The Last 10 Amino Acid Residues beyond the Hydrophobic Motif Are Critical for the Catalytic Competence and Function of Protein Kinase Ca*

The segment C-terminal to the hydrophobic motif at the V5 domain of protein kinase C (PKC) is the least conserved both in length and in amino acid identity among all PKC isozymes. By generating serial truncation mutants followed by biochemical and functional analyses, we show here that the very C terminus of PKCα is critical in conferring the full catalytic competence to the kinase and for transducing signals in cells. Deletion of one C-terminal amino acid residue caused the loss of ~60% of the catalytic activity of the mutant PKCα, whereas deletion of 10 C-terminal amino acid residues abrogated the catalytic activity of PKCα in immune complex kinase assays. The PKCα C-terminal truncation mutants were found to lose their ability to activate mitogen-activated protein kinase, to rescue apoptosis induced by the inhibition of endogenous PKC in COS cells, and to augment melatonin-stimulated neurite outgrowth. Furthermore, molecular dynamics simulations revealed that the deletion of 1 or 10 C-terminal residues results in the deformation of the V5 domain and the ATP-binding pocket, respectively. Finally, PKCα immunoprecipitated using an antibody against its C terminus had only marginal catalytic activity compared with that of the PKCα immunoprecipitated by an antibody against its N terminus. Therefore, the very C-terminal tail of PKCα is a novel determinant of the catalytic activity of PKC and a promising target for selective modulation of PKCα function. Molecules that bind preferentially to the very C terminus of distinct PKC isozymes and suppress their catalytic activity may constitute a new class of selective inhibitors of PKC.

Protein kinase C (PKC)³ is a family of at least 11 members of serine/threonine protein kinases that play a key role in trans-
matured PKC is kept inactive via autoinhibition in which the pseudosubstrate segment binds to the substrate-bind site. Finally, the catalytically competent but inactive PKC is released to the cytosol and is ready to be activated by secondmessengers generated by the stimulation of extracellular cues.

Despite remarkable achievements in the understanding of the biochemistry and cell biology of PKC in the last 3 decades, the molecular details underlying how PKCs are regulated are still far from complete. One of the less studied domains of PKC is the V5 domain, which is a segment of about 50–70 amino acid residues C-terminal to the catalytic core (C3 and C4 domains). Although it contains the highly conserved turn motif and hydrophobic motif (FXXF/S(T/F/Y)), the V5 domain is apparently the least conserved domain in PKC (8). This low conservation of sequence homology has led some authors to postulate that the C-terminal portion of PKC has little importance in the biology of PKC (9). However, more recent studies have shown that the V5 domain is not only important in the maturation of PKC (2), but it also constitutes at least part of the determinants of isozyme-specific functions of PKC (10–12).

Analysis of the C-terminal sequence of cPKCs, PKCζ (representative of aPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC), PKCζ (representative of nPKC).
ing System (Fuji Photo Film) equipped with a cooled CCD camera that registers light emitted from chemiluminescence but not the radiation from $^{32}$P. The chemiluminescence signals were quantified using ImageGauge 4 software (Fuji Photo Film).

**Estimation of Protein Half-life**—Twenty hours post-transfection, the medium was replaced with a fresh medium containing 200 μg/ml cycloheximide or solvent control (0.1% (v/v)). In these experiments, the cycloheximide used was from a stock solution of cycloheximide that was dissolved in tissue culture grade dimethyl sulfoxide (catalog numbers C7698 and D2650, respectively; Sigma). At each time point, cells in 12-well plates were lysed with 100 μl of 1× SDS-PAGE loading buffer, boiled for 5 min, and centrifuged at 12,000 × g for 10 min. Thirty seven μl of supernatant for each sample was used for Western analysis. The zero point of the time course was defined as 4 h after the addition of cycloheximide. The amounts of PKCα in each sample were normalized by that of endogenous β-actin in Western blotting using an anti-FLAG antibody. The half-life was derived by plotting the amount of protein remaining against time on a semi-logarithmic graph as described previously (17).

**Subcellular Fractionation**—For preparation of total cell membranes, cells cultured in a 60-mm dish were lysed in 1 ml of isotonic buffer without detergent (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 5 mM EGTA, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 μM okadaic acid, and a mixture of protease inhibitors) by 30 strokes of a tight-fitting Dounce homogenizer. After 10 min of centrifugation at 1,000 × g at 4 °C, the resulting supernatant was further centrifuged at 300,000 × g for 1 h at 4 °C. Two hundred μl of the supernatant after the second centrifugation was concentrated using a chloroform/methanol precipitation method (18) and was designated as the cytosolic fraction. The pellet obtained after the ultracentrifugation was designated as total membranes (particulate fraction).

**Immune Complex Kinase Assay**—Total cell lysate was incubated with paramagnetic Dynabeads that had been coated with anti-FLAG antibodies. The immunoprecipitate complexes were washed twice with buffer B (phosphate-buffered saline (PBS) containing 1% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride), three times with buffer C (10 mM Tris, pH 7.5, and 0.5 M LiCl), twice with buffer D (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 50 mM NaCl) and once with the kinase buffer (20 mM MOPS, pH 7.4, 5 mM MgCl₂, and a mixture of protease inhibitors) by 30 strokes of a tight-fitting Dounce homogenizer. After 10 min of centrifugation at 1,000 × g at 4 °C, the resulting supernatant was further centrifuged at 300,000 × g for 1 h at 4 °C. Two hundred μl of the supernatant after the second centrifugation was concentrated using a chloroform/methanol precipitation method (18) and was designated as the cytosolic fraction. The pellet obtained after the ultracentrifugation was designated as total membranes (particulate fraction).

