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Phosphorylation at Conserved Carboxyl-terminal Hydrophobic Motif Regulates the Catalytic and Regulatory Domains of Protein Kinase C*

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Mature protein kinase C is phosphorylated at a conserved carboxyl-terminal motif that contains a Ser (or Thr) bracketed by two hydrophobic residues; in protein kinase C βII, this residue is Ser-660 (Keranen, L. M., Dutil, E. M., and Newton, A. C. (1995) *Curr. Biol.* 5, 1394–1403). This contribution examines how negative charge at this position regulates the function of protein kinase C. Specifically, Ser-660 in protein kinase C βII was mutated to Ala or Glu and the enzyme's stability, membrane interaction, Ca^{2+} regulation, and kinetic parameters were compared with those of wild-type protein phosphorylated at residue 660. Negative charge at this position had no significant effect on the enzyme's diacylglycerol-stimulated membrane interaction nor the conformational change accompanying membrane binding. In contrast, phosphate caused a 10-fold increase in the enzyme's affinity for Ca^{2+} and a comparable increase in its affinity for phosphatidylserine, two interactions that are mediated by the C2 domain. Negative charge also increased the protein's thermal stability and decreased its K_m for ATP and peptide substrate. These data indicate that phosphorylation at the extreme carboxyl terminus of protein kinase C structures the active site so that it binds ATP and substrate with higher affinity and structures determinants in the regulatory region enabling higher affinity binding of Ca^{2+}. The motif surrounding Ser-660 in protein kinase C βII is found in a number of other kinases, suggesting interactions promoted by phosphorylation of the carboxyl terminus may provide a general mechanism for stabilizing kinase structure.

Phosphorylation is a widely used mechanism for reversibly regulating protein structure and function. Conformational or electrostatic changes promoted by phosphorylation modulate the enzymatic activity and macromolecular interactions of a plethora of cellular proteins (1). Since the discovery 4 decades ago that phosphorylation activates phosphorylase kinase (2), it has been clearly established that phosphorylation serves as a general mechanism for regulating kinase function (1, 3, 4). Best characterized is the requirement for negative charge on the activation loop of kinases that renders the kinase core competent for catalysis (3, 4).

Phosphorylation has recently been shown to play an essential role in regulating the protein kinase Cs (5, 6). These enzymes transduce the myriad of signals promoting phospholipid hydrolysis (7). They are recruited to membranes upon the production of diacylglycerol and, for the conventional isoforms, increased Ca^{2+} concentrations. Binding of these cofactors results in a conformational change that removes an autoinhibitory (pseudosubstrate) domain from the active site, thus promoting substrate binding and phosphorylation (8). It was recently established that protein kinase C is multiply phosphorylated *in vivo* and that these phosphorylations are required to process newly synthesized protein kinase C into the mature, cofactor-responsive enzyme that has been extensively studied over the past 2 decades (5, 6).

Mass spectrometric analysis established that the protein kinase C present in the detergent-soluble fraction of cell or tissue extracts is phosphorylated at three conserved positions, with non-phosphorylated or partially phosphorylated forms partitioning exclusively in the detergent-insoluble fraction (5). In protein kinase C βII, these phosphorylation sites are Thr-500, Thr-641, and Ser-660 (5, 6). Based on distinct electrophoretic mobility shifts resulting from these phosphorylations and the use of antibodies selective for dephosphorylated Ser-660, the order of phosphorylations was established to be Thr-500, followed by Thr-641, followed by Ser-660 (5). Each of these phosphorylatable residues has an analogue in other protein kinases: Thr-500 aligns with the activation loop phosphorylatable residue found in many protein kinases (including Thr-197 in protein kinase A (4)), Thr-641 has an analogous site on protein kinase A (S338) (9), and Ser-660 is part of a hydrophobic phosphorylation motif present in diverse kinases such as S6 kinase and protein kinase B (Akt kinase) that has the consensus sequence F*XX*F(*S*/*T*)(F/Y) (10, 11).

The first phosphorylation event, occurring on the activation loop, appears to be catalyzed by an unidentified protein kinase C kinase (5, 12), whereas the two carboxyl-terminal phosphorylations are autophosphorylations (13–15). Phosphorylation at the activation loop is required to initiate the processing of inactive protein kinase C to the cofactor-activable, mature form (12, 16, 17). However, once the mature, carboxyl-terminal phosphorylated enzyme is formed, the requirement for negative charge at the activation loop is relieved (5). The second phosphorylation event, occurring on the first carboxyl-terminal site, Thr-641, has been proposed to be critical to the catalytic function of protein kinase C (5), to allow the release of protein kinase C from the detergent-insoluble fraction of cells (18), and to stabilize protein kinase C (19). Although most of the phosphorylation sites are conserved throughout the protein kinase C family, some isozymes contain a Glu in place of a phosphorylatable residue in the hydrophobic motif (5). Furthermore, protein kinase C δ, which has the most divergent activation

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loop sequence, does not require phosphorylation of the residue corresponding to Thr-500 in protein kinase C βII for the formation of mature, activable enzyme (20).

This contribution explores the role of the final phosphorylation event, that at position Ser-660, in regulating protein kinase C βII. Specifically, Ser-660 was mutated to Glu (present in two other isozymes (5)) to mimic the effect of phosphate or Ala to mimic the neutral, non-phosphorylated residue. Kinetic and binding analyses reveal that negative charge at this position markedly increases the stability of the enzyme, its affinity for substrate, and its affinity for Ca^{2+}. These data reveal that phosphorylation at this carboxyl-terminal hydrophobic motif plays a role in structuring both the kinase core and determinants in the regulatory domain.

MATERIALS AND METHODS

Bovine brain L-α-phosphatidylserine, 1-palmitoyl-2-oleoyl-phosphatidylcholine, 1-palmitoyl-2-oleoyl-phosphatidylserine, and *sn*-1,2-dioleoylglycerol were purchased from Avanti Polar Lipids, Inc. Dithiothreitol (DTT),[1] HEPES, EGTA, trypsin (type XIII from bovine pancreas 10 units μg^{-1}), and ATP were from Sigma. [γ-^{32}P]ATP (3000 Ci mmol^{-1}) and [^{3}H]dipalmitoyl phosphatidylcholine 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. Chemiluminescence SuperSignal substrates were from Pierce. Lipofectin reagent was purchased from Life Technologies, Inc. and BaculoGold DNA was from Pharmingen. A protein kinase C-selective peptide (Ac-FKKSFKL-NH_2; (21)) was synthesized by the Indiana University Biochemistry Biotechnology Facility. All other chemicals were reagent grade. A polyclonal antibody against the bacterially expressed catalytic domain of protein kinase C βII was a gift from Drs. Andrew Flint and Daniel E. Koshland, Jr.

