Minireview

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Protein Kinase C: Structure, Function, and Regulation*

Alexandra C. Newton

From the Department of Pharmacology, University of California at San Diego, La Jolla, California 92093-0640

The protein kinase C family of enzymes transduces the myriad of signals promoting lipid hydrolysis. The prevalence of this enzyme family in signaling is exemplified by the diverse transduction mechanisms that result in the generation of protein kinase C's activator, diacylglycerol. Signals that stimulate members of the large families of G protein-coupled receptors, tyrosine kinase receptors, or non-receptor tyrosine kinases can cause diacylglycerol production, either rapidly by activation of specific phospholipase Cs or more slowly by activation of phospholipase D to yield phosphatidic acid and then diacylglycerol (1–3). In addition, fatty acid generation by phospholipase A2 activation modulates protein kinase C activity (3). Thus, multiple receptor pathways feeding into multiple lipid pathways have the common end result of activating protein kinase C by production of its second messenger.

Phorbol esters, potent tumor promoters, can substitute for diacylglycerol in activating protein kinase C (1–3). Unlike diacylglycerol, phorbol esters are not readily metabolized, and treatment of cells with these molecules results in prolonged activation of protein kinase C. As a result, phorbol esters have proved invaluable in dissecting out protein kinase C-catalyzed phosphorylations *in vivo*.

In addition to regulation by diacylglycerol or phorbol esters, all isozymes of protein kinase C require phosphatidylserine, an acidic lipid located exclusively on the cytoplasmic face of membranes, and some isozymes require Ca^{2+} for optimal activity (4–7). This review discusses the structure of the protein kinase C family, its enzymatic function, and how structure and function are regulated by 1) cofactors and 2) phosphorylation.

Structure

Members of the protein kinase C family are a single polypeptide, comprised of an N-terminal regulatory region (approximately 20–40 kDa) and a C-terminal catalytic region (approximately 45 kDa) (Fig. 1). Cloning of the first isozymes in the mid-1980s revealed four conserved domains: C1–C4 (8). Each is a functional module, and many unrelated proteins have one or the other (9). The function of each of these domains has been established by extensive biochemical and mutational analysis; the C1 domain contains a Cys-rich motif, duplicated in most isozymes, that forms the diacylglycerol/phorbol ester binding site (Fig. 1, *orange*) (7); this domain is immediately preceded by an autoinhibitory pseudosubstrate sequence (Fig. 1, *green*) (10); the C2 domain contains the recognition site for acidic lipids and, in some isozymes, the Ca^{2+}-binding site (Fig. 1, *yellow*) (9). The C3 and C4 domains form the ATP- and substrate-binding lobes of the kinase core (Fig. 1, *pink* and *cyan*) (11). The regulatory and catalytic halves are separated by a hinge region that becomes proteolytically labile when the enzyme is membrane-bound (6); the proteolytically generated kinase domain (protein kinase M), freed of inhibition by the pseudosubstrate, is constitutively active (12).

To date, 11 protein kinase C isozymes have been identified and classified into three groups based on their structure and cofactor regulation (3). The best characterized and first discovered are the conventional protein kinase Cs: α, two alternatively spliced variants βI and βII, and γ. This class distinguishes itself from the others in that function is regulated by Ca^{2+}; its C2 domain contains a putative Ca^{2+}-binding site (see below). The next well characterized are the novel protein kinase Cs: δ, ϵ, η (L), θ, and μ. These isozymes are structurally similar to the conventional protein kinase Cs, except that the C2 domain, while maintaining structural residues, does not have the functional groups that appear to mediate Ca^{2+} binding (see below). The least understood isozymes are the atypical protein kinase Cs: ζ and λ (I). These differ significantly in structure from the other two classes; first, the C1 domain contains only one Cys-rich motif (not two), and second, key residues that maintain the C2 fold do not appear to be present. Furthermore, these isozymes have been reported not to respond to phorbol esters *in vivo* or *in vitro* (3). Perhaps adding to the three groups, two kinases with a C2 domain similar to that of novel protein kinase Cs, but with no C1 domain, have been identified (13, 14).