**Flow Cytometry**—About 1 × 10⁶ COS-1 cells were collected by trypsinization. After microcentrifugation at 200 × g for 5 min, the cell pellet was resuspended in ice-cold PBS. A single cell suspension was obtained by repeated pipetting or vortexing. Cells were fixed by dropwise addition of ice-cold ethanol (final concentration of 70%) while vortexing. Ethanol-fixed cells were stored at 4 °C overnight in the dark. Prior to propidium iodide staining, cells were centrifuged and washed with PBS. Cells were then treated with 1 ml of PBS containing 40 μl/ml propidium iodide, 1 mg/ml DNase-free RNase A, and 0.1% Triton X-100 (v/v) at 37 °C for 30 min. Stained cells were held at 4 °C for at least 1.5 h before analysis. Immediately prior to analysis, cells were passed through a Falcon 35-μm nylon mesh cell strainer cap (BD Biosciences) to remove aggregated cells. Flow cytometry analysis was performed by operators without the knowledge of the identity of each sample using an EPICS Elite flow cytometer from Beckman Coulter (Fullerton, CA) with EXPO32 software. For each histogram, at least 10,000 cells were assayed. The percentages of DNA fragmentation reflecting apoptotic cells were determined by measuring the fraction of nuclei containing a hypodiploid DNA content (sub-G₀ fraction), which is a characteristic feature of apoptosis (20).

**Homology Modeling of Bovine PKCα**—A structural alignment of human PKCα at 2.0 Å (PDB code 1XJD (21)), human PKBβ at 1.6 Å (PDB code 1O6L (22)), the catalytic subunit of bovine PKA at 2.04 Å (PDB code 1SMH (23)), and the turn/hydrophobic motif from the recently solved structure of human PKCα at 3.0 Å (24) was generated by superposing the three-dimensional coordinates according to a ClustalW (21) align-
ment of their sequences, followed by manual manipulation using QUANTA (Accelrys, San Diego, CA). To this alignment, the sequence for the catalytic domains of bovine PKCα (residues 333–672) was added and aligned. Insertions and deletions relative to the known structures were positioned in the loop regions, keeping the secondary structure elements intact. The program MODELLER (25) was then used to generate 20 initial homology models of bovine PKCα-(333–672) based on this alignment. The model with the lowest objective function was chosen as the representative model for further study.

To provide some structural basis for the positioning of the final nine residues, the PKCα sequence was also used to search the PDB using LOOOP (26) and BLAST (27). The human G protein-coupled receptor kinase 2 (28) was identified from the LOOOP search, and a short segment from isopenicillin-N synthetase (29) was identified from the BLAST search.

Models of the two deletion mutants PKCα-Δ672 and PKCα-Δ663 were generated by removing 1 and 10 residues, respectively, followed by terminal re-patching. The three models were then further optimized using CHARMM (30) to add hydrogen atoms to the protein and phosphate atoms to the known phosphorylated residues, followed by a restrained energy minimization to remove any remaining steric clashes.

**Molecular Dynamics Simulation of PKCα Models**—To probe the possibility of structural changes in the native and mutant models, the three models were subjected to a 10-ns molecular dynamics simulation using NAMD software (31) with the CHARMM22 force field.

The model structures were solvated to a distance of 8 Å from all protein atoms in a rectangular box of water. Fifteen sodium cations were added to each box to neutralize the overall negative charge of the structures. Molecular dynamics simulations were performed with a 2-fs time step at a constant temperature of 300 K and a constant pressure of 1 atm under periodic boundary conditions. The Particle Mesh Ewald method was used for electrostatics, and a 12 Å cutoff was used for van der Waals interactions. The TIP3P water model (32) was used to model the solvent.

The ensemble was minimized for 1500 steps using the conjugate gradient method. This was followed by a heating protocol where the ensemble temperature was increased from 0 to 300 K over 5 ps. Afterward, the backbone atoms of the structure were fixed, whereas the side chains and solvent were allowed to move unrestrained for a further 30 ps, followed by totally restrained equilibration for 10 ps. The production run was then performed for 10 ns, with the SHAKE algorithm (33) implemented to restrain all hydrogen motions. Coordinates were saved every 1 ps.

**Statistical Analysis**—One-way analysis of variance was used for statistical analysis. Tukey’s honestly significant difference test was used to calculate differences between means for N1E-115 neurite outgrowth. Differences were considered significant when \( p < 0.05 \).

**RESULTS**

**Generation and Characterization of PKCα Mutants**—A full-length bovine PKCα with a FLAG epitope tag fused to the second amino acid residue was cloned into a mammalian expression vector pcDNA3. A PCR-based mutagenesis strategy was used to create all the truncation and point-mutation constructs. The truncation mutants were generated by introducing an in-frame stop codon at the designated positions. For example, the PKCα-Δ672 mutant carried a stop codon at amino acid residue 672 with the C terminus of the mutant being amino acid residue 671. Table 1 shows a schematic representation of all PKCα constructs used in this study. All the PKCα constructs expressed well in COS-1 cells (data not shown). Because it had been proposed that PKC mutants lacking the C-terminal segment might be insoluble (2), it is important that we first analyzed the solubility of all our PKCα truncation and point-mutation proteins. To do this, cells transiently transfected with PKCα were lysed with buffers containing 1% Triton X-100. Detergent-soluble and -insoluble fractions were obtained after centrifugation and were then subjected to Western analysis. Consistent with reports from others (34, 35), all PKCα mutants were found in the soluble fraction, although there was a slightly increased detergent-insoluble fraction for truncation mutants in COS cells (data not shown). We further analyzed the in vivo stability of the truncation mutants of PKCα by measuring the half-life of these proteins. As shown in Table 1, removal of one amino acid residue from the C terminus had minimal impact on the apparent half-life of PKCα-Δ672, whereas the removal of 3–10 more amino acid residues resulted in a slight decrease of the apparent half-life of PKCα-Δ667, PKCα-Δ666, and PKCα-Δ663. Only the removal of 11 amino acid residues from the C terminus caused a severe reduction in the apparent half-life of PKCα-Δ662. We concluded that the truncation of the last 10 amino acid residues from the C terminus does not significantly alter the detergent solubility or the in vivo stability of the PKCα mutants.
C-terminal Activity/Function Determinant of PKCα

