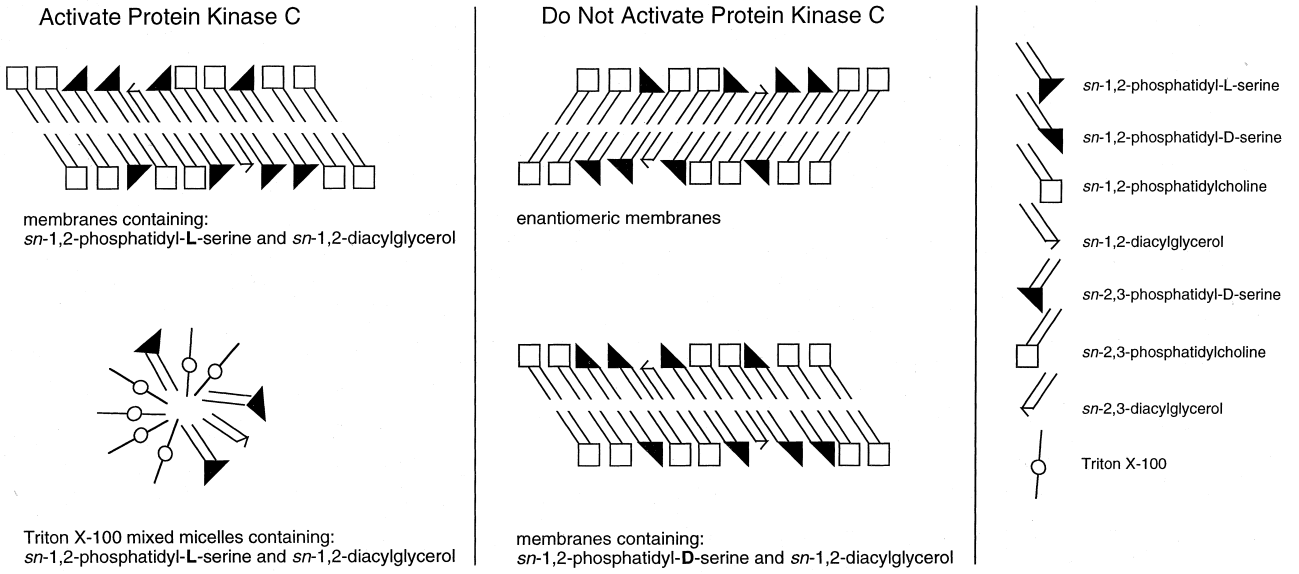

Fig. 3. Protein kinase C's lipid interaction depends on lipid structure rather than membrane structure. Left panel: Maximal activation of protein kinase C requires *sn*-1,2-phosphatidylserine and *sn*-1,2-diacylglycerol, and is not sensitive to whether the lipid is presented in the form of bilayers or detergent:lipid mixed micelles. Middle panel: Protein kinase C does not bind with high affinity and is not activated by enantiomeric membranes containing *sn*-2,3-phosphatidyl-D-serine, *sn*-2,3-phosphatidylcholine, and *sn*-2,3-diacylglycerol (top), nor does it interact with membranes containing *sn*-1,2-lipid isomers but the D-serine headgroup (bottom). Right panel: Key describing lipid structures.

cific diacylglycerol binding site is now clearly established by elucidation of C1 domain structure showing the ligand binding pocket [26]. However, whether the specific interaction with phosphatidylserine arises from the presence of a distinct binding site for this lipid on protein kinase C has been the subject of much debate over the years. One school of thought is that protein kinase C does not interact specifically with phosphatidylserine. Rather, there is a unique property of phosphatidylserine such that it is able to optimally present diacylglycerol to the C1 domain. In other words, membrane structure, rather than lipid structure, is what protein kinase C recognizes. A second school of thought is that protein kinase C has specific determinants on its surface that selectively bind phosphatidylserine over other phospholipids.