The crystal structure of the second Cys-rich repeat from the C1 domain of protein kinase C δ was solved recently with (Fig. 2*A*) and without bound phorbol ester by Hurley and co-workers (15), as was the NMR structure of the corresponding repeat from protein kinase C α, in the absence of ligand (16). Strikingly, this β sheet-rich domain undergoes no conformational change upon ligand binding. Rather, binding of phorbol ester plugs the hydrophilic binding site (a groove formed by two unzipped β strands), so that the top third of the domain displays a contiguous hydrophobic surface (15).

The crystal structure of the C2 domain of synaptotagmin, elucidated by Sprang and co-workers (17), reveals how the other half of the regulatory region of protein kinase C folds. Fig. 2*B* shows the core of this domain ("C2 key"): 5 aspartate residues form the Ca^{2+}-binding site (*pink*); on the back face of this cleft are bulky aromatics (*purple*) adjacent to a basic surface formed by two β strands (*blue*). Sossin and Schwartz (18) noted that novel protein kinase Cs contain a C2 domain. The solved structure elucidates how these protein kinase Cs can have this domain without being Ca^{2+}-regulated; the C2 domain of novel protein kinase Cs has the conserved residues that maintain the fold of the domain (*e.g.* Fig. 2*B*, *orange*), but the coordinating oxygens in the Ca^{2+}-binding site are mainly absent (9).

A modeled structure of the catalytic domain of protein kinase C βII, with bound pseudosubstrate, based on the crystal structure of protein kinase A with bound inhibitory peptide (19) is shown in Fig. 2*C* (20). The primary sequence of the kinase core of conventional protein kinase Cs is approximately 40% identical to that of protein kinase A's core. The N-terminal residue of the model is just before the hinge region; the peptide chain would continue on to the C2 and then C1 domains and then connect to the pseudosubstrate. Modeling of the latter in the substrate binding cavity reveals that it is held there, in part, by a cluster of acidic residues that is unique to the protein kinase C family (20). The pseudosubstrate sequence was identified by House and Kemp (10) based on the ability of a synthetic peptide of this sequence to inhibit protein kinase C.

Function

Enzymology—The chemistry of protein kinase C's catalytic core is similar to that of the archetypal kinase, protein kinase A. The kinase uses MgATP as substrate, with a K_m for ATP in the low μM range (21). The enzyme's maximal catalytic rate and K_m for a peptide substrate based on its pseudosubstrate have been reported to be 8 μmol of phosphate hydrolyzed per min per mg of protein (corresponding to 10 reactions/s) and 0.2 μM, respectively (10). This corresponds to a k_{cat}/K_m of 5×10^7 s^{-1} M^{-1}, revealing remarkable efficiency. Most other synthetic peptides have K_m values in the low μM range and V_{max} values typically ranging from 1 to 8 μmol min^{-1} mg^{-1} (22–26), suggesting that a k_{cat}/K_m of 10^6 s^{-1} M^{-1} is more representative of this family of enzymes. The k_{cat}/K_m of protein kinase A for kemptide, a synthetic peptide based on the kinase's consensus phosphorylation sequence (27), is comparable (28).

Protein kinase C typically phosphorylates serine or threonine

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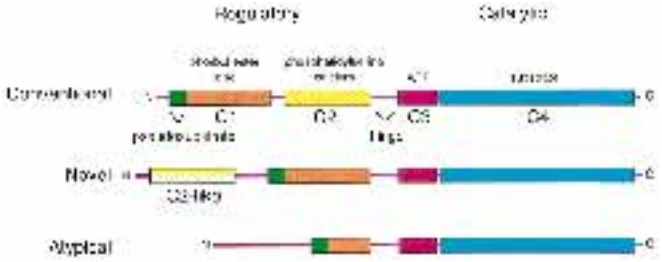