The Last 10 Amino Acid Residues Are Essential for the Catalytic Competence of PKCα—Next, we proceeded to analyze the catalytic activity of these mutants. After transient transfection, the FLAG-tagged PKCα was immunoprecipitated from the COS cell lysate, washed extensively with 0.5 M lithium chloride, and subjected to an in vitro immune complex kinase assay. We first tested the wild-type PKCα and truncation mutants using protamine sulfate as a substrate, as this arginine-rich protein binds PKC and releases the pseudosubstrate and thus serves an excellent cofactor-independent substrate of PKC (36–38). As shown in Fig. 3A, removal of only one amino acid at the C terminus resulted in a PKCα-Δ672 mutant that lost 60% catalytic activity. Truncating away seven amino acid residues at the C terminus produced a PKCα-Δ666 mutant that had a catalytic activity slightly lower than that of the PKCα-Δ672 mutant. Further truncation of two more amino acid residues resulted in a PKCα-Δ664 mutant with ~10% of the catalytic activity as the wild-type PKCα. Removal of the last 10 amino acid residues resulted in a PKCα-Δ663 mutant that had lost more than 95% of its catalytic activity. Given that protamine sulfate maximally activates cPKC (38), the PKCα-Δ663 mutant is a practically catalytically inactive mutant of PKCα. There was little detectable catalytic activity for the PKCα-Δ662 mutant.

We further analyzed the ability of these PKCα mutants to be activated by cofactors in assays using histone H1 as a substrate. Since the analysis of C-terminal sequences of PKCα, it is evident that a phenylalanine residue five amino acid residues C-terminal to the hydrophobic phosphorylation motif is found in all cPKC (Fig. 1). Having demonstrated that the last 10 amino acid residues are essential for the catalytic activity of PKCα, we studied whether the phenylalanine at position 663, which is 10 residues away from the C terminus, is absolutely required for the catalytic competence of PKCα. Interestingly, substitution of Phe-663 with conserved amino acid residues with aromatic side chains resulted in PKCα-F663W or PKCα-F663Y with 50% reduction of their catalytic activities toward protamine sulfate (Fig. 4A). Mutation of Phe-663 to amino acid residues with smaller side chains resulted in PKCα-F663A or PKCα-F663L with greater than 80% reduction in their catalytic activities (Fig. 4A). In contrast, mutation of Tyr-658, which constitutes the C-terminal boundary of the hydrophobic phosphorylation motif and is the least conserved among the three residues with aromatic side chain in the motif, to either Phe or Trp led to the PKCα-Y658F or PKCα-Y658W mutants with very similar catalytic activity as the wild-type enzyme (Fig. 4B). These observations, together with those presented in Fig. 3, suggest that although a phenylalanine at position of 663 makes significant contribution to the catalytic activity, it is the last nine amino acid residues at the C terminus plus Phe-663 that are collectively responsible for the full catalytic competence of PKCα.

FIGURE 3. The very C terminus of PKCα is critical for the catalytic competence of the kinase. Expression constructs encoding the wild-type or various deletion mutants of PKCα as indicated were transiently transfected into COS-1 cells. Twenty hours after transfection, the FLAG-tagged recombinant PKCα was immunoprecipitated after lysing cells with 1% Triton X-100, A, the specific activity of PKCα and truncation mutants was determined in an in vitro immune complex kinase assay using 0.67 mg/ml protamine sulfate as a substrate without cofactors after normalization of protein levels as described under “Experimental Procedures.” Results shown are mean ± S.E. of at least six independent experiments and are represented as percentage of the activity of the wild-type PKCα. A representative immunoblot used to derive specific activities is shown at the bottom. B, cofactor-activated specific activity of PKCα and truncation mutants was determined in an immune complex kinase assay with 200 μg/ml histone H1 as a substrate in the presence of 300 μM CaCl2, 40 μg/ml phosphatidylserine, 108 μg/ml phosphatidyicholine, and 1.6 μg/ml diacylglycerol after normalization of protein levels as described under “Experimental Procedures.” Results shown are mean ± S.E. of at least five independent experiments and are represented as percentage of the activity of wild-type PKCα without cofactors. Representative immunoblots indicating amount of each PKCα construct in the corresponding kinase reaction are shown at the bottom.
The C-terminal Segment beyond the Hydrophobic Motif Is Required for the Phosphorylation of the Activation Loop of PKCα—The phosphorylation of a conserved threonine residue at the activation loop in isoforms of the PKC superfamily is the first and rate-limiting step in the maturation process of this group of serine/threonine kinases (2). It has been proposed that the hydrophobic motif of PKC isoforms serves as an anchor for docking with 3-phosphoinositide-dependent kinase-1 (39). As the deletion of the C-terminal segment beyond the hydrophobic motif led to either reduced or diminished catalytic activity of PKCα (Fig. 3), we investigated the phosphorylation status at the activation loop of these mutants. To this end, the FLAG-tagged PKCα C-terminal truncation mutants were transfected into COS cells, and the truncated proteins were immunoprecipitated. Following extensive washing, Western analysis was carried out using the P500 antibody, which only reacts with a PKC if the conserved threonine at its activation loop is phosphorylated (14, 15). As shown in Fig. 5, truncation of either one or nine amino acid residues at the C terminus did not cause any overt change in the extent of phosphorylation of the activation loop of the PKCα truncation mutants (Fig. 5, 2nd to 5th lanes, top panel). Truncation of 10 or more amino acid residues at the C terminus resulted in marked underphosphorylation of the activation loop in PKCαΔΔ663, PKCαΔ662, and PKCαΔΔ661 (middle panel). Thus, Phe-663 that is five amino acid residues C-terminal to the hydrophobic motif is necessary for the productive phosphorylation or for the prevention of excessive dephosphorylation of the activation loop in PKCα.

