Deletion of the Regulatory Domain of Protein Kinase C α Exposes Regions in the Hinge and Catalytic Domains that Mediate Nuclear Targeting

Guy James and Eric Olson

Department of Biochemistry and Molecular Biology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

Abstract. Members of the protein kinase C (PKC) family are characterized by an NH_2-terminal regulatory domain containing binding sites for calcium, phosphatidylserine, and diacylglycerol (or tumor-promoting phorbol esters), a small central hinge region and a COOH-terminal catalytic domain. We have constructed fusion proteins in which the regulatory domain of PKCα was removed and replaced by a 19-amino acid leader sequence containing a myristoylation consensus or by the same sequence in which the amino-terminal glycine was changed to alanine to prevent myristoylation. The goal was to generate constitutively active mutants of PKC that were either membrane bound, due to their myristoylation, or cytoplasmic. Western blotting of fractions from COS cells transfected with plasmids encoding wild-type and mutant proteins revealed that PKCα resided entirely in a Triton X-100 soluble (TS) fraction, whereas both the myristoylated and nonmyristoylated mutants were associated primarily with the nuclear envelope fraction. A similar mutant that lacked the 19 amino acid leader sequence was also found almost entirely in the nuclear envelope, as was a truncation mutant containing only the regulatory domain, hinge region, and a small portion of the catalytic domain. However, an additional truncation mutant consisting of only the regulatory domain plus the first one-third of the hinge region was almost entirely in the TS fraction. A nonmyristoylated fusion protein containing only the catalytic domain was also found in the nuclear envelope. Immunostaining of cells transfected with these constructs revealed that both the myristoylated and nonmyristoylated mutants were localized in nuclei, whereas wild-type PKCα was primarily cytoplasmic and perinuclear. Phorbol dibutyrate treatment of PKCα-transfected cells resulted in increased perinuclear and nuclear staining. The results are consistent with a model in which activation of PKC, by phorbol esters or by deletion of the regulatory domain, exposes regions in the hinge and catalytic domains that interact with a PKC "receptor" present in the nuclear envelope, and may explain the ability of wild-type PKC to be translocated to the nucleus under certain conditions.

PROTEIN kinase C alpha (PKCα)[1] belongs to a family of serine/threonine protein kinases activated by calcium, phospholipid, and the tumor promoting phorbol esters (Coussens et al., 1986; Knopf et al., 1986). Members of this family are characterized by an amino-terminal regulatory domain containing co-factor–binding sites and a carboxy-terminal catalytic domain (Bell and Burns, 1991; Kikkawa et al., 1989). A conserved pseudosubstrate sequence has also been identified in the regulatory domain, near the extreme amino terminus (House and Kemp, 1987).

Current knowledge concerning the regulation of PKC catalytic activity is consistent with a model in which the inactive enzyme exists in a folded conformation, with the amino-terminal pseudosubstrate sequence occupying the active site (Bell and Burns, 1991; House and Kemp, 1987). Binding of cofactors to the regulatory domain is believed to dislodge the pseudosubstrate sequence from the active site, followed by intramolecular autophosphorylations which can occur at three separate regions of the molecule, indicating remarkable flexibility (Flint et al., 1990). This sequence of events presumably leaves the enzyme membrane bound in an active conformation, poised for interaction with the appropriate substrates. Previous models have envisioned binding of activated PKC to membranes as being mediated by complex interactions between the regulatory domain, cofactors, and the membrane bilayer (Bell, 1986; Bell and Burns, 1991).

Although the critical substrates for PKC have traditionally been considered to be plasma membrane and cytoplasmic proteins, Blackshear and co-workers (Halsey et al., 1987) observed translocation of PKC to the perinuclear region following treatment of 3T3-L1 fibroblasts with phorbol ester. More recently, a PKCα-specific mAb revealed phorbol ester-induced translocation of PKCα to the nuclear envelope in NIH 3T3 cells (Leach et al., 1989). In HL60 cells, which express PKCα and PKCβ_{II}, bryostatin treatment caused selective translocation of PKCβ_{II} to the nuclear membrane

1. *Abbreviations used in this paper*: PDBu, phorbol dibutyrate; PKC, protein kinase C; TI, Triton X-100 insoluble; TS, Triton X-100 soluble.

(Hocevar and Fields, 1991). In rat embryo fibroblasts PKCα was found to be associated with focal contacts in a nonionic detergent- and chelator-resistant manner, suggesting that PKC resides in the particular fraction due to protein–protein as well as protein–lipid interactions (Jaken et al., 1989). Consistent with this notion is the recent identification of two proteins of 30 and 33 kD in a Triton X-100 insoluble (TI) fraction (cytoskeleton plus nuclei) that interact specifically and saturably with activated PKC (Mochly-Rosen et al., 1991). Binding of PKC to these "receptor" proteins was not inhibited by preincubation with a peptide substrate, implying that this observation did not simply reflect an enzyme–substrate recognition event and that the domain of PKC involved in the interaction lies outside the active site. The presence of such receptors would explain the ability of activated PKC to interact tightly with the detergent-insoluble fraction.

Prolonged treatment of cells with phorbol esters leads to disappearance of immunoreactive PKC because of an increased rate of proteolysis (Young et al., 1987). In vitro proteolysis of purified PKC with trypsin or the calcium-dependent protease calpain generates a lipid-binding regulatory domain and a constitutively active catalytic fragment (PKM) that is no longer able to associate with membranes (Lee and Bell, 1986; Melloni et al., 1985; Mizuta et al., 1985). The site of proteolysis in vitro has been mapped to a region of nonconserved sequence between the regulatory and catalytic domains, referred to as the hinge region (Kishimoto et al., 1989).

