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Dual Role of Pseudosubstrate in the Coordinated Regulation of Protein Kinase C by Phosphorylation and Diacylglycerol*

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The activity of protein kinase C is reversibly regulated by an autoinhibitory pseudosubstrate, which blocks the active site of the enzyme in the absence of activators. However, before it can be allosterically regulated, protein kinase C must first be processed by three ordered phosphorylations, the first of which is modification of the activation loop catalyzed by the phosphoinositide-dependent kinase-1 (PDK-1). Here we use limited proteolysis to show that 1) newly synthesized protein kinase C adopts a conformation in which its pseudosubstrate sequence is removed from the active site, and 2) this exposure is essential to allow PDK-1 to phosphorylate the enzyme. Precursor (unphosphorylated) protein kinase C βII obtained by 1) *in vitro* transcription and translation, 2) expression of a phosphorylation-deficient mutant (T500V), or 3) *in vivo* labeling with a pulse of [^{35}S]cysteine/methionine is cleaved at the amino-terminal pseudosubstrate by the endoproteinase Arg-C. In marked contrast to mature (phosphorylated) enzyme, proteolysis occurs in the absence of lipid activators, revealing that precursor protein kinase C has its pseudosubstrate sequence removed constitutively. Additionally, we show that PDK-1 is unable to phosphorylate protein kinase C when the active site is sterically blocked by a peptide substrate. Neither can mature enzyme be dephosphorylated when the active site is blocked by binding either the pseudosubstrate sequence or a heterologous substrate. Thus, the accessibility of the activation loop to both phosphorylation and dephosphorylation requires an exposed pseudosubstrate. In summary, newly synthesized protein kinase C adopts a conformation in which its pseudosubstrate sequence is removed from the active site, rendering the activation loop accessible to phosphorylation by PDK-1. Phosphorylation serves as a conformational switch to position the pseudosubstrate so that it blocks the active site, a conformation that is maintained until stimulus-dependent membrane binding releases it, thus activating the enzyme.

The protein kinase Cs comprise a family of enzymes that are regulated by two distinct mechanisms: phosphorylation and membrane targeting (reviewed in Ref. 1). In the first regulatory mechanism, protein kinase C isozymes are rendered catalytically competent by a series of ordered phosphorylation events, the first of which has recently been shown to be catalyzed by PDK-1[1] (2–4). PDK-1 phosphorylates a segment near the entrance to the active site referred to as the activation loop; the phosphorylated position corresponds to Thr^{500} in the conventional protein kinase C βII (5). This phosphorylation serves to correctly position residues for catalysis and triggers the autophosphorylation of two conserved positions at the carboxyl terminus, a turn motif (Thr^{641} in protein kinase C βII), and a hydrophobic motif (Ser^{660} in protein kinase C βII) (6). The phosphorylated "mature" species localizes to the cytosol where it is maintained in an inactive conformation by an autoinhibitory pseudosubstrate sequence (7, 8). In the second regulatory mechanism, generation of diacylglycerol causes mature (phosphorylated) protein kinase C to translocate to the plasma membrane where it undergoes a conformational change in which the pseudosubstrate sequence is removed from the active site (8). Freed of steric constraints, protein kinase C is able to phosphorylate targets and elicit a cellular response. Dual regulation, first by phosphorylation to "prime" the enzyme and second by cofactor binding to remove autoinhibition, allows for ultrasensitive regulation of activity.

Autoinhibitory sequences regulate the activity of a large number of kinases, including the archetypal kinase, protein kinase A, where binding of cAMP to the regulatory subunit of this kinase causes dissociation of the catalytic subunit (9). Such conformational regulation of kinase activity by intra- or intermolecular autoinhibitory sequences provides an exquisite switch to kinase function. However, conformational regulation of substrates can be equally critical in controlling phosphorylation activity. In particular, conformational changes, which expose phosphorylation sites, can dictate whether or not phosphorylation takes place.

