

## Carboxyl-terminal Phosphorylation Regulates the Function and Subcellular Localization of Protein Kinase C $\beta$ II\*

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**Protein kinase C is processed by three phosphorylation events before it is competent to respond to second messengers. Specifically, the enzyme is first phosphorylated at the activation loop by another kinase, followed by two ordered autophosphorylations at the carboxyl terminus (Keranen, L. M., Dutil, E. M., and Newton, A. C. (1995) *Curr. Biol.* 5, 1394–1403). This study examines the role of negative charge at the first conserved carboxyl-terminal phosphorylation position, Thr-641, in regulating the function and subcellular localization of protein kinase C  $\beta$ II. Mutation of this residue to Ala results in compensating phosphorylations at adjacent sites, so that a triple Ala mutant was required to address the function of phosphate at Thr-641. Biochemical and immunolocalization analyses of phosphorylation site mutants reveal that negative charge at this position is required for the following: 1) to process catalytically competent protein kinase C; 2) to allow autophosphorylation of Ser-660; 3) for cytosolic localization of protein kinase C; and 4) to permit phorbol ester-dependent membrane translocation. Thus, phosphorylation of Thr-641 in protein kinase C  $\beta$ II is essential for both the catalytic function and correct subcellular localization of protein kinase C. The conservation of this residue in every protein kinase C isozyme, as well as other members of the kinase superfamily such as protein kinase A, suggests that carboxyl-terminal phosphorylation serves as a key molecular switch for defining kinase function.**

The protein kinase C family of enzymes transduce the myriad of extracellular signals that promote phospholipid hydrolysis (1). Generation of diacylglycerol, typically in the plasma membrane, activates most members of this class of kinases by recruiting them to membranes, where they are activated by interaction with the phospholipid, phosphatidylserine (2). Binding of both diacylglycerol and phosphatidylserine to the protein results in a conformational change that removes an autoinhibitory pseudosubstrate domain from the active site, thus allowing substrate binding and catalysis (3).

Despite 2 decades of studying the regulation of protein kinase C, it has only recently been appreciated that the enzyme must be phosphorylated before it is competent to respond to second messengers. Pulse-chase experiments by Fabbro and co-workers (4) in the late 1980s provided the first evidence that protein kinase C is phosphorylated *in vivo*. Specifically, they showed that protein kinase C is first synthesized as an inac-

tive, dephosphorylated precursor with an apparent molecular mass of 74 kDa that associates with the detergent-insoluble fraction of cells; this species was chased to a transient 77-kDa phosphoform and then to the final 80-kDa mature form that localized to the cytosol (4). Comparison with protein kinase A suggested that one of the phosphorylation sites was a conserved Thr on the activation loop that is located near the entrance to the active site; phosphorylation on this loop controls the function of a large number of kinases (5). In support of this, replacement of the potential phosphorylated Thr on the activation loop of protein kinase C  $\alpha$  with Ala, or of protein kinase C  $\beta$ II with Val, resulted in inactive enzyme (6, 7), whereas replacement with Glu in protein kinase C  $\beta$ II resulted in activable enzyme (7).

Mass spectrometric analysis later established that protein kinase C  $\alpha$  and  $\beta$ II are phosphorylated at three positions *in vivo* (8): in protein kinase C  $\beta$ II these correspond to Thr-500 on the activation loop and Thr-641 and Ser-660 on the enzyme's carboxyl terminus (8, 9) (see Fig. 1). These residues are conserved among all protein kinase C isozymes, with the exception of two isozymes (protein kinase C  $\zeta$  and  $\iota$ ) which have a Glu at the position corresponding to Ser-660 (8). Although phosphorylation on the activation loop is required to process mature protein kinase C, phosphatase treatment of the mature enzyme revealed that phosphate on Thr-500 is no longer required for activity once Thr-641 has been phosphorylated. Specifically, kinase activity is retained upon dephosphorylation of Thr-500 and Ser-660 but not Thr-641. In contrast, additional dephosphorylation of Thr-641 results in complete loss of activity (8, 10).

The first phosphorylation event of protein kinase C  $\beta$ II, that of Thr-500, is mediated by another protein kinase (8, 11), with recent studies showing that the phosphoinositide-dependent protein kinase, PDK-1, can mediate this phosphorylation both for conventional (12) and atypical (13, 14) protein kinase Cs. This phosphorylation is followed by rapid phosphorylations at the two carboxyl-terminal positions, with *in vivo* data suggesting that Thr-641 precedes phosphorylation of Ser-660 (8). Mutagenesis of Ser-660 to Glu or Ala recently revealed that phosphate at this position is not critical for function; rather, it structures determinants in both the active site and regulatory region thereby allowing tighter binding of both substrates and cofactors (15). Similar mutagenesis of Ser-657 in protein kinase C  $\alpha$  showed that, in addition to increasing the stability of the protein, negative charge at this position increases the phosphatase resistance of protein kinase C (16). This residue is part of an unusual hydrophobic phosphorylation motif (FXXF(S/T)(F/Y)) that is found in a number of other kinases such as S6 kinase and protein kinase B (Akt kinase) (17, 18).

Dephosphorylation studies implicate Thr-641 as being the key switch for locking protein kinase C in a catalytically competent conformation; for mature enzyme previously processed

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by phosphorylation, phosphate at this position is required and is sufficient for maximal catalysis (8, 10). However, mutagenesis studies have provided conflicting results on the role of this residue. Mutagenesis studies with protein kinase C  $\beta$ I suggested that phosphorylation here is essential for activity (19), whereas similar studies with protein kinase C  $\alpha$  concluded that this residue is not essential for activity (20). The possibility of compensating phosphorylations was not taken into account in these studies.

Protein kinase C has also been shown to autophosphorylate by an intramolecular reaction at a number of non-conserved residues *in vitro* (21, 22). One such residue in protein kinase C  $\beta$ II is Thr-634, which neighbors the *in vivo* site, Thr-641. It is noteworthy that neither the *in vivo* nor *in vitro* autophosphorylation sites conform to "consensus" phosphorylation motifs for protein kinase C (23); for intramolecular reactions, the high local concentration of substrate may ablate the necessity for optimal substrate binding.

This study explores the role of phosphorylation of Thr-641 in regulating protein kinase C  $\beta$ II. Analysis of phosphorylation site mutants reveals that lack of negative charge at this position 1) results in expression of inactive enzyme, 2) prevents autophosphorylation at the second carboxyl-terminal site, 3) causes association of the protein with the detergent-insoluble cell fraction, and 4) prevents the enzyme from translocating to the plasma membrane in response to phorbol esters. As part of this work, we discovered that the mutation of both Thr-634 and Thr-641 to Ala results in a compensating phosphorylation at a neighboring residue that mimics the effect of phospho-Thr-641, underscoring the importance of negative charge on the carboxyl terminus for the function of protein kinase C. Thus, this residue serves as a key molecular switch in regulating protein kinase C. Its conservation in a number of other kinases suggests that protein kinases are regulated by two phosphorylation switch mechanisms: the well characterized phosphorylation of the activation loop, and phosphorylation events at the carboxyl terminus described in this report.