The ability to generate a constitutively active form of PKC in vitro led to speculation that the phorbol ester-induced proteolysis of PKC in vivo might result in formation of a soluble kinase (PKM) capable of phosphorylating cytoplasmic proteins (Kikkawa et al., 1989; Melloni et al., 1985; Mizuta et al., 1985). Although calcium- and phospholipid-independent kinase activity has been detected in cell extracts (Tapley and Murray, 1984), numerous investigators have reported an inability to detect the catalytic fragment of PKC by Western blotting (presumably because of an extremely short half-life), casting doubt on the physiological relevance of "PKM" (Kikkawa et al., 1989; Ways et al., 1991). Thus, it remains unclear whether the effects of chronic exposure to phorbol esters are due to a reduced level of native PKC, continuous generation of a constitutively active, yet short lived, catalytic fragment, or some cellular process unrelated to PKC.

To distinguish among these possibilities and further define the properties of the PKC catalytic fragment in vivo, we constructed mutants of PKCα in which the regulatory domain was removed and replaced by either a 19-amino acid leader sequence containing a myristoylation consensus, or the same sequence in which the amino-terminal glycine residue was changed to alanine to prevent myristoylation. The myristoylation consensus contains sufficient sequence information to direct co-translational myristoylation (Wilcox et al., 1987) and based on previous studies would be predicted to target PKC to the membrane fraction (see James and Olson, 1990 for review). The goal was to generate constitutively active PKC mutants that were either membrane bound, due to their myristoylation, or cytoplasmic. Surprisingly, subcellular fractionation of transiently transfected COS cells followed by Western blotting revealed that both the myristoylated and nonmyristoylated mutants were tightly associated with the nuclear envelope fraction. Further deletion analyses revealed that at least two segments, one in the hinge region and another in the catalytic domain, are responsible for nuclear localization. These findings are consistent with a model in which activation of PKC results in the unfolding of the molecule and the exposure of regions in the hinge and catalytic domains that have high affinity for one or more receptor proteins present in the nuclear envelope. These observations may explain the ability of PKC to be translocated to the nucleus under certain conditions.

Materials and Methods

Cloning and Mutagenesis

SRαPKC, an expression vector containing a cDNA encoding bovine PKCα, was obtained from Dr. Paul Simpson (University of California at San Francisco, San Francisco, CA) (23). The murine MARCKS cDNA was cloned from an NIH 3T3 library using an oligonucleotide corresponding to a region of the bovine MARCKS (80K) cDNA sequence (Stumpo et al., 1989). The first 19 amino acids of the murine and bovine MARCKS proteins are identical.[2] Site-directed mutagenesis, using a single-stranded template as described (Brennan et al., 1991), was used to change the second codon of this cDNA, GGT (glycine), to GCC (alanine), thus preventing myristoylation of the protein (Graff et al., 1989).[2] The mutation was confirmed by DNA sequencing (Sequenase, United States Biochemical Corporation, Cleveland, OH). This mutant protein will be referred to as A1, for alanine at position 1 (following removal of the initiating methionine). A 220-bp fragment from the 5′ end of the MARCKS (80K) and A1 cDNAs, encoding the first 19 amino acids of each protein, was subcloned into pUC19 between the HindIII and BamHI sites. These plasmids were denoted 5′80K/pUC and 5′A1/pUC, and were used as cassettes into which various portions of the bovine PKCα cDNA were inserted in-frame, resulting in myristoylated or nonmyristoylated fusion proteins.

For expression in COS cells, all constructs were subcloned into a derivative of pCDM8 (obtained from Dr. Randy Legerski, M.D. Anderson Cancer Center, Houston, TX), a vector in which expression is driven from the cytomegalovirus promoter and which has the SV-40 origin of replication, allowing amplification in COS cells (Seed, 1987). We constructed a derivative of this vector (pCDM8*) by removing the 400-bp sequence between the cytomegalovirus promoter and the SV-40 polyadenylation signal and inserting a portion of the pBluescript KS polylinker, from the HindIII to the NotI sites, resulting in seven unique cloning sites.

PKC1 and PKC2 were constructed by inserting a fragment of ~2,100 bp, extending from the NarI site at nucleotide 83 to the StuI site at nucleotide 2,180 in the 3′-untranslated region of the cDNA, into the SmaI site of 5′80K/pUC and 5′A1/pUC, respectively. The NarI site at the 5′ end of the fragment was blunt ended using S1 nuclease. Plasmids with inserts in the correct orientation were identified by restriction mapping, and these were sequenced to verify the reading frame. These constructs encode fusion proteins in which the first 11 amino acids of PKCα have been deleted and replaced by the first 19 amino acids of MARCKS or A1. PKC7 and PKC8 were constructed using a 1,370-bp fragment extending from the SacI site at nucleotide 948 to the XbaI site at the 3′ end of the cDNA. This fragment was blunt ended with S1 nuclease and inserted into the BamHI site (blunt ended with the Klenow fragment of DNA polymerase I) of 5′80K/pUC and 5′A1/pUC. Plasmids with inserts in the correct orientation and reading frame were identified as described above. These constructs encode fusion proteins beginning at amino acid 302 of PKCα. PKCΔ12-253 was made by excising a 723-bp fragment between the NarI site at nucleotide 83 and the XmnI site at nucleotide 806. The 5′ overhang at the NarI site was removed with S1 nuclease and the plasmid was religated. The correct reading was verified by sequencing. PKC14 consists of an 1,100-bp fragment extending from the NcoI site at nucleotide 1201 to the 3′ end of the cDNA. The 5′ overhangs on this fragment were filled and it was cloned into the BamHI site of 5′A1/pUC, as described for PKC8. PKC16 was made by inserting the 723-bp XhoII fragment (blunt ended) from nucleotides 1,153 to 1,876 into the BamHI site of 5′A1/pUC, as described for PKC8. For PKCα-TM385, the 1,152-bp NcoI fragment from nucleotides 49 to 1,201 was isolated, blunt ended with Klenow, and cloned into the SmaI site of pCDM8*. Plasmids with inserts in the correct orientation were identified by restriction map-