Akt (protein kinase B) and p70 S6 kinase are two examples of kinases whose activity is regulated by conformation-dependent phosphorylation. Similar to protein kinase C, these kinases are regulated by phosphorylation at the activation loop by PDK-1 (10–12). This phosphorylation depends on specific conformational changes that render the activation loop accessible to phosphorylation. In the case of Akt, binding of polyphosphoinositides to its pleckstrin homology domain is required to expose the activation loop phosphorylation site (13–15). Activation loop phosphorylation of S6 kinase by PDK-1 also requires a distinct conformational change: this kinase is first phosphorylated on a set of proline residues located within its carboxyl-terminal pseudosubstrate sequence. This set of phosphorylation events is essential to allow PDK-1 phosphorylation, presumably by exposing the activation loop (12, 16, 17). Allosteric regulation of substrates of PDK-1 may be particularly critical because the intrinsic catalytic activity of PDK-1 does not appear to be tightly regulated (18).

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[1] The abbreviations used are: PDK-1, phosphoinositide-dependent kinase 1; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

In this communication, we addressed whether the phosphorylation of protein kinase C by PDK-1 is conformationally regulated. We show that 1) the pseudosubstrate of protein kinase C does not occupy the active site when the enzyme is in the unphosphorylated conformation and 2) this open conformation is critical in allowing PDK-1 to phosphorylate the activation loop and initiate the maturation of the enzyme. Once protein kinase C is fully phosphorylated, the pseudosubstrate sequence gains occupancy of the active site, locking the enzyme in an inactive conformation. In the presence of diacylglycerol, protein kinase C translocates to the membrane, resulting in renewed release of the pseudosubstrate from the active site. Thus, the pseudosubstrate plays two roles: a novel one in which it regulates the phosphorylation of precursor enzyme and the well characterized one in which it regulates the activity of the mature enzyme.

MATERIALS AND METHODS

Bovine brain L-α-phosphatidylserine and *sn*-1,2-dioleoylglycerol were obtained from Avanti Polar Lipids, Inc. [γ-^{32}P]ATP (3000 Ci mmol^{-1}), translation grade [^{35}S]methionine (1175 Ci mmol^{-1}), and Easy Tag [^{35}S]methionine/cysteine (1175 Ci mmol^{-1}) were purchased from NEN Life Science Products. Polyvinylidene difluoride membranes were from Millipore. Endoproteinase Arg-C, microcystin, Triton X-100, and peroxidase-conjugated goat anti-rabbit antibodies were from Calbiochem. The chemiluminescence SuperSignal kit was purchased from Pierce. The TNT® T7 Quick Coupled Transcription/Translation System kit was from Promega. A protein kinase C-selective peptide (^{159}FKKSFKL-NH_2) (19) was synthesized in the laboratory of E. Komives at the University of California, San Diego. Protamine sulfate was purchased from Sigma. Protein kinase C βII was purified from the baculovirus expression system, as described previously (8). Glutathione *S*-transferase -tagged PDK-1 was expressed in *Escherichia coli* and purified as described previously (2). A histidine-tagged construct of the catalytic domain of protein kinase C βII (residues 286–673; histidine tag at the amino terminus) was expressed in *E. coli* and purified on Probond nickel resin (Invitrogen) and stored at −20 °C in 50 mM Tris buffer, pH 7.5, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and 150 mM Imidazole. Recombinant protein phosphatase 1 catalytic subunit α isoform (CS1) purified from *E. coli* was a generous gift from Dr. Anna DePaoli-Roach (Indiana University). Polyclonal antibodies against the carboxyl terminus of protein kinase C βII were from Calbiochem and Santa Cruz Biotechnology. A polyclonal antibody (P500) directed against the phosphorylated activation loop of protein kinase C βII was generated by immunizing rabbits with a phosphopeptide based on the sequence: Asp^{494}-Gly^{495}-Val^{496}-Thr^{497}-Thr^{498}-Lys^{499}-phospho-Thr^{500}-Phe^{501}-Cys^{502}-Gly^{503}-Thr^{504}-Pro^{505}-Asp^{506}. The specificity for the activation loop phosphate has been previously described (2). A polyclonal antibody against a peptide comprising residues 645–673 of protein kinase C βII was provided by Eli Lilly. The cDNA for rat protein kinase C βII was a gift from Daniel E. Koshland, Jr. (University of California, Berkeley) and that for rat protein kinase C α was from Yusuf Hannun (Medical University of South Carolina). All other chemicals were reagent grade.

In Vitro Transcription/Translation—Protein kinase C βII was transcribed and translated using the TNT® T7 Quick Coupled Transcription/Translation System (Promega). Protein kinase C βII DNA in pcDNA3 (200 ng) was added to 80 μl of TNT® Quick Master Mix in the presence of 20 μCi of [^{35}S]methionine (translation grade) and nuclease-free water (Promega) for 90 min at 30 °C. Samples were then subjected to proteolysis as described below.