Mutagenesis—Expression vectors encoding the cDNA sequence of protein kinase C βII with mutation of Ser-660 to Ala or Glu were made by polymerase chain reaction using protein kinase C βII in the pBluescript vector (pbluePKC) as the template. The sense primer used for both mutants was GGAGCATGCATTTTTCCG and contains an *Nsi*I restriction site. Antisense primers corresponding to the sequence around the codon for Ser-660 and containing the necessary nucleic acid changes to encode the desired mutation were CAGAGTTAACAAAG-**GC**AAATCCTTCGAATTCTG (S660A mutation) and CAGAGTTAA-CAAA**CTC**AAATCCTTCGAATTCTG (S660E mutation). These antisense primers contain an *Hpa*I restriction site. The polymerase chain reaction product and the pbluePKC template were digested with *Nsi*I and *Hpa*I (unique sites in pbluePKC), and the products were gel-purified and ligated together. The mutant protein kinase C gene was then subcloned into the pVL1393 (Invitrogen) baculovirus transfer vector using *Xba*I and *Sma*I (see below). The sequence of all mutants was verified by DNA sequencing.

Expression of Mutant Protein Kinase Cs in Sf21 Cells—Sf21 insect cells were cotransfected with the baculoviral transfer vectors encoding the protein kinase C mutants and linearized wild-type baculovirus DNA (BaculoGold, Pharmingen) by liposome-mediated transfection. Isolated recombinant baculovirus was obtained by plaque purification and amplified by two rounds of propagation in insect cells as described in a manual from Pharmingen for the BaculoGold expression system (Pharmingen, San Diego). Sf21 insect cells were then infected with high titer (1×10^8 plaque-forming units ml^{-1}) baculovirus encoding wild-type protein kinase C βII or its mutants. The cells were harvested after 3 days at 27 °C and lysed by homogenization in buffer containing 50 mM HEPES (pH 7.4), 0.2% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 85 μM leupeptin, 2 mM benzamidine, and 0.2 mM phenylmethanesulfonyl fluoride (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 (TLA 120.2, Beckman). The pellet was resuspended in lysis buffer, and lysate, supernatant (detergent-soluble), and pellet (detergent-insoluble) fractions were diluted 2-fold in glycerol and stored at −20 °C. All experiments were performed using the high speed, detergent-soluble supernatants.

Electrophoresis and Western Blots—Aliquots of cell lysate, detergent-soluble supernatant, or detergent-insoluble pellet from Sf21 cells expressing wild-type protein kinase C βII or the Ser-660 mutants were analyzed by SDS-polyacrylamide gel electrophoresis (7% polyacrylamide unless otherwise stated). The proteins were then transferred to polyvinylidene difluoride (Immobilon-P, Millipore) and probed with antibodies to the catalytic domain of protein kinase C βII and peroxidase-conjugated secondary antibodies. Labeling was detected using chemiluminescence.

Protein Kinase C Activity—Protein kinase C activity in 1–3 μl 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^{2+}, as described (22). The reaction mixture contained 50 μM (or indicated amounts) protein kinase C-selective peptide in 20 mM HEPES (pH 7.5 at 30 °C), 1 mM DTT, 100 μM [γ-^{32}P]ATP (unless otherwise indicated), 5 mM $MgCl_2$, and either 0.5 mM Ca^{2+} and lipid (sonicated dispersion of brain phosphatidylserine (140 μM) and diacylglycerol (3.8 μM), prepared as described (22)) or 0.5 mM EGTA in a final volume of 80 μl. Samples were incubated at 30 °C for 4–6 min and quenched by the addition of 25 μl of a solution containing 0.1 M ATP and 0.1 M EDTA (pH 8–9). Aliquots (85 μl) were spotted on P81 ion-exchange chromatography paper and washed four times with 0.4% (v/v) phosphoric acid, followed by a 95% ethanol rinse, and ^{32}P incorporation was detected by liquid scintillation counting in 5 ml of scintillation fluid (Biosafe II, Research Products International Corp.).

Protein Kinase C Membrane Binding—Sucrose-loaded large unilamellar vesicles composed of 40 mol % 1-palmitoyl-2-oleoyl-phosphatidylserine, 55 or 60 mol % 1-palmitoyl-2-oleoyl-phosphatidylcholine, and 0 or 5 mol % diacylglycerol and containing trace [^{3}H]dipalmitoyl phosphatidylcholine were prepared by extrusion as described (23). Stock phospholipid concentrations were determined by phosphate analysis (24). Concentrations of lipids recovered after extrusion were calculated from radioactivity. The interaction of protein kinase C with sucrose-loaded vesicles was measured as described by Rebecchi *et al.* (25) and adopted for protein kinase C (26). Briefly, protein kinase C (15–20 μl of detergent-soluble supernatant containing wild-type or mutant protein kinase C) was incubated with vesicles (75 μM total lipid) in the presence of 100 nM to 1 mM Ca^{2+}, in buffer containing 20 mM HEPES (pH 7.5), 0.3 mg ml^{-1} bovine serum albumin, and 100 mM KCl for 5 min at 22 °C. Vesicle-bound enzyme was separated from free enzyme by centrifugation of the vesicle/enzyme mixture at $100,000 \times g$ for 30 min at 25 °C. Aliquots from the supernatant and pellet of the binding experiment were assayed under identical conditions (1–2 μl of detergent-soluble supernatant per assay), and the vesicle-associated kinase activity was calculated as described (23).

Proteolysis—Protein kinase C's sensitivity to trypsin was determined by incubating the Sf21 cell supernatants containing 10–20 ng of wild-type or mutant protein kinase Cs in a total volume of 90 μl (containing 20 mM HEPES, 0.3 mM Ca^{2+}, with or without brain phosphatidylserine (250 μM) and diacylglycerol (7 μM)) in the presence of 0–20 units ml^{-1} trypsin, as indicated in the legend to Fig. 5. Proteolysis was carried out for 10 min at 30 °C and stopped by addition of 30 μl of SDS loading buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (9% polyacrylamide) and followed by Western blot analysis using antibodies that recognize the catalytic domain of protein kinase C βII.

Free Calcium Determinations—Concentrations of free Ca^{2+} were calculated using a program provided by Dr. Claude Klee (27) that takes into account pH, Ca^{2+}, Mg^{2+}, K^+, Na^+, EGTA, EDTA, and ATP concentrations.

Data Analysis—The dependence of protein kinase C's membrane binding on Ca^{2+} concentration was analyzed by a nonlinear least-squares fit to the Hill equation using the program Sigma Plot. The dependence of protein kinase C activity on ATP or substrate concentrations was analyzed by Michaelis-Menten kinetics using the program Sigma Plot. Apparent membrane association constraints were calculated by determining the ratio of bound protein kinase C:free protein kinase C divided by the total lipid concentration, as described (26).

RESULTS

Wild-type protein kinase C and the two carboxyl-terminal phosphorylation site mutants, S660A and S660E, were expressed in insect cells to characterize the effect of charge at position 660 on the biochemical properties of protein kinase C. The Western blot in Fig. 1 shows that all three proteins were expressed at comparable levels in Sf21 cells; the antibody used labels phosphorylated and dephosphorylated protein kinase C equally (5). Lysate of cells expressing wild-type protein kinase C (L) contained a major band migrating with an apparent

[1] The abbreviation used is: DTT, dithiothreitol.

