Regulation of protein kinase C

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Protein kinase C has been in the spotlight since the discovery two decades ago that it is activated by the lipid second messenger diacylglycerol. Despite protein kinase C's enduring stage presence, the regulation and specific roles of its isozymes in defined cellular processes are still under intense investigation. Elucidation of the structures of protein kinase C's regulatory modules, the discovery that phosphorylation regulates the enzyme, and the identification of targeting mechanisms have made the past year a significant one for unveiling how this ubiquitous class of enzymes operates.

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## Introduction: protein kinase C in the limelight

Protein kinase Cs transduce the myriad of signals mediated by phospholipid hydrolysis. Activation of G protein coupled receptors, tyrosine kinase receptors, and nonreceptor tyrosine kinases can lead to protein kinase C activation by stimulation of either phospholipase Cs to yield diacylglycerol, or phospholipase D to yield phosphatidic acid and then diacylglycerol. Diacylglycerol is the key 'on' switch for most protein kinase Cs. In addition, conventional protein kinase Cs are also modulated by Ca^{2+}; these isozymes are particularly sensitive to hydrolysis of phosphatidylinositol bisphosphate because it generates an additional signal, inositol trisphospnate, which mobilizes intracellular Ca^{2+}. This review focuses on advances in the past year in understanding the structure and regulation of protein kinase C.

Protein kinase C structure

Protein kinase C isozymes consist of a single polypeptide chain that contains an amino-terminal regulatory region (of 20–70 kDa) and a carboxy-terminal kinase domain (of approximately 45 kDa) (reviewed in [1••,2]) (Fig. 1). The isozymes have been categorized into three subclasses, as follows: conventional protein kinase Cs (α, β and γ) which are regulated by diacylglycerol, phosphatidylserine, and Ca^{2+}; novel protein kinase Cs (δ, ε, η and θ) which are regulated by diacylglycerol and phosphatidylserine; and atypical protein kinase Cs (ζ, ι and λ) whose regulation has not been clearly established, although their activity is stimulated by phosphatidylserine. Protein kinase μ (human) and its murine homolog, protein kinase D, form a distinct class in that the kinase core is

Figure 1
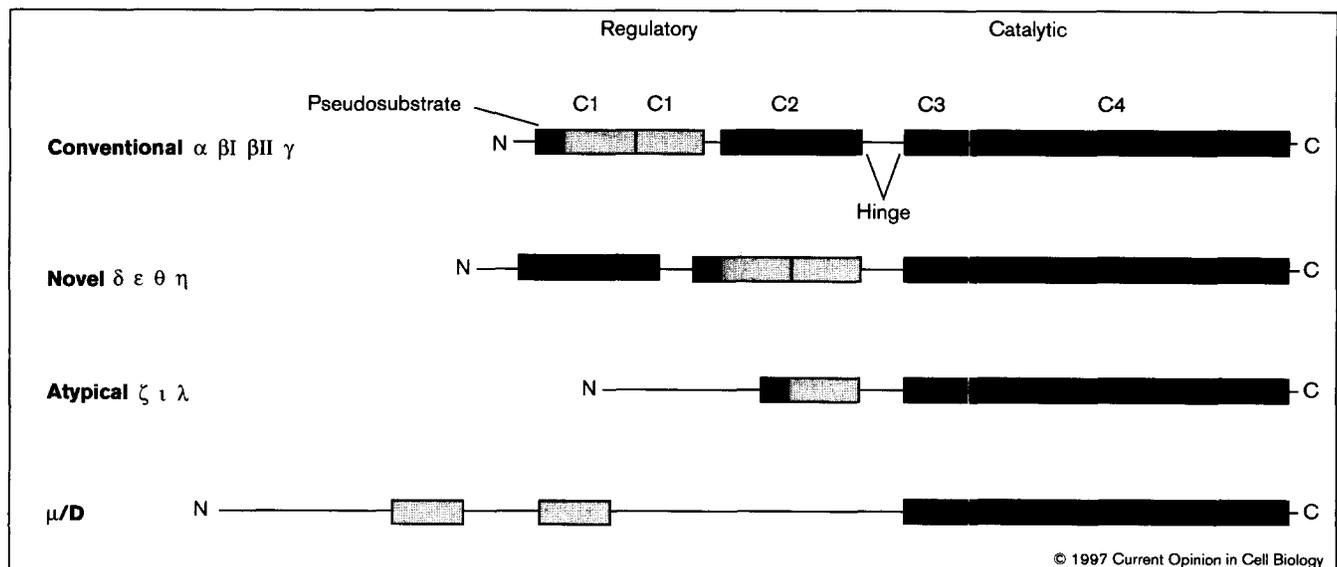
Schematic representation of the primary structures of protein kinase C isozymes. Indicated are the pseudosubstrate (black), the C1 domain which binds diacylglycerol or phorbol esters (recently redefined to describe a single cysteine-rich motif [5•]), the C2 domain which binds acidic lipids and, for conventional protein kinase Cs, Ca^{2+}, and the C3 and C4 domains which comprise the ATP- and substrate-binding lobes of the kinase core. Members of each isozyme subclass are listed at the left. N, amino terminus; C, carboxyl terminus.