#### MATERIALS AND METHODS

Bovine brain L- $\alpha$ -phosphatidylserine and *sn*-1,2-dioleoylglycerol were purchased from Avanti Polar Lipids, Inc. Dithiothreitol (DTT),<sup>1</sup> HEPES, PDBu, PMA, and ATP were from Sigma. [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci mmol<sup>-1</sup>) and [<sup>35</sup>S]methionine (1175 Ci mmol<sup>-1</sup>) were from NEN Life Science Products, and calcium chloride (analytical grade) was from J. T. Baker, Inc. Peroxidase-conjugated goat anti-rabbit antibodies and bovine serum albumin were obtained from Boehringer Mannheim. Antibodies to protein kinase C were purchased from Santa Cruz Biotechnologies ( $\beta$ II carboxyl-terminal antibodies) and Transduction Laboratories ( $\alpha$ -hinge antibodies; recognize protein kinase C  $\alpha$  and  $\beta$ II). Chemiluminescence SuperSignal substrates were from Pierce. Fluorescein isothiocyanate- or Texas Red-conjugated antibodies were from Molecular Probes. Lipofectin reagent was purchased from Life Technologies, Inc., and BaculoGold DNA was from PharMingen. *In vitro* transcription/translation kit (TNT) was purchased from Promega. A protein kinase C-selective peptide (Ac-FKKSFKL-NH<sub>2</sub> (24)) was synthesized by Dr. Elizabeth Komives, University of California, San Diego. All other chemicals were reagent grade.

**Mutagenesis**—Expression vectors encoding the cDNA sequence of protein kinase C  $\beta$ II with mutation of Ser-660 to Ala or Glu were made as described previously (15). The T634A/T641A double mutant in pBluescript and in baculovirus was a generous gift of Drs. A. J. Flint and D. E. Koshland, Jr. The T641E and T634A/T641A/S654A mutants were made by polymerase chain reaction using wild-type protein kinase C  $\beta$ II or the Thr-634/Thr-641 mutant, respectively, in the pBlueScript vector (pBluePKC) as the template. The sense primer used for both mutants was GGAGCATGCATTTTTCCG and contained a *Nsi*I restriction site. Antisense primers corresponding to the sequence around

the codon for Thr-641 or Ser-654 and containing the necessary nucleic acid changes to encode the desired mutation were ATCCTTCGAATTC-TGCTTGGTCAATATTCCTG (S654A mutation) and ATCCTTCGAAT-TCTGATTGGTCAATATTCCTGATGACTTCCTGGTCAAGAGGTTCT-AGGACTGGTGG (T641E mutation). These antisense primers contained a *Bst*BI restriction site. The polymerase chain reaction product and the pBluePKC template were digested with *Nsi*I and *Bst*BI (unique sites in pBluePKC), and the products were gel-purified and ligated together. The mutant protein kinase C gene was then subcloned into pcDNA3 (for mammalian expression) using *Xho*I/*Xba*I or into the pVL1393 baculovirus transfer vector (Invitrogen) as described (15). The sequence of all mutants was verified by DNA sequencing.

**Expression of Protein Kinase C in COS-7 Cells and Subcellular Fractionation**—DNA (5–10  $\mu$ g per 10-cm plate) encoding wild-type protein kinase C  $\beta$ II or its mutants was introduced into COS-7 cells by calcium-phosphate transfection. Cells were lysed by sonication in 50 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 85  $\mu$ M leupeptin, 2 mM benzamide, and 100 mM microcystin (lysis buffer). A portion of the lysate was retained, and the remainder was centrifuged at 100,000  $\times$  g for 20 min at 4 °C. The supernatant (cytosol) was saved, and the pellet was resuspended in lysis buffer containing 1% Triton X-100. The resuspended pellet was centrifuged (100,000  $\times$  g, 20 min, 4 °C), and the supernatant (membrane) and pellet (detergent-insoluble) fractions were saved. All fractions were diluted 2-fold in glycerol and stored at -20 °C. For some experiments, cells were treated with 200 nM PDBu for 20 min prior to cell lysis.

**Expression of Mutant Protein Kinase Cs in Sf21 Cells**—Sf21 insect cells were infected with baculovirus encoding wild-type protein kinase C  $\beta$ II or its mutants, and the detergent-soluble supernatants were analyzed for protein kinase C activity as described (15).

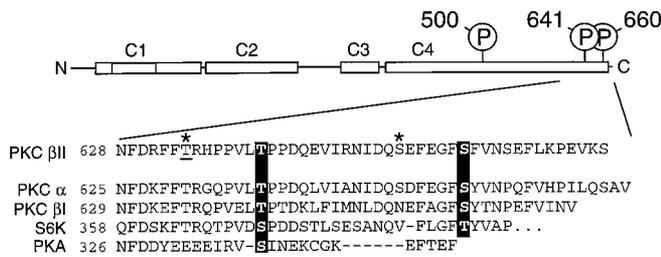
**Electrophoresis and Western Blots**—Aliquots of cell lysate, cytosol, detergent-soluble supernatant (membrane), or detergent-insoluble pellet from COS-7 cells expressing wild-type protein kinase C  $\beta$ II or the phosphorylation site mutants were analyzed by SDS-polyacrylamide gel electrophoresis (7% polyacrylamide). The proteins were then transferred to polyvinylidene difluoride (Immobilon-P, Millipore) and probed with antibodies to the carboxyl terminus of protein kinase C  $\beta$ II and peroxidase-conjugated secondary antibodies. Labeling was detected using chemiluminescence.

**Cell-free Translation**—cDNA encoding wild-type protein kinase C  $\beta$ II (50 ng) or the T641AAA mutant (1  $\mu$ g) in pcDNA3 were incubated with 20  $\mu$ l of the cell-free transcription/translation lysate provided by Promega, in the presence of 0.4  $\mu$ M [<sup>35</sup>S]methionine (1175 Ci mmol<sup>-1</sup>) following the manufacturer's protocol. After 90 min incubation at 30 °C, lysates were centrifuged at 100,000  $\times$  g for 20 min at 4 °C, and the resulting supernatant and pellet were analyzed by SDS-polyacrylamide gel electrophoresis followed by detection of radioactivity using a Bio-Rad Imager or autoradiography.