2. James, G., and E. Olson, unpublished data.

ping. PKCα-TM310 was obtained by partial digestion to allow isolation of an ~1,000-bp fragment extending from a HindIII site in the pCDM8* polylinker to the HindIII site at nucleotide 975 of the PKCα cDNA. This fragment was cloned into the HindIII site of pCDM8*, and the orientation was determined by restriction mapping. PKC-TM385 and PKC-TM310 have 36 and 43 amino acids, respectively, of non-PKC sequence at their carboxy-termini derived from vector sequences on the 3′ side of the cloning site.

In vitro transcription and translation was performed as described previously (Brennan et al., 1991).

Cell Culture, Metabolic Labeling, and Transfections

Cells were maintained in DME containing 10% FBS, as described previously (James and Olson, 1989*a*).

For transient transfections, equal numbers COS-1 cells were seeded in 10-cm dishes (Falcon, Lincoln Park, NJ) at ~50% confluency and were transfected by calcium-phosphate precipitation 16–24 h after seeding, as described (Sternberg et al., 1988). Cells were refed with medium containing 10% FBS 16 h after transfection, and were harvested 24–32 h later.

Metabolic labeling with [^{3}H]myristate was performed as described previously (James and Olson, 1989*a*; Wilcox et al., 1987), with the label (0.5 mCi/10-cm dish) being added 8 h before harvesting.

Subcellular Fractionation, SDS-PAGE, and Western Blotting

Cells were harvested by scraping in PBS and collected by centrifugation, as described (James and Olson, 1989*a*).

For preparation of Triton X-100 soluble (TS) and TI fractions, cell pellets were resuspended in 50 μl of lysis buffer A, consisting of 20 mM Tris HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and protease inhibitors (0.5 mM PMSF, 5 μg/ml each of leupeptin, pepstatin, and aprotinin). The suspension was vortexed, incubated on ice for 5 min, and centrifuged for 30 s at 12,000 *g*. The supernatant was removed and designated the TS fraction. Pellets (TI fraction) were washed once in lysis buffer A and the wash was discarded. Protein concentrations were determined using the Coomassie protein assay reagent from Pierce Chemical Co. (Rockford, IL). SDS sample buffer was added to each fraction, samples were boiled for 5 min and vortexed, and equal amounts were subjected to SDS-PAGE as described (James and Olson, 1989*b*).

Nuclear envelope and intranuclear protein fractions were prepared as described by Kaufmann et al. (1983). Lamin B and p38 chicken antisera were obtained from Dr. Scott Kaufmann (Johns Hopkins Oncology Center (Baltimore, MD) and have been described previously (Kaufmann, 1989; Fields et al., 1986). These were detected using alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma Chemical Co., St. Louis, MO). Color development was performed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt) according to the manufacturer's instructions (Southern Biotechnology Associates, Inc., Birmingham, AL).

Proteins were transferred to nitrocellulose as described (James and Olson, 1989*b*). Blots were incubated with Blotto (James and Olson, 1989*b*) for 30 min to block unbound sites, and primary antibody (1:100 dilution in Blotto) was added for 3–4 h. PKC antibodies used were either MC5, a mAb (Amersham Corp., Arlington Heights, IL) whose epitope has been mapped to amino acid residues 296–309 of PKCα (MC5 also recognizes PKCβ), or a PKCα-specific anti-catalytic domain mAb available from Upstate Biotechnology, Inc. (Lake Placid, NY). A polyclonal antibody from Oncogene Science, Inc. (Manhasset, NY) that recognizes amino acids 520–525 was also used in some experiments. Blots were rinsed briefly and then incubated with ^{125}I-labeled goat anti-mouse or donkey anti-rabbit secondary antibody (Amersham Corp.) at 2 × 10^6 cpm/ml for 1–2 h. After being washed for 15–30 min in Blotto, with several changes, blots were placed on film for the indicated lengths of time.

Immunofluorescence

COS cells were seeded in 35-mm dishes and transfected with the indicated plasmid as described above. 48 h after transfection, cells were rinsed with PBS and fixed with an ice-cold mixture of acetone/ethanol (1:1) for 3 min. After being rinsed with PBS, dishes were incubated for 30 min with PBS containing 2% BSA. MC5 or anti-catalytic domain antibody, diluted 1:100 in PBS/1% BSA, was added for 2 h. Dishes were rinsed 3 × 5 min with PBS/1% BSA, and fluorescein-conjugated goat anti-mouse secondary antibody (Sigma Chemical Co.) was added for 1 h at a dilution of 1:100 in PBS/1% BSA. Dishes were rinsed 3 × 5 min with PBS/1% BSA and 2 × 5 min with PBS. Immunostained cells were visualized at 495-nm wavelength and photographed using Ektachrome 400 color film (Eastman Kodak Co., Rochester, NY).