COS7 Cell Expression—COS7 cells were transfected with 5 μg of protein kinase C βII (pcDNA3) or T500V protein kinase C βII (pcDNA3) using the Superfect transfection method (Qiagen). DNA was added to 300 μl of serum-free DMEM and 30 μl of Superfect reagent and incubated at 22 °C for 5 min. The DNA-containing mixture was added to cells plated at 70% confluency on 10-cm tissue culture dishes containing 3 ml of DMEM + 10% fetal bovine serum. Cells were incubated for 3 h at 37 °C and then washed with DMEM + 10% fetal bovine serum. Cells were pulsed after 24 h as described below or harvested after 48 h in 600 μl of lysis buffer containing 20 mM HEPES buffer, pH 7.5, 1% Triton X-100, 1 mM DTT, 300 μM phenylmethylsulfonyl fluoride, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, and 100 nM microcystin. Lysates were centrifuged at 100,000 × *g* for 20 min at 4 °C; supernatants were stored in 50% glycerol at −20 °C.

Pulse Labeling—COS7 cells were plated on a 10-cm tissue dish and transfected with protein kinase C α DNA as described above. At 24 h post-transfection, cells were incubated in methionine/cysteine-deficient DMEM + 10% fetal bovine serum for 15 min at 37 °C and then pulsed with 7 mCi of [^{35}S]methionine/cysteine for 7 min at 37 °C. Cells were then washed with DMEM + 10% fetal bovine serum containing 5 mM unlabeled methionine and 5 mM unlabeled cysteine and lysed in 1 ml of lysis buffer containing 20 mM HEPES buffer, pH 7.5, 1% Triton X-100, 1 mM DTT, 300 μM phenylmethylsulfonyl fluoride, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, and 100 nM microcystin. Cell lysates were centrifuged at 100,000 × *g* for 20 min at 4 °C, and protein kinase C α in the supernatant was immunoprecipitated with protein kinase C α antibodies (Transduction Laboratories) as described (2).

Arg-C Proteolysis—In vitro translated protein kinase C βII (described above) was incubated with 2 units ml^{-1} of Arg-C for 1–60 min at 30 °C. Aliquots were removed at indicated times into 1/5 volume SDS-PAGE sample buffer. COS7 cell lysates containing protein kinase C βII or T500V protein kinase C were incubated with 0, 10, 20, or 35 units ml^{-1} Arg-C in 10 mM HEPES, pH 7.5, 1 mM DTT, and 600 nM microcystin in the presence or absence of 140 μM phosphatidylserine, 3.8 μM diacylglycerol vesicles, and 100 μM $CaCl_2$ for 30 min at 30 °C. Reactions were terminated by the addition of 1/5 volume SDS-PAGE sample buffer. Protein A/G-agarose beads with bound immunoprecipitated pulse-chased protein kinase C were treated with 2.5 units ml^{-1} Arg-C in 10 mM HEPES, pH 7.5, 1 mM DTT, and 600 nM microcystin in the presence or absence of 140 μM phosphatidylserine, 3.8 μM diacylglycerol, and 100 μM $CaCl_2$ for 10 min at 30 °C. Reactions were quenched by the addition of 1/5 volume SDS-PAGE sample buffer. All samples were separated by SDS-PAGE (7%) and electrophoretically transferred to polyvinylidene difluoride membrane. *In vitro* translated and pulse-labeled protein kinase C were visualized by exposing membrane on a Bio-Rad Molecular Imager System screen for 18 or 72 h, respectively. A Western blot of *in vitro* translated samples was then performed by probing the membrane with Santa Cruz Biotechnology protein kinase C βII primary antibodies, incubating with peroxidase-conjugated secondary antibodies, and detecting labeling with chemiluminescence.

Phosphatase Incubations—Protein kinase C βII (20 nM) was incubated with the catalytic subunit of protein phosphatase 1 (25 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 in the presence or absence of 140 μM phosphatidylserine and 4 μM diacylglycerol for 20 min at 22 °C. Where indicated, reactions were carried out in the presence of 100 μg ml^{-1} of protamine sulfate or selective peptide. Reactions were quenched by the addition of 1/5 volume SDS-PAGE sample buffer and analyzed by SDS-PAGE (7%) and silver staining (21). In one experiment, histone H1 was phosphorylated by protein kinase C and used as a substrate for the catalytic subunit of protein phosphatase 1.