To elucidate whether protein kinase C's phospholipid specificity arises from protein determinants, or whether it arises from unique properties of phosphatidylserine membranes, the interaction of protein kinase C with enantiomeric membranes was recently examined (Johnson, Daleke, and Newton, in preparation). If protein kinase C specifically recognizes *sn*-1-2-phosphatidyl-L-serine, then it should not bind or be activated by membranes containing the enantiomeric *sn*-2,3-phosphatidyl-D-serine. In contrast, if phosphatidylserine endows unique physical properties to membranes, these unique physical properties will be identical in enantiomeric membranes (Fig. 3). Specifically, membranes composed of *sn*-2,3-phosphatidylcholine, *sn*-2,3-phosphatidyl-D-serine, and *sn*-2,3-diacylglycerol have the exact same physical properties of membranes containing the naturally occurring *sn*-1,2-phosphatidylcholine, *sn*-1,2-phosphatidyl-L-serine, and *sn*-1,2-diacylglycerol.

Binding measurements showed that protein kinase C does not bind to, and is not activated by, enantiomeric membranes. Specifically, the enzyme does not interact with membranes containing 40 mol % *sn*-2,3-phosphatidyl-D-serine, 55 mol % *sn*-2,3-phosphatidylcholine, and 5 mol % of *sn*-2,3-diacylglycerol (or 5 mol % *sn*-1,2-diacylglycerol); in striking contrast, it binds membranes containing the naturally occurring enantiomers with an apparent membrane affinity of 10^5 M^{-1} (Johnson, Daleke, and Newton, in preparation). These data reveal that protein kinase C

specifically interacts with *sn*-1,2-phosphatidyl-L-serine: it recognizes determinants on the structure of this molecule and these contacts are essential in activating protein kinase C.

5.3. Role of diacylglycerol/phorbol esters in mediating membrane binding

The most pronounced result of treating cells with phorbol esters is the 'translocation' of protein kinase C from the cytosol to the membrane. Ever since its discovery in the early 1980s [94–96], translocation has served as the hallmark for protein kinase C activation. Extensive biochemical studies, coupled with recent structural studies, have defined the molecular basis for this translocation.

5.3.1. C1 ligands act as 'molecular glue' to recruit protein kinase C to membranes

Phorbol esters, and to a lesser extent diacylglycerols, serve as hydrophobic anchors to recruit protein kinase C to membranes: they cause a dramatic increase in protein kinase C's membrane affinity that is linearly proportional to the mol fraction C1 ligand in the membrane [32]. Specifically, the dissociation constant of phorbol myristate acetate from membrane-bound protein kinase C, separated from all other interactions, is 1.5×10^{-5} mol % relative to membrane lipids. The dissociation constant of diacylglycerol is approximately 250 times higher. Stated differently, 1 mol % phorbol myristate acetate increases protein kinase C's membrane affinity by a remarkable four orders of magnitude.

The interaction of protein kinase C with phorbol esters is reversible both in vitro [38] and in vivo [97]; this interaction obeys the law of mass action and membrane dissociation can be achieved by diluting membranes. In vitro, protein kinase C has been shown to exchange rapidly between membranes (e.g. enzyme bound to large unilamellar vesicles exchanges rapidly to multilamellar vesicles) [38]. In vivo, protein kinase C that has translocated to cellular membranes has been shown to redistribute to the cytosol upon extraction of phorbol esters by washing cells with solution containing BSA [97]. Earlier reports suggesting irreversible membrane binding likely resulted from the enormously tight membrane binding that phorbol esters mediate [98,99].

5.3.2. Phorbol ester binding and Ca^{2+} binding are independent events

Since the discovery that phorbol esters activate protein kinase C, an apparent synergistic activation by Ca^{2+} and phorbol esters was noted [100]. That is, the greater the phorbol ester concentration, the lower the concentration of Ca^{2+} required to promote translocation or activation of protein kinase C and, conversely, the greater the Ca^{2+} concentration, the lower the concentration of phorbol ester/diacylglycerol needed to activate protein kinase C. Because Ca^{2+} increases the affinity of protein kinase C for anionic lipids, and because C1 domain ligands increase the affinity of protein kinase C for phosphatidylserine, it was not clear whether the apparent synergism reflected a direct allosteric interaction between the phorbol and Ca^{2+} sites.