**Protein Kinase C Activity**—Protein kinase C activity in 3–5  $\mu$ l of detergent-soluble fractions was assayed by measuring the rate of phosphorylation of a synthetic peptide in the presence or absence of brain phosphatidylserine, diacylglycerol, and Ca<sup>2+</sup>, essentially as described (25). The reaction mixture (80  $\mu$ l) contained 50  $\mu$ M protein kinase C-selective peptide (see "Materials and Methods") in 20 mM HEPES (pH 7.5 at 30 °C), 1 mM DTT, 0.1 mM [ $\gamma$ -<sup>32</sup>P]ATP (0.1  $\mu$ Ci mmol<sup>-1</sup>), 5 mM MgCl<sub>2</sub>, and either 0.5 mM Ca<sup>2+</sup> and lipid (sonicated dispersion of brain phosphatidylserine (140  $\mu$ M) and diacylglycerol (3.8  $\mu$ M), prepared as described (21)) or 0.5 mM EGTA. Samples were incubated at 30 °C for 4–6 min and quenched by the addition of 25  $\mu$ l of a solution containing 0.1 M ATP and 0.1 M EDTA (pH 8–9). Aliquots (85  $\mu$ l) were spotted on P81 ion-exchange chromatography paper and washed 4 times with 0.4% (v/v) phosphoric acid, followed by a 95% ethanol rinse, and <sup>32</sup>P incorporation was detected by liquid scintillation counting in 5 ml of scintillation fluid (Biosafe II, Research Products International Corp.).

**Immunocytochemistry**—COS-7 cells were grown to confluency on glass coverslips and transfected with 1–2  $\mu$ g of the indicated protein kinase C  $\beta$ II plasmid cDNA expression constructs using the calcium phosphate precipitation method. Cells were incubated with the DNA for 16 h at 37 °C under 5% CO<sub>2</sub>, washed with phosphate-buffered saline (PBS), and incubated for an additional 48 h in fresh growth medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum, 1% penicillin/streptomycin). In some cases, cells were incubated with 100 nM PMA for 15 min at 37 °C under 5% CO<sub>2</sub>. Cells were then washed twice with PBS, fixed for 10 min in PBS containing 3.7% formaldehyde, washed with PBS, and permeabilized for 1 min in ice-cold acetone. Cells were washed with PBS and blocked for 30 min in PBS, 0.2% bovine serum albumin. Cells were incubated for 1 h with primary antibody (1:500

<sup>1</sup> The abbreviations used are: DTT, dithiothreitol; PDBu, phorbol dibutyrate; PMA, phorbol myristate acetate; PBS, phosphate-buffered saline.



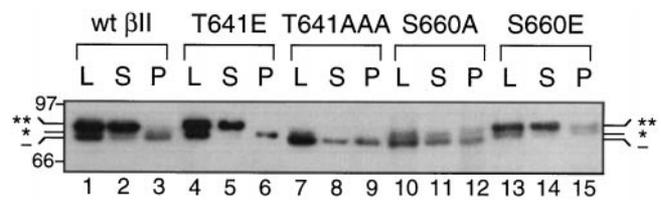
**FIG. 1. Schematic of protein kinase C showing conserved carboxyl-terminal phosphorylation sites.** *Top*, cartoon representation of the primary structure of protein kinase C showing ligand binding domains (C1 and C2) in regulatory moiety, and ATP and substrate-binding moieties (C3 and C4) in kinase core (reviewed in Ref. 2). The three *in vivo* phosphorylation sites are indicated as follows: Thr-500 in the activation loop and Thr-641 and Ser-660 in the carboxyl terminus (protein kinase C  $\beta$ II numbering) (8). *Bottom*, alignment of carboxyl termini of protein kinase C (PKC)  $\beta$ II,  $\alpha$ , and  $\beta$ I, S6 kinase (S6K), and protein kinase A (PKA) showing conserved phosphorylation sites (black rectangles). Asterisks indicate potential compensating phosphorylation sites in protein kinase C  $\beta$ II; underlined residue with asterisk is *in vitro* autophosphorylation site of protein kinase C  $\beta$ II (22). Sequences shown are for rat protein kinase C  $\beta$ I and  $\beta$ II (43), bovine  $\alpha$  (44), rat S6 kinase (45), and mouse protein kinase A (46).

dilution of mouse anti-protein kinase C  $\alpha/\beta$ , Transduction Laboratories; 1:100 dilution of rabbit anti-calreticulin, Affinity Bioreagents, Inc.) and washed three times with PBS, 0.2% bovine serum albumin. The cells were then incubated for 1 h with fluorescent secondary antibodies (donkey anti-mouse Texas Red or donkey anti-rabbit Cy5, Jackson ImmunoResearch; fluorescein isothiocyanate-phalloidin, Molecular Probes), followed by washing with PBS, 0.2% bovine serum albumin. The coverslips were then mounted on glass slides using the Prolong system (Molecular Probes), and indirect immunofluorescent staining was detected in successive focal planes using a UV-visible laser-scanning confocal microscope system (Bio-Rad).

## RESULTS

**Phosphorylation Site Mutant Mimicking Dephosphorylated Thr-641**—A series of mutants at each of the two carboxyl-terminal phosphorylation sites in protein kinase C  $\beta$ II, Thr-641 and Ser-660, were constructed in order to assess how phosphorylation at these positions modulates the function of protein kinase C. Hydroxyl-containing residues were replaced with either Glu as a mimic of phosphate (26) or the neutral, non-phosphorylatable residue, Ala.

Initial studies focused on analyzing a protein kinase C mutant in which Thr-641 (*in vivo* autophosphorylation site) and Thr-634 (*in vitro* autophosphorylation site) were mutated to Ala (Fig. 1). However, biochemical analysis revealed that this mutant did not mimic protein dephosphorylated on Thr-641. First, the double mutant T634A/T641A displayed the same electrophoretic mobility as wild-type enzyme. Because phosphorylation of Thr-641 causes an approximately 2-kDa shift in electrophoretic mobility (8), this result indicated that incorporation of a phosphate at an additional (compensating) site was causing an equivalent mobility shift (8). Second, phosphatase treatment caused the same effects on the T634A/T641A mutant and wild-type enzyme. Specifically, protein phosphatase 1 caused a large electrophoretic mobility shift and complete loss of activity; in wild-type enzyme, this large shift and loss of activity result from dephosphorylation at two carboxyl-terminal positions, Thr-641 and Ser-660. In addition, protein phosphatase 2A caused a small electrophoretic mobility shift with no loss of activity; in wild-type enzyme this smaller shift results from loss of phosphate at one carboxyl-terminal position, Ser-660 (10). These data revealed that mutation of position 634 and 641 to Ala resulted in a compensating phosphorylation that mimicked the effect of phosphorylation of Thr-641 in all parameters tested. Analysis of the sequence around Thr-641 indicated a possible candidate for such a compensating phospho-