Protein Kinase C Assays

Plasmids were transfected into COS cells in quadruplicate as described above. PKC mutants were partially purified using a protocol similar to that described by Housey et al. (1988). Cells were harvested after 48 h and cell pellets were resuspended in 0.5 ml of a buffer (lysis buffer B) consisting of 20 mM Tris HCl (pH 7.5), 5 mM EDTA, 5 mM EGTA, 15 mM 2-mercaptoethanol, 0.1% Triton X-100, and protease inhibitors as described above. Cells were disrupted by 50 strokes in a 1 ml Dounce homogenizer, and the lysate was transferred to a 1.5-ml Eppendorf tube (Brinkmann Instruments Inc., Westbury, NY). The homogenizer was rinsed with 0.5 ml of buffer and the rinse was added to the lysate. Lysates were centrifuged 30 s at 12,000 *g*, and the supernatants were transferred to a fresh tube, diluted to 2 ml with buffer, and applied to 0.4-ml DEAE Sephacel columns previously equilibrated with lysis buffer B. Columns were washed with 20 vol of buffer B and the PKC mutants were eluted with buffer B containing 200 mM NaCl. Fractions were assayed for their ability to phosphorylate a peptide corresponding in sequence to a region surrounding threonine 654 in the EGF receptor, a site that is highly specific for PKC in vivo and in vitro (Housey et al., 1988). Reactions were performed in the absence of cofactors, and specific activities were calculated as described in the protein kinase C assay kit from Amersham Corp. All reagents used in the assays were from this kit. Protein concentrations were determined as described above. Approximately 1% of each mutant protein was solubilized under these conditions (see Results).

Results

Construction of Myristoylated and Nonmyristoylated PKCα Mutants

Our initial aim was to compare the properties of a soluble and membrane-bound PKC catalytic domain in vivo. We therefore created fusion proteins in which the first 19 amino acids of the murine MARCKS (80K) protein or of a non-myristoylated mutant (A1) of this protein were fused in-frame to various portions of PKCα. PKC1 and PKC2 consist of the first 19 amino acids of MARCKS and A1, respectively, fused to amino acid 12 of PKCα (Fig. 1). These proteins therefore retained the pseudosubstrate sequence, which lies between amino acids 19 and 36 (House and Kemp, 1987). These constructs were prepared as controls to test the effect of myristoylation on the properties of full-length PKCα. PKC7 (myristoylated) and PKC8 (nonmyristoylated) began at residue 302, which is just beyond the end of conserved region C2, 12 amino acids into the hinge region.

The MARCKS protein is myristoylated at its amino-terminal glycine residue, and a peptide consisting of the first eight amino acids of this protein was previously shown to serve as an efficient substrate for *N*-myristoyltransferase in vitro (Graff et al., 1989). However, in certain cases, favorable interactions between this enzyme and its substrates require structural information that lies beyond the first eight residues (Duronio et al., 1991). Thus, it was important to verify that the first 19 amino acids of the MARCKS protein were recognized by *N*-myristoyltransferase when present outside of their normal context.

To study the properties of these mutants in vivo, constructs were subcloned into a derivative of the expression vector pCDM8 and transiently transfected into COS cells. PKCα is the only PKC isoform known to be expressed in these cells (Ohno et al., 1990). Fig. 2 represents an experiment in which PKCα, PKC1, PKC2, PKC7, and PKC8 were trans-

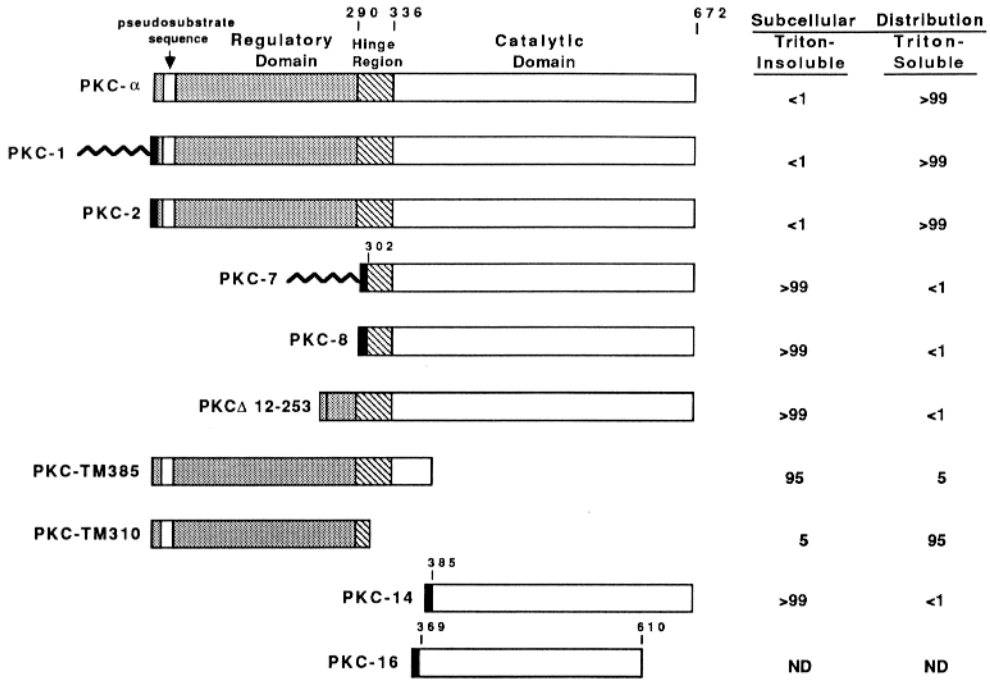

Figure 1. Diagram of wild-type and mutant PKC constructs. The top bar shows the domain organization of PKCα, with the pseudosubstrate sequence (amino acids 19–36) and hinge region (amino acids 290–336) also denoted. The fusion proteins and deletion mutants were constructed as described in Materials and Methods. The black bar at the amino terminus of the fusion proteins indicates the presence of the first 19 amino acids of the MARCKS (odd-numbered constructs) or A1 proteins (even-numbered constructs). The myristate moiety is shown diagrammatically at the end of PKC1 and PKC7. Subcellular distributions were determined by scanning densitometry of the Western blot shown in Fig. 3. *ND*, not detectable.