The molecular basis for this apparent synergism was recently addressed by taking advantage of the finding that the binding to phorbol esters is so strong that membrane binding can be achieved in the complete absence of anionic lipids, and hence C2 domain interactions [32]. Specifically, the binding of protein kinase C to membranes containing 2 mol % phorbol myristate acetate and 98 mol % of the neutral lipid phosphatidylcholine was examined as a function of Ca^{2+} concentration; note that protein kinase C does not ordinarily bind neutral membranes, however in this case binding was driven by the relatively large phorbol ester concentration and was presumably mediated by C1 domain interactions alone. Importantly, altering the Ca^{2+} concentration by 5 orders of magnitude had no effect on protein kinase C's phorbol ester-dependent membrane interaction [32]. Thus, the phorbol ester site in the C1 domain does not interact allosterically with the Ca^{2+} site in the C2 domain. Thus, the apparent synergism arises because both Ca^{2+} and phorbol esters/diacylglycerol, by separate mechanisms, independently increase protein kinase C's affinity for phosphatidylserine.

5.4. Role of acidic lipids and Ca^{2+} in mediating membrane binding

Ca^{2+} was the original cofactor reported to activate the pro-enzyme of the proteolytically generated, constitutively active protein kinase M, hence the naming of this ubiquitous class of kinases [69]. Research over

the past decade has established that Ca^{2+} binding requires anionic lipids, that Ca^{2+} increases protein kinase C's affinity for anionic lipids, and that Ca^{2+}-dependent binding to anionic lipids does not require C1 ligands [54,56,91,101]. The Ca^{2+}-dependent increase in membrane affinity shows no selectivity for headgroup beyond the requirement for negative charge [56].

Mosior and Epand have shown that protein kinase C's affinity for anionic membranes increases linearly with Ca^{2+} concentration from the submicromolar to low mM range; with the dissociation constant of Ca^{2+} from membrane-bound protein kinase C calculated to be 0.7 μM (below this Ca^{2+} concentration, membrane binding is insensitive to Ca^{2+} concentration), and the dissociation constant of Ca^{2+} from soluble protein kinase C estimated to be 3 mM [53].

Curiously, the Ca^{2+} requirement for membrane binding and for activation is not the same [80]. For example, half-maximal binding of protein kinase C βII to large unilamellar vesicles containing 40 mol % phosphatidylserine and 5 mol % diacylglycerol requires 1 μM Ca^{2+}, whereas half-maximal activation requires 40 μM Ca^{2+} [80]. One possibility is that sufficiently tight membrane binding is required to supply the energy to release the pseudosubstrate from the active site.

5.5. Role of phosphatidylserine

It has been almost two decades since Nishizuka and coworkers reported that the pro-enzyme of the protease-activated kinase they had just discovered was activated by phosphatidylserine [69]. Since then, a plethora of studies have been aimed at unveiling the molecular details for the remarkably specific interaction of protein kinase C with this phospholipid.

5.5.1. Specificity for phosphatidylserine

Activity measurements in the 1980s revealed that activation of protein kinase C specifically required phosphatidyl-L-serine [102,103]. Bell and coworkers showed that alterations in headgroup stereochemistry, distance between amino and carboxyl groups, and blocking of either the amine or carboxyl group, resulted in lipids not able to significantly activate protein kinase C [92].

Binding measurements in the past few years revealed that, surprisingly, this selective recognition of phosphatidylserine occurs only in the presence of C1 domain ligands [56,87,90]. In the absence of diacylglycerol/phorbol esters, protein kinase C binds all mono-anionic lipids with equal affinity. However, the presence of diacylglycerol results in a remarkably selective increase in affinity for *sn*-1,2-phosphatidyl-L-serine. This diacylglycerol-dependent increase in affinity for phosphatidylserine occurs independently of membrane structure: protein kinase C binds phosphatidyl-L-serine dispersed in Triton X-100 mixed micelles [56] or present in lipid bilayers (Mosior and Newton, unpublished data) over an order of magnitude more tightly than phosphatidyl-D-serine in these same structures (i.e. provided diacylglycerol is present).