**FIG. 2. Negative charge at position 641 or 660 alters electrophoretic mobility and subcellular partitioning of protein kinase C  $\beta$ II.** Western blot of whole cell lysate (L), detergent-soluble supernatant (S), and detergent-insoluble pellet (P) from COS-7 cells expressing wild-type protein kinase C  $\beta$ II (*wt*  $\beta$ II), T641E mutant, T634A/T641A/S654A (T641AAA) mutant, S660A mutant, or S660E mutant; blot was probed with a polyclonal antibody against protein kinase C  $\beta$ II's carboxyl terminus that recognizes phosphorylated and dephosphorylated protein kinase C with equal affinity. Lanes 1–6 and 13–15 contained sample from approximately  $2 \times 10^4$  cells; lanes 7–12 contained sample from approximately  $4 \times 10^4$  cells. Double asterisk indicates the position of protein kinase C with negative charge at both carboxyl-terminal positions (Thr-641 and Ser-660); single asterisk denotes the position of protein kinase C with a single negative charge on the carboxyl-terminal phosphorylation sites, and the dash indicates the position of protein kinase C with no phosphates on the carboxyl terminus, as determined previously by mass spectrometry (8). Note that the faint slower migrating band in lane 7 is not protein kinase C  $\beta$ II and is not labeled by other protein kinase C-specific antibodies; it is a protein endogenous to COS cells whose cross-reactivity is apparent when higher amounts of cell extract are loaded on gels (see text).

phorylation, Ser-654. To eliminate any potential compensating phosphorylation at residues near Thr-641, we constructed a triple mutant T634A/T641A/S654A. This mutant is referred to as T641AAA henceforth.

**Lack of Negative Charge at Carboxyl-terminal Phosphorylation Sites Results in Association of Protein Kinase C with the Detergent-insoluble Cell Fraction**—Wild-type protein kinase C and four phosphorylation site mutants, T641E, T641AAA, S660A, and S660E, were expressed in COS-7 cells, and their subcellular partitioning and electrophoretic mobilities were compared in Fig. 2. As reported previously, wild-type enzyme from cell lysate migrated as two major species (lane 1): the slowest migrating species partitioned in the detergent-soluble fraction (lane 2, \*\*) and the fastest migrating species partitioned in the detergent-insoluble fraction (lane 3, -). We have previously established that the slower migrating form is quantitatively phosphorylated at Thr-641 and Ser-660,<sup>2</sup> and the fastest migrating species is not phosphorylated at the carboxyl terminus (8). A minor intermediate species was also visible (lane 3, \*); previous analyses showed that this intermediate species is phosphorylated at Thr-641 but not Ser-660 (8). Under the expression conditions in Fig. 1, approximately 10% of the total protein kinase C  $\beta$ II migrated as the dephosphorylated form partitioning in the detergent-insoluble fraction (-); however, this percentage depended on protein expression levels, with increased expression typically resulting in increased representation of the dephosphorylated species.

Replacement of Thr-641 with Glu resulted in expression of protein whose electrophoretic mobility and subcellular fractionation pattern was similar to that of wild-type enzyme except that the fastest migrating form was absent. Specifically, T641E in cell lysate migrated as two species (lane 4), with the slowest species co-migrating with fully phosphorylated wild-type enzyme (\*\*) and, like mature wild-type enzyme, partitioned in the detergent-soluble fraction (lane 5). The fastest migrating form had an apparent mass of 78 kDa (\*), co-migrating with wild-type enzyme containing a single phosphate on its

<sup>2</sup> This species is approximately 60% phosphorylated on Thr-500; negative charge at this position does not alter the protein's electrophoretic mobility (8).

carboxyl terminus and partitioned in the detergent-insoluble fraction (*lane 6*). These data reveal that Glu at position 641 mimics phosphate in causing a decrease in the electrophoretic mobility of the enzyme.

The T641AAA mutant migrated as a single band corresponding to completely dephosphorylated protein kinase C (-); this mutant partitioned equally in the detergent-soluble (*lane 8*) and detergent-insoluble (*lane 9*) fractions. The lack of bands co-migrating with partially phosphorylated species of protein kinase C indicated that the mutant was not phosphorylated on Ser-660 nor at any other position on the carboxyl terminus that could alter the electrophoretic mobility of the protein. Note that a minor slower migrating band is visible in the lysate (*lane 7*) and, to a lesser extent, in the pellet (*lane 9*). This band is not protein kinase C  $\beta$ II; first, it is present in control COS cells transfected with pcDNA3 vector alone, and second, it is not labeled with a different antibody that labels the kinase domain in a phosphorylation-independent manner (data not shown). Because the Ala mutants expressed less well than wild-type or Glu mutants, twice as much cell extract of the former is loaded on gels.

Expression of S660A in COS-7 cells resulted in the appearance of two bands in the lysate. Fig. 2, *lane 10*, shows a major band co-migrating with dephosphorylated wild-type enzyme (-) and a minor band co-migrating with wild-type enzyme modified at only one carboxyl-terminal position (\*). Both species were present in the detergent-soluble (*lane 11*) and detergent-insoluble (*lane 12*) fractions. The S660E mutant also migrated as two bands in the lysate (*lane 13*). However, the mobilities of the bands were shifted by approximately 2 kDa compared with the Ala mutant, so that the fastest migrating band co-migrated with wild-type enzyme with one carboxyl-terminal phosphate (\*) and the slowest migrating band co-migrated with wild-type enzyme with two carboxyl-terminal phosphates (\*\*). Thus, Ala at position 660 mimicked protein with no phosphate at that position, whereas Glu at that position mimicked protein with phosphate at that position, as reported previously based on baculovirus expression studies (15). In addition, the ratios and subcellular fractionation patterns of the two species were comparable to that of wild-type enzyme; the upper band was the major species and partitioned in the detergent-soluble fraction (*lane 14*) and lower band was the minor species and localized to the detergent-insoluble fraction (*lane 15*).

In summary, the T641E and S660E mutants did not contain species co-migrating with the lowest 76-kDa band of wild-type enzyme, consistent with negative charge at either of these positions causing an apparent 2 kDa in electrophoretic mobility. Both T641E and S660E mutants contained species that co-migrated with the 78- and 80-kDa bands of wild-type protein, representing forms with negative charge at one (78 kDa) or both (80 kDa) carboxyl-terminal sites. The alanine mutants, on the other hand, lacked species co-migrating with the uppermost 80-kDa band, consistent with lack of negative charge at the indicated phosphorylation site. Furthermore, the T641AAA mutant migrated as only one band which corresponded to the fastest migrating form of protein kinase C. Thus, the T641AAA was not phosphorylated on Ser-660.