actually most similar to that of calmodulin-dependent kinases and no pseudosubstrate motif has been identified (reviewed in [3]).

Regulatory domains

The regulatory half of protein kinase Cs contains an autoinhibitory domain, that is, the pseudosubstrate, and one or two membrane-targeting motifs, namely, the C1 domain, which is present in all isozymes, and the C2 domain, which is present in conventional and novel protein kinase Cs (Fig. 1). Each domain, separately, is present in a number of other proteins and comprises a discrete structural and functional module (reviewed in [4]). The C1 domain binds diacylglycerol and phorbol esters, functional analogs of diacylglycerol, in all but the atypical protein kinase Cs; this domain is present as a tandem repeat in most protein kinase Cs, and has been recently redefined to describe one (not both) of these repeats [5•]. The C2 domain binds acidic phospholipids and also, in conventional protein kinase Cs, Ca^{2+}.

The C1 domain is a cysteine-rich sequence that coordinates two Zn^{2+} atoms; it folds into a globular structure with two pulled-apart β sheets forming the ligand-binding cavity. Hurley and coworkers [6••] elucidated the crystal structure of the C1 domain with and without bound phorbol and found that the domain undergoes no significant conformational change upon phorbol binding. Rather, the ligand caps the hydrophilic binding site so that the top third of the domain displays a contiguous hydrophobic surface. Thus, membrane targeting is achieved by ligand altering the surface of the protein to promote hydrophobic interactions. On the basis of structural and mutagenesis studies (e.g. [7]), key residues that define the ligand-binding cavity have been identified and used to classify C1 domains as typical or atypical, for those that do or do not bind phorbol esters, respectively [5•]. Elucidation of an atypical C1 domain structure, that of Raf, revealed that the phorbol-binding site is, indeed, compromised by the lack of consensus residues that form a hydrophobic face of the pocket in typical C1 domains [8•].

Elucidation of the structure of C2 domains from synaptotagmin [9••] and phospholipase C [10••,11] revealed a β sheet rich domain with a novel Ca^{2+}-binding pocket; two loops comprising sequences at the amino and carboxyl termini of the C2 core come together to form an aspartate-lined mouth that coordinates Ca^{2+}. For the C2 domains studied, NMR and crystallographic data have shown that this pocket coordinates two metal ions [10••,12]. Metal ion binding produces a significant conformational change in the phospholipase Cδ C2 domain, exposing three lysine residues on the back face of the Ca^{2+}-binding site [10••] that could potentially bind acidic lipids. This conformational change supports biochemical data showing allosteric interactions between Ca^{2+} and lipid binding (reviewed in [2]). A number of proteins with C2 domains do not bind Ca^{2+}, notably the novel protein kinase Cs, and

sequence alignment reveals that key aspartates involved in Ca^{2+} coordination are not present in these proteins [4,13•].