The molecular basis for why protein kinase C shows no selectivity for anionic lipids in the absence of C1 ligands, but selectively recognizes phosphatidylserine in the presence of C1 ligands remains to be elucidated. However, the finding that protein kinase C does not bind enantiomeric membranes (see Section 5.2) reveals that this selective recognition arises not because of unique physical properties of phosphatidylserine, but rather because protein kinase C recognizes molecular determinants on the L-serine headgroup (Johnson, Daleke and Newton, in preparation). Thus, a specific binding site for phosphatidylserine must be present somewhere in the structure of protein kinase C. Determinants mediating the binding to phosphatidylserine are unlikely to reside exclusively in the C2 domain, since recruitment to membranes by that domain alone (i.e. in the absence of C1 ligands) shows no selectivity for phosphatidylserine. They are also unlikely to reside exclusively in the C1 domain since Quest and Bell noted only a very modest effect of phosphatidylserine on the binding of GST-C1 domain constructs to membranes [104]. One possibility is that the juxtaposition of membrane-bound C1 and membrane-bound C2 domains provides an interface that binds phosphatidylserine. Alternatively, determinants that bind phosphatidylserine may not be positioned optimally to bind this lipid unless both the C1 and C2 domains are membrane-bound. Note that the additional binding energy that arises when both diacylglycerol and phosphatidylserine are bound to protein kinase C

could be partly accounted for by the availability of the exposed pseudosubstrate to bind anionic lipids (see Section 4.4).

Curiously, the fluorescent lipid probe, dansyl-phosphatidylethanolamine has been reported to mimic phosphatidylserine in inducing high-affinity membrane binding in the presence, but not absence, of diacylglycerol [105]. This suggests that the determinants on protein kinase C that bind the L-serine headgroup are able to recognize properties of the dansyl-modified ethanolamine headgroup to effect high-affinity binding. This is particularly intriguing given the enzyme's low tolerance for any alterations in the structure of the serine headgroup, with particular sensitivity to modification of the amine group [92].

5.5.2. Protein kinase C interacts with multiple phosphatidylserine molecules

The binding of protein kinase C to phosphatidylserine displays positive cooperativity both in detergent micelles and in bilayers [18,56,89,90,101,106], revealing that protein kinase C interacts with multiple phosphatidylserine molecules. The actual number of phosphatidylserine molecules that interact with protein kinase C has recently been calculated to be on the order of 8 (Mosior and Newton, in preparation). Whether the enzyme has one specific binding site for phosphatidylserine with multiplicity arising from additional non-specific interactions with anionic lipid binding surfaces, or whether it contains multiple sites is unknown. However, given that the structures of the C1 and C2 domain modules are now known, it seems unlikely that the regulatory domain could accommodate multiple specific lipid binding sites. Thus, it may be that a unique phosphatidylserine binding site exists, with additional molecules perhaps interacting with the C2 domain's anionic lipid-binding determinants. This latter possibility is consistent with phosphatidylserine's propensity to form domains in membranes [107]. Indeed, protein kinase C's interaction with acidic membranes has been shown to cause extensive segregation of acidic phospholipids [108]. Related to this, Glaser and coworkers have recently shown that domain formation modulates protein kinase C phosphorylation of the MARCKS peptide [109].

5.6. Involvement of other lipids in regulating protein kinase C

Protein kinase C's activity is sensitive to other amino or anionic phospholipids and to a variety of amphipathic membrane intercalators, including fatty acids and anesthetics. The enzyme's requirement for phosphatidylserine is reduced in the presence of other anionic lipids or phosphatidylethanolamine [18,110].

Protein kinase C is activated by cis-unsaturated fatty acids. In vitro, this activation is stimulated by diacylglycerol and Ca^{2+}, suggesting that fatty acids may function as C2 domain ligands, substituting for anionic lipids [111,112]. In this regard, oleic acid has been shown to decrease the concentration of phosphatidylserine required to activate protein kinase C [113]. Thus, membrane targeting and activation may result from the same C1/C2 anchoring mechanism that occurs when anionic lipids recruit protein kinase C to membranes. Fatty acids are also effective at causing protein kinase C to translocate to membranes in vivo [114,115].

A number of studies have shown that the polyphosphoinositides are also effective activators of protein kinase C [116–118]. Bell and coworkers showed that these lipids reduce the concentration of phosphatidylserine required to activate protein kinase C, with their efficacy directly proportional to the negative charge of the headgroup [118]. Particular interest has been accorded to the finding that phosphatidylinositol-3,4,5-trisphosphate stimulates the phosphatidylserine-dependent activation of the protein kinase Cs given the recently discovered role of this lipid in signal transduction [119–121]. For most protein kinase Cs, this activation could arise from the ability of the high negative charge of the polyphosphoinositides to interact with the anionic lipid-binding determinants of the C2 domain. However, for protein kinase C μ, this activation may result from specific binding to its PH domain [15]. Whether alterations in the levels of this lipid in vivo modulate the membrane recruitment and hence activation of protein kinase C is an intriguing possibility.