fected into COS cells. The cells were labeled with [^{3}H] myristate for 8 h before harvesting, and equal amounts of whole-cell lysates were then electrophoresed in duplicate. The proteins in one gel were transferred to nitrocellulose and analyzed by Western blotting with a mAb (MC5) that recognizes amino acids 296–309 of PKCα. As shown in Fig. 2 *a*, transfected PKCα, PKC1, and PKC2 co-migrated with endogenous PKCα (~77 kD). PKC7 and PKC8 migrated at ~41 kD, consistent with their predicted molecular mass. Only those fusion proteins with the MARCKS amino terminus (PKC1 and PKC7) were labeled with myristate (Fig. 2 *b*), indicating that the first 19 amino acids of the MARCKS protein are sufficient to direct myristoylation in vivo.

Subcellular Distribution of Myristoylated and Nonmyristoylated Mutants

Since the wild-type MARCKS protein is predominantly membrane bound (James and Olson, 1989*a*) whereas the nonmyristoylated mutant (A1) is almost entirely cytoplasmic (Graff et al., 1989),[2] PKC2 and PKC8 would be expected to reside mainly in the cytoplasm. PKC1 and PKC7 should be able to associate with membranes, at least to a certain extent, because of their myristoylation. Preliminary experiments, however, indicated that PKC7 and PKC8 were associated almost entirely with the 12,000 *g* nuclear pellet following lysis of cells by freeze/thaw in hypotonic buffer containing 1 mM EDTA and EGTA. PKCα and PKC2 were found predominantly in the soluble (200,000 *g* supernatant) fraction. In contrast, PKC1 was mostly in the crude membrane (200,000-*g* pellet) fraction, presumably a result of its myristoylation (data not shown).

We next prepared TS and TI fractions by fractionating transfected COS cells in buffer containing EDTA, EGTA, and 1% Triton X-100. The TS fraction contains cytoplasmic and peripheral plasma membrane proteins, whereas the TI fraction consists primarily of cytoskeletal proteins and nuclei (Ben-Ze'ev et al., 1979). Western blotting of these fractions indicated that PKCα, PKC1, and PKC2 were found entirely in the TS fraction (Fig. 3). Based on previous studies, PKCα is expected to be completely soluble under these conditions (1 mM EDTA, 1 mM EGTA). PKC1 and PKC2 are also expected to be soluble, since both the MARCKS and A1 proteins are found entirely in the TS fraction following their transfection into COS cells (data not shown). In contrast, >99% of PKC7 and PKC8 were tightly associated with the TI pellet (Fig. 3) and they remained associated with this fraction following extraction of the pellet with buffer containing 0.5 M NaCl (data not shown). One interpretation of this result is that deletion of the regulatory domain exposes a region within the hinge or catalytic domains that interacts with some component of the cytoskeleton and/or nucleus.

To demonstrate that the subcellular localization observed for PKC7 and PKC8 was because of a conformational change in the hinge/catalytic domain and not to structural features present in the MARCKS and A1 amino termini, we constructed a mutant in which amino acids 12 to 253 were deleted (PKCΔ12-253, see Fig. 1). This mutant is missing a large portion of the regulatory domain, including the pseudosubstrate sequence, and would therefore be expected to assume a conformation similar to PKC7 and PKC8. However, it contains the PKCα amino terminus instead of that from the MARCKS or A1 proteins. The distribution of PKCΔ12-253 was identical to that of PKC7 and PKC8 (Fig. 3), indicating that the region responsible for localization of these mutants to the TI fraction lies between amino acid 302 and the COOH terminus. Additional mutants have revealed that deletion of as few as the first 186 amino acids from the PKCα amino terminus is sufficient to induce distribution to the TI fraction (data not shown).

Previous in vitro studies have shown that proteolysis of PKC by calpain occurs in the hinge region, resulting in a constitutively active catalytic fragment that is unable to associate with membranes (Kikkawa et al., 1989). Based on this