PDK-1 Phosphorylation of Protein Kinase C—Purified protein kinase C catalytic domain (30 nM) was incubated with 25 nM purified PDK-1 in the presence of 10 mM HEPES, pH 7.5, 500 μM ATP, 50 μM $MgCl_2$, 1 mM DTT, and 0, 80, 175, or 350 μM selective peptide for 1 h at 30 °C. Reactions were quenched by the addition of 1/5 volume SDS-PAGE sample buffer, separated by SDS-PAGE (7%), and electrophoretically transferred to polyvinylidene difluoride. Western blots were performed by probing membrane with indicated primary antibodies, incubating with peroxidase-conjugated secondary antibodies, and detecting labeling with chemiluminescence.

RESULTS AND DISCUSSION

Molecular modeling studies suggested that the phosphorylation site on the activation loop of protein kinase C is effectively masked when the pseudosubstrate is bound in the substrate-binding cavity (22). Specifically, an invariant basic residue (Lys^{29} in protein kinase C βII) within the pseudosubstrate sequence lies over the phosphorylation site (Thr^{500} in protein kinase C βII). The apparent blocking of this key regulatory switch by the pseudosubstrate led us to explore whether the pseudosubstrate is, in fact, out of the active site in precursor (unphosphorylated) protein kinase C. To this end, we used the endoproteinase Arg-C as a tool to probe whether the pseudosubstrate of newly synthesized protein kinase C is exposed. We have previously shown that this protease cleaves Arg^{19} of the pseudosubstrate sequence of protein kinase C βII when the enzyme is in the active but not inactive conformation (8, 23). The amino terminally truncated product migrates with

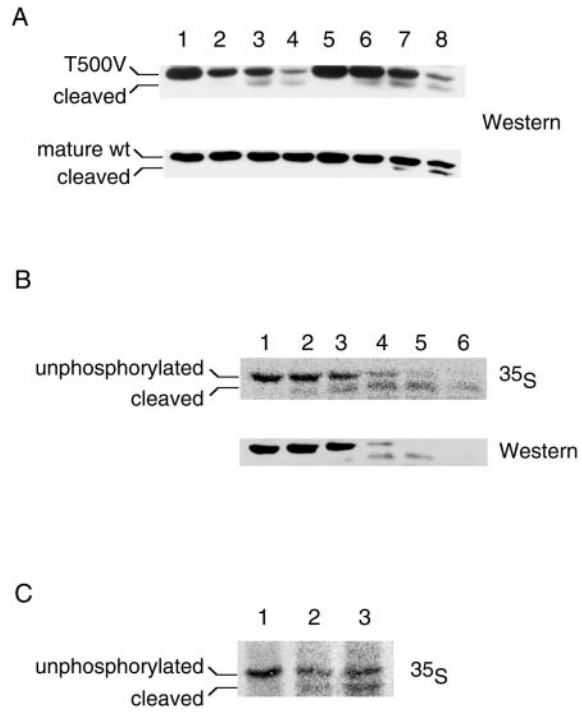

FIG. 1. **Unphosphorylated, precursor protein kinase C is proteolytically labile at its amino terminus.** *A,* Western blot showing Arg-C proteolysis of T500V protein kinase C (*top*) and wild-type protein kinase C βII (*bottom*) expressed in COS7 cells. Triton X-100 soluble supernatants were incubated with 0, 10, 20, or 35 units ml^{-1} of Arg-C (*lanes 1* and *5*, *2* and *6*, *3* and *7*, and *4* and *8*, respectively) in the absence (*lanes 1–4*) or presence (*lanes 5–8*) of phosphatidylserine, diacylglycerol, and Ca^{2+}. Western blots were probed with antibodies raised against the carboxyl terminus of protein kinase C βII (Calbiochem). Electrophoretic mobility of Arg-C proteolysis product is denoted as cleaved. *B,* autoradiograph (*top*) and Western blot (*bottom*) of *in vitro* transcribed/translated protein kinase C βII treated with 2 units ml^{-1} Arg-C for 0, 5, 10, 20, 40, or 60 min (*lanes 1, 2, 3, 4, 5*, and *6*, respectively). Western blot was probed with βII antibodies, which recognize the carboxyl terminus of the enzyme (Santa Cruz Biotechnology). Electrophoretic mobility of the unphosphorylated protein kinase C and the Arg-C proteolysis product are denoted as unphosphorylated and cleaved, respectively. *C,* Arg-C proteolysis product of pulse-labeled protein kinase C α. Pulse-labeled, precursor protein kinase C α incubated in the absence (*lane 1*) or presence (*lanes 2* and *3*) of 2.5 units ml^{-1} Arg-C and in the presence (*lane 2*) or absence (*lane 3*) of 140 μM phosphatidylserine and 4 μM diacylglycerol. Precursor protein kinase C and Arg-C proteolysis product are denoted unphosphorylated pulse-labeled and cleaved, respectively. These data are representative of three independent experiments.