Protein kinase C can also be maximally activated by short-chained phospholipids, but only at the critical micelle concentration of these lipids. This unusual mode of activation requires diacylglycerol and

Ca^{2+} [122] and is accompanied by (1) exposure of the hinge region to proteolysis, a hallmark of the C2 domain's membrane interaction, and (2) release of the pseudosubstrate [67], a hallmark of the active conformation. Although the mechanism of this activation is unclear, it must involve unique surface properties attendant to the critical micelle concentration that are somehow able to mimic the effect of the C2 domain's membrane interaction (since C1 domain ligands are still required for activity, and the conformational change that results from C2 domain interactions occurs).

5.7. *Modulation of protein kinase C by membrane properties*

Although the maximal rate of catalysis of protein kinase C (approximately 10 reactions per s) depends on lipid structural properties, protein kinase C activity is modulated by membrane structural properties [123–125]. Sando and coworkers showed that protein kinase C activity is sensitive to acyl chain saturation, with unsaturation required somewhere in the membrane structure [126]. For example, membranes composed solely of saturated phospholipids, whether or not phosphatidylserine and diacylglycerol are present, do not activate protein kinase C. However, unsaturation on any phospholipid in the bilayer, not necessarily phosphatidylserine, results in membranes competent to activate protein kinase C. The effects of unsaturation likely arise from alterations in headgroup spacing rather than by increasing membrane fluidity: under conditions where cholesterol decreases membrane fluidity, protein kinase C activity is increased [126,127].

Protein kinase C activity is also sensitive to phosphatidylethanolamine which, like diacylglycerol, forms non-lamellar membrane structures at high

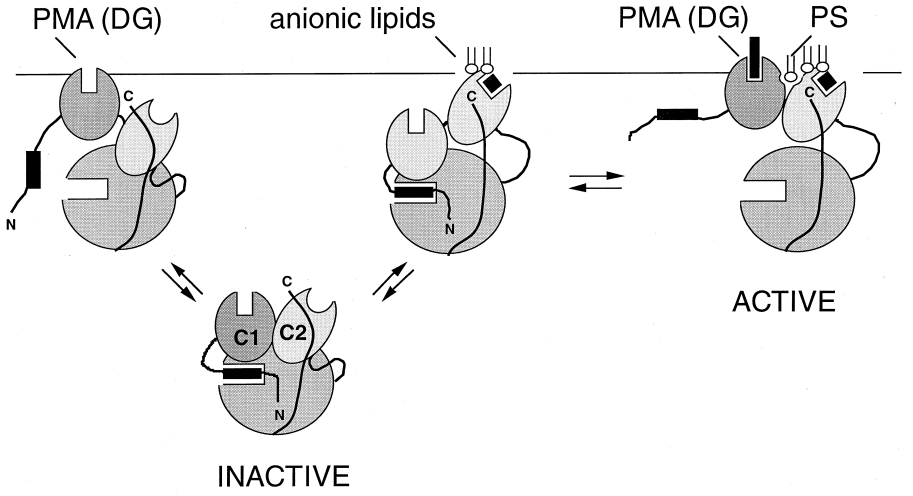

Fig. 4. Protein kinase C is regulated by two membrane-targeting modules. In solution, the pseudosubstrate (black rectangle) occupies the active site (open rectangle) of the kinase domain (large grey circle) of protein kinase C, thus maintaining it in an inactive form. In this conformation, the hinge region separating the regulatory domains from the kinase domain is masked to proteolysis (S-shaped line). The enzyme can be recruited to membranes by either the C1 domain (left) or C2 domain (middle right). Left: Recruitment by the C1 domain is mediated by phorbol esters, occurs in the absence of anionic lipids, does not cause a detectable change in the conformation of the hinge, and results in only low activation of protein kinase C (note that this interaction is unlikely to be physiologically relevant because all cell membranes contain anionic lipids). Middle right: Recruitment by the C2 domain is mediated by anionic lipids, is regulated by Ca^{2+} (■) for conventional protein kinase Cs, and results in a membrane interaction that is too weak to release the pseudosubstrate from the active site. This interaction is driven primarily by electrostatic interactions, does not depend on lipid headgroup beyond the requirement for negative charge, and promotes exposure of the hinge region. Far right: Activation of protein kinase C results when both the C1 and C2 domains bind membranes. The interaction energy is greater than that obtained by summing up the C1 domain and C2 domain interactions alone, because when both domains are membrane bound there is a selective and additional recognition of phosphatidylserine. Part of the additional binding energy may result from binding of the basic pseudosubstrate to anionic membranes [71]. In summary, protein kinase C can be recruited to membranes by either the C1 or the C2 domain, but both domains must be membrane-bound in order to release the pseudosubstrate from the active site.