Catalytic domain

The catalytic domain of protein kinase C has high similarity to that of protein kinase A [14•], and this has been taken advantage of to model the kinase cores of protein kinase C βII [15] and protein kinase C α [16]. Protein kinase C is maintained in an inactive state by interaction with the pseudosubstrate. Modeling of this basic autoinhibitory domain in the kinase core suggests that electrostatic contacts with acidic surfaces in the cavity are important determinants in the interaction [15].

Characterization of optimal peptide sequences to which protein kinase Cs bind, using an oriented peptide library, recently confirmed the importance of basic residues to the amino- and carboxy-terminal ends of the phosphoacceptor site of the peptide, and underscored the importance of a basic residue at the P−3 position in particular [17••]. In addition, a strong preference for a hydrophobic residue at the P+1 position was established, and structural models of the kinase domain suggest that, as in protein kinase A, this position contacts a hydrophobic groove in the substrate-binding cavity [17••]. Subtle differences in sequence preferences were noted amongst the isozymes, with the exception of distinct differences in the requirements of protein kinase C μ. At most positions, selectivity for particular residues was typically twofold. The moderate substrate selectivity of protein kinase C *in vitro* suggests the importance of targeting and accessibility in the *in vivo* operation of the enzyme.

Related kinases

A group of kinases whose catalytic core shares considerable sequence similarity with that of the protein kinase Cs has recently been characterized, and its members are called protein kinase N (PKN) or protein kinase C related kinases (PRKs) [3]. The amino-terminal half of these proteins contains a leucine zipper in place of the C1 domain, but does have a C2-like domain analogous to that of the novel protein kinase Cs. Consistent with possessing a C2-like domain, the activity of these proteins is stimulated by acidic phospholipids [18]. The small GTPase Rho has recently been shown to bind to and activate PKN in the presence of GTP [19,20], highlighting the importance of protein–protein interactions in PKN/PRK regulation.

Regulation by cofactors

Activation of protein kinase C requires the removal of the autoinhibitory pseudosubstrate domain from the active site. This conformational change is achieved by highly specific binding of 1,2-*sn*-diacylglycerol and phosphatidyl-L-serine to the two membrane-targeting domains, C1 and C2 (Fig. 2). Binding of ligand to either domain is sufficient to recruit the enzyme to membranes by a low-affinity interaction; however, both domains must be membrane-bound for the high-affinity

interaction that results in pseudosubstrate removal and maximal activation (reviewed in [2,21]). The use of two membrane-targeting domains to ensure specificity and high-affinity interactions is turning out to be a common theme in the regulation of many amphitrophic signal transducers, where combinations of pleckstrin homology (PH), C1, C2, and myristoylated domains define regulatory mechanisms.

Phosphatidylserine and other anionic lipids

In the absence of diacylglycerol, protein kinase C binds anionic membranes with no discrimination between phospholipid headgroup beyond the requirement for negative charge. However, the presence of diacylglycerol induces remarkable specificity for phospholipid head-group: when diacylglycerol is present, the enzyme binds membranes containing phosphatidyl-L-serine with at least

an order of magnitude higher affinity than membranes containing other acidic lipids (reviewed in [2,21]). Although biochemical and mutational analyses have clearly established that the C2 domain binds acidic lipids, specific binding determinants for phosphatidylserine have not been identified. On the basis of reactivity with phosphatidylserine anti-idiotypic antibodies, a putative phosphatidylserine-binding motif was proposed to reside in the C2 domain [22]; however, mutation of this motif has no effect on phospholipid regulation (JE Johnson, AC Newton, unpublished data).

