Communication

In Vivo Regulation of Protein Kinase C by Trans-phosphorylation Followed by Autophosphorylation*

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Dephosphorylation by the catalytic subunits of protein phosphatases 1 (CS1) and 2A (CS2) reveals that mature protein kinase C is phosphorylated at two distinct sites. Treatment of protein kinase C βII with CS1 causes a significant increase in the protein's electrophoretic mobility (approximately 4 kDa) and a coincident loss in catalytic activity. The CS1-dephosphorylated enzyme cannot autophosphorylate or be phosphorylated by mature protein kinase C, indicating that a different kinase catalyzes the phosphorylation at this site. The loss of activity is consistent with dephosphorylation on protein kinase C's activation loop (Orr, J. W., and Newton, A. C., (1994) *J. Biol. Chem.* 269, 27715–27718). Treatment with CS2 results in a smaller shift in electrophoretic mobility (approximately 2 kDa) and no loss in catalytic activity. Furthermore, the CS2-dephosphorylated form can autophosphorylate and thus regain the electrophoretic mobility of mature enzyme, consistent with dephosphorylation at protein kinase C's carboxyl-terminal autophosphorylation site, which is modified *in vivo* (Flint, A. J., Paladini, R. D., and Koshland, D. E., Jr. (1990) *Science* 249, 408–411). In summary, two phosphorylations process protein kinase C to generate the mature form: a trans-phosphorylation that renders the kinase catalytically competent and an autophosphorylation that may be important for the subcellular localization of the enzyme.

Protein kinase Cs transduce the plethora of signals provoking phospholipid hydrolysis (3). Production of diacylglycerol results in a high affinity protein kinase C:membrane interaction that is mediated by specific binding to both the lipid second messenger and phosphatidylserine (4, 5). These two lipids cause a conformational change that removes the enzyme's autoinhibitory pseudosubstrate domain from the active site (6, 7). Thus, protein kinase C is allosterically regulated by lipid.

Several recent reports have revealed an additional mode of regulation of protein kinase C: phosphorylation (1, 8–10). In contrast to protein kinase C's unique regulation by lipid second messengers, regulation by phosphorylation is shared by most other members of the protein kinase superfamily (11). In particular, elucidation of the crystal structure of protein kinase A revealed a lip at the entrance to the catalytic core whose phosphorylation is important in correctly aligning residues involved in catalysis (12). The crystal structures of Cdk2 (13) and mitogen-activated protein kinase (14) support the requirement for negative charge on this lip for catalytically competent enzyme, hence the naming of this lip as the activation loop (11). For protein kinase A, phosphorylation on the activation loop is structural (15). For Cdk2 and mitogen-activated protein kinase, the phosphorylations are transient and regulate the kinases (16, 17).

Modeling of the catalytic core of protein kinase C βII revealed that it has a threonine (conserved in all isozymes of protein kinase C) at the position of the phospho-Thr on the activation loop of protein kinase A (7). Phosphorylation at Thr^{500} would provide the same electrostatic contacts that the phospho-Thr of protein kinase A provides with residues in the catalytic core. Supporting the requirement for phosphorylation at Thr^{500} for catalytically competent protein kinase C, mutation of this residue revealed that negative charge at this position is required to produce an activatable protein kinase C (1). Similarly, mutation of the corresponding Thr to Ala in protein kinase C α (Thr^{497}) results in an inactive kinase (10). Consistent with the requirement for phosphate at the activation loop, Fabbro and co-workers (8) found that protein kinase C is first synthesized as a dephosphorylated, inactive, membrane-bound precursor with an apparent mobility on SDS-PAGE[1] of 74 kDa. Pulse-labeling experiments revealed that the 74-kDa form is chased into a 77-kDa form and finally into the 80-kDa "mature" form. That at least one of these phosphorylations is catalyzed by a different kinase is suggested by the inability to express catalytically active protein kinase C in bacteria (10, 18).