mol fractions [105,128]. Although activity of protein kinase C does not directly correlate with the presence of non-lamellar phases, the possibility exists that disruption of bilayer structure promotes protein kinase C's membrane interaction. Protein kinase C activity is also sensitive to microdomains: formation of both diacylglycerol-rich domains [129,130] and phosphatidylserine-rich domains [109] stimulate the enzyme's activity. In addition, enzyme activity is modulated by membrane intercalators such as anesthetics and alcohols [131].

A common theme that emerges from the above studies is that protein kinase C activity is sensitive to defects in headgroup packing, either caused by curvature strain resulting from non-lamellar-forming lipids or present at boundaries of microdomains. Whether disruption in headgroup interactions facilitates intercalation of protein kinase C into the hydrophobic membrane (e.g. C1 domain) or facilitates headgroup:protein interactions remains to be determined. What is becoming evident, however, is that the enzyme has specific binding sites for diacylglycerol and phosphatidylserine, but recognition of these lipids is facilitated by optimal membrane presentation.

6. Conclusions

Structural, biophysical, kinetic, and molecular biological studies over the past few years have illuminated key details into the molecular mechanism of how protein kinase C function is regulated by its second messenger, diacylglycerol. Importantly, while the molecular details are unique to protein kinase C, the general mechanism of relying on two membrane-targeting motifs to achieve specificity in activation turns out to be shared by many other signaling proteins.

6.1. Model of protein kinase C's membrane interaction

Fig. 4 presents a model for protein kinase C's regulation based on our current knowledge of protein kinase C. In solution, protein kinase C adopts a conformation such that the pseudosubstrate occupies the active site, thus sterically blocking substrate binding; in this conformation, the hinge separating the regulatory domains from the kinase core (Fig. 1) is masked to proteolysis. The enzyme can be recruited to membranes by either the C1 domain or C2 domain, resulting in a membrane interaction that is of too low an affinity to provide the energy to displace the pseudosubstrate significantly. That is, high concentrations of phorbol esters are able to recruit protein kinase C to neutral membranes, in the absence of C2 domain interactions (left panel of Fig. 4). This interaction does not involve a conformational change at the hinge region and results in only modest activity (Mosior and Newton, in preparation). High concentrations of anionic lipids and Ca^{2+} are able to recruit protein kinase C to membranes via the C2 domain alone (middle right of Fig. 4); this interaction is driven primarily by electrostatic interactions, is relatively weak, results in a significant unmasking of the now proteolytically labile hinge region, but does not result in significant pseudosubstrate exposure and hence activation. This interaction is insensitive to phospholipid headgroup structure, beyond the requirement for negative charge.

Recruitment of protein kinase C to membranes by both the C1 and C2 domains results in a remarkably high-affinity interaction that depends on the presence of both diacylglycerol (or phorbol esters) and phosphatidylserine (far right panel of Fig. 4). This extraordinarily tight binding results in a release of the pseudosubstrate from the active site, thus allowing substrate binding and catalysis.

6.2. Protein kinase C's membrane interaction as a paradigm for the membrane interaction of other amphitropic proteins

Regulation of protein kinase C's function by two membrane-targeting modules provides ultra-sensitivity in activating protein kinase C. Thus, it is not surprising that nature has used this 'double-anchor' as a general regulatory mechanism in signal transduction. Through a variety of membrane tethers, from protein domains to lipid modifications, great diversity is found in the molecular details for each signaling protein; nonetheless, the theme of two membrane anchors with one being reversibly regulated appears to be a tried and true rule that nature uses again and again.

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