**Lack of Negative Charge at Carboxyl-terminal Phosphorylation Sites Does Not Cause Protein Insolubility**—The fractionation studies above revealed that lack of negative charge at Thr-641 caused protein kinase C to partition with the detergent-insoluble fraction of cells. One possibility is that this insolubility reflects an intrinsic property of the protein (*i.e.* misfolded and aggregated). Alternatively, it could result from a specific interaction of the dephosphorylated protein kinase C



FIG. 3. Precursor protein kinase C and T641AAA mutant are soluble proteins when expressed in cell-free translation system. Autoradiogram showing  $^{35}\text{S}$ -labeled wild-type protein kinase C  $\beta$ II and T641AAA mutant in the supernatant (S; lanes 1 and 3) and pellet (P; lanes 2 and 4) fractions following high speed centrifugation of *in vitro* transcription/translation reaction mixtures. Details are presented under "Materials and Methods."

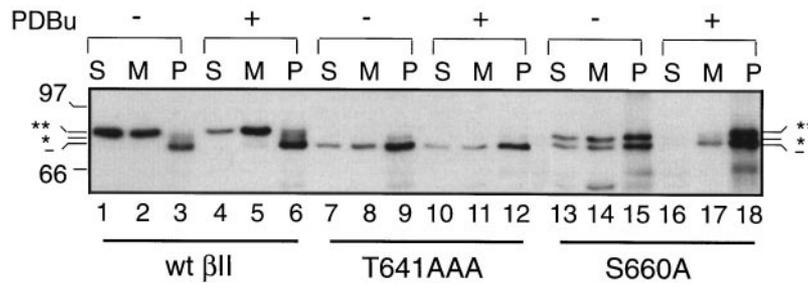
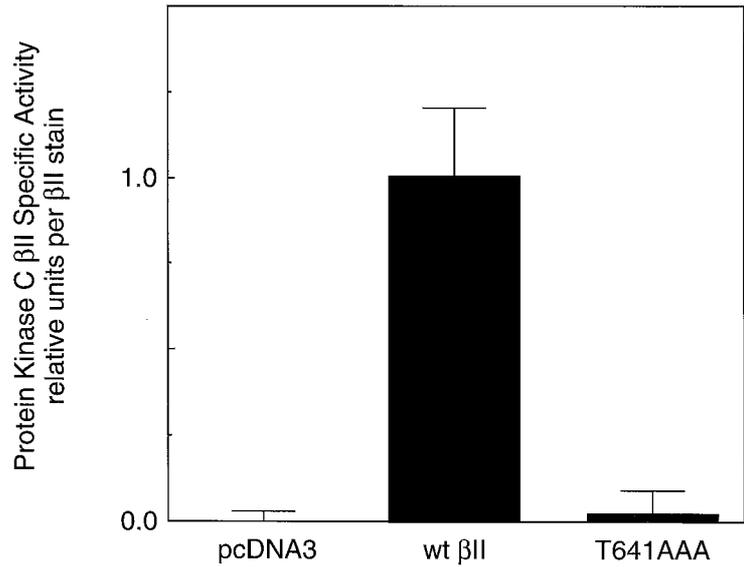
with its environment (*i.e.* binding to component in detergent-insoluble fraction). To distinguish between these possibilities, we asked whether dephosphorylated protein kinase C was soluble when expressed in a cell-free *in vitro* transcription/translation system. Protein kinase C expressed in this system is not phosphorylated; it co-migrates with completely dephosphorylated protein kinase C and is not recognized by phosphorylation-specific antibodies (data not shown).

Wild-type protein kinase C and T641AAA were expressed in a cell-free transcription/translation system, and the reaction mixtures were then centrifuged at  $100,000 \times g$  for 20 min. Fig. 3 shows that both wild-type protein kinase C (corresponding to dephosphorylated precursor enzyme; *lane 1*) and T641AAA mutant (*lane 3*) remained in the supernatant. Thus, differences in the milieu of where protein kinase C is expressed, rather than intrinsic properties of the protein, appear to dictate its solubility. This suggests that the detergent insolubility of the *in vivo* expressed proteins lacking phosphate at the carboxyl terminus results from interaction with cellular components and does not reflect an intrinsic property of the isolated protein.

**Requirement of Negative Charge at Position 641 for Catalytic Function**—The effect of negative charge at position 641 on catalytic activity of protein expressed in COS-7 cells was examined in Fig. 4. For the data presented, the activity contributed by endogenous protein kinase C  $\alpha$  was subtracted from the total activity; it accounted for 50% of the activity observed when wild-type protein kinase C  $\beta$ II was transfected. When assayed in the presence of saturating cofactor concentrations for wild-type enzyme (see legend to Fig. 4), no significant activity of the T641AAA mutant was detected. Similarly, no activity of the T641AAA mutant was detected when expressed in insect cells, where there is no endogenous protein kinase C, even in the presence of ATP and peptide concentrations 20-fold higher than the  $K_m$  for these substrates (data not shown). In insect cells, the lipid-dependent activity of the T641E mutant was  $55 \pm 9\%$  that of wild-type enzyme; this reduced activity likely arose from thermal instability of the enzyme, because addition of glycerol increased the activity of the mutant to levels similar to those of wild-type enzyme. Glycerol, however, was unable to promote the activity of the T641AAA mutant.

**Phorbol Ester-induced Membrane Translocation Depends on Carboxyl-terminal Phosphorylation**—The effect of phorbol ester treatment on the subcellular localization of wild-type and phosphorylation site mutants of protein kinase C was examined both biochemically and immunocytochemically. Fig. 5 shows the effect of PDBu treating COS-7 cells expressing wild-type protein kinase C or phosphorylation site mutants on the partitioning of the enzyme in the cytosolic fraction (S), the membrane fraction (M), or the detergent-insoluble pellet fraction (P). As discussed above, the mature wild-type enzyme partitions in the detergent-soluble fraction (*e.g.* Fig. 2, *lane 2*).

**FIG. 4. Negative charge at position 641 is required for catalytic activity.** Protein kinase C activity in the detergent-soluble supernatant from COS-7 cells expressing wild-type protein kinase C  $\beta$ II or T641AAA was measured in the presence of  $\text{Ca}^{2+}$  (500  $\mu\text{M}$ ) and sonicated dispersions of phosphatidylserine (140  $\mu\text{M}$ ) and diacylglycerol (3.8  $\mu\text{M}$ ). ATP and peptide concentrations were 0.1 mM and 50  $\mu\text{M}$ , respectively. Data are expressed as protein kinase C  $\beta$ II activity (total activity minus endogenous protein kinase C  $\alpha$  activity) divided by the amount of protein kinase C  $\beta$ II based on Western blot analysis with protein kinase C  $\beta$ II-specific antibodies (mean  $\pm$  S.E. for one representative experiment performed in triplicate).