In addition to phosphorylation at Thr^{500}, mature protein kinase C is also phosphorylated at the COOH terminus. Koshland and co-workers (2) showed that protein kinase C βII purified from baculovirus was phosphorylated *in vivo* at Thr^{634} and Thr^{641}; these residues could also be autophosphorylated *in vitro*. Phosphorylation at the latter site appears to be important for the correct subcellular localization of protein kinase C (19). *In vitro*, protein kinase C βII also autophosphorylates at its amino terminus and hinge region (2).

In this communication, we have used the catalytic subunits of phosphatases 1 (CS1) and 2A (CS2) to dissect the roles of specific phosphorylations in processing mature protein kinase C. Our data support a model in which protein kinase C is activated by trans-phosphorylation at the activation loop followed by autophosphorylation at the COOH terminus.

MATERIALS AND METHODS

Bovine brain L-α-phosphatidylserine and *sn*-1,2-dioleoylglycerol were obtained from Avanti Polar Lipids, Inc. Microcystin was purchased from ICN. Triton X-100 (10% (w/v) aqueous solution) was from Pierce, and [γ-^{32}P]ATP (3000 Ci mmol^{-1}) was from DuPont NEN. A protein kinase

[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; CS1, catalytic subunit of protein phosphatase 1; CS2, catalytic subunit of protein phosphatase 2A; DTT, dithiothreitol.
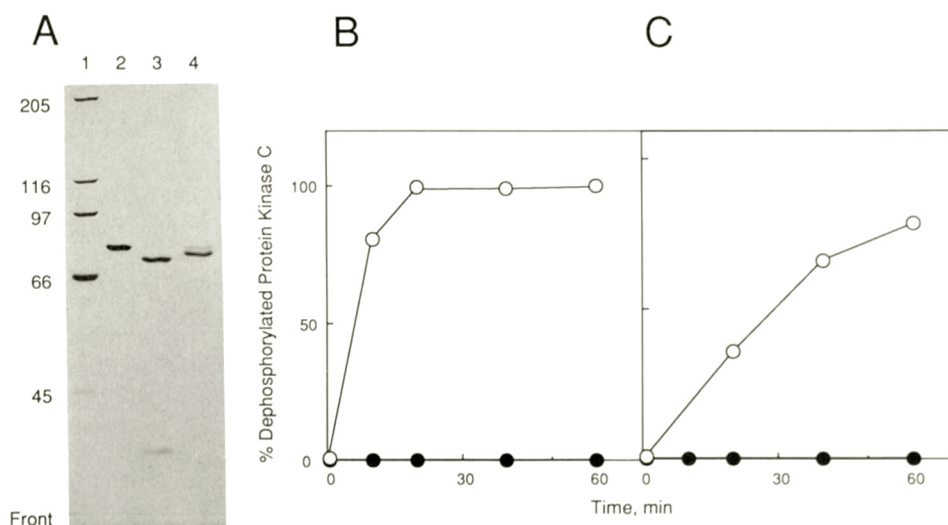
FIG. 1. **Dephosphorylation of protein kinase C catalyzed by CS1 or CS2 results in a specific shift in electrophoretic mobility and requires the membrane-bound conformation of protein kinase C.** *A*, silver-stained gel showing protein kinase C before (*lane 2*) and after incubation with CS1 (23 units ml^{-1}) for 45 min (*lane 3*) or CS2 (10 units ml^{-1}) for 90 min (*lane 4*). Indicated molecular size standards are shown in *lane 1*. The bands migrating below the 45-kDa marker in *lanes 3* and *4* represent CS1 and CS2, respectively. *B*, protein kinase C (20 nM) was incubated with CS1 (23 units ml^{-1}) for the indicated times, in the presence (○) or absence (●) of 140 μM phosphatidylserine and 4 μM diacylglycerol. The percentage of dephosphorylated protein was obtained by integrating the amount of dephosphorylated (faster migration on SDS-PAGE compared with mature) and mature protein kinase C on silver-stained gels. *C*, protein kinase C (20 nM) was incubated with CS2 (5 units ml^{-1}) for the indicated times, in the presence (○) or absence (●) of 140 μM phosphatidylserine and 4 μM diacylglycerol. Data represent the amount of dephosphorylated enzyme, determined as in *B*. Results are representative of at least four separate experiments.