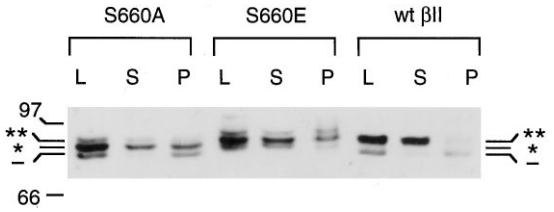

FIG. 1. **Negative charge at residue 660 alters protein kinase C βII's electrophoretic mobility.** Western blot of whole cell lysate (*L*), detergent-soluble supernatant (*S*), and detergent-insoluble pellet (*P*) from Sf21 cells expressing the S660A mutant, S660E mutant, or wild-type protein kinase C βII; blot was probed with a polyclonal antibody against protein kinase C βII's catalytic domain that recognizes phosphorylated and dephosphorylated protein kinase C with equal affinity. Each lane contained sample from approximately 5×10^3 cells. *Double asterisk* indicates the position of protein kinase C phosphorylated at both carboxyl-terminal positions (Thr-641 and Ser-660); *single asterisk* indicates the position of protein kinase C with a single phosphate on the carboxyl terminus, and the *dash* indicates the position of protein kinase C with no phosphates on the carboxyl terminus, as determined previously by mass spectrometry (5).

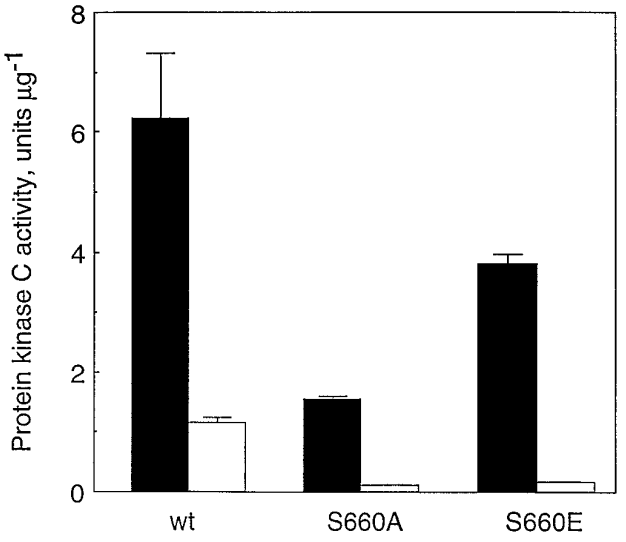

FIG. 2. **Mutation of Ser-660 decreases protein kinase C βII's specific activity measured under standard conditions.** Protein kinase C activity in the detergent-soluble supernatant from Sf21 cells was measured in the presence (*solid bars*) or absence (*open bars*) of Ca^{2+} (300 μM) and sonicated dispersions of phosphatidylserine (140 μM) and diacylglycerol (3.8 μM). ATP and peptide concentrations were 0.1 mM and 50 μM, respectively. One unit is defined as 1 nmol of phosphate incorporated per min onto the protein kinase C-selective peptide; the amount of protein kinase C was quantified by Western blot analysis relative to a standard curve with known amounts of purified protein kinase C βII. Data are expressed as mean ± S.E. for one representative experiment performed in triplicate. *wt*, wild type.

molecular mass of 80 kDa (*double asterisk*), and a minor faster-migrating form with an apparent mass of 76 kDa (*dash*). The upper band partitioned in the detergent-soluble fraction and the lower band partitioned in the detergent-insoluble fraction. Mass spectrometric analysis previously established that the upper band is quantitatively phosphorylated at the two carboxyl-terminal phosphorylation sites, Thr-641 and Ser-660, and that the lower band is not phosphorylated at either of these positions (5) (an intermediate-migrating band, not labeled detectably in wild-type lysate in Fig. 1, contains phosphate at Thr-641 but not Ser-660). We have also shown previously (5) that phosphorylation at each carboxyl-terminal position results in a distinct electrophoretic shift. In contrast, phosphorylation of Thr-500, the first phosphorylation event, does not result in a change in protein kinase C's electrophoretic mobility; in addition, only about half of the mature protein kinase C (upper-migrating band) is phosphorylated at this position (5).

The S660A mutant and S660E mutant in cell lysates (L) resolved into two main bands. For the S660A mutant, the upper main band migrated with an apparent mass of 78 kDa (indicated by *single asterisk* in Fig. 1), just below that of the fully phosphorylated (80 kDa) wild-type enzyme. This is the position where protein phosphorylated at Thr-641 but not Ser-660 migrates (5), indicating that substitution of Ser-660 with Ala mimics the electrophoretic mobility of wild-type protein with no phosphate at position 660. The lowest S660A band comigrated with the lowest band for wild type (*dash*), again indicating the equivalence of dephosphorylated Ser and Ala at position 660 in affecting the mobility of protein kinase C. For the S660E mutant, the upper band comigrated with the upper band of wild-type protein kinase C (*double asterisk*), revealing that Glu mimics phosphate in inducing an electrophoretic mobility shift. Consistent with this, the lowest S660E band migrated with an intermediate mobility (*single asterisk*). Thus, negative charge at position 660 decreases the electrophoretic mobility of protein kinase C, with Glu or phosphate causing the same mobility change. Curiously, a faintly stained band above the mature protein kinase C was apparent in Western blots of S660A and S660E, suggesting the possibility of additional phosphorylation on a small fraction of the mutant protein kinase C population.

The slower-migrating band for both mutants was preferentially, but not exclusively, localized in the detergent-soluble fraction (S). In particular, half of the slower-migrating (mature) band of S660A partitioned in the detergent-soluble fraction and half partitioned in the detergent-insoluble fraction (P). This contrasted with the slowest-migrating (mature) form of wild-type protein kinase C which partitioned almost exclusively in the detergent-soluble fraction. Thus, Ala at position 660 decreased the solubility of mature protein kinase C. As observed for wild-type enzyme, the faster migrating form of S660A, containing no phosphate at Thr-641, partitioned almost entirely in the detergent-insoluble fraction. A fraction of the mature S660E also partitioned in the detergent-insoluble fraction (P). However, in marked contrast to both wild-type enzyme and the S660A mutant, a significant fraction (65%) of the fastest migrating band of S660E partitioned in the detergent-soluble fraction. This indicates that protein with charge at Ser-660, but not Thr-641 (*i.e.* faster migrating S660E band), has an increased solubility compared with non-phosphorylated wild-type enzyme; conversely, protein with negative charge at Thr-641 but not Ser-660 (*i.e.* slower migrating S660A band), has a decreased solubility compared with fully phosphorylated wild-type enzyme. Lysis and fractionation in the absence of detergent revealed similar partitioning of the mature wild-type protein kinase C in the soluble fraction, revealing that it is localized in the cytosol (data not shown). Thus, negative charge at position 660 promotes the release of protein kinase C from the detergent-insoluble fraction into the cytosol.

In the following experiments, the biochemical properties of wild-type and mutant protein kinase Cs recovered in the detergent-soluble fraction of cell lysates were characterized. Importantly, the wild-type protein kinase C in these extracts was quantitatively phosphorylated on Ser-660, as assessed by its migration as a single 80-kDa band on SDS-polyacrylamide gel electrophoresis and as confirmed previously by mass spectrometry (5).