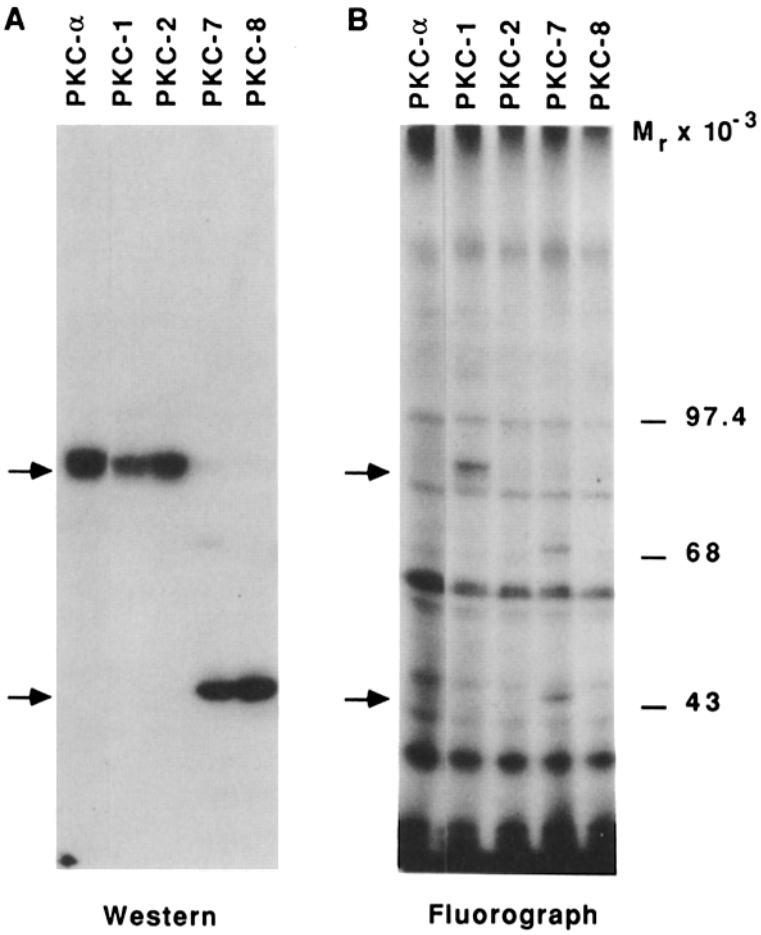

Figure 2. Fusion proteins containing the MARCKS amino terminus are myristoylated. COS cells in 10-cm dishes were transiently transfected with 15 μg each of plasmids encoding the indicated proteins and labeled with [^{3}H]myristate as described in Materials and Methods. Cell pellets were lysed in buffer A, and equal amounts of total cell protein were mixed with SDS sample buffer and electrophoresed in duplicate in 8% SDS–polyacrylamide gels (150 μg/lane). The proteins in one gel were analyzed by Western blotting (*A*) using the MC5 mAb and ^{125}I-labeled goat anti–mouse secondary antibody (24-h exposure). The other gel was treated with EN^3-HANCE, dried, and placed on film for 14 d (*B*). Positions of PKCα, PKC1, PKC2, and PKC7, and PKC8 are indicated by the upper and lower arrows, respectively. Migration of molecular weight standards is indicated at right.

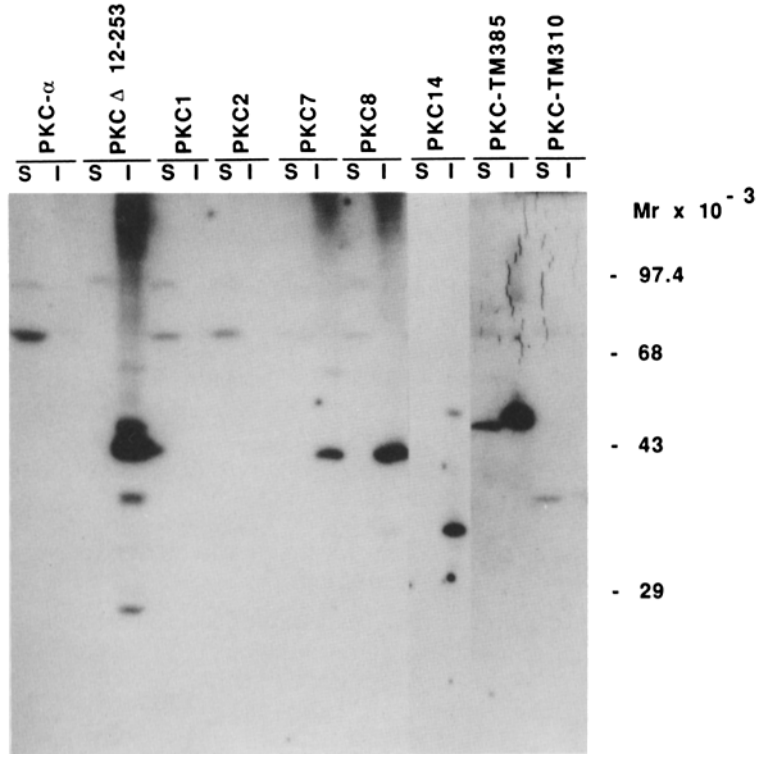

Figure 3. Subcellular distribution of wild-type and mutant PKC proteins. COS cells in 10-cm dishes were transiently transfected with 15 μg each of plasmids encoding the indicated proteins. TS (*S*) and TI (*I*) fractions were prepared as described in Materials and Methods. The entire fractions, containing ~150 (*S*) and 75 μg (*I*) of protein each, were electrophoresed in 10% SDS–polyacrylamide gels and analyzed by Western blotting. PKC14 was detected with a mixture of the anti-catalytic domain mAb and a polyclonal antibody recognizing amino acids 520–525, followed by anti-mouse and anti-rabbit ^{125}I-labeled secondary antibodies. All other lanes were probed with MC5, followed by anti-mouse ^{125}I-labeled secondary antibody. Exposure times were 48 h except for PKC14 (24 h), PKC-TM385, and PKC-TM310 (96 h). Migration of molecular weight standards is indicated at right. The distribution of all proteins was analyzed at least twice, and in some cases up to 10 times, with nearly identical results.

information the region responsible for localization to the TI fraction would be expected to be contained within the hinge region, NH_2-terminal to the catalytic domain. To address this possibility, we constructed two truncation mutants, PKC-TM385 and PKC-TM310, consisting of the first 385 and first 310 amino acids of PKCα, respectively. As shown in Fig. 3, PKC-TM385 exhibited a distribution almost identical to PKC7 and PKC8. PKC-TM310, however, was found almost entirely in the TS fraction, suggesting that the region between amino acids 310 and 385 mediates association with the TI fraction. This region consists of approximately two-thirds of the hinge region and 49 amino acids from the beginning of conserved region C3, with the consensus ATP binding site starting at amino acid 346 (Parker et al., 1986). Although equal amounts of PKC-TM385 and PKC-TM310 plasmid were transfected, much less of the latter protein was detected by Western blotting, suggesting that the regulatory domain was rapidly degraded in the cytoplasm.