In addition to phosphatidylserine, a number of other lipids stimulate the diacylglycerol-dependent activation. The stimulation by fatty acids, notably arachidonic acid, has been well characterized (reviewed in [23]), as has the stimulation by highly acidic lipids such as the

Figure 2
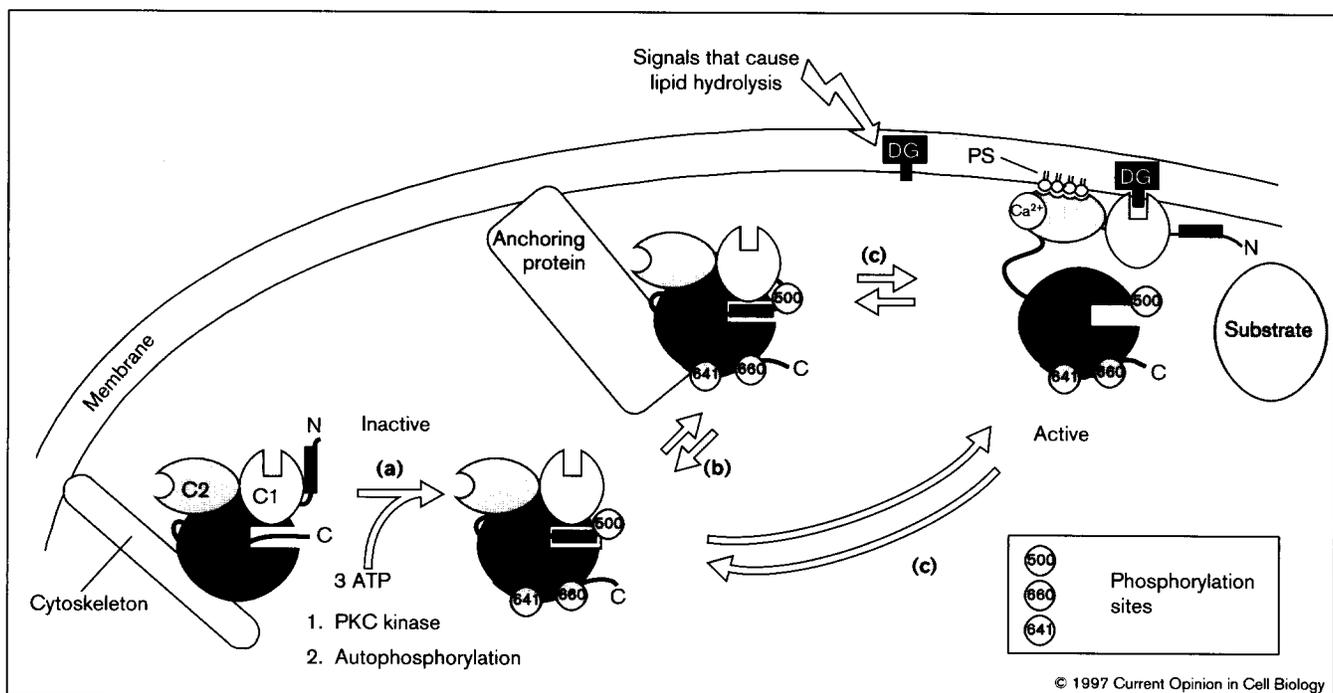
Model for the regulation of protein kinase C by phosphorylation, targeting proteins, and cofactors. Domains (C1–C4) of protein kinase C are those described in Figure 1. Newly synthesized protein kinase C associates with the cytoskeleton (far left), in a conformation that exposes the activation loop (top right point of active site, which is represented by a gap in C4) and tethers the carboxyl terminus (C) near the active site. **(a)** Phosphorylation by a putative protein kinase C (PKC) kinase on the exposed activation loop (at Thr500 in protein kinase C βII) correctly aligns residues for catalysis, allowing autophosphorylation at two carboxy-terminal positions (Thr641 and Ser660 in protein kinase C βII). Phosphorylation at the first of these (Thr641 in protein kinase C βII) locks the kinase in a catalytically competent conformation, an event that may involve removal of the carboxyl terminus from the active site and insertion, instead, of the pseudosubstrate (black rectangle). Subsequent phosphorylation at the second carboxy-terminal site (Ser660 in protein kinase C βII) releases mature protein kinase C into the cytosol. **(b)** Isozyme-specific targeting of mature protein kinase C may be regulated by specific anchoring proteins: interaction with these poises the enzyme for rapid response to its second messenger, diacylglycerol (DG). **(c)** Binding of diacylglycerol to the C1 domain and phosphatidylserine (PS) to the C2 domain results in removal of the pseudosubstrate from the active site. (The binding to diacylglycerol and phosphatidylserine may or may not require, or be facilitated by, step [b].) Although protein kinase C can bind to membranes by either the C1 or the C2 domain alone, both must be membrane-bound for the high-affinity interaction that results in pseudosubstrate release. The C2 domain of conventional protein kinase Cs also has a binding site for Ca^{2+}, which allosterically increases this domain's affinity for phosphatidylserine. Interaction of this activated enzyme with other targeting proteins, such as RACKS (receptors for activated C kinase; not shown), may position the enzyme close to its substrates. N, amino terminus.