Fig. 2 shows that the baculovirus-expressed mutants displayed lipid-dependent activity when assayed under standard conditions (see "Materials and Methods"); however, the specific activity of the mutants was reduced relative to wild type. Under these assay conditions, S660A phosphorylated a syn-

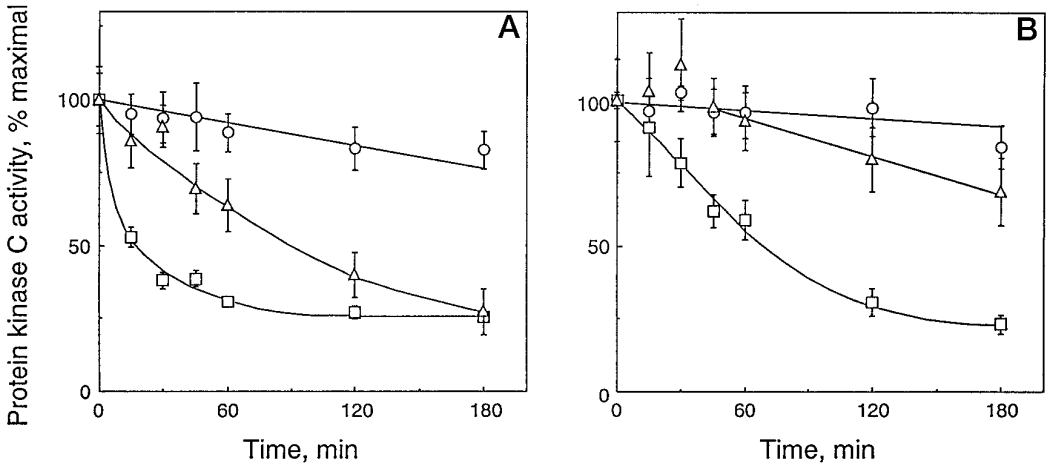

FIG. 3. **Mutation of Ser-660 affects protein kinase C βII's thermal stability.** Activity of wild-type protein kinase C βII (○), S660A (□), and S660E (△) toward the protein kinase C-selective peptide (0.5 mM) was measured after the indicated incubation times, at 22 °C, in buffer containing 0.3 mM free Ca^{2+} in the presence (*A*) or absence (*B*) of phosphatidylserine (140 μM) and diacylglycerol (3.8 μM) membranes. The ATP concentration was 0.1 mM. Data are expressed as the weighted average of three separate experiments performed in triplicate.

thetic peptide based on the myristoylated alanine-rich C kinase substrate (MARCKS) protein (protein kinase C-selective peptide (21)) at approximately 25% of the rate of wild-type enzyme, and S660E phosphorylated the peptide at approximately 61% of the rate of wild-type enzyme.

To explore whether the reduced activity of the mutants arose because of decreased thermal stability, activity was examined as a function of incubation time in the presence or absence of lipid cofactors. Fig. 3*A* shows that wild-type protein kinase C (○) was relatively stable when incubated at 22 °C, losing only 20% of its activity after a 3-h incubation; no significant difference was observed in the presence or absence of lipid cofactors. In contrast, S660A (□) was thermally labile, losing half of its activity after a 20-min incubation in the presence of lipid (*A*) and 60 min in the absence of lipid (*B*). S660E (△) displayed intermediate stability, losing half of its activity after approximately 90 min incubation in the presence of lipid (*A*); as with S660A, the rate of activity loss of S660E was approximately three times slower in the absence of lipid (*B*). This loss of activity did not result from proteolysis, as assessed by analysis on polyacrylamide gels, nor did it result from dephosphorylation: inclusion of microcystin in incubation mixtures had no effect on the loss of activity (data not shown). Thus, Ala at position 660 markedly decreased the thermal stability of protein kinase C, with Glu at that position partially protecting against destabilization. In addition, the active, lipid-bound conformation of protein kinase C was significantly more thermally labile than the inactive, unbound conformation.

To address whether the 660 mutants were impaired catalytically, or whether the decreased activity observed in Fig. 2 arose because the enzyme was so labile, activity was measured under conditions promoting protein stability: in the presence of increasing concentrations of glycerol. Fig. 4 shows that the specific activity of both mutants approached that of wild-type as glycerol concentrations were raised. Specifically, inclusion of 20% glycerol in the reaction mixture increased the specific activity of S660A and S660E to 70 ± 10 and 90 ± 20%, respectively, of the wild-type specific activity assayed under the same conditions. Similarly, the specific activities of the mutants were closer to that of wild-type when assayed on ice (data not shown). Thus, the decreased activity in Fig. 2 arises because of instability of the mutants rather than impaired catalysis.

We next tested whether the mutants underwent the same conformational change upon membrane binding as wild-type protein kinase C. The hinge separating the regulatory and

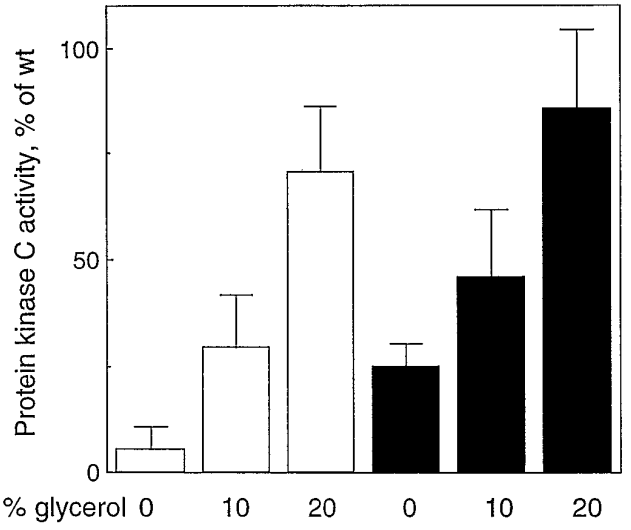

FIG. 4. **Glycerol stabilizes Ser-660 mutants.** Protein kinase C activity in the detergent-soluble fraction of cells expressing S660A (*open bars*) or S660E (*solid bars*) was measured in the presence of the indicated amounts of glycerol and in the presence of 1 mM ATP and 2.6 mM protein kinase C-selective peptide. Data were normalized to the amount of protein kinase C based on Western blot analysis and are expressed as the percent of wild-type specific activity assayed under identical conditions. Data are the means ± S.E. for a representative experiment performed in triplicate.

catalytic moieties of protein kinase C is sensitive to trypsin, and this sensitivity increases at least 10-fold upon membrane binding (28).[2] Fig. 5*A* shows that when wild-type, S660A, or S660E enzymes were incubated in the presence of lipid, 0.2 units ml^{-1} trypsin resulted in proteolysis of most of the native enzyme and the appearance of the approximately 50-kDa catalytic domain fragment (*lane 3*); no intact protein kinase C was apparent after incubation with 2 units ml^{-1} trypsin (*lane 4*). In the absence of lipid, higher concentrations of trypsin were required to observe similar proteolysis; intact protein kinase C was still apparent after incubation with 2 units ml^{-1} trypsin (*lane 9*) but was not apparent after incubation with 20 units ml^{-1} trypsin (*lane 10*). Thus, the hinge of the S660A and S660E

[2] The binding-dependent increase in hinge proteolytic sensitivity is greater for pure protein kinase C (up to 100-fold increase) compared with that for protein kinase C in cell extracts (typically 10-fold increase).