The Last 10 Amino Acid Residues of PKCα Are Required for the Full Activation of Mitogen-activated Protein Kinase (MAPK)—By having demonstrated the importance of the very C terminus in conferring the catalytic activity to PKCα in vitro, we proceeded to study its contribution to the cellular function of PKCα. PKCα has been shown to activate MAP kinase via Raf-1 in COS-1 cells (19, 40). Therefore, we examined whether the very C-terminal segment beyond the hydrophobic motif contributes to the ability of PKCα to activate MAPK in cells. The FLAG-tagged wild-type or truncation mutants of PKCα and Myc-p42 MAPK were co-transfected into COS-1 cells.
After treating cells with TPA to activate PKC, Myc-p42 MAPK was immunoprecipitated, and its activity was assessed in an in vitro kinase assay using MBP as a substrate. Consistent with a previous report (40), activation of PKC by TPA in COS cells resulted in at least a 12-fold increase in the activity of co-transfected Myc-ERK1/2 (Fig. 6). However, under the same experimental conditions, truncating one to six amino acid residues at the C terminus of PKC/H9251 resulted in at least a 3-fold reduction in the activation of ERK1/2 by PKCαΔ672, PKCαΔ670, and PKCαΔ667 (Fig. 6). Truncation of 10 or more amino acid residues from the C terminus markedly diminished the capacity of PKCαΔ663 or PKCαΔ662 to activate ERK1/2 upon treatment of TPA (Fig. 6 and data not shown). Thus, the last 10 amino acid residues of PKCα are not only required for the catalytic competence of kinase in vitro but also critical for the activation of MAPK by PKCα in cells.
The Very C Terminus of PKCα Is Required for Providing Survival Signals—As an additional test of the requirement for the C-terminal segment beyond the hydrophobic motif of PKCα in cellular functions, we adopted a cellular model of rescuing apoptosis caused by the expression of a dominant-negative PKCα (41). Whelan and Parker (41) have shown that transfection of a dominant-negative PKCα mutant (T/A)_3 in which all the three threonine residues in the activation loop had been substituted with alanines induced apoptosis in COS-1 cells. Co-expression of wild-type PKCα can rescue the apoptotic response (41). We therefore utilized this system to study whether the last few amino acid residues at the C terminus of PKCα are required for the rescue of apoptosis. For comparison, we used the same experimental parameter, e.g. the appearance of the sub-G₁ DNA peak, as an estimation of cell death as used by Whelan and Parker (41). Consistent with previous results (41), transfection of wild-type PKCα alone reduced the base-line cell death as compared with the vector-only control, whereas the introduction of the dominant-negative PKCα mutant (T/A)_3 into COS-1 cells induced cell death (Fig. 7, A and B). When trans-

![Homology model of the catalytic domain of PKCα](image)

**FIGURE 9.** Homology model of the catalytic domain of PKCα. A, schematic representation of the catalytic domain of bovine PKCα. The N-lobe is shown in yellow, the C-lobe in red, and the V5 domain in blue. Also shown are the N-terminal Arg-333, the activation loop (green), the glycine-rich ATP-binding loop (orange), and the phosphorylated residues (red). B and C, close-ups of the interactions between the N-lobe and the V5 domain (same color scheme is used as with A). Hydrogen bonds are shown as black dotted lines. Residues involved in interactions are labeled. D, plot of RMSF of C-α atoms against the amino acid residues for the molecular dynamics simulations (10 ns) of the catalytic domain models of wild-type PKCα (red), PKCα-Δ672 (blue), and PKCα-Δ663 (green). The changes at the activation loop and the Mg²⁺ positioning loop (DFG motif, circled) observed in the molecular dynamics simulations are depicted in the inset in D.
ected alone, the PKCα-Δ672 mutant caused marginal cell death, whereas PKCα-Δ670, PKCα-Δ662, and PKCα-Δ663 induced substantial apoptosis (Fig. 7, A and B). We found that co-transfecting the wild-type PKCα could indeed suppress cell death caused by PKCα-(T/A)₃ (Fig. 7, A and B). Interestingly, the co-transfection of the PKCα-Δ672 mutant, which is only one amino acid residue shorter at the C terminus, could rescue only half of the cell death compared with its wild-type counterpart (Fig. 7, A and B). However, the PKCα-Δ670 mutant, with its last three amino acid residues deleted, could hardly rescue cell death induced by the co-transfected PKCα-(T/A)₃, whereas the PKCα-Δ663 and PKCα-Δ662 mutants were unable to rescue the cell death (Fig. 7, A and B). These results suggest that the last three amino acid residues at the C terminus are essential for PKCα to provide survival signals in COS-1 cells under the condition of loss of function of PKC.