polyphosphoinositides. Isozyme specificity in regulation by phosphatidylinositol 3,4,5-trisphosphate has been suggested, although isozyme and isomer specificity has not been uniformly observed (reviewed in [21]). Recently, the modulation of diacylglycerol-dependent activation by lysophosphatidic acid has been described [24]. These acidic molecules may mimic phosphatidylserine, presumably by binding the C2 domain and thus increasing protein kinase C's membrane affinity.

Diacylglycerol and phorbol esters

Structural, biophysical, and mutagenesis studies in the past year have resulted in much headway into the search for the molecular mechanism for the diacylglycerol/phorbol ester dependent membrane association that has served as a hallmark for protein kinase C activation *in vivo*. Complementing the structural determination showing that phorbol binding changes the surface hydrophobicity of the C1 domain, in the absence of a conformational change [6••], binding measurements revealed that phorbol esters and diacylglycerol activate protein kinase C by the same mechanism: they increase protein kinase C's membrane affinity linearly with respect to the molar fraction of ligand in the membrane. A membrane dissociation constant of 1.5×10^{-5} mol % describes the remarkably high affinity of membrane-bound protein kinase C for membrane-bound phorbol myristate acetate [25•]. The dissociation constant of diacylglycerol is two orders of magnitude lower [25•], possibly because the increased flexibility of this ligand results in a greater entropic loss upon binding. Consistent with this, conformationally constrained analogs of diacylglycerol have a significantly increased affinity for protein kinase C [26]. Thiotriazole, a farnesylcysteine derivative that modulates superoxide release in neutrophils, has recently been identified as another functional analog of diacylglycerol [27].

The interaction of protein kinase C with phorbol esters has been shown to be so strong that it can target protein kinase C to neutral membranes (i.e. in the absence of acidic lipids and hence presumably in the absence of C2 domain interactions) [25•]; indeed, recombinant C1 domains were shown to bind phorbol esters in solution, albeit with very low affinity [28•].

The interaction of protein kinase C with diacylglycerol/phorbol esters has also been shown to be unaffected by changing Ca^{2+} concentrations over five orders of magnitude [25•], revealing no allosteric interactions between the Ca^{2+}-binding site in the C2 domain and the phorbol site in the C1 domain. Thus, Ca^{2+} appears to 'synergize' with diacylglycerol/phorbol esters [29], because this cation increases protein kinase C's affinity for phosphatidylserine, resulting in lower amounts of the C1 domain ligand required to effect membrane binding.

The stoichiometry of binding of phorbol esters to protein kinase C is 1:1 (see [25•] and references therein) despite

the presence of two C1 domains in most protein kinase C isozymes. Furthermore, expression studies reveal that the first and second C1 domains bind phorbol esters with comparable affinity when expressed separately (see discussion in [30••]). Although the presence of two C1 domains is still not understood, work by Blumberg and coworkers [30••] established that these domains are functionally distinct on the protein: transfection studies in NIH 3T3 cells using protein kinase C δ mutants revealed that it is the second C1 domain that is responsible for phorbol ester dependent translocation of protein kinase C. Whether the first C1 domain mediates a separate function, perhaps involving protein–protein interactions, remains to be determined.