FIG. 1. **Schematic representation of the primary structure of conventional, novel, and atypical protein kinase Cs.** Indicated are the pseudosubstrate domain (*green*), C1 domain comprising one or two Cys-rich motifs (*orange*), C2 domain (*yellow*) in the regulatory half, and the ATP-binding lobe (C3, *pink*) and substrate-binding lobe (C4, *teal blue*) of the catalytic region. The C2 domain of novel protein kinase Cs lacks amino acids involved in binding calcium but has key conserved residues involved in maintaining the C2 fold (hence its description as "C2-like"). Atypical protein kinase Cs have only one Cys-rich motif, and phorbol ester binding has not been detected.

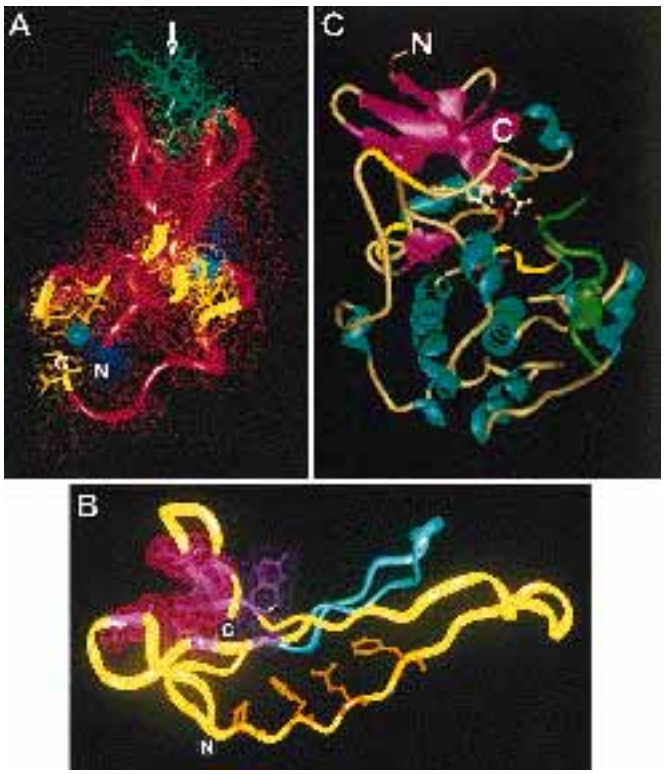

FIG. 2. **Structures of protein kinase C's domains.** *A*, C1 domain. The ribbon and surface diagram of amino acids 231–280 in the second Cys-rich domain of protein kinase C δ with bound phorbol ester (*green*) based on the coordinates of Zhang *et al.* (15) is shown. Conserved Cys (*yellow*) and His (*purple*) that coordinate the two zinc atoms of each cysteine-rich repeat (78) (*green balls*) are indicated. The *arrow* indicates the C12 position of the phorbol ester that is fatty acylated in bioactive phorbol esters (51). *B,* C2 domain. The ribbon diagram of residues 167–240 from the C2 domain of synaptotagmin based on the coordinates of Sutton *et al.* (17) is shown. The five aspartates in the Ca^{2+}-binding site are indicated in *pink*, the bulky hydrophobics on the back face in *purple*, and the adjacent two β strands that are positively charged and likely constitute the acidic lipid-binding surface are in *blue*. Residues shown in *orange* are conserved in all C2 domains (9). *C*, catalytic (C3 and C4) domain. The modeled structure of residues 340–632 of protein kinase C βII with bound pseudosubstrate (residues 9–28) (20) is shown. The upper lobe, involved primarily in nucleotide binding, is mainly β sheet (*pink*) and the lower lobe, containing the substrate-binding cavity, is predominantly α helix (*teal blue*). Indicated are ATP (*cream*), two Mn^{2+} atoms (*red dots*), and the pseudosubstrate (*green*) with the *orange dot* representing the alanine at the phosphoacceptor position. The *yellow loop* at the entrance to the catalytic site (below ATP) is the activation loop (11); phosphorylation here aligns residues for catalysis (75). Reproduced from Ref. 20.