C-selective peptide (FKKSFKL-NH$_2$; Ref. 20) was synthesized by the Indiana University Biochemistry Biotechnology Facility. Homogeneously pure protein kinase C βII, α, or ε was purified from a baculovirus expression system as described (6); a mixture of isozymes was partially purified from rat brain by DEAE-Sepharose chromatography as described (21). Protein kinase C was stored at –20 °C in 10 mM Tris buffer, pH 7.5 (4 °C), 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT, and 50% glycerol. Recombinant protein phosphatase 1 catalytic subunit α isoform (CS1) was purified to homogeneity from *Escherichia coli* by a procedure[2] similar to that of Zhang *et al.* (22); purified enzyme was stored at –20 °C in 50 mM imidazole, pH 7.4, 0.1 M NaCl, 1 mM EDTA, 2 mM DTT, 2 mM MnCl$_2$, and 50% glycerol (45 μg ml^{-1}; 20,000 nmol min^{-1} mg^{-1} using phosphorylase *a* (1 mg ml^{-1}) as a substrate in the presence of 5 mM caffeine). Protein phosphatase 2A catalytic subunit (CS2) was purified to homogeneity from rabbit skeletal muscle by a modification[3] of the procedure of Tung *et al.* (23); purified enzyme was stored in 20 mM Tris, pH 7.2, 5 mM EDTA, 2 mM EGTA, and 10% glycerol (10 μg ml^{-1}; 10,000 nmol min^{-1} mg^{-1} using phosphorylase a (2 mg ml^{-1}) as a substrate in the presence of 5 mM caffeine). The activity of the phosphatases is presented in units ml^{-1} where 1 unit is 1 nmol of phosphate removed from phosphorylase *a* in 1 min under the conditions described above for each phosphatase. All other chemicals were reagent-grade.

Lipid—Sonicated dispersions of phosphatidylserine (1.4 mM) and diacylglycerol (38 μM) or Triton X-100 (1.0%, w/v) mixed micelles containing phosphatidylserine (15 mol %) and diacylglycerol (5 mol %) were prepared as described (21). Phosphatidylserine concentrations were verified by assay for phosphate concentration (24).

Phosphatase Incubations—Protein kinase C (20 nM) was incubated with CS1 (23 units ml^{-1}) in 20 mM HEPES buffer, pH 7.5, containing 200 μM MnCl$_2$, 1 mM DTT, 0.5 mM CaCl$_2$, and 0.04 mM EDTA for 0.25 to 60 min, 22 °C. Incubations with CS2 (5 or 10 units ml^{-1}) were similar except that chelator concentrations were 250 μM EDTA and 100 μM EGTA, and CaCl$_2$ was present at a concentration of 1 mM. Unless otherwise stated, incubations included 140 μM phosphatidylserine and 4 μM diacylglycerol. Dephosphorylation reactions were quenched by addition of 1 mM microcystin to yield a final concentration of 30 μM or by addition of 0.25 volume of SDS-PAGE sample buffer. Control incubations (*i.e.* no phosphatase) were as described above except that phosphatase was omitted; concentrations of all other reagents were the same. Experiments in which control incubations included phosphatase and 30 μM microcystin yielded identical results to those obtained with no phosphatase (not shown). Samples quenched in sample buffer were analyzed by SDS-PAGE (7.5% polyacrylamide) and silver staining (25).