**FIG. 5. Negative charge at the carboxyl terminus modulates the phorbol ester-dependent redistribution of protein kinase C  $\beta$ II.** Western blot of cytosol (S), membrane (M), and detergent-insoluble pellet (P) from COS-7 cells expressing wild-type protein kinase C  $\beta$ II, the T641AAA mutant, or the S660A mutant; blot was probed as described in the legend to Fig. 2. Cells were treated without (–) or with (+) 200 nM PDBu for 20 min at 37 °C prior to cell lysis, as described under “Materials and Methods.” Lanes 1–6 contained sample from approximately  $2 \times 10^4$  cells; lanes 7–18 contained sample from approximately  $4 \times 10^4$  cells. The barely visible slower migrating band in lanes 9 and 12 is not protein kinase C, as discussed in the legend to Fig. 3.

Further fractionation of the detergent-soluble fraction into the cytosolic and membrane fractions revealed that, under the conditions of cell culture, half the mature protein partitioned in the cytosol (Fig. 5, lane 1) and half was associated with the membrane (Fig. 5, lane 2). As observed in Fig. 2, faster migrating species of protein kinase C partitioned in the detergent-insoluble fraction (lane 3). Phorbol ester treatment caused the wild-type protein kinase C in the cytosolic fraction to redistribute to the membrane (lanes 4 and 5); in addition, a slight increase in the amount of partially phosphorylated/dephosphorylated species in the pellet was observed (lane 6). In marked contrast, the T641AAA mutant was refractory to phorbol ester treatment. Although approximately 10% of the protein was present in the cytosolic fraction, no significant change was noted upon phorbol ester treatment. The majority of the protein was associated with the detergent-insoluble fraction, and this distribution did not change upon phorbol ester treatment (compare lanes 9 and 12).

Unlike the T641AAA mutant, the S660A mutant responded detectably to phorbol ester treatment. In unstimulated cells, approximately half the protein was in the cytosolic fraction (lane 13) and half in the membrane fraction (lane 14). Phorbol ester treatment caused protein kinase C to disappear from the cytosol and, for the slower mobility species and also the membrane, redistribute to the detergent-insoluble fraction (lane 18).

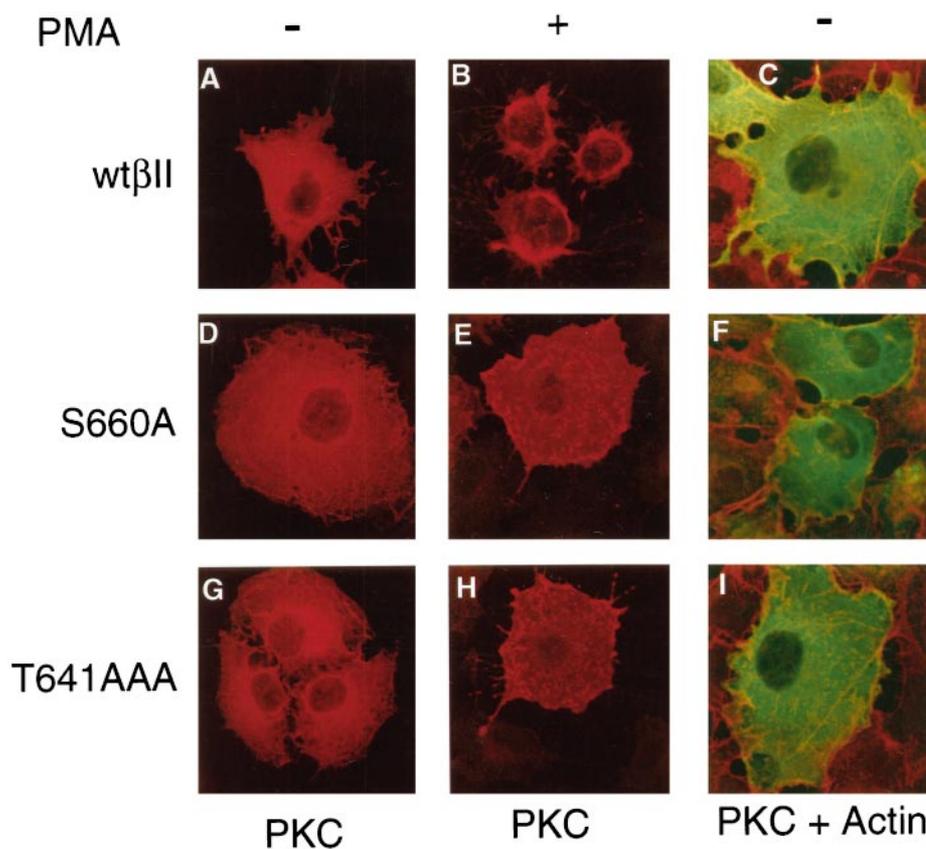
The subcellular partitioning of T641E and S660E, in the presence or absence of phorbol esters, was indistinguishable from that of wild-type enzyme (data not shown).

The above biochemical studies revealed that the T641AAA and S660A mutants exhibited different subcellular fractionation patterns from wild-type protein kinase C  $\beta$ II. Therefore, the intracellular locations of these mutants were analyzed immunocytochemically. COS-7 cells were transiently transfected with wild-type protein kinase C  $\beta$ II or phosphorylation site mutants, and the subcellular distribution of the proteins was monitored immunocytochemically using a monoclonal antibody that recognizes protein kinase C  $\alpha$  and  $\beta$ II independently of phosphorylation state.

Fig. 6A shows that wild-type protein kinase C  $\beta$ II displayed diffuse cytoplasmic distribution and staining of the plasma membrane, consistent with results of fractionation studies. This staining was distinct from that observed with the actin (Fig. 6C) or endoplasmic reticulum (not shown) markers, and composite analysis revealed that protein kinase C did not co-distribute with either the endoplasmic reticulum or the actin-based cytoskeleton (Fig. 6C).

The immunostaining pattern of both T641AAA (Fig. 6D) and S660A (Fig. 6G) differed from that of wild-type enzyme in two aspects: staining of the plasma membrane was absent, and the cytoplasmic staining appeared less diffuse and more punctate.

**FIG. 6. Immunocytochemical analysis of wild-type protein kinase C  $\beta$ II and carboxyl-terminal phosphorylation mutants.** Confocal microscopy of COS-7 cells transfected with wild-type protein kinase C  $\beta$ II (A–C), S660A mutant (D–F), or T641AAA mutant (G–I). Cells were treated without (A, C, D, F, G, and I) or with (B, E, and H) 100 nM PDBu for 15 min prior to fixation, as described under “Materials and Methods.” Transfected cells were stained immunocytochemically to visualize protein kinase C in all panels and also with phalloidin to visualize F-actin (C, F, and I). Fluorescent staining was imaged by laser scanning confocal microscopy. The images shown are representative focal planes. Co-staining of protein kinase C  $\beta$ II (red) and F-actin (green) are shown as composite images in C, F, and I. The data shown are representative from at least three separate transfection experiments for each construct.