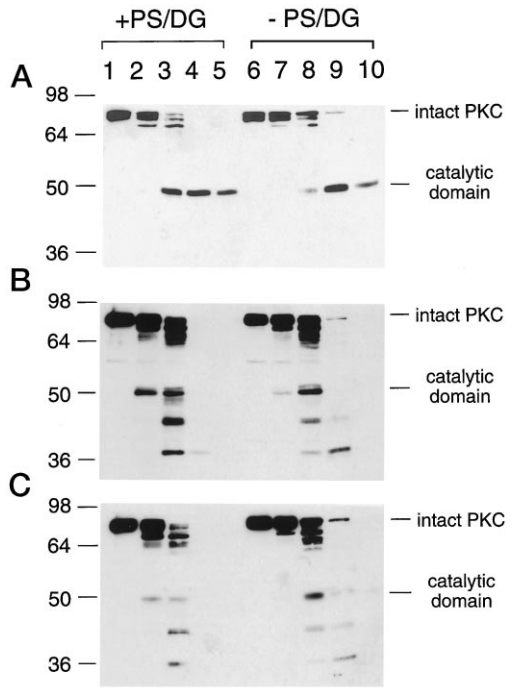

FIG. 5. **Proteolytic sensitivity of wild-type and mutant protein kinase Cs.** Western blots showing the detergent-soluble fraction from cells expressing wild-type protein kinase C βII (*A*), S660A (*B*), or S660E (*C*) protein kinase C (*PKC*) after treatment with 0 (*lanes 1* and *6*), 0.02 units ml^{-1} (*lanes 2* and *7*), 0.2 units ml^{-1} (*lanes 3* and *8*), 2 units ml^{-1} (*lanes 4* and *9*), or 20 units ml^{-1} (*lanes 5* and *10*) trypsin in the presence (*lanes 1–5*) or absence (*lanes 6–10*) of lipid cofactors (250 μM phosphatidylserine and 7 μM diacylglycerol, *PS/DG*). Blots were probed with a polyclonal antibody that recognizes the catalytic domain of protein kinase C, described in the legend to Fig. 1. Molecular weight markers are indicated on the *left*.

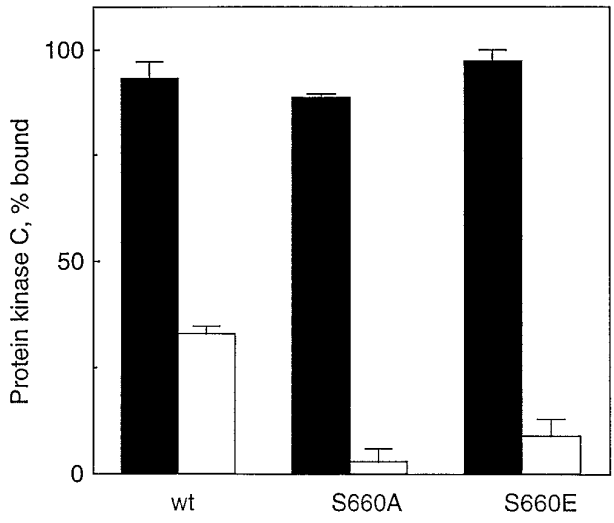

FIG. 6. **Effect of negative charge at residue 660 on protein kinase C βII's membrane interaction.** The binding of protein kinase C to large unilamellar vesicles containing 40 mol % phosphatidylserine and 60 mol % phosphatidylcholine (*open bars*) or 40 mol % phosphatidylserine, 55 mol % phosphatidylcholine, and 5% diacylglycerol (*solid bars*) was measured as described under "Materials and Methods." Data show the percent of total protein kinase C associated with the vesicles and are expressed as the mean ± range of two separate experiments. *wt*, wild type.

mutants undergoes the same membrane binding-dependent exposure as the hinge of protein phosphorylated at Ser-660. One difference between the mutants and wild-type protein kinase C was, however, noted: the cleaved catalytic domain was considerably more sensitive to further proteolysis for the mutants compared with wild-type enzyme. Although the cleaved catalytic fragment of wild-type protein kinase C was relatively resistant to further proteolysis (see Fig. 5*A, lanes 3–5*), the catalytic fragment of both mutants was rapidly degraded (Fig. 5, *B* and *C, lanes 3–5*). In summary, the hinge of both mutants became exposed in the presence of phosphatidylserine and diacylglycerol, consistent with undergoing the same conformational change as wild-type enzyme upon membrane binding. However, the proteolytic sensitivity of the cleaved catalytic domain of the mutants was increased, indicating that phosphate on Ser-660 stabilizes the kinase core.

The effect of mutation of Ser-660 on protein kinase C's affinity for membranes was examined in Fig. 6. Wild-type, S660A, and S660E bound phosphatidylcholine vesicles containing 5 mol % diacylglycerol and 40 mol % phosphatidylserine to comparable levels (Fig. 6, *solid columns*); analysis of the binding data in Fig. 6 revealed apparent membrane association constants on the order of 10^5 M^{-1} for all three protein kinase Cs. In the absence of diacylglycerol, membrane binding was significantly decreased for wild-type protein kinase C (*open columns*); analysis of data revealed a 30-fold drop in the apparent membrane association constant, consistent with previous reports (26, 29, 30). This decrease was even more pronounced for the two mutants; these displayed an approximately 250-fold drop in membrane affinity in the absence of diacylglycerol. Thus, mutation of Ser-660 to Ala or Glu caused an approximately 10-fold decrease in protein kinase C's low affinity membrane interaction that occurs in the absence of diacylglycerol but did not alter the biologically relevant high affinity interaction that is induced by diacylglycerol.

Fig. 7 shows that mutation of Ser-660 to Ala caused a marked decrease in protein kinase C's affinity for Ca^{2+}. Specifically, the concentration of Ca^{2+} required for half-maximal binding to membranes was 7-fold higher for S660A (1.8 ± 0.3 μM) compared with wild-type (0.25 ± 0.03 μM) protein kinase C (Fig. 7*A*). Glu partially mimicked the effect of phosphate in increasing protein kinase C's Ca^{2+} affinity, with half-maximal binding requiring 0.67 ± 0.05 μM Ca^{2+}. Similarly, the concentration of Ca^{2+} eliciting half-maximal activation was higher for the S660A mutant compared with wild-type enzyme (Fig. 7*B*). These data reveal that the negative charge on the carboxyl terminus of protein kinase C contributes to the interaction of protein kinase C with Ca^{2+}.