residues in basic sequences but displays significantly less specificity than protein kinase A (29). First, unlike protein kinase A (29), no clear requirements for positive charge at specific positions are apparent from analysis of sequences around phosphorylation sites (30–32) or from analysis of synthetic peptide substrates (23, 26, 33). Second, protein kinase C displays lower stereospecificity than protein kinase A (25), phosphorylating both D- and L-stereoisomers of α configurational isomers of a number of alcohols (24). Lawrence and co-workers (24) have suggested that protein kinase C's lack of stereospecificity could reflect substrate binding in either direction (*i.e.* C to N or N to C) in the substrate-binding cavity (24).

Protein kinase C also autophosphorylates *in vitro* (34, 35) by an intramolecular mechanism (36) at the N terminus, hinge, and C terminus (37); the latter site is a poor *in vitro* site because it is almost quantitatively phosphorylated *in vivo* (see below).

In addition to catalyzing phosphorylation reactions, protein kinase C has ATPase and phosphatase activity. The enzyme catalyzes a cofactor-dependent and substrate-stimulated hydrolysis of ATP (38), and it can work backwards (*i.e.* as a phosphatase) in the presence of excess ADP.[1]

Biological Function—Given the plethora of substrates and the effectiveness of phorbol esters in modulating diverse cellular responses, a multiplicity of functions have been ascribed to protein kinase C (4). Recurring themes are that protein kinase C is involved in receptor desensitization, in modulating membrane structure events, in regulating transcription, in mediating immune responses, in regulating cell growth, and in learning and memory among many other functions. These and the functions of specific isozymes are described in a number of excellent reviews (1–4, 39).

A key regulator of protein kinase C function *in vivo* is likely to be subcellular distribution of both the enzyme and substrate (40). Protein kinase C isozymes are distributed differentially throughout the cell (and differently among many cell types) (39), and a number of targetting proteins have been described (40).

Deciphering the specific functions of isozymes likely awaits the development of isozyme-specific inhibitors (41). The application of combinatorial chemistry toward this goal has provided the first isozyme-specific inhibitor (42); similar specificity using antisense DNA has been demonstrated for *in situ* studies (43).

Regulation

The function of protein kinase C is regulated by two equally important mechanisms. First, the enzyme is rendered catalytically competent by phosphorylations that correctly align residues for catalysis and localize protein kinase C to the cytosol. Second, binding of ligands or, in some cases, substrate activates the enzyme by removing the pseudosubstrate from the substrate-binding site.

Pseudosubstrate Regulation

Biochemical experiments have established that, as predicted (10), activation of protein kinase C is accompanied by removal of its pseudosubstrate from the kinase core (20, 44). Specifically, the basic pseudosubstrate is protected from proteolysis when the enzyme is not catalytically active but becomes highly sensitive to proteolysis by trypsin or endoproteinase Arg-C upon activation (44). Importantly, the pseudosubstrate is unmasked whether protein kinase C is activated by conventional (phosphatidylserine, diacylglycerol, and Ca^{2+}), non-conventional (*e.g.* short chained phosphatidylcholines (45)), or cofactor-independent substrates (*e.g.* protamine (46)) (20). Consistent with this, incubation of protein kinase C with an antibody directed against the pseudosubstrate was shown to activate the enzyme, presumably by removing the pseudosubstrate from the active site (47).

Diacylglycerol and Phorbol Esters—Since the discovery that phorbol esters cause protein kinase C to "translocate" to membranes (48–50), this and the accompanying activation by these molecules and diacylglycerols have been the subject of extensive investigations (7, 51). Taken together with recent molecular biological (52–56), structural (15), and biophysical studies (57–59), a fairly good understanding of the mechanism for the effects of these C1 ligands on protein kinase C function has emerged.