a molecular mass approximately 4 kDa smaller than that of the native enzyme on SDS-PAGE (8).

Newly synthesized, unphosphorylated protein kinase C (precursor protein kinase C) was obtained by three independent methods. First, we examined the protease sensitivity of the activation loop mutant, T500V, expressed in COS7 cells. The lack of negative charge at the activation loop site prevents autophosphorylation at the two carboxyl-terminal phosphorylation sites (6). Therefore, similar to precursor protein kinase C, the T500V mutant is not phosphorylated at any of the three *in vivo* positions. The Western blot in Fig. 1*A* shows that treatment of the T500V mutant with endoproteinase Arg-C resulted in the removal of ~4 kDa. This cleavage occurred at the amino terminus of the enzyme because the product was recognized by an antibody directed toward the carboxyl terminus of protein kinase C βII (residues 660–673). The T500V mutant was proteolyzed both in the presence or absence of phosphatidylserine and diacylglycerol (Fig. 1*A, top panel*, *lanes 1–4* and *5–8*, respectively). The rate of proteolysis was not significantly different with or without lipid (compare *lanes 4* and *8* where the ratio of native:cleaved protein kinase C is similar). In contrast, cleavage of wild-type (fully phosphorylated) protein kinase C expressed in COS7 cells is dependent on the presence of phosphatidylserine and diacylglycerol (Fig. 1*A, bottom panel*; *lanes 1–4*). The requirement of wild-type enzyme for lipid activators (Fig. 1*A*, *bottom panel*; *lanes 5–8*) is consistent with previous studies showing that the enzyme must be in the active conformation in order for the pseudosubstrate to be proteolytically labile (23). Thus, the phosphorylation-defective mutant, T500V, has a constitutively exposed pseudosubstrate.

In a second approach, the precursor conformation was obtained by *in vitro* transcription/translation of protein kinase C βII. The expressed protein is not processed by phosphorylation; it co-migrates with dephosphorylated enzyme and is not recognized by phospho-specific antibodies (24). Treatment of the *in vitro* transcribed/translated protein kinase C with Arg-C, in the absence of phosphatidylserine and diacylglycerol, resulted in the time-dependent generation of a proteolytic product, which migrated with an apparent molecular mass 4 kDa smaller than the starting material (Fig. 1*B*, *top panel*). This proteolytic product was labeled by antibodies generated against a peptide derived from the carboxyl terminus of protein kinase C (*lower panel*) but not antibodies directed against the first 19 amino acids of the protein (data not shown). Thus, the proteolytic site was at the amino terminus, consistent with the characterized cleavage at the pseudosubstrate.

Lastly, we investigated the endoproteinase Arg-C sensitivity of newly synthesized protein kinase C from pulse-labeled COS7 cells expressing wild-type protein kinase C α. *Lanes 1–3* in Fig. 1*C* show [^{35}S]methionine/cysteine-labeled protein kinase C one minute after pulse labeling COS7 cells. Protein kinase C obtained in this manner has the same electrophoretic mobility as both the *in vitro* transcribed/translated, unphosphorylated protein kinase C and the phosphorylation-deficient mutants of protein kinase C (data not shown). This precursor form is chased into the fully phosphorylated, activable form with a half-time of 20 min.[2] Thus, the pulse-labeled protein kinase C in Fig. 1*C* represents precursor protein kinase C that has not yet been processed by phosphorylation. Fig. 1*C* shows that this unphosphorylated species is proteolyzed both in the presence (*lane 2*) and absence (*lane 3*) of phosphatidylserine and diacylglycerol to a product which migrates approximately 4 kDa faster than untreated enzyme. Consistent with the analysis of pulse-labeled precursor protein kinase C, incubation of crude cell lysates with Arg-C resulted in a lipid-independent proteolysis of the faster migrating unphosphorylated protein kinase C and a lipid-dependent proteolysis of the slower migrating phosphorylated protein kinase C (data not shown). These data reveal that precursor, unphosphorylated protein kinase C adopts a conformation in which the pseudosubstrate is removed from the active site.