The data in Fig. 7*B* reveal that the Ser-660 mutants had comparable activity to wild-type enzyme in this experiment. In addition to the increased activity promoted by having saturating concentrations of peptide substrate, we have found that the mutants are considerably more active when bound to the large unilamellar vesicles used in the assay in Fig. 7. Thus, binding to membranes containing 40 mol % phosphatidylserine and 5 mol % diacylglycerol stabilizes the Ser-660 mutants; this stabilization is not observed when protein kinase C interacts with the multilamellar phosphatidylserine in the sonicated lipid dispersion used in standard assays (*e.g.* Fig. 2).

The effect of mutation of Ser-660 on protein kinase C's K_m for ATP was explored in Fig. 8. Analysis of data from six separate experiments revealed that mutation of Ser-660 to Ala caused a 3-fold increase in the K_m of the enzyme for ATP, from 37 ± 1 to 100 ± 10 μM. In contrast, mutation of this residue to Glu had no significant effect on the K_m (44 ± 3 μM). The K_m for ATP for all three proteins was the same whether measured in the presence of 50 μM substrate (Fig. 8) or 500 μM substrate (data not shown). The K_m for peptide substrate was also increased upon removal of the negative charge at Ser-660 (Fig. 9). Compilation of data from six separate experiments revealed that mutation of Ser-660 to Ala resulted in a 5-fold increase in the K_m for

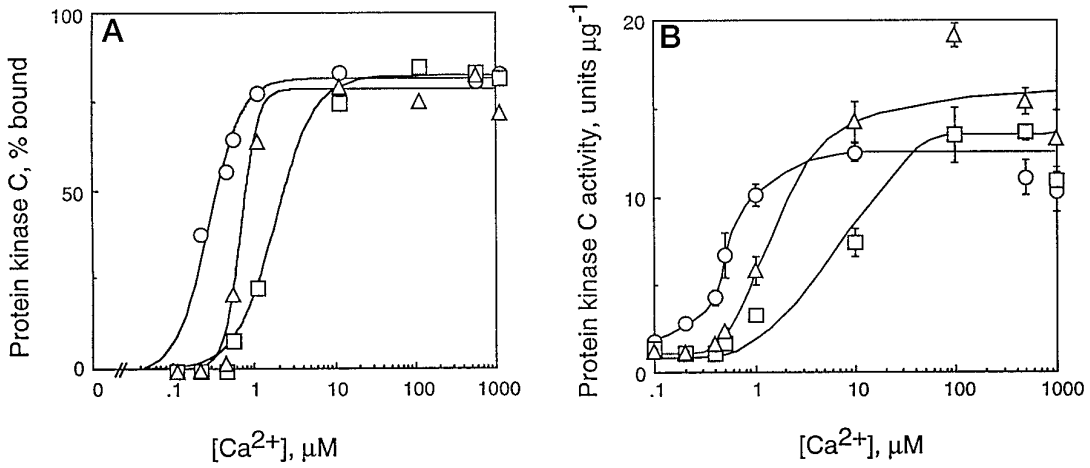

FIG. 7. **Negative charge at residue 660 increases protein kinase C's affinity for Ca^{2+}.** Membrane binding (*A*) and activity of wild-type protein kinase C βII (*B*) (○), S660A (□), and S660E (△) were measured as a function of the indicated free Ca^{2+} concentrations, in the presence of large unilamellar vesicles (see "Materials and Methods") containing 5 mol % diacylglycerol, 40 mol % phosphatidylserine, and 55 mol % phosphatidylcholine. Activity was measured in the presence of 0.1 mM ATP and 0.5 mM protein kinase C-selective peptide. Data are expressed as units of activity per μg of protein kinase C, as described in the legend of Fig. 2; points show the mean ± S.E. of a representative experiment assayed in triplicate.

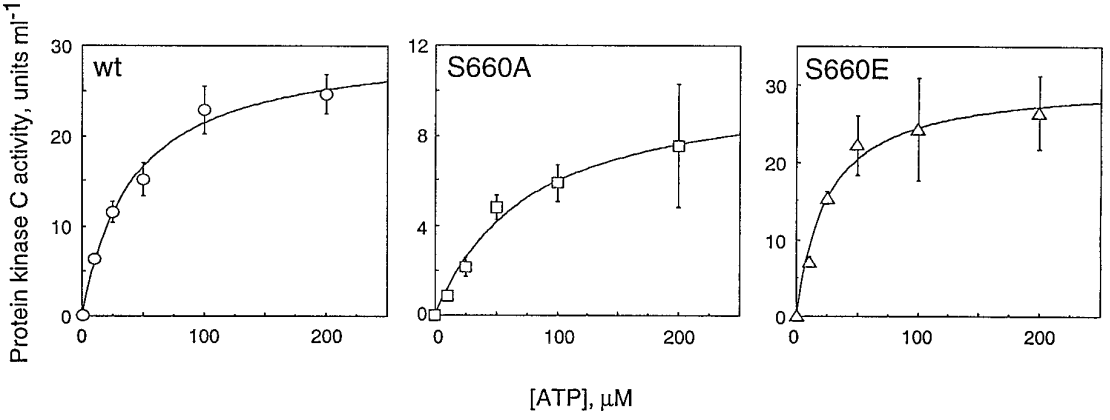

FIG. 8. **Negative charge at residue 660 increases protein kinase C's affinity for ATP.** Protein kinase C activity in the detergent-soluble supernatant from Sf21 cells was measured as a function of ATP concentration in the presence or absence of lipid (140 μM phosphatidylserine and 38 μM diacylglycerol) and Ca^{2+} (0.3 mM). The protein kinase C-selective peptide concentration was 50 μM. Data show the cofactor-dependent activity, expressed as units of activity per ml of supernatant, and represent the mean ± S.E. for a representative experiment performed in triplicate.

peptide substrate (280 ± 20 μM for S660A compared with 57 ± 3 μM for wild-type), whereas mutation to Glu had no significant effect on the K_m for substrate (45 ± 3 μM). Thus, negative charge at position 660 increases the apparent binding of both ATP and peptide substrate, either by increasing the enzyme's affinity for these substrates or by increasing the catalytic rate constant.

DISCUSSION

The foregoing studies reveal that phosphorylation at the conserved hydrophobic motif, F*XX*F(*S*/*T*)(F/Y), on protein kinase C's carboxyl terminus affects both the kinase core and regulatory domain of protein kinase C. Specifically, phosphate at position 660 in protein kinase C βII increases the thermal stability of the kinase, increases the proteolytic stability of the kinase core, and increases the enzyme's apparent affinity for ATP and peptide substrate. In addition to these changes involving the kinase domain, phosphate at Ser-660 increases the enzyme's affinity for Ca^{2+} by almost an order of magnitude. It also increases the enzyme's affinity for phosphatidylserine but does not alter the enzyme's high affinity membrane interaction that is induced by binding diacylglycerol. Thus, phosphorylation at this conserved motif appears to play a key role in structuring protein kinase C for higher affinity binding of substrates to the active site, for higher affinity binding of Ca^{2+} and phosphatidylserine to determinants in the regulatory region, and for increased stability.