The punctate staining did not result from protein kinase C binding the actin-based cytoskeleton, because the phalloidin stain was distinct from that of the protein kinase C stain, and co-distribution was not apparent in composite pictures (Figs. 6, F and I). Although the protein kinase C staining pattern shared some features in common with that of the endoplasmic reticulum staining, composite analysis revealed only minimal co-distribution of the two proteins (not shown). Thus, the phosphorylation site mutants associated with a detergent-insoluble component that was neither actin-based nor a result of association with the endoplasmic reticulum.

In response to PMA, wild-type protein kinase C redistributed to the plasma membrane, as evidenced by the stronger staining of the cell periphery (Fig. 6B). Phorbol ester treatment also promoted changes in cell morphology, as evidenced by induction of filopodia and actin distribution; no changes in the endoplasmic reticulum were apparent (data not shown). Composite analysis suggested that the altered distribution of protein kinase C resulting from PMA treatment did not reflect association of the enzyme with actin or the endoplasmic reticulum.

PMA treatment of both T641AAA and S660A-expressing cells caused a marked difference in the staining pattern of the protein kinase C mutants (Fig. 6, E and H). Specifically, staining became considerably more punctate for both the S660A and T641AAA mutants. The punctate staining did not represent co-distribution of protein kinase C with either actin or the endoplasmic reticulum (not shown), as illustrated by composite analysis. COS-7 cells expressing the phosphorylation site mutants did not undergo significant phorbol ester-dependent changes in cell morphology.

In summary, biochemical fractionation studies and immunocytochemical analysis revealed that negative charge at the carboxyl-terminal phosphorylation sites is required for the cytosolic localization of protein kinase C. Mutants lacking negative charge at these positions associate with a detergent-insol-

uble fraction that does not appear to be the endoplasmic reticulum or to be actin-based. Although phorbol esters do not alter the detergent insolubility of these mutants, they do cause the proteins to redistribute in this detergent-insoluble fraction, suggesting that the phorbol-binding site of the mutants remains exposed and able to bind ligand. However, the strength of the interaction with the detergent-insoluble fraction dominates over the binding energy provided by phorbol esters in recruiting protein kinase C to the plasma membrane.

#### DISCUSSION

This study establishes that phosphorylation of Thr-641 in protein kinase C  $\beta$ II is essential for both the catalytic function and correct subcellular location of protein kinase C. Specifically, lack of negative charge at this position results in expression of inactive enzyme, incapable of phosphorylating at Ser-660, that displays a different subcellular distribution and phorbol ester responsiveness compared with wild-type enzyme.

This study also establishes that mutation of the key residue, Thr-641, is not adequate to examine the role of its phosphorylation in protein kinase C function because the enzyme compensates by incorporating phosphate at adjacent residues that are functionally similar. This finding underscores the need for careful analysis of phosphorylation site mutants.

*Carboxyl-terminal Phosphorylation Is Required for Catalysis*—Analysis of phosphorylation site mutants reveals that negative charge at position 641 is required for the catalytic competence of protein kinase C. Replacing Thr-641 as well as potential compensating phosphorylation sites with Ala results in expression of protein that is inactive both in COS-7 cells (data not shown) and in insect cells. This inactivity is unlikely to result from unusually high protein instability, since addition of protein stabilizers such as glycerol are unable to promote activity. The finding that negative charge at Thr-641 is required for catalysis is consistent with data from phosphatase

sensitivity experiments which revealed that protein phosphorylated at Thr-641, but not Thr-500 and Ser-660, is catalytically active. In contrast, dephosphorylation at all three positions inactivates protein kinase C (8, 10).

Parker and co-workers (20) recently mutated Thr-638 in protein kinase C  $\alpha$ , corresponding to Thr-641 in protein kinase C  $\beta$ II, to Ala and reported that the protein retained significant activity. Based on our results with protein kinase C  $\beta$ II, this activity could have resulted from a compensating phosphorylation that partially mimicked the effect of phosphate on Thr-638. Both the compensating phosphorylation sites found in protein kinase C  $\beta$ II, Thr-634, and Ser-654, are conserved in protein kinase C  $\alpha$ . Consistent with this, the migration of the T638A mutant on SDS-polyacrylamide gel electrophoresis did not appear to differ detectably from that of fully phosphorylated wild-type protein kinase C  $\alpha$  (20), suggesting the same number of carboxyl-terminal phosphorylations as present on wild-type enzyme.

In protein kinase C  $\beta$ I, mutation of Thr-642 to Ala, corresponding to Thr-641 in protein kinase C  $\beta$ II, results in expression of inactive protein that associates with the detergent-insoluble fraction (19), consistent with the results reported here for the T641AAA mutant. For the  $\beta$ I isozyme, the proposed compensating phosphorylation site, Ser-654, is absent.

The crystal structure of protein kinase A reveals that its carboxyl terminus is tethered to the small, ATP-binding lobe of the kinase core by a phosphorylated residue, Ser-338 (27, 28); this residue corresponds to that of Thr-641 in protein kinase C  $\beta$ II (Fig. 1). Mutagenesis studies established that negative charge at position 338 is required for protein kinase A stability, presumably by strengthening the interaction of the carboxyl terminus with the small lobe (29). Molecular modeling suggests that phosphorylation of Thr-641 on protein kinase C  $\beta$ II could also tether the carboxyl terminus away from the active site, thus stabilizing the kinase core (30). In support of an electrostatic contact mediated by phosphate at position 641, Glu, with a single negative charge, is much less effective at allowing activation. Thus, a weaker potential electrostatic contact formed by Glu at position 641 compared with phospho-Thr, may not structure the core adequately for maximal catalysis. This would account for the decreased thermal stability of Glu mutations at this position both in protein kinase C  $\beta$ II (above) and protein kinase C  $\alpha$  (20).

*Lack of Negative Charge on Thr-641 Prevents Phosphorylation of Ser-660*—Previously we showed that protein kinase C effectively autophosphorylates on Ser-660 *in vitro*; specifically, pure protein kinase C that has been quantitatively dephosphorylated on Thr-500 and Ser-660 by treatment with protein phosphatase 2A stoichiometrically re-autophosphorylates on Ser-660 upon addition of MgATP (8, 10). The finding that the catalytically incompetent T641AAA mutant is not phosphorylated on Ser-660 in COS-7 cells indicates that this modification is also an autophosphorylation *in vivo*. In support of Ser-660 being an autophosphorylation event, other kinase-dead constructs of protein kinase C are also not phosphorylated at Ser-660.<sup>3</sup>

The inability of the T641AAA mutant to become phosphorylated on Ser-660 *in vivo* supports the hypothesis that phosphorylation of Thr-641 precedes that of Ser-660 (8). This hypothesis was based on the finding that Ser-660 is not phosphorylated in any of the partially phosphorylated forms that associate with the detergent-insoluble fraction, indicating that its phosphorylation coincides with release of the fully phosphorylated enzyme into the cytosol.