Protein Kinase C Activity Assay—Aliquots from phosphatase or control incubations were diluted 8-fold into an 80-μl reaction volume containing protein kinase C selective peptide (FKKSFKL-NH$_2$, 50 μg ml^{-1} in assay), 20 mM HEPES buffer (pH 7.5 at 30 °C), 1 mM DTT, 50 μM [γ-^{32}P]ATP (150 Ci mol^{-1}), 10 mM MgCl$_2$, and either 500 μM CaCl$_2$ and Triton X-100 (0.1%, w/v) mixed micelles containing 15 mol % phosphatidylserine and 5 mol % diacylglycerol (activating conditions), or 100 μM EDTA, 100 μM EGTA, and Triton X-100 (0.1%, w/v) (non-activating conditions). Because the 8-fold dilution of the samples from the first incubation (± phosphatase) into the reaction mixture containing 0.1% Triton X-100 resulted in dilution of the lipid from the first incubation to non-activating concentrations (1.0 mol % phosphatidylserine and 0.03 mol % diacylglycerol in Triton X-100 mixed micelles in final reaction mixture). Samples were incubated at 30 °C for 5 min and quenched and analyzed as described previously (6). ^{32}P incorporation into substrate was linear with time under the conditions employed.

Autophosphorylation—Autophosphorylation of protein kinase C (40 nM) was carried out essentially as described for substrate phosphorylation except that selective peptide was omitted.

RESULTS AND DISCUSSION

Treatment of mature protein kinase C βII, isolated from the baculovirus expression system, with the catalytic subunit (α isoform) of protein phosphatase 1 (CS1) or the catalytic subunit of protein phosphatase 2A (CS2) resulted in an increase in the relative mobility of the protein on SDS-PAGE. Fig. 1A shows that incubation with CS1 caused protein kinase C to migrate with an apparent molecular mass 4 kDa lower (*lane 3*) than the native 80-kDa protein (*lane 2*). Fig. 1A also shows that incubation with CS2 altered the relative mobility of protein kinase C; however, the shift corresponded to an apparent molecular mass change of only 2 kDa (*lane 4*). Extensive CS2 treatment could not shift the mass of the dephosphorylated enzyme (78 kDa) to the 76-kDa form, nor could any 78-kDa intermediate be detected in the CS1-catalyzed dephosphorylation of protein kinase C. Western blot analysis revealed similar increases in electrophoretic mobility of protein kinase C α, βII, and ε resulting from phosphatase treatment of partially purified rat brain enzyme (data not shown).

Fig. 1 (*B* and *C*) shows that the sensitivity of protein kinase C to dephosphorylation by either phosphatase was markedly enhanced in the presence of phosphatidylserine and diacylglyc-

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[2] I. K. Park and A. A. DePaoli-Roach, unpublished results.
[3] A. A. DePaoli-Roach, unpublished results.