Diacylglycerol and phorbol esters serve as hydrophobic anchors to recruit protein kinase C to the membrane; they cause a dramatic increase in the enzyme's membrane affinity[2] that is linearly related to the mol fraction C1 ligand in the bilayer, is reversible, and

[1] A. C. Newton, unpublished data.

[2] The dissociation constant of phorbol myristate acetate from membrane-bound protein kinase C, separated from all other interactions, has recently been measured as 1.5×10^{-5} mol % relative to membrane lipids; that for diacylglycerol is 250 times higher (M. Mosior and A. C. Newton, *Biochemistry*, in press).

can occur in the absence of acidic lipids and C2 domain interactions[3] (55, 57–61). Differences in the biological action of these two classes of ligands are accounted for by the 2 orders of magnitude increased potency of phorbol esters compared with diacylglycerol (58) and the long life of phorbol esters in cells. The phorbol ester domain structure suggests how the membrane anchor works; by capping the hydrophilic ligand groove, phorbol ester binding alters the surface hydrophobicity of the domain, thus promoting the membrane interaction in the absence of conformational changes (15).

In addition to increasing protein kinase C's membrane affinity, C1 ligands may also stabilize the active conformation of protein kinase C. Diacylglycerol doubles the catalytic efficiency of enzyme that is bound to phosphatidylserine (57, 59, 62, 63); it also stimulates the activation promoted by fatty acids (3) and short chained phosphatidylcholines (45).

C1 ligands markedly reduce the concentration of Ca^{2+} required for the phosphatidylserine-dependent activation of protein kinase C (64). The molecular basis for this does not arise from allosteric interactions between the C1 and C2 domain sites; Ca^{2+} has no effect on protein kinase C's affinity for either C1 ligand[3] (59, 65). Rather, the apparent synergy between these two activators arises because each, by separate mechanisms, increases the affinity of protein kinase C for membranes. Consistent with no allosteric interactions, the structure of the phorbol ester-binding domain is unchanged by phorbol ester binding (15).

Phosphatidylserine—The requirement for a "membrane factor" to activate protein kinase C was established shortly after the discovery of the enzyme (46). Pioneering work by Bell and coworkers in the mid-1980s established the remarkable specificity for the serine headgroup for activation (7). The mechanism for the phosphatidylserine-dependent stimulation is now well characterized as a result of binding measurements that have allowed the effect of phospholipid headgroup structure, diacylglycerol, and Ca^{2+} on the interaction of protein kinase C with membranes to be dissected out (6). Studies with lipid in bilayers or detergent-lipid mixed micelles have established that the phospholipid regulation and accompanying conformational changes depend on phospholipid structure rather than membrane structure (6), although the latter does modulate enzyme activity (5, 66).

In the absence of C1 ligands, protein kinase C binds acidic lipids with little selectivity for the headgroup beyond the requirement for negative charge (59) (this interaction is Ca^{2+}-regulated for conventional protein kinase Cs; see next section). This binding is of relatively low affinity, is sensitive to ionic strength, is accompanied by a conformational change that exposes the hinge region to proteolysis, and is typically not accompanied by much activation or pseudosubstrate exposure (Fig. 3, *top middle*) (44, 59, 60). Note that the hinge exposure is independent of the active state of the kinase, reflecting rather the "membrane-bound conformation" of the enzyme (67).

The presence of diacylglycerol causes a striking and selective increase in conventional and novel protein kinase C's affinity for phosphatidylserine that is accompanied by activation and pseudosubstrate release (Fig. 3, *top left*) (6). This high affinity interaction is 1 order of magnitude stronger for surfaces containing phosphatidyl-L-serine compared with other acidic lipids such as phosphatidyl-D-serine (59). Thus, specific structural elements of the L-serine headgroup are required for the high affinity binding of protein kinase C to membranes containing C1 ligands. Because phosphatidylserine promotes the binding of phorbol esters to a single recombinant Cys-rich domain (55), the specificity may arise from additional interactions of the L-serine headgroup with the C1 domain or with new surfaces created at the C1-C2 interface. Kinetic studies suggest that protein kinase C interacts cooperatively with multiple phosphatidylserine molecules (6, 7).