The cofactor regulation within each isozyme class is similar, suggesting that mechanisms additional to second messenger binding fine-tune isozyme-specific function. These mechanisms could include regulation by phosphorylation and regulation by targeting proteins.

Regulation by phosphorylation

Protein kinase C has recently been shown to be phosphorylated at three positions in the kinase core *in vivo*, both when isolated from mammalian tissue extracts and when expressed in insect cells [31•,32••]. In protein kinase C βII, these residues correspond to Thr500 in the activation loop, and Thr641 and Ser660 at the carboxyl terminus. Hydroxyl-containing residues (or acidic residues at the equivalent position of Ser660 in protein kinase Cs ζ and ι) at corresponding positions are conserved throughout the protein kinase C family, with the exception of in protein kinase μ whose carboxy-terminal phosphorylation sites are not apparent from sequence comparison. Protein kinase C recovered from the detergent-soluble fraction of cells (mammalian or insect) is completely phosphorylated at the two carboxy-terminal positions and half the population is also phosphorylated at the activation-loop site [32••]; this protein kinase C migrates as a single band on SDS-PAGE (at approximately 80 kDa for conventional protein kinase Cs). The protein kinase C in the detergent-insoluble fraction contains faster-migrating species that correspond to nonphosphorylated enzyme or enzyme phosphorylated on the activation loop (both migrating with an apparent molecular weight of 76 kDa for conventional protein kinase Cs), and enzyme phosphorylated on the activation loop and Thr641 or its equivalent (migrating with an apparent molecular weight of 78 kDa for conventional protein kinase Cs). Biochemical analyses of protein kinase C dephosphorylated at selective positions, coupled with analysis of phosphorylation-site mutants, revealed that each site has a specific function [32••].

Activation loop phosphate: transphosphorylation site?

Mutation of the activation loop threonine residue in protein kinase C βII or protein kinase C α to neutral, nonphosphorylatable residues results in an inactivatable enzyme [33,34]. In contrast, mutation to glutamate to

mimic the effect of phosphate results in a cofactor-activatable enzyme [34]. Modeling studies suggest that, as with protein kinase A, a negative charge at the activation loop correctly aligns residues in the active site for catalysis. Importantly, protein kinase C is unable to autophosphorylate by either an intramolecular or an intermolecular reaction at this site, suggesting that another kinase phosphorylates at this position [32••].

The activation loop is the first site phosphorylated in the processing of protein kinase C [32••]. From modeling studies, this phosphorylation site appears to be masked when the pseudosubstrate occupies the active site, suggesting that the pseudosubstrate must be out of the active site for phosphorylation to occur (Fig. 2). Consistent with this hypothesis, dephosphorylation of the activation loop requires the active (pseudosubstrate-out) conformation of protein kinase C [32••].

Carboxy-terminal phosphorylation sites
Following phosphorylation at the activation loop, protein kinase C becomes phosphorylated at Thr641 (for protein kinase C βII). Both the proximity of the carboxyl terminus to the active site and the finding that neighboring residues are phosphorylated by an intramolecular reaction in vitro suggest that the reaction is an autophosphorylation (see [32••] and references therein). This phosphorylation appears to lock protein kinase C in a catalytically competent conformation, perhaps by anchoring the carboxyl terminus out of the active site. Subsequent selective dephosphorylation at the activation loop position results in retention of catalytic activity [32••]. In contrast, dephosphorylation at Thr641 in protein kinase C βII [32••], or mutation of the corresponding residue to alanine in protein kinase C βI [35], results in inactivatable enzyme. Curiously, mutation of the corresponding residue in protein kinase C α results in an activatable enzyme, although it displays reduced stability [36]. However, this could reflect a compensatory phosphorylation on this key stretch of the enzyme. In this regard, mutation of Thr641 to alanine in protein kinase C βII results in a compensatory phosphorylation at neighboring serine and threonine residues; thus, mutation of Thr641, Thr634, and Ser654 is required to prevent phosphorylation in this region (AS Edwards, LM Keranen, AC Newton, unpublished data).