**The Very C terminus of PKCα Is Required in Augmenting Melatonin-stimulated Neurite Outgrowth**—In the above MAPK activation assay, the pharmacological agent TPA was employed. To establish further the functional importance of the very C terminus of PKCα in a more physiological setting, we performed a third analysis. The mouse neuroblastoma cells (N1E-115) have been shown to rapidly respond to melatonin stimulation and form neurites within 24 h (42, 43). Treatment of N1E-115 cells with melatonin elicited a transient and reversible vimentin inter-five neurites within 24 h (42, 43). These results suggest that the last three amino acid residues at the C terminus are essential for PKCα to provide survival signals in COS-1 cells under the condition of loss of function of PKC.

**Computer Modeling of the Three-dimensional Structure of the Catalytic Domain of Bovine PKCα**—To understand the structural basis of the C terminus that confers the full catalytic competence of PKCα, we generated a comparative model of the catalytic domain of bovine PKCα based on the sequence homology to the known kinase structures of PKA, PKBβ, and PKCθ as well as the turn/hydrophobic motifs of PKCα. The overall structure of the model (Fig. 9A) was closely related to the template structures. A superposition of the model against these structures showed a root mean square deviation of 0.54, 0.91, 0.99, and 1.08 Å for the model against PKCθ, PKBβ, PKCα, and PKA, respectively.

The final energy-minimized model of the catalytic domain of bovine PKCα (Fig. 9A) consisted of residues 333–672. The N-lobe (residues 333–421) included one central 5-stranded β-sheet and two α-helices, one of which being the αC-helix that is required to be properly aligned for substrate binding and catalysis in the AGC family of kinases (46, 47). The C-lobe (residues 422–597) consisted of seven helices as well as the activation loop. The V5 domain (residues 598–672) extended from the C-lobe and wrapped around the C- and N-lobes on the opposite side from the catalytic cleft of the protein.

The active site of the protein was located in a large cleft between the N- and C-lobes with the following key components: the glycine-rich phosphate-binding loop (residues 346–351), the activation loop (residues 481–499), as well as the catalytic residues Lys-368, Asp-463, and Asp-481. The activation loop was stabilized in an active conformation by the phosphorylated Thr-497 that formed salt bridge interactions with the side chains of Arg-462 and Lys-486 and a hydrogen bond with the hydroxyl group of Thr-495 (not shown in figure).

The turn motif was stabilized against the N-lobe through the phosphorylated residue Thr-638 that formed salt bridges with the side chains Lys-362, Lys-371, and Arg-412 (not shown on figure) in an almost identical manner to that seen in the structure of PKCζ (48). It then formed a short helix immediately before the hydrophobic motif.

Because of the lack of structural data in the templates for the positioning of the nine C-terminal residues, we performed a search of the PDB and identified GRK2 that possesses an α-helix immediately after the hydrophobic domain. This was followed by a BLAST search of the last 20 residues of the PKCα sequence against the PDB where we identified a short helical section from residues 114 to 118 of the isopenicillin N synthase (HPRIQ) that was similar to residues 665–669 of PKCα (HIPILQ). Based on this, we decided to model the last nine residues as a helix (Fig. 9, A and B).

The structural stability of the C-terminal V5 domain of PKCα against the catalytic domain was mediated by several key interactions among residues in the V5 domain hydrophobic motif and the N-lobe (Fig. 9, B and C). The hydrophobic motif interacted with the N-lobe through two phenylalanine residues (Phe-653 and Phe-656) protruding into the hydrophobic core. Also, the phosphorylated Ser-657 on the hydrophobic motif was observed to form a salt bridge with the N-terminal Arg-333 side chain (Fig. 9B). Residues 658–660 assumed an anti-parallel β-strand conformation and formed main chain hydrogen bonds with residues 404–406 on a β-strand in the N-lobe, extending the N-lobe β-sheet by another strand. The side chains of Asn-660 and Gln-662 were also involved in the interactions that cemented the V5 domain to the N-lobe, with the amide nitrogens of both residues forming hydrogen bonds to a carboxylate oxygen atom of Asp-395 on the αC-helix (Fig. 9C).

The amide nitrogen of Asn-660 also formed a hydrogen bond to the backbone carbonyl of Leu-403. The second carboxylate oxygen atom of Asp-395 in turn formed a hydrogen bond with the peptide nitrogen of Leu-403.

**Structural Basis Underlying the Impact of the C-terminal Deletion on the Catalytic Competence of PKCα**—Molecular dynamics simulations were performed on the solvated, neutral-
ized models of PKCα-(333–672) (WT–PKCα), PKCα-Δ672, and PKCα-Δ663 for a time scale of 10 ns under constant pressure and temperature conditions. The root-mean-square fluctuation (RMSF) plot (Fig. 9D) shows the relative fluctuations of the C-α atoms across the length of the simulations in relation to their starting positions.

Simulation of the catalytic domain of the wild-type PKCα revealed that overall the structural integrity was maintained with relatively low RMSF across the length of the sequence, although such fluctuations increased for residues 600 onward (the V5 domain) (Fig. 9D). This agrees with previous reports on the crystal structures of members of the ABC kinase family in that the very C terminus beyond the hydrophobic motif in the native V5 domain is missing from the observed electron density maps (21, 22).

The activation loop was observed to undergo small changes in conformation (Fig. 9D, inset). The fluctuations of a loop in the PKCα-Δ663 mutant C-terminal to the DFG motif (residues 481–483) were much greater than those of the wild-type kinase and the PKCα-Δ672 mutant (Fig. 9D, inset). The DFG motif, which is known to function in positioning Mg2+ and bridging the β- and γ-phosphates of ATP (49), is completely conserved among all the ABC family kinases (13). It is conceivable that such dynamic changes in the activation loop and in the DFG motif region could impinge on the catalytic activities of the PKCα truncation mutants.