To determine whether the hinge region was entirely responsible for the subcellular distribution of PKC7 and PKC8, or if portions of the catalytic domain also contribute to their localization, we constructed an additional mutant that consisted of the A1 amino terminus fused to amino acid 385 of PKCα (Fig. 1). This fusion protein, designated PKC14, lacked the 75-amino acid segment defined in the above experiments as being required for localization to the TI fraction, but contained the remainder of the catalytic domain intact. Surprisingly, this mutant was also tightly associated with the TI pellet (Fig. 3), suggesting that a second region present in the catalytic domain also participates in determining subcellular distribution.

Since the core of the catalytic domain is involved in catalysis and is presumably unavailable for mediating localization to the TI fraction, we felt that the extreme COOH terminus was a likely candidate for this second binding region. We therefore constructed a mutant similar to PKC14 but lacking the last 62 amino acids at the COOH terminus (PKC16, see Fig. 1). PKC16 yielded a protein of the expected molecular mass when transcribed and translated in vitro, but we were unable to detect it in either fraction following transfection into COS cells, suggesting that it is extremely unstable (data not shown). Although the precise epitope for the anti-catalytic domain mAb is not known, we were also unable to detect PKC16 with a polyclonal antibody that recognizes amino acids 520–525, indicating that our inability to detect this mutant was not due to deletion of the antibody epitope (data not shown). Based on the apparent instability of PKC-TM310, which resides in the TS fraction, we believe that PKC16 may also be unable to associate with the TI fraction and is thus rapidly degraded in the cytoplasm.

Since a majority of the above mutants were found in a TI pellet fraction, we considered the possibility that their apparent distribution might be a result of an inherent insolubility caused by their altered conformation. To address this, we transcribed and translated all the mutants in vitro and then centrifuged the translation products at 200,000 *g* for 30 min. Analysis of the supernatant and pellet fractions by electrophoresis and fluorography revealed that the mutant proteins remained in the supernatant (data not shown), suggesting that their distribution in vivo is not due to inherent insolubility.

PKC Mutants in the TI Fraction Are Localized to the Nuclear Envelope

To determine more precisely where the mutants in the TI fraction were located, we subfractionated nuclei according to the method of Kaufmann et al. (1983). COS cells were transfected with plasmids encoding the indicated proteins and nuclei were isolated. Following treatment with DNAse and RNAse, intranuclear proteins were separated from nuclear envelopes by extraction in buffer containing 1.6 M NaCl. Intranuclear proteins (1.6 M NaCl) and nuclear envelope fractions were electrophoresed in triplicate and analyzed by Western blotting using either PKC antibodies (Fig. 4 *a*), anti-lamin B antisera (Fig. 4 *b*), or antisera to a 38-kD nucleolar protein (Fig. 4 *c*) that is expected to be in the high salt extract (Fields et al., 1986). The five mutant PKC proteins found in the TI fraction (see Fig. 3) were localized almost entirely to the nuclear envelope fraction (Fig. 4 *a*; data not shown for PKC7). Small amounts were detected in the 1.6 M NaCl (Fig. 4 *a*) and DNAse/RNAse extracts (data not shown). The 38-kD nucleolar protein was predominantly in the 1.6 M NaCl extract (Fig. 4 *c*), whereas lamin B was consistently found in both the intranuclear and nuclear envelope fractions (Fig. 4 *b*). Neither lamin B nor p38 were detected in the postnuclear supernatant, and only residual levels were found in the DNAse/RNAse extract (data not shown). Thus, the PKC mutants found in the TI fraction were localized primarily to the nuclear envelope.

Immunostaining of COS Cells Expressing PKC Mutants

To confirm the results of biochemical fractionation and Western blotting, we examined the subcellular distribution of the PKC mutants by indirect immunofluorescence. As shown in Fig. 5, COS cells transiently transfected with PKC7, PKC8, PKCΔ12-253, and PKC14 displayed intense perinuclear and nuclear staining with little or no staining in the cytoplasm. Staining was not observed when the primary antibody was omitted from the procedure (data not shown), and nuclear staining was never seen in cells expressing wild-type PKCα in the absence of PDBu (see below). Some cells expressing PKC-TM385 occasionally yielded perinuclear staining only, whereas in others the entire nucleus was stained. However, the distribution of PKC-TM385 was indistinguishable from the other mutants when examined by subcellular fractionation and Western blotting (Fig. 4). PKC1 and PKC2 yielded cytoplasmic and perinuclear staining that was clearly distinct from that observed for the nuclear envelope–localized mutants (data not shown) and was indistinguishable from that detected in cells expressing PKCα in the absence of PDBu (see below). We have also observed nuclear localization of the truncation mutants in C2 myoblasts, indicating that their ability to localize to the nucleus is not restricted to COS cells (data not shown).