*Lack of Negative Charge at Carboxyl Terminus Tethers Protein Kinase C to Detergent-insoluble Cell Fraction*—Subcellular fractionation studies reveal that lack of negative charge at the carboxyl-terminal phosphorylation positions causes protein kinase C to associate with the detergent-insoluble cell fraction. Both non- or partially phosphorylated forms of wild-type enzyme and Ala mutants (T641AAA and S660A) partition with the detergent-insoluble cell fraction. This insolubility is unlikely to reflect an intrinsic property of the protein, because both unphosphorylated wild-type protein and the T641AAA mutant are soluble when expressed in a cell-free translation system. Rather, the insolubility appears to result from a specific interaction with a detergent-insoluble cellular component.

One possibility to account for the detergent insolubility is that the phosphorylation site mutants are retained in the endoplasmic reticulum; for example, the detergent insolubility of methionyl-tRNA synthetase arises from its tight association with the endoplasmic reticulum (31). However, confocal images of COS-7 cells stained for T641AAA or S660A and for the endoplasmic reticulum marker, calreticulin, suggest that protein kinase C mutants are not retained in this subcellular compartment. This suggests that the detergent-insoluble component is cytoskeletal. One obvious candidate could be the actin-based cytoskeleton; however, confocal microscopy indicated that protein kinase C does not appear to co-distribute with actin. Consistent with this, disruption of the actin-based cytoskeleton does not alter the subcellular distribution of protein kinase C  $\beta$ II in COS-7 cells.<sup>4</sup> Despite eliminating potential sites for sequestration of the kinase, the nature and mechanism of the cytoskeletal tether for non- or partially phosphorylated protein kinase C remain to be determined.

*Phorbol Esters Do Not Release Cytoskeletal-bound Protein Kinase C but Do Cause Soluble Protein Kinase C to Redistribute to the Cytoskeleton*—As has been extensively documented over the past 15 years, phorbol ester treatment of cells causes a dramatic redistribution of protein kinase C from the cytosol to the membrane (32–34). This translocation arises because phorbol esters act as molecular glue, altering the membrane affinity of protein kinase C by many orders of magnitude (for example, 1 mol % PMA increases membrane affinity of protein kinase C by 4 orders of magnitude (35)). The structural basis for this effect results from phorbol esters capping a hydrophilic ligand-binding pocket in the C1 domain, thus altering the surface hydrophobicity of the domain such that its top third now constitutes a continuous hydrophobic surface (36).

In marked contrast to its effects on wild-type protein kinase C  $\beta$ II, fractionation studies revealed that phorbol esters do not cause the carboxyl-terminal phosphorylation site mutants, T641AAA and S660A, to associate with the membrane. In unstimulated cells, the T641AAA mutant is primarily associated with the detergent-insoluble fraction and phorbol ester treatment has no significant effect on this fractionation pattern. Interestingly, a significant fraction of the S660A mutant is soluble (present both in cytosolic and membrane fraction), yet phorbol ester treatment promotes the association of this soluble protein with the detergent-insoluble pellet. Thus, at the time point measured, phorbol ester treatment did not recruit the phosphorylation site mutants to the membrane. Rather, for the mutant that displayed some solubility, the effect of phorbol esters was to target the protein to the detergent-insoluble fraction.

The phorbol ester-dependent relocation to the detergent-insoluble fraction noted for S660A was also observed, to a lesser

<sup>3</sup> A. Behn-Krappa and A. C. Newton, unpublished data.

<sup>4</sup> E. M. Dutil and A. C. Newton, manuscript in preparation.

extent, for wild-type enzyme. In this case, however, redistribution to the cytoskeleton was accompanied by dephosphorylation of the protein as assessed by its increased electrophoretic mobility. The latter observation is consistent with a report by Parker and co-workers (37) showing that phorbol ester treatment promotes the dephosphorylation of protein kinase C  $\alpha$ . Our results indicate that this dephosphorylation is accompanied by targeting of the kinase to the detergent-insoluble fraction. Thus, dephosphorylation of the carboxyl terminus may regulate the binding of protein kinase C to a cytoskeletal component. Wild-type enzyme appears to be first targeted to the membrane in response to phorbol esters, followed by dephosphorylation and association with the detergent-insoluble fraction. The S660A mutant may also first translocate to the membrane; however, increased phosphatase sensitivity (16) could have resulted in a much more rapid redistribution to the detergent-insoluble fraction such that membrane translocation was not detected in our experiments.

Phorbol esters did, however, affect the nature of the phosphorylation site mutants' interaction with the cytoskeleton. Specifically, confocal microscopy studies revealed that phorbol esters caused the staining of T641AAA and S660A to become more punctate. This suggests that interaction of the protein with the detergent-insoluble fraction is altered, without actually releasing the proteins from this compartment.

Phorbol ester treatment caused marked changes in the morphology of untransfected COS-7 cells or ones transfected with functional protein kinase C  $\beta$ II. Curiously, these changes were not apparent in cells transfected with the inactive phosphorylation site mutants (e.g. T641AAA), suggesting that these inactive protein kinase Cs may be acting as dominant negatives in preventing protein kinase C-mediated cell morphological changes.

**Conclusions**—Phosphorylation provides two key switches in regulating protein kinase C: 1) transphosphorylation at the activation loop, which likely structures the active site for catalysis, and 2) autophosphorylation at the carboxyl terminus which locks the enzyme in a catalytically competent conformation and dictates its subcellular localization. The activation loop switch has been well characterized in many kinases (5, 38). Now, the growing number of reports of kinases phosphorylated on their carboxyl terminus suggests that the mechanism described for protein kinase C may also provide a general regulatory switch for the kinase superfamily. For example, S6 kinase and protein kinase B (Akt kinase) both require carboxyl-terminal phosphorylation as part of their activation (39–41). Most striking, mutagenesis of Ser-371 to Ala (or Asp) in S6 kinase, corresponding to Thr-641 in protein kinase C  $\beta$ II, abolishes activity in this kinase also (42). In addition, the archetypal kinase, protein kinase A requires carboxyl-terminal phosphorylation to stabilize the kinase (29). Thus, carboxyl-terminal phosphorylation may provide an electrostatic anchor that structures the kinase and, in addition, alters the surface of the kinase to promote or disrupt protein-protein interactions.

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