For most parameters examined, Glu was an effective mimic of phosphate. However, for some parameters, the S660E mutant displayed properties intermediate between those of the S660A mutant and the phosphorylated wild-type. Thus, the single negative charge of the carboxylate, and perhaps distance and orientational constraints, may have decreased the strength of stabilizing interactions normally formed by the phosphorylated carboxyl terminus.

Negative Charge at Position Ser-660 Alters Protein Kinase C's Electrophoretic Mobility—Analysis of the Ser-660 mutants revealed that the negative charge of Glu mimics the effect of phosphate in decreasing the electrophoretic mobility of protein kinase C. The precursor (non-phosphorylated) S660A in the detergent-insoluble fraction of cells comigrates with the wild-type precursor, whereas the S660E precursor migrates with a slower mobility, mimicking one phosphorylation event. Similarly, the mature S660A (in this case phosphorylated at Thr-500 (no effect on electrophoretic mobility) and Thr-641, with no modification possible at Ser-660) migrates faster than mature wild-type protein kinase C, presumably because there is no

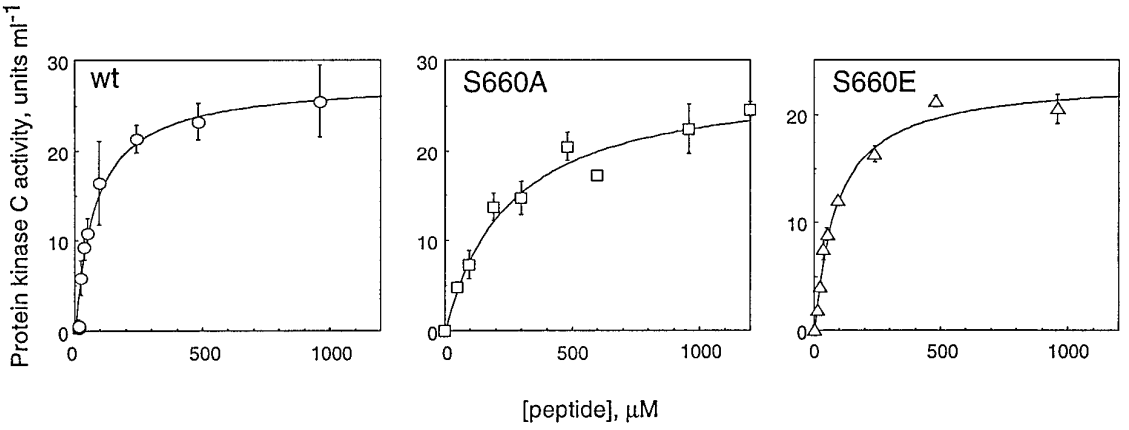

FIG. 9. **Negative charge at position 660 increases protein kinase C's affinity for peptide substrate.** Protein kinase C activity in the detergent-soluble supernatant from Sf21 cells was measured as a function of selective peptide (see "Materials and Methods") concentration; phosphorylation mixtures contained 0.1 mM ATP and phosphatidylserine (140 μM) and diacylglycerol (3.8 μM). Activity in the absence of peptide was subtracted from each point; this activity accounted for less than 10% of the activity in the presence of the highest peptide concentration. Data are expressed as units of activity per ml of supernatant and are the mean ± S.E. of a representative experiment performed in triplicate.

negative charge at position 660. Consistent with this, mature S660E comigrates with mature wild-type protein kinase C, presumably because S660E and wild-type have negative charge at position 660. Using selective dephosphorylation and antibodies selective for dephosphorylated Ser-660, we showed previously that phosphorylation of Ser-660 causes a decrease in protein kinase C's electrophoretic mobility. The present study confirms that one of the mobility shifts observed during the processing of protein kinase C arises from a negative charge on residue 660.

Negative Charge on Ser-660 Affects Protein Kinase C's Partitioning—Fractionation studies revealed that negative charge at position 660 promotes the partitioning of protein kinase C in the detergent-soluble fraction of cells. In particular, a fraction of the precursor S660E (lowermost band, consistent with no phosphorylation of Thr-641) partitioned in the detergent-soluble fraction, revealing increased solubility as a result of negative charge at position 660. Conversely, a fraction of the mature S660A (uppermost band, consistent with phosphorylation of Thr-500 and Thr-641) partitioned in the detergent-insoluble fraction, indicating decreased solubility. These results are consistent with the final phosphorylation event, phosphorylation of Ser-660, regulating the release of protein kinase C from a cytoskeletal anchorage into the cytosol.

Pulse-chase experiments by Fabbro and coworkers (31) showed that protein kinase C α is initially associated with the detergent-insoluble cell fraction as a faster migrating, dephosphorylated form and is chased into a slower migrating form that partitions in the detergent-soluble fraction. However, Parker and coworkers (32) have recently suggested that newly synthesized (non-phosphorylated) protein kinase C localizes to the cytosol where it is phosphorylated, and subsequent dephosphorylation of mature enzyme results in association with the detergent-insoluble fraction. Consistent with the former finding, mutation of the first carboxyl-terminal phosphorylation site to Ala in protein kinase C βI (Thr-642, corresponding to Thr-641 in protein kinase C βII) traps precursor kinase in the detergent-insoluble fraction (18). A similar result is observed when all potential phosphorylation sites around Thr-641 are mutated in protein kinase C βII; mutation to Ala of Thr-634, Thr-641, and Ser-654 (absent in protein kinase C βI) traps precursor enzyme as a faster migrating form in the detergent-insoluble fraction. Mutation of only Thr-634 and Thr-641 results in a compensatory phosphorylation as evidenced by an additional electrophoretic mobility shift and phosphatase sensitivity experiments[3]; this compensatory phosphorylation could account for a report showing that mutation of the corresponding Thr-638 to Ala does not significantly affect the function of protein kinase C α and results in mature protein with the same electrophoretic mobility as wild-type enzyme (19). Although it is possible that these carboxyl-terminal mutations generate an insoluble enzyme because it is not properly folded, the increased partitioning of partially phosphorylated S660E in the detergent-soluble fraction described in this contribution is consistent with this final phosphorylation event releasing protein kinase C into the cytosol.

Negative Charge on Ser-660 Stabilizes the Kinase Core—Kinetic analyses revealed that negative charge at position 660 has a marked effect on parameters relating to the structure of the enzyme. That is, negative charge provided by phosphate or Glu at this position decreased the enzyme's K_m for ATP and peptide substrate, suggesting that it participates in structuring the active site. Further evidence that phosphorylation of Ser-660 structures the kinase core derives from the finding that mutation of Ser-660 increases the proteolytic sensitivity of the kinase domain, as well as decreasing the thermal stability of the intact enzyme. It is noteworthy that Glu was a poor mimic of phosphate in stabilizing the cleaved catalytic domain from further proteolysis; Glu did, however, partially protect protein kinase C from thermal denaturation.