A

B

FIG. 2. **Dephosphorylation catalyzed by CS1, but not CS2, results in loss of protein kinase C activity.** Protein kinase C (20 nM) was incubated in the presence or absence of CS1 (23 units ml⁻¹) (*A*) or CS2 (5 units ml⁻¹) (*B*). At the indicated times, aliquots were quenched with microcystin (30 μM) and the activity toward phosphorylation of a protein kinase C selective peptide was monitored in the presence or absence of phosphatidylserine, diacylglycerol, and Ca²⁺. Activity is expressed as percent of control (*i.e.* relative to activity of enzyme incubated in the absence of phosphatase) (*B*). *Inset*, silver-stained gel of phosphatase-treated samples quenched with microcystin at 15 s (*lane 1*), 11 min (*lane 2*), 30 min (*lane 3*), 45 min (*lane 4*), or 60 min (*lane 5*).
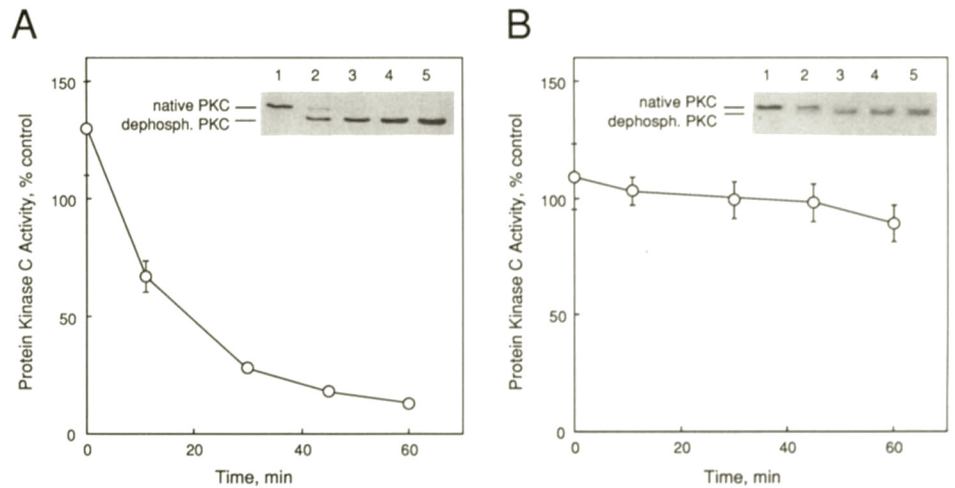
erol. Because these lipids did not alter significantly the rate of dephosphorylation of phosphorylase *a* by either phosphatase (data not shown), the requirement for lipid to dephosphorylate protein kinase C indicates that the phosphorylated residues are considerably more exposed in the membrane-bound conformation of the enzyme. Consistent with the membrane-bound conformation exposing the phosphorylated sites, protein kinase C activated by short-chained phosphatidylcholines, but not by protamine sulfate, had enhanced phosphatase sensitivity (data not shown). The former exposes protein kinase C's hinge region, mimicking the membrane-bound conformation, whereas protamine activates protein kinase C in the absence of this conformational change (7). The CS1-catalyzed dephosphorylation of protein kinase C from human breast cancer cells has also been reported to depend on lipid (8).

Dephosphorylation of mature protein kinase C by CS1 was accompanied by loss of enzymatic activity. Fig. 2*A* shows the substrate-directed activity of protein kinase C after incubation with CS1 for increasing amounts of time; data represent the activity of phosphatase-treated enzyme relative to enzyme treated with the same buffer but without the phosphatase (similar data were obtained when the control enzyme was treated with phosphatase but in the presence of microcystin). Loss of activity correlated with dephosphorylation of the enzyme (see *inset*). CS1 treatment of a mixture of protein kinase C isozymes from rat brain resulted in loss of both Ca²⁺-dependent and Ca²⁺-independent activity (data not shown), indicating that both conventional and novel isozymes of protein kinase C must be phosphorylated at the CS1 site for catalysis.

In contrast to the effects of CS1, incubation with CS2 had no significant effect on the activity of protein kinase C. Fig. 2*B* shows that protein kinase C retained its ability to phosphorylate a synthetic peptide even when completely dephosphorylated (*inset, lane 5*). In these assays, phosphatidylserine and diacylglycerol were presented in the form of Triton X-100 mixed micelles. Under these conditions, CS2-dephosphorylated protein kinase C cannot rephosphorylate at the CS2 site (not shown); thus, the full catalytic activity in the micelle assay reveals that the dephosphorylated form has the same V_{max} as the control enzyme when measured using a synthetic peptide as substrate.