One region in the V5 domain that had a low RMSF consisted of residues 656–660, which corresponds to the hydrophobic motif known to interact with the hydrophobic patches at the back of the N-lobe as described previously (21, 46, 50). Another region with a low root mean square deviation centered around the turn motif residue Thr-638 (Fig. 9D and Fig. 10A). These interactions could account for the low RMSF observed in the molecular dynamics simulation for this region. The C-terminal tail region of the V5 domain was observed to be flexible across the 10-ns time scale with the modeled helix unwinding, and the C-terminal carboxylate group of Val-672 forming a salt bridge with the N-terminal amino group (Fig. 10A).

The truncation of one C-terminal residue in the PKCα-Δ672 mutant increased fluctuations in both the N-lobe and the V5 domain (Fig. 9D, blue trace). In the N-lobe, increased fluctuations were observed in turns between the anti-parallel strands (Gly-360 and Val-410). Such fluctuations could affect the catalytic activities of the enzyme. Like the wild-type PKCα (Fig. 9D), the C terminus of the V5 domain was also found to be very flexible, with a minimal fluctuation around Phe-626 and the hydrophobic motif region. The very C terminus was observed to rapidly adopt a stable interaction with the N-lobe within 2.5 ns, through hydrogen bonding of Ser-670 to Asp-339 (data not shown). A large fluctuation observed in the V5 domain between residues 643 and 648 that formed a small helical section after the turn motif caused the small helix to begin to unfold. Similar fluctuation was also found in the hydrophobic motif (Fig. 10B). We speculate that the bigger fluctuations in the extreme C terminus were caused by the lack of a salt bridge between Arg-333 and the C-terminal carboxylate group, allowing greater movements of the hydrophobic motif. Such motions were then transmitted throughout the rest of the V5 domain.

In the PKCα-Δ663 simulation, the truncation of 10 residues resulted not only in the further deformation of the V5 domain between the turn motif and the hydrophobic motif, with the small helix now completely unfolded, but also in the extension of the deformation in the region before the turn motif (Fig. 10C, blue trace). This is evident from the RMSF plot showing greater fluctuations in the V5 domain of the PKCα-Δ663 mutant (Fig. 9D, green trace). The major change in this region was brought about by the repositioning of Phe-626 that forms part of the lining of the ATP-binding pocket in the wild-type kinase. Because of the motions in the more extensively deformed V5 domain in PKCα-Δ663, Phe-626 was abstracted from the residues packing in the pocket. The movement of Phe-626 caused a major conformational change in the V5 domain before the turn motif (Fig. 10C). The small helix from residues 643–648 was found to completely unfold. The distortion of the three-dimensional structure of the kinase was further exacerbated by the closure of the ATP-binding loop, with the resultant closed loop being held by a salt bridge between Lys-338 and Asp-468 (data not shown). The higher fluctuations in the V5 domain were probably because of the motion of the last two residues at the C terminus of the PKCα-Δ663 mutant, Pro-661 and Gln-662, which did not form stable interactions with the rest of the structure during the simulation. These two residues were observed to move freely in solution. Therefore, the increased motions of the C terminus of PKCα-Δ663 were transmitted to the rest of the V5 domain, causing an overall increase in atomic fluctuation and structural deformation. As a result, the catalytic activity of the PKCα-Δ663 was lost.

**The Extreme C Terminal of PKCα Controls the Catalytic Activity of the Kinase**—If the extreme C-terminal segment of PKCα beyond the hydrophobic motif interacts with the key residues in αC-helix as suggested in our homology modeling (Fig. 9), this C-terminal tail of PKCα may be involved in the control of the catalytic activities of the kinase in addition to its known roles in the determination of the subcellular localization of PKC. If this is the case, we envisage that molecules that bind to the extreme C terminus of PKCα would interfere with the catalytic activities of this cPKC. As most commercially available isozyme-specific antibodies are generated using a peptide corresponding to the very C terminus of PKC isozymes, we immunoprecipitated the ectopically expressed wild-type PKCα either with an antibody that binds to its N terminus or with an antibody to its C terminus. This was followed by an immune complex kinase assay. As shown in Fig. 11, the catalytic activity of PKCα immunoprecipitated by the antibody against the C terminus was 5-fold lower than that of the same PKCα immunoprecipitated by the antibody against the N terminus. Given that the anti-N-terminal antibody (anti-FLAG) does not abolish the autoinhibitory effect of the N terminus of PKCα (Fig. 3B), our results suggest that the extreme C terminus of PKCα constitutes a novel determinant that controls the full catalytic activities of this cPKC.