Calcium Ions—Ca^{2+} increases the affinity of conventional protein kinase Cs for negatively charged lipids (69), with no selectivity for headgroup other than the requirement for negative charge (59). This increase varies linearly with Ca^{2+} concentration in the low μM to submillimolar range (65), consistent with the single Ca^{2+}-binding site apparent in the C2 domain structure (17). The dissociation constant of Ca^{2+} from membrane-bound protein kinase C has been calculated to be approximately 700 nM, and that from soluble protein kinase C has been estimated to be 3 mM (65).

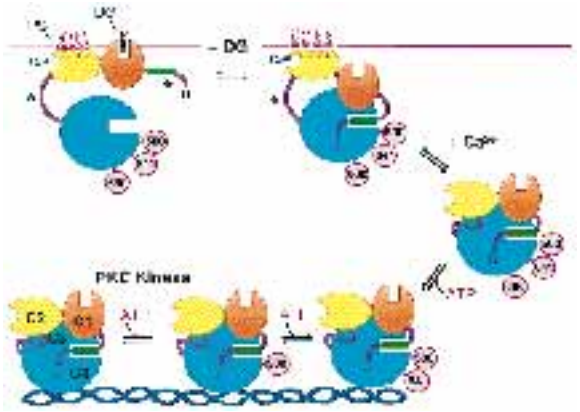

FIG. 3. **Model for the regulation of protein kinase C by 1) phosphorylation and 2) membrane binding and pseudosubstrate release.** Newly synthesized protein kinase C (*PKC*) associates with the detergent-insoluble fraction of cells (72) (*bottom left*). It is processed to the mature, cytosolic form by three functionally distinct phosphorylations: transphosphorylation at the activation loop to render the kinase catalytically competent (Thr-500 in βII); an autophosphorylation at the C terminus (Thr-641 in βII) that stabilizes the catalytically competent conformation (73); and a second autophosphorylation at the C terminus (Ser-660 in βII) that releases protein kinase C into the cytosol (73). This triple phosphorylated mature form is inactive because the pseudosubstrate occupies the substrate-binding cavity (*middle*). Generation of diacylglycerol (*DG*) causes the affinity of protein kinase C for membranes to increase dramatically. Membrane translocation is mediated by diacylglycerol binding to the C1 domain and phosphatidylserine (*PS*) binding to the C2 domain (*top right*). The affinity for acidic lipids is increased by Ca^{2+} for conventional protein kinase Cs, likely by structuring the lipid-binding surface, but not for novel protein kinase Cs, whose lipid-binding surface may already be structured. Protein kinase C can bind to membranes with low affinity with either C1 domain ligands (not shown) or with C2 domain ligands (*top middle*). However, it is the high affinity binding (*top left*) mediated by both domains that results in pseudosubstrate release and maximal activation. *Asterisks* indicate the exposed hinge, which becomes proteolytically labile upon membrane binding (independently of pseudosubstrate release (67)), and the exposed pseudosubstrate, which becomes proteolytically labile upon activation (independently of membrane binding (20)).

The C2 domain structure provides tantalizing insight into how Ca^{2+} might increase the affinity of conventional, but not novel, protein kinase Cs for acidic lipids. For conventional protein kinase Cs, binding of Ca^{2+} to the aspartate-lined "mouth" (Fig. 2*B*) might clamp together the upper and lower lobes, thus orienting the bulky aromatics on the back face of the mouth to interact with the membrane and orienting the basic face of the β sheet behind the site to interact with lipid headgroups. For novel protein kinase Cs (such as ϵ), the presence of an Arg instead of an Asp at one of the positions in the site (9) might cause the mouth to adopt the closed conformation, so that the domain is already structured to bind acidic lipids. C1 ligands would then target novel protein kinase Cs to membranes, with the reduction in dimensionality promoting the binding of the C2 domain to acidic lipids. Consistent with this, the lipid regulation of novel protein kinase Cs is the same as that for conventional protein kinase Cs, except that it occurs in the absence of Ca^{2+} (70).