We next explored the conformational and steric requirements necessary for the phosphorylation sites of protein kinase C to be accessible. We have previously shown that the pseudosubstrate must be exposed in order for protein kinase C to be dephosphorylated by either protein phosphatase 1 or protein phosphatase 2A (25). Mass spectrometric analysis established that protein phosphatase 1 dephosphorylates all three *in vivo* phosphorylation sites, resulting in a large increase in electrophoretic mobility (equivalent to ~4 kDa), whereas protein phosphatase 2A dephosphorylates the activation loop (Thr^{500}) and hydrophobic site (Ser^{660}) resulting in an intermediate shift in electrophoretic mobility (equivalent to ~2 kDa). In this study, we investigated whether occupancy of the active site masked the phosphorylation sites.

[2] E. M. Dutil and A. C. Newton, manuscript in preparation.

Fig. 2 shows that the catalytic subunit of protein phosphatase 1 was unable to dephosphorylate purified protein kinase C βII in the absence of lipid activators (*lane 2*). However, the presence of phosphatidylserine and diacylglycerol resulted in dephosphorylation of the enzyme, as evidenced by the increase in electrophoretic mobility (*lane 3*). This lipid-dependent dephosphorylation was quantitatively blocked in the presence of protamine sulfate (*lane 5*). Similarly, the presence of another substrate, a heptapeptide based on the MARCKS protein, also inhibited dephosphorylation (*lane 7*). Under the conditions of the assay, the heptapeptide had no significant effect (± 3%) on the activity of protein phosphatase 1 toward dephosphorylation of ^{32}P-phosphorylated histone; protamine reduced histone phosphorylation by 50%. In addition, dephosphorylation of the *in vitro* autophosphorylation sites on protein kinase C was unaffected by the presence of peptide and only partially inhibited (30%) by the presence of protamine (data not shown). Thus, the ability of the peptide to reduce the dephosphorylation of protein kinase C was specific to the *in vivo* sites, including the activation loop, and not the *in vitro* sites. Although it is not possible to entirely rule out some direct effects of protamine on phosphatase activity, the quantitative inhibition of dephosphorylation of the *in vivo* protein kinase C sites but only partial inhibition of histone phosphorylation or *in vitro* autophosphorylation sites suggests affects beyond merely inhibiting the phosphatase. Thus, occupancy of the active site by either pseudosubstrate (*lane 2*) or substrate (*lanes 5* and *7*) reduced dephosphorylation.

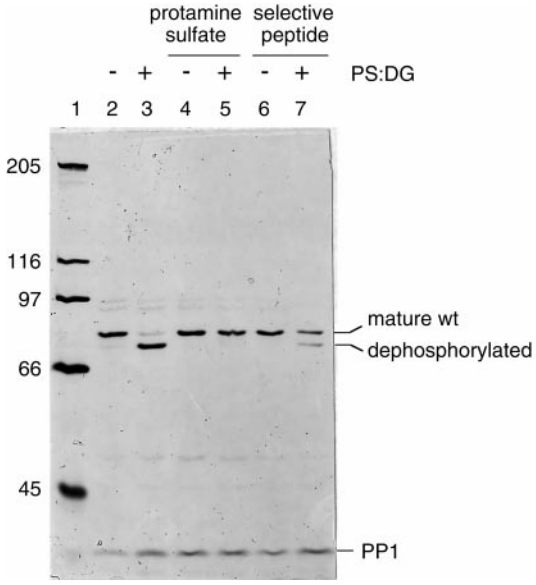

FIG. 2. **The phosphorylation sites of protein kinase C are resistant to dephosphorylation when the active site is sterically blocked.** Silver-stained gel of protein kinase C βII dephosphorylated by the catalytic subunit of protein phosphatase 1 (*PP1*) in the absence (*lanes 2*, *4*, and *6*) or presence (*lanes 3, 5*, and *7*) of phosphatidylserine (*PS*) and diacylglycerol (*DG*) and in the presence of 100 μg ml^{-1} protamine sulfate (*lanes 4* and *5*) or selective peptide (*lanes 6* and *7*). The electrophoretic mobility of phosphorylated (mature wild type (*wt*)) and dephosphorylated protein kinase C (dephosphorylated) is indicated.