Wild-type PKCα Is Translocated to the Nucleus in Response to PDBu Stimulation

As stated above, previous studies have documented the phorbol ester–induced nuclear translocation of PKC in other cell types. We wished to determine whether nuclear localization of PKC truncation mutants (Figs. 4 and 5) reflected the pres-

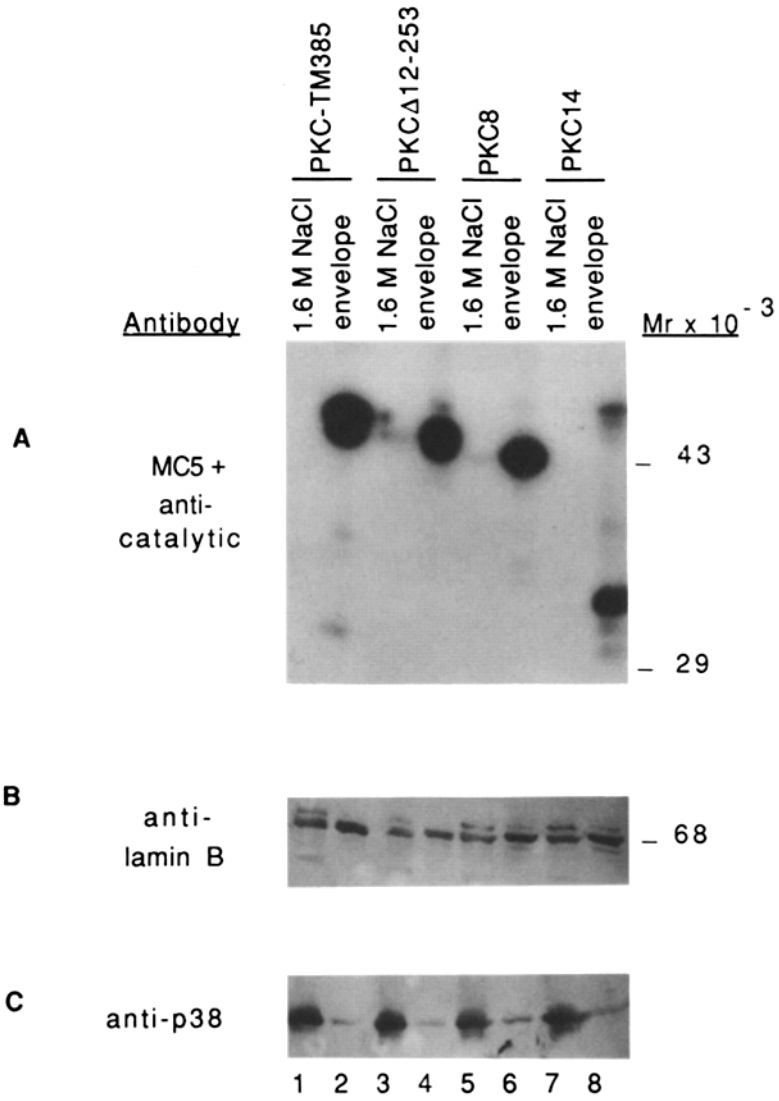

Figure 4. PKC mutants reside in the nuclear envelope. COS cells in 10-cm dishes were transiently transfected with 15 μg each of plasmids encoding the indicated proteins. Intranuclear (1.6 M NaCl) and nuclear envelope (*envelope*) fractions were prepared (see Materials and Methods) and electrophoresed in triplicate in 10% SDS–polyacrylamide gels, followed by Western blotting. The blot *a* (48-h exposure) was probed with a mixture of MC5 and anti-catalytic domain mAbs, followed by ^{125}I-labeled anti-mouse secondary antibody. *b* and *c* were probed with chicken antisera to lamin B and p38, respectively, and bands were visualized as described in Materials and Methods. Migration of molecular weight standards is indicated at right of *a* and *b*. This experiment was performed three times with identical results.

ence of a similar mechanism in COS cells. Cells were transiently transfected with plasmid encoding PKCα and the subcellular distribution of the enzyme was examined by immunostaining at various time points following stimulation of cells with PDBu. As shown in Fig. 6, untreated cells displayed cytoplasmic and perinuclear staining, whereas nuclei were clearly unstained. An identical pattern of staining was observed in cells treated with PDBu for 1 and 2 min (data not shown). However, after 5 min of PDBu stimulation, immunostaining began to appear within nuclei. Nuclear staining was still apparent after 10 min and localization to a juxtanuclear position was also evident. After 20 min, staining was almost entirely nuclear and juxtanuclear, and the cells overexpressing PKC had begun to display a morphology similar to that observed in other cell types after exposure to phorbol ester (Housey et al., 1988). Nearly identical patterns of staining were observed with anti-regulatory domain (MC5) and anti-catalytic domain antibodies.

PKC7 and PKC8 Are Constitutively Activated

We wished to determine whether PKC7 and PKC8 possessed kinase activity independently of the normally required cofactors. As shown in Fig. 4, however, these mutants reside primarily in the nuclear envelope and we were unable to reconstitute kinase activity following their solubilization by addition of SDS. However, we found that ~1% of each mutant protein was solubilized following lysis of cells in a Dounce homogenizer in the presence of Triton X-100 (data not shown). Thus, plasmids encoding PKC7 and PKC8 were transfected into COS cells and the mutant proteins present in the soluble fraction were partially purified by DEAE Sephacel chromatography. Fractions were assayed for kinase activity towards a synthetic peptide substrate corresponding to a region of the EGF receptor surrounding threonine 654. As presented in Table I, transfection with PKC7 and PKC8 resulted in an 8- to 11-fold increase, respectively, in cofactor-independent kinase activity in the soluble fraction, compared with transfection of vector alone. PKC7 and PKC8 are therefore clearly constitutively activated, suggesting that deletion of the regulatory domain results in a conformational change in the catalytic domain. These data are consistent with a previous study in which deletion of a portion of the regulatory domains of PKCα and PKCβ was found to induce