In the case of protein kinase A, phosphorylation of a carboxyl-terminal residue, Ser-338 (analogous to Thr-641 in protein kinase C βII), also plays a key role in structuring the catalytic subunit. Determination of the crystal structure revealed that phosphate at this position anchors the carboxyl terminus to the top of the ATP-binding lobe of the kinase, maintaining it away from the active site (33). Mutation of this Ser to Ala, and to a lesser extent Glu, markedly decreases the enzyme's stability (9). By analogy, the corresponding phosphorylated position in protein kinase C βII (Thr-641), perhaps assisted by the phosphate on Ser-660, may anchor protein kinase C's carboxyl terminus away from the active site (see Fig. 10). For protein kinase C, autophosphorylation of these residues appears to be intramolecular (13, 14), indicating that prior to phosphorylation the carboxyl terminus accesses, or possibly binds, the active site. Thus, a prime function of carboxyl-terminal phosphorylation of protein kinases, in general, may be to stabilize

[3] A. S. Edwards, L. M. Keranen, and A. C. Newton, unpublished data.

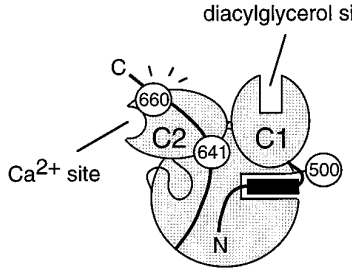

FIG. 10. **Schematic showing possible role of protein kinase C's carboxyl-terminal phosphorylations in stabilizing the kinase core and participating in Ca^{2+} binding in the regulatory domain.** The two membrane-targeting modules in the regulatory moiety of protein kinase C are indicated as follows: the C1 domain that binds diacylglycerol and the C2 domain that binds Ca^{2+} (for conventional protein kinase Cs) and acidic phospholipids. The *thin S-shaped line* depicts the hinge that connects the kinase domain to the C2 domain. The *large circle* represents the kinase core, and the *open rectangle* represents the substrate-binding cavity; the amino-terminal pseudosubstrate (*black rectangle*) occupies this site in the absence of ligand binding to the C1 and C2 domains (8). The first phosphorylation event, phosphorylation of Thr-500 near the entrance to the active site, has been proposed to correctly align residues for catalysis (8). The subsequent phosphorylations on the carboxyl terminus are proposed to tether this stretch of peptide away from the active site (by analogy with protein kinase A), with phosphate on Ser-660 contributing both to stabilizing the compact structure of the kinase core as well as participating directly (by contributing coordinating ligand) or indirectly (by structuring Ca^{2+} site) to Ca^{2+} binding.

the kinase core by tethering the carboxyl terminus away from the active site.

Curiously, treatment of mature protein kinase C with protein phosphatase 2A dephosphorylates Ser-660 (and Thr-500) to yield a protein with phosphate only on Thr-641 that is fully active under standard assay conditions (15). The finding that the mature S660A mutant (*i.e.* phosphorylated on Thr-641) is so thermally labile that only partial activity is observed under standard assay conditions suggests that mature protein that has been dephosphorylated on Ser-660 is not entirely equivalent to protein that has never had negative charge at Ser-660. Possibly tethering of the carboxyl terminus proposed above requires phosphorylation at both carboxyl-terminal positions, and once tethered, dephosphorylation of Ser-660 does not have as significant an effect on the interaction as having no charge at that position during the maturation. It should be noted, however, that wild-type enzyme dephosphorylated at Ser-660 is unable to re-autophosphorylate when lipid is presented in the form of Triton X-100 mixed micelles (15), consistent with the reduced affinity for cofactors reported for the S660A mutant in this contribution.

Negative Charge on Ser-660 Regulates Ca^{2+} Binding Site—A surprising finding from this research was that phosphorylation of Ser-660 affects determinants in the regulatory region of protein kinase C. Specifically, phosphate at position 660 increases protein kinase C's affinity for Ca^{2+} by almost an order of magnitude compared with Ala at that position and about 2-fold compared with Glu. In addition, phosphate at this position increases protein kinase C's low affinity interaction with phosphatidylserine by an order of magnitude. These data suggest that phosphate on Ser-660 may form stabilizing contacts with determinants in the C2 domain of protein kinase C (see Fig. 10). This domain contains the Ca^{2+} binding site, an aspartate-lined mouth formed by two ends of a β strand-rich domain, as well as determinants for binding acidic phospholipids (8). In contrast, there is no significant difference in membrane affinity for the mutants and wild-type enzyme in the presence of diacylglycerol. Diacylglycerol's binding site is in the C1 domain, a separate membrane-targeting domain that is not allosterically regulated by the Ca^{2+} site (34); presumably when this domain drives the membrane interaction, the contribution of Ser-660 in allowing the C2 domain to bind to membranes is too small to be apparent or, alternatively, the binding of diacylglycerol may compensate for conformational changes ordinarily mediated by the negative charge at position 660.

One possible explanation for the effect of negative charge at Ser-660 on C2 domain interactions is that the phosphate (or Glu) at position 660 structures part of the Ca^{2+} binding site by providing coordinating ligands for Ca^{2+}. Another possibility is that phosphorylation of Ser-660 induces a conformational change that structures the Ca^{2+} binding site for higher affinity binding of Ca^{2+}. The possibility that the carboxyl terminus regulates the Ca^{2+} binding site is supported by two findings. First, protein kinase C βI and βII, which differ only in the carboxyl-terminal 50 residues, have different Ca^{2+} affinities.[4] Second, a regulatory domain-directed RNA inhibitor binds protein kinase C βI and βII with different affinities ((35),[4] suggesting this molecule binds a surface containing determinants shared by the regulatory domain and carboxyl terminus. The findings that low affinity membrane binding and Ca^{2+} binding, both of which are mediated by the C2 domain, are of decreased affinity for the S660A mutant are consistent with carboxyl-terminal interactions regulating the Ca^{2+} binding (C2) domain.

Hydrophobic Phosphorylation Motif—The phosphorylation motif of a Ser or Thr bracketed by two aromatic hydrophobic residues occurs at the carboxyl terminus of a number of kinases, presenting the possibility that phosphorylation here might provide a general mechanism for structuring protein kinases. For example, protein kinase B (Akt) is markedly stimulated by phosphorylation of the Ser in its hydrophobic motif (11). Unlike protein kinase C, this phosphorylation results from cell stimulation and is catalyzed by another kinase. Similarly, S6 kinase has a hydrophobic phosphorylation motif at the carboxyl terminus of its kinase core; rapamycin treatment of Swiss 3T3 cells promotes the dephosphorylation of the Ser at this position, an event that is accompanied by inactivation of the kinase (10). Thus, phosphorylation at this hydrophobic phosphorylation motif may provide one mechanism for anchoring the carboxyl termini of kinases in such a way as to lock the enzymes in a catalytically favorable conformation.

Conclusion—In summary, studies with protein kinase C βII reveal that the role of the hydrophobic phosphorylation motif F*XX*F(*S*/*T*)(F/Y) is to stabilize both the kinase core and regulatory moiety of protein kinase C. The possibility that phosphate here tethers the carboxyl terminus away from the active site, providing interactions with determinants in the regulatory moiety, is supported by the finding that phosphate at Ser-660 increases the enzyme's affinity for Ca^{2+} and promotes substrate binding.

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