We next examined whether pseudosubstrate exposure was essential for the first step in the processing of protein kinase C, phosphorylation by PDK-1 on the activation loop. Specifically, we examined whether the ability of PDK-1 to phosphorylate precursor protein kinase C was impaired by occupancy of the active site. We used the catalytic domain of protein kinase C, expressed in *E. coli*, for these experiments because this form of the enzyme is not phosphorylated at any of the three *in vivo* positions and is an effective substrate for PDK-1 (4). In the absence of the protein kinase C substrate, the selective peptide, PDK-1, effectively phosphorylated the activation loop of the catalytic domain of protein kinase C (Fig. 3*A*, *lane 1*) as assessed using an antibody that specifically labels phosphorylated Thr^{500} (Fig. 3*A*, *upper panel*). However, increasing concentrations of selective peptide resulted in increased inhibition of activation loop phosphorylation by PDK-1 (Fig. 3*A*, *lanes 2–4*). The selective peptide had no effect on the intrinsic activity of PDK-1; the autophosphorylation of PDK-1 was unaffected by the peptide (Fig. 3*B*). Nor did PDK-1 directly phosphorylate the selective peptide (data not shown). Thus, the inhibition of activation loop phosphorylation did not result from the selective peptide affecting PDK-1 activity or from substrate competition. These data reveal that peptide binding to the unphosphorylated kinase domain prevents PDK-1 phosphorylation of the activation loop. Although the mechanism of substrate binding to unphosphorylated kinase domain is not known, these data suggest that the pseudosubstrate must be removed from the active site in order for PDK-1 to phosphorylate the activation loop.

Conclusions—This study reveals exquisite conformational control of protein kinase C not only by allosteric activators, but also by phosphorylation. Specifically, we show that 1) precursor, unphosphorylated protein kinase C, adopts a conformation in which the pseudosubstrate is removed from the active site

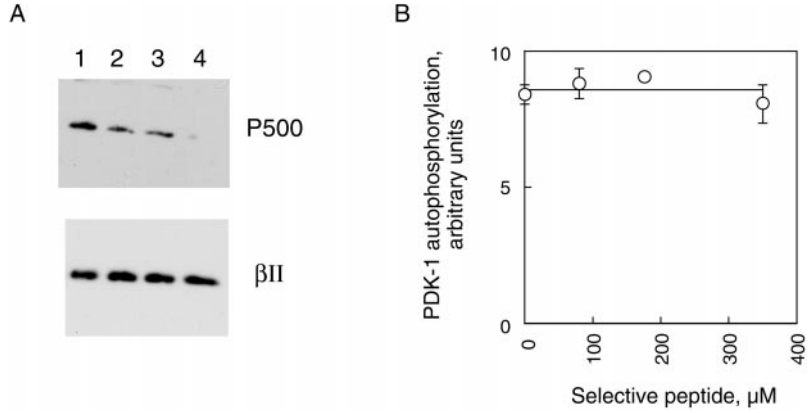

FIG. 3. **Activation loop phosphorylation of protein kinase C by PDK-1 is inhibited by sterically blocking the active site of protein kinase C.** *A,* Western blot of the catalytic domain of protein kinase C (100 ng) after incubation with Mg^{2+}-ATP and PDK-1 (150 ng) in the presence of 0, 80, 175, or 350 μM selective peptide (*lanes 1–4*, respectively) probed with P500 antibodies (*top*) or Santa Cruz Biotechnology βII antibodies. *B,* PDK-1 activity toward autophosphorylation is unaffected by selective peptide. PDK-1 (25 nM) was incubated with Mg^{2+}-ATP in the presence of 0, 80, 175, or 350 μM selective peptide. PDK-1 autophosphorylation was quantitated using the Bio-Rad Molecular Imaging System. The data represent the mean ± S.E. of triplicate assays.