Substrates—Protein kinase C phosphorylates a number of Arg-rich proteins in a cofactor-independent manner (46, 71). These substrates, alone, are able to displace the pseudosubstrate from the kinase core (20). One possibility is that these Arg-rich peptides neutralize the acidic patch that appears to maintain the pseudosubstrate in the active site (20), thus releasing the basic pseudosubstrate by competing for contacts. In this regard, the ability of Arg-rich peptides to promote protein kinase C autophosphorylation (rather than compete with it) led to the suggestion that Arg-rich molecules bind to a separate site from the active site (71). An intriguing possibility is that the acidic cluster interacts with Arg-rich sequences of cytoskeletal proteins, thus allowing activation distal from the lipid bilayer.

Regulation by Phosphorylation in Vivo

Pulse-chase experiments by Fabbro and co-workers (72) provided the first evidence that protein kinase C is phosphorylated *in vivo*. Specifically, they showed that protein kinase C is first synthesized

[3] M. Mosior and A. C. Newton, *Biochemistry,* in press.

as an inactive, dephosphorylated precursor with an apparent M_r of 74 kDa; this was chased to a transient 77-kDa phospho-form and then to the final 80-kDa mature form. Mass spectrometry has recently revealed that protein kinase C is modified by three phosphorylations *in vivo* (73, 74). The differential dephosphorylation of these sites by protein phosphatases 1 and 2A (75), as well as analysis of phosphorylation site mutants (76, 77), has allowed the function of each phosphorylation to be identified (73).

A model consistent with biochemical data is presented in the *lower half* of Fig. 3. Newly synthesized protein kinase C associates with a detergent-insoluble cell fraction (72); it is rendered catalytically competent upon phosphorylation by a putative protein kinase C kinase on its activation loop (Fig. 2*C*). Negative charge on this loop at the entrance to the active site correctly aligns residues involved in catalysis in diverse kinases (11); replacement of the phosphorylated residue (Thr-500) with Glu in protein kinase C βII results in activatable enzyme (77) whereas replacement with neutral non-phosphorylatable residues in this isozyme (77) or protein kinase C α (76) results in kinase that cannot be activated. The first consequence of the transphosphorylation appears to be autophosphorylation at the C terminus of the kinase; this residue is Thr-641 in protein kinase C βII (9 residues removed from the C terminus of the model in Fig. 2*C*) (73, 74). Phosphorylation here likely stabilizes the catalytically competent conformation of the kinase as it replaces the requirement for negative charge at the activation loop (73); this phosphorylation causes the first detectable shift in electrophoretic mobility (73, 75). Last, the enzyme autophosphorylates further along the C terminus (Ser-660 in protein kinase C βII) in a motif shared by several other kinases (73); this phosphorylation causes the final shift in electrophoretic mobility and releases the mature enzyme into the cytosol (73, 75). It is this 80-kDa form, localized to the detergent-soluble fraction, that has been extensively purified and studied *in vitro*. Curiously, it is only half-phosphorylated at the activation loop (but quantitatively phosphorylated at the two C-terminal sites) (73), suggesting that dephosphorylation/transphosphorylation at this position may regulate the kinase in response to stimuli. The mature form then translocates to the membrane and undergoes the pseudosubstrate regulation discussed above.

Conclusion

Protein kinase C is regulated by two distinct mechanisms: by phosphorylation which regulates the active site and subcellular localization of the enzyme, and by second messengers which promote protein kinase C's membrane association and resulting pseudosubstrate exposure. Regulation by two independent mechanisms may provide exquisite fine-tuning for this family of enzymes, ensuring low basal activity in the midst of complex intracellular signaling pathways.

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