The third in vivo phosphorylation occurs at Ser660 and is an autophosphorylation reaction [32••]. This final phosphorylation correlates with release of protein kinase C into the cytosol, suggesting that its role is to direct the subcellular location of protein kinase C. The two autophosphorylation reactions at the carboxyl terminus appear to be triggered by phosphorylation at the activation loop; thus, phosphorylation by the putative protein kinase C kinase initiates this phosphorylation cascade.

Most of the protein kinase C in unstimulated cells is the triple-phosphorylated, mature form, suggesting that

phosphorylation at these conserved sites is involved in the processing of the enzyme rather than in modulating the function of the enzyme in response to specific signals. However, protein kinase C's subcellular localization and function may be modulated by dephosphorylation at these key positions: ceramide has recently been reported to inactivate protein kinase C α, probably by activating a phosphatase [37•].

Other phosphorylation sites
Additional phosphorylation at nonconserved residues may provide a mechanism for isozyme-selective and stimulus-specific regulation of protein kinase Cs. So far, one isozyme, protein kinase C δ, has been shown to be phosphorylated on tyrosine in response to activation [38–40].

Regulation by localization
Immunocytochemical analysis has clearly established that different protein kinase C isozymes localize to different subcellular compartments (reviewed in [41•]). Subcellular targeting by interaction with specific proteins provides an attractive mechanism for isozyme-specific regulation (reviewed in [42]). A number of proteins have been identified that bind protein kinase C; these proteins include the RACKs (receptors for activated C kinase), a group of proteins that are proposed to bind to the active conformation of protein kinase C [43••]. Binding sites for these proteins have been localized to both conserved and variable regions in the protein kinase C sequence by peptide competition, and peptides based on these sequences effectively disrupt the targeting of protein kinase C [44,45•]. Binding proteins that serve as substrates have also been identified [46]. In addition, Scott and coworkers [47••] showed recently that protein kinase Cs α and βII bind a multi-enzyme scaffold protein, AKAP 79 (for a kinase-anchoring protein). This scaffold also coordinates at least two other signal transducers, namely protein kinase A and the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin. Isozyme-selective binding to a number of proteins has been reported recently; protein kinase C ε binds to 14-3-3 proteins [48], to cytoskeletal components via its hinge or pseudosubstrate [49], and to actin via a sequence between the first and second C1 domains [50•]. Protein kinase C βII has also been reported to bind to actin, in this case via its carboxyl terminus [51•].

Positioning protein kinase C at specific locations in the cell is probably central to its ability to, firstly, respond efficiently to second messengers and, secondly, have ready access to substrates (Fig. 2). The recurring theme that protein kinase C is involved in lipid signalling pathways exemplifies its need to be positioned near the membrane, for rapid access to not only diacylglycerol but also its substrates. For example, protein kinase C regulates phospholipase D, both catalytically and by a novel noncatalytic mechanism [52–55,56••]. The enzyme is also involved in lipid-mediated apoptosis; Spiegel and coworkers [57••] reported that protein kinase C activation

could inhibit ceramide-mediated apoptosis by activating sphingosine kinase, an event that presumably occurs at the membrane interface.

Conclusions

Elucidating the precise functions of the protein kinase Cs has been confounded by their broad substrate specificity *in vitro*, the abundant effects of phorbol esters *in vivo*, and the existence of multiple isoforms within single cell types. However, our growing understanding of the structures and molecular mechanisms of this central family of players will allow the use of new approaches to dissect its role in cellular functions; for example, the use of peptides [45•] or entire domains [58] of protein kinase C to inhibit cofactor or substrate access is showing much promise for determining isozyme function. Thus, with increasing insight into the regulation by ligands, by phosphorylation, and by macromolecular interactions involved in localization, the field is now poised to unravel the *raison d'être* for this ubiquitous family.

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