To address whether the phosphorylations at the CS1 and CS2 sites were mediated by another kinase, or whether protein kinase C could rephosphorylate these sites, protein kinase C was first dephosphorylated (in the presence of lipid and Ca²⁺) and then incubated with [³²P]ATP and MgCl₂. Fig. 3*A* shows that the relative mobility of protein kinase C dephosphorylated by CS1 did not change upon incubation with ATP. Thus, the kinase was unable to autophosphorylate at the residue dephos-
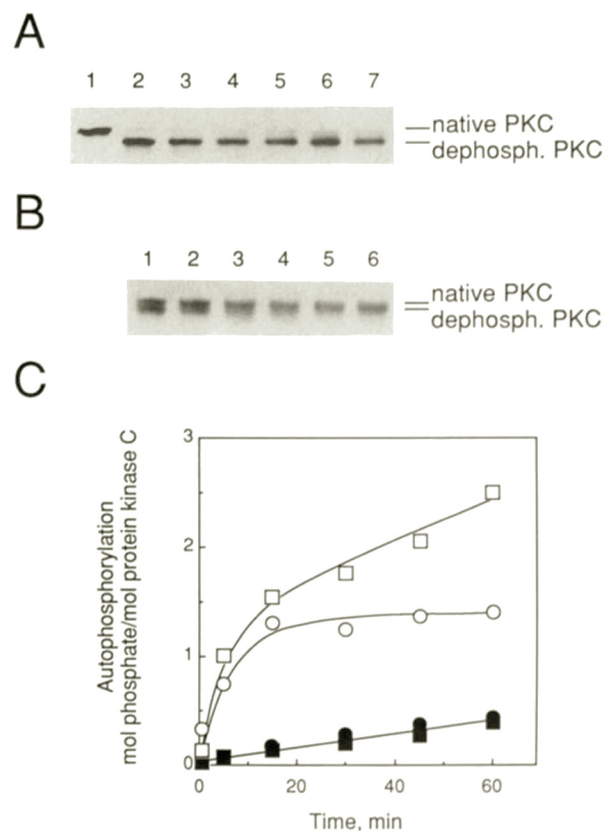
A

B

C

FIG. 3. **Protein kinase C dephosphorylated by CS2, but not CS1, can re-autophosphorylate.** *A*, silver-stained gel of protein kinase C first dephosphorylated with CS1 (23 units ml⁻¹, 20 min) and then incubated with [³²P]ATP and MgCl₂ in the presence of phosphatidylserine, diacylglycerol, and Ca²⁺ for 0.5 min (*lane 2*), 5 min (*lane 3*), 15 min (*lane 4*), 30 min (*lane 5*), 45 min (*lane 6*), or 60 min (*lane 7*); *lane 1* shows sample before dephosphorylation. *B*, silver-stained gel of protein kinase C first dephosphorylated with CS2 (5 units ml⁻¹, 25 min) and then incubated with [³²P]ATP and MgCl₂ in the presence of phosphatidylserine, diacylglycerol, and Ca²⁺ for 0.5 min (*lane 1*), 5 min (*lane 2*), 15 min (*lane 3*), 30 min (*lane 4*), 45 min (*lane 5*), or 60 min (*lane 6*). *C*, graph showing phosphate incorporation into control protein kinase C (*circles*) or protein kinase C first dephosphorylated by CS2 (*squares*) measured in the presence of phosphatidylserine, diacylglycerol, and Ca²⁺ (*open symbols*) or in the presence of chelator (*closed symbols*).

phorylated by CS1 and thus regain its original electrophoretic mobility. Furthermore, addition of mature enzyme to the CS1-dephosphorylated enzyme did not result in any significant phosphorylation of the dephosphorylated enzyme (data not
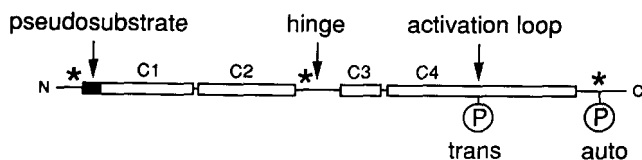
Fig. 4. **Cartoon of primary structure of protein kinase C showing the position of the** *in vivo* **trans-phosphorylation site on the activation loop (Thr^{500} for protein kinase C βII; Ref. 1) and the COOH-terminal autophosphorylation site (Thr^{641} for protein kinase C βII; Refs. 2 and 19).** The former is dephosphorylated by CS1 resulting in an increase in apparent mobility equivalent to 4 kDa. The latter is dephosphorylated by CS2 resulting in an increase in apparent mobility equivalent to 2 kDa. Dephosphorylation at both sites requires the membrane-bound conformation. Indicated with *asterisks* are the positions of the *in vitro* autophosphorylation sites (2); note that phosphorylation at the COOH-terminal site *in vitro* is weak because this residue is modified primarily *in vivo* (2). The conserved C1 and C2 regions in the regulatory domain and conserved C3 and C4 regions in the catalytic domain are indicated. Also indicated are the variable hinge region, separating the regulatory and catalytic domains, and the auto-inhibitory pseudosubstrate domain.