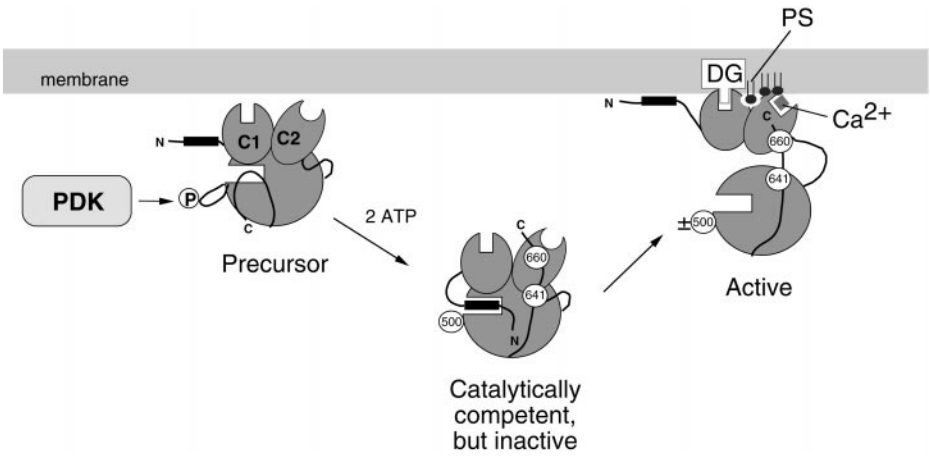

FIG. 4. **Model showing the dual role of the pseudosubstrate in the processing and regulation of protein kinase.** Newly synthesized protein kinase C (*left*) adopts a conformation in which the pseudosubstrate sequence (*black rectangle*) is removed from the active site (open rectangle); the pseudosubstrate of this unphosphorylated, precursor protein kinase C is cleaved by Arg-C in a lipid-independent manner. The activation loop is exposed in this conformation, enabling PDK-1 to phosphorylate it. Substrate binding, which sterically blocks the active site, inhibits activation loop phosphorylation by PDK-1. Furthermore, phosphate accessibility to phosphatases requires an unoccupied active site. Once protein kinase C is phosphorylated at all three *in vivo* positions, it localizes to the cytosol where it is competent to respond to second messengers. In response to diacylglycerol (*DG*) production, protein kinase C translocates to the plasma membrane and adopts a conformation in which its pseudosubstrate sequence is removed from the active site. *PS*, phosphatidylserine.

and 2) this conformation is required to free steric hindrance at the active site and allow PDK-1 to catalyze the initial phosphorylation event on the activation loop. Once protein kinase C is phosphorylated, the pseudosubstrate is able to block the active site, presumably by occupying the substrate-binding cavity. The conformation of the pseudosubstrate of the phosphorylated enzyme is then under allosteric control by lipid activators.

The requirement for pseudosubstrate release in the activation loop phosphorylation of protein kinase C is similar to the conformational regulation of a related PDK-1 substrate, Akt. For the latter kinase, the pleckstrin homology domain must be engaged with the membrane to allow access of PDK-1 to the activation loop (13, 15, 26). Similarly, phosphorylation of the activation loop of the Ca^{2+}/calmodulin-dependent kinase I by its upstream kinase requires calmodulin binding to release the pseudosubstrate sequence from the active site (20). Note that the regulation of the the latter kinases by activation loop phosphorylation contrasts with that of protein kinase C in that binding of allosteric regulators triggers phosphorylation. For protein kinase C, the phosphorylation primes the enzyme for subsequent allosteric regulation by diacylglycerol.

A model for the dual regulation of the pseudosubstrate by phosphorylation and cofactors is presented in Fig. 4. The pseudosubstrate of newly synthesized protein kinase C is displaced from the active site, allowing PDK-1 to access the phosphorylation site on the activation loop. Phosphorylation at this exposed site by PDK-1 is followed by rapid autophosphorylation at the carboxyl-terminal sites (6). These latter phosphorylations cause the pseudosubstrate to become engaged in the active site (*middle panel*), locking the enzyme in a catalytically competent but inactive conformation. Generation of diacylglycerol in the plasma membrane recruits the enzyme to the membrane, where binding of its membrane-targeting domains provides the energy to release the pseudosubstrate from the active site, allowing substrate phosphorylation (8). Thus, the pseudosubstrate has a novel role in the regulation of protein kinase C; in addition to serving as an autoinhibitory sequence for activity, it serves as a switch to allow the phosphorylation/dephosphorylation of protein kinase C.

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