**DISCUSSION**

The C terminus of PKCα has been known to be involved in the interaction with other cellular proteins and to contribute to subcellular localization and isozyme-specific functions (2). For
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was a concern that the truncation of the V5 domain of PKC might render such mutants insoluble (2). However, we found all the C-terminal truncation mutants of PKCα used in this study are largely soluble, which is consistent with the observations made by others (34, 55). It is surprising to find that the truncation of only one amino acid residue from the C terminus resulted in the mutant PKCα-Δ672 that lost 60% of its catalytic activity. Furthermore, the removal of the last 10 amino acid residues resulted in the mutant PKCα-Δ663 that was practically catalytically inactive (Fig. 3). Our results are in sharp contrast to some previous reports (9, 34). Su et al. (9) reported that the bovine PKCα with the deletion of 11 amino acid residues at the C terminus retained 30% catalytic activity when expressed in Saccharomyces cerevisiae. This discrepancy could derive from the different organisms used to express the bovine PKCα and the fundamentally different experimental protocols being employed. First, a mammalian PKCα expressed in the yeast may not be properly post-translationally modified. For example, recombinant glycoproteins expressed in S. cerevisiae are of the high mannose type and could be hyperglycosylated (56). Second, Su et al. (9) used crude yeast lysate containing the ectopically expressed PKC as the source of the enzyme although we used the immunoprecipitated PKCα in the in vitro kinase assays. Third, we analyzed specific activity of the truncated PKCα, but Su et al. (9) used the crude yeast lysate without normalizing the protein mass of the PKCα truncation mutants expressed in yeast. Finally, different PKC substrates were used. Although the conclusions reached are disparate, however, the differences between the measured kinase activities of PKCα truncation mutants in our study and that of Su et al. (9) are of quantitative nature, which are not necessarily mutually exclusive.

In another separate study on the regulation of phospholipase D1 (PLD1) by PKCα, it was found that a PKCα mutant with its C-terminal 10 amino acid residues deleted could not phosphorylate or activate PLD1 (34). However, the authors reported that both the PKCα-Δ9 or PKCα-Δ10 mutants (equivalent to our PKCα-Δ664 or PKCα-Δ663, respectively) still retained kinase activity. This could be due to the methodologies used to assess the catalytic activity of PKCα and its mutants after TPA treatment, i.e. by transfecting PKCα constructs into COS-7 cells and measuring the increased immunoreactivity of the total cellular proteins to an anti-phospho-Thr antibody in Western analysis (34). It is known, however, that COS cells express endogenous PKCα and PKCβ that are activated by TPA (41, 57). The endogenous PKCs and other diacylglycerol-responsive kinases (58) could be activated by TPA to phosphorylate many cellular proteins, including PLD1, and thus obscure the defects in the catalytic activity of PKCα-Δ9 or PKCα-Δ10 mutants (34). In contrast, we assessed the exogenously introduced PKCα

example, PKCα interacts with an anchoring protein PICK through its last four amino acid residues, and such interaction is essential for selective targeting of PKCα to mitochondria and supports cerebellar long term synaptic depression (35, 51, 52). Within the V5 domain, there are two highly conserved sequences, the turn motif and the hydrophobic motif. It has been established that the phosphorylation for Thr-638 at the turn motif and for Ser-657 at the hydrophobic motif is not essential for catalysis. Rather, the phosphorylation of these two sites at the C terminus controls the duration of activation of PKCα by maintaining a thermally stable and phosphatase-resistant conformation, thus regulating the rate of dephosphorylation and inactivation of the kinase (53, 54). As such, it is generally thought that the V5 domain of PKCα plays a regulatory role in fine-tuning the location and the extent of the signaling. In this study, however, we provide the first evidence that the very C-terminal segment beyond the hydrophobic motif is absolutely required for the catalytic activity of PKCα.

To examine the contribution of the very C terminus of PKCα to the catalytic activity of the kinase, we generated a series of C-terminal truncation mutants of PKCα. In the literature, there

FIGURE 11. The anti-V5 antibody inhibits the catalytic activity of the wild-type PKCα. The N-terminally FLAG-tagged wild-type PKCα was transiently transfected into COS-1 cells. Twenty hours after transfection, the cells were lysed with 1% Triton X-100, and the FLAG-tagged PKCα was immunoprecipitated with either a monoclonal anti-FLAG antibody that binds to the N terminus of FLAG-PKCα or with a polyclonal anti-V5 antibody (catalog number P4334, Sigma) that binds to the C terminus of FLAG-PKCα. The specific activity of FLAG-PKCα was determined in an in vitro immune complex kinase assay using 0.67 mg/ml protamine sulfate as a substrate without cofactors after normalization of protein levels of immunoprecipitated FLAG PKCα. Results are mean ± S.E. of three independent experiments and are represented as percentage of the activity of the FLAG-PKCα immunoprecipitated using the anti-FLAG antibody. A representative immunoblot used to derive specific activities is shown at the bottom.

FIGURE 10. Dynamic motions of the catalytic core and the V5 domain. Molecular dynamics simulation of the catalytic domain of PKCα and C-terminal truncation mutants is shown. Only the V5 domain and the N-lobe are selected for the presentation. The initial models are shown in schematic representation (brown), whereas the motions of the V5 domain are shown as C-α trace snapshots of molecular dynamics simulations at 2.5-ns (red), 5.0-ns (yellow), 7.5-ns (green), and 10-ns (blue) intervals along the time scale. A, motions of the WT-PKCα V5 domain. Also shown are the N-terminal Arg-333, which formed a permanent salt bridge with Ser-657 (green dotted line) and a transient salt bridge with Val-672 (black dotted line), as well as the relatively low motions of Phe-626 in the ATP-binding pocket. B, motions of the PKCα-Δ672 V5 domain showing the structural deformation domain between the turn motif (Thr-638) and the hydrophobic motif (Ser-657). The extended Arg-333 side chain (red) relative to that for the initial model is illustrated with an arrow. C, motions of the PKCα-Δ663 V5 domain showing the structural deformation extending to the region before the turn motif Ser-638 (blue bracket). Also shown is the Phe-626 residue, which moved out of the ATP-binding pocket.
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activity by direct analysis of the specific activities of immunoprecipitated wild-type and truncation mutants of PKCα (Fig. 3).

Irrespective of the differences in the assessment of the catalytic activities of PKCα truncation mutants, our data on the cellular functions of such mutants in mammalian cells are entirely consistent with the published data. For example, it has been shown that PLD1 is activated by the wild-type PKCα but not the PKCα-Δ10 or PKCα-F663A mutants (34, 59). We found that the corresponding FLAG-PKCα-Δ663 and FLAG-PKCα-F663A had minimal catalytic activity (Figs. 3 and 4A). Therefore, regardless of whether PKCα activates PLD1 by directly phosphorylating PLD1 or other protein kinases/phosphatases that in turn affect the activity or subcellular localization of PLD1, our findings that PKCα-Δ663 (equivalent to the PKCα-Δ10) and PKCα-F663A lost their catalytic competence are consistent with the fact that the catalytic activity of PKCα is required for the activation of PLD1 (34, 60, 61). Therefore, deficiency in catalytic activity could constitute one of the major biochemical mechanisms underlying the inability of PKCα-Δ10 and PKCα-F663A in the activation of PLD1 (34, 59). Taken together, our findings point to the critical roles played by the very C-terminal segment not only in conferring the full catalytic competence of PKCα but also in mediating the known cellular functions of this important PKC isozyme.

It is interesting to note that the effects of C-terminal deletion on the catalytic activities of the truncated PKCα (Fig. 3) did not parallel the extent of the phosphorylation of the activation loop (Fig. 6). Although the phosphorylation of the activation loop is critical for the initial maturation of the newly synthesized PKC, the removal of the phosphate from the consensus threonine at the activation loop does not necessarily affect the catalytic activities of PKC adversely once PKC has matured (2). Thus, our data are consistent with previous findings that once PKC has matured, the degree of phosphorylation at the activation loop of PKC is not directly linked to its catalytic activity; it is rather an indicator of the accessibility of the loop to phosphatases (2, 4, 62). On the other hand, the molecular dynamics simulations revealed small but detectable changes in the positioning of Thr-497 as well as the conformation of the activation loop of PKCα-Δ672 and in PKCα-Δ663. Furthermore, the conformation of the conserved DFG motif and the nearby loop was also altered. These conformational changes might not affect the extent of phosphorylation at the activation loop but are most likely to have an adverse effect on the catalytic activities of the truncation mutants.

Another interesting observation is that the deletion of a few C-terminal residues had more profound effects on the ability of the PKCα mutants to rescue apoptosis (Fig. 7) and to promote neurite outgrowth (Fig. 8) than on the catalytic activities of the mutant kinase per se (Fig. 3). It has been shown recently that some biological functions of protein kinases are independent of the catalytic activities of the kinases (63, 64). Furthermore, the catalytic domains of some protein kinases are known to possess noncatalytic functions (65, 66). It is possible that rescuing apoptosis and promoting neurite outgrowth are regulated by PKCα via the noncatalytic functions of PKCα. Therefore, the observed catalytic activities of PKCα truncation mutants did not correspond to these cellular functions, as the latter may not be causally linked to the catalytic activities of the kinase.

How does the very C terminus beyond the hydrophilic motif in PKCα control its catalytic activity? Our computer modeling and molecular dynamics simulation results suggest that although this C-terminal tail is very flexible, it is actually involved in stable interactions with the N-lobe in the catalytic core (Fig. 10A). Truncation of the extreme C terminus results in a number of changes, including the disruption of its interaction with the N-lobe, the deformation of the rest of the V5 domain, as well as bigger structural fluctuations within the N-lobe (as can be seen in the ATP-binding loop and the activation loop) (Fig. 9D, inset). Such structural changes observed for the truncation mutants may manifest as a reduction or total loss of the catalytic competency of the mutant enzymes (Fig. 3). The observation of the importance of the very C terminus in controlling PKCα activities presented here is consistent with our recent finding that the very C terminus beyond the hydrophilic motif is essential for full lipid responsiveness for PRK1/PKN, a member of the PKC superfamily (67). Thus, the critical role of the very C terminus in conferring the catalytic competence of PKCα uncovered in this study may be a silent feature shared by several other, if not all, isozymes in the PKC superfamily. The definitive structural basis underlying this feature awaits a high resolution three-dimensional structure of PKCα, including details of the backbone packing and side chain interactions of the residues at the very C terminus.

One of the goals in PKC research is to develop strategies for specific modulation of the activity and function of individual PKC isozymes. However, most of the available PKC inhibitors lack desired specificity (68, 69). Our findings point to a new direction for generating PKC isozyme-specific pharmacological agents. The very C terminus of PKC is not only the least conserved in sequence but is likely to be exposed on the surface of folded PKC as judged from the fact the most PKCs can be readily immunoprecipitated using isozyme-specific antibodies that are generated against the very C terminus. Most importantly, in a proof-of-principle experiment, we found that PKα immunoprecipitated using an antibody against its C terminus displayed markedly diminished catalytic activity compared with that of PKCα immunoprecipitated by an antibody against its N terminus (Fig. 11). Therefore, monoclonal antibodies, DNA or RNA aptamers, or small molecules that are able to interact the very C terminus of PKCα and suppress its catalytic activity may become invaluable tools in the study of the biology of this kinase. Moreover, such agents could represent a new generation of drugs for treating diseases in which the activation of PKCα is implicated, such as in heart failure and sepsis/endotoxemia (70, 71).

Acknowledgments—We thank A. Toker (Harvard Medical School), C. J. Marshall (Cancer Research UK), and A. Newton (University of California, San Diego) for providing bovine PKCα cDNA, Myc-p42-ERK1/2 plasmid, and P500 antibody, respectively. We also thank Dr. Y. Yuan for editing.