shown). Thus, the site dephosphorylated by CS1 is not a substrate for protein kinase C.

In marked contrast to the CS1-treated enzyme, protein kinase C that had been dephosphorylated by CS2 was capable of autophosphorylation. Fig. 3C shows the time-dependent incorporation of phosphate into enzyme that had been dephosphorylated by 50% (*open squares*) compared with the phosphate incorporation into control enzyme (*open circles*). The autophosphorylation of the control enzyme represents the intramolecular autophosphorylation (26) at the amino terminus and at the hinge region (2); this typically results in the incorporation of 1 or 2 phosphates/protein kinase molecule (27). Protein kinase C that had first been dephosphorylated by CS2 incorporated twice as many phosphates as control enzyme in a cofactor-dependent manner. This is consistent with autophosphorylation at the site that had been made available by dephosphorylation by CS2 treatment. Fig. 3B shows that the lipid-stimulated re-autophosphorylation of dephosphorylated enzyme was accompanied by a shift in the relative mobility of the enzyme to the slower migrating form (*i.e.* same as before dephosphorylation). In summary, protein kinase C dephosphorylated by CS2 re-autophosphorylates in the presence of lipid and Ca^{2+}.

Conclusions—The foregoing data reveal that mature protein kinase C is phosphorylated at two distinct sites, which are selectively dephosphorylated by CS1 and CS2. CS1 dephosphorylates the enzyme to yield an inactive enzyme that cannot be rephosphorylated by protein kinase C, consistent with dephosphorylation at the activation loop (Fig. 4). Supporting this, CS1 did not alter the electrophoretic mobility of the activation loop mutants T500E or T500V of protein kinase C βII (data not shown). In contrast, CS2 dephosphorylates the enzyme to yield a form that is capable of re-autophosphorylation, consistent with dephosphorylation on the COOH-terminal autophosphorylation site that is modified *in vivo* (2).

A model consistent with our data and previous data is that protein kinase C is first synthesized as an inactive precursor that is membrane-bound (8). The kinase is then recognized by the putative protein kinase C kinase (8, 9), which phosphorylates it on the activation loop (1, 10, 29). Electrostatic inter-

actions with the phosphate on the activation loop and residues in the catalytic core correctly position residues for catalysis (1). The membrane-bound enzyme is then stimulated by phosphatidylserine to autophosphorylate at the COOH terminus (see above). This autophosphorylation may decrease the enzyme's membrane affinity so that it partitions into the cytosol; consistent with this, expression of a mutant protein kinase C βI with Ala replacing the COOH-terminal autophosphorylated Thr resulted in expression of an insoluble protein (19). The activation loop-phosphorylated, COOH-terminal autophosphorylated enzyme is thus the mature, diacylglycerol-regulatable enzyme that has been extensively studied *in vitro*.

The identity of the kinase that mediates the trans-phosphorylation is unknown. It is, however, likely to be a kinase that is localized near the membrane because this appears to be the site of trans-phosphorylation. Whether this kinase is, in turn, regulated by second messengers (*e.g.* Ca^{2+}) and whether different isozymes of protein kinase C are activated by specific protein kinase C kinases remain to be established. The requirement for both trans-phosphorylation and diacylglycerol may provide a mechanism of ensuring low background noise in the midst of complex intracellular signaling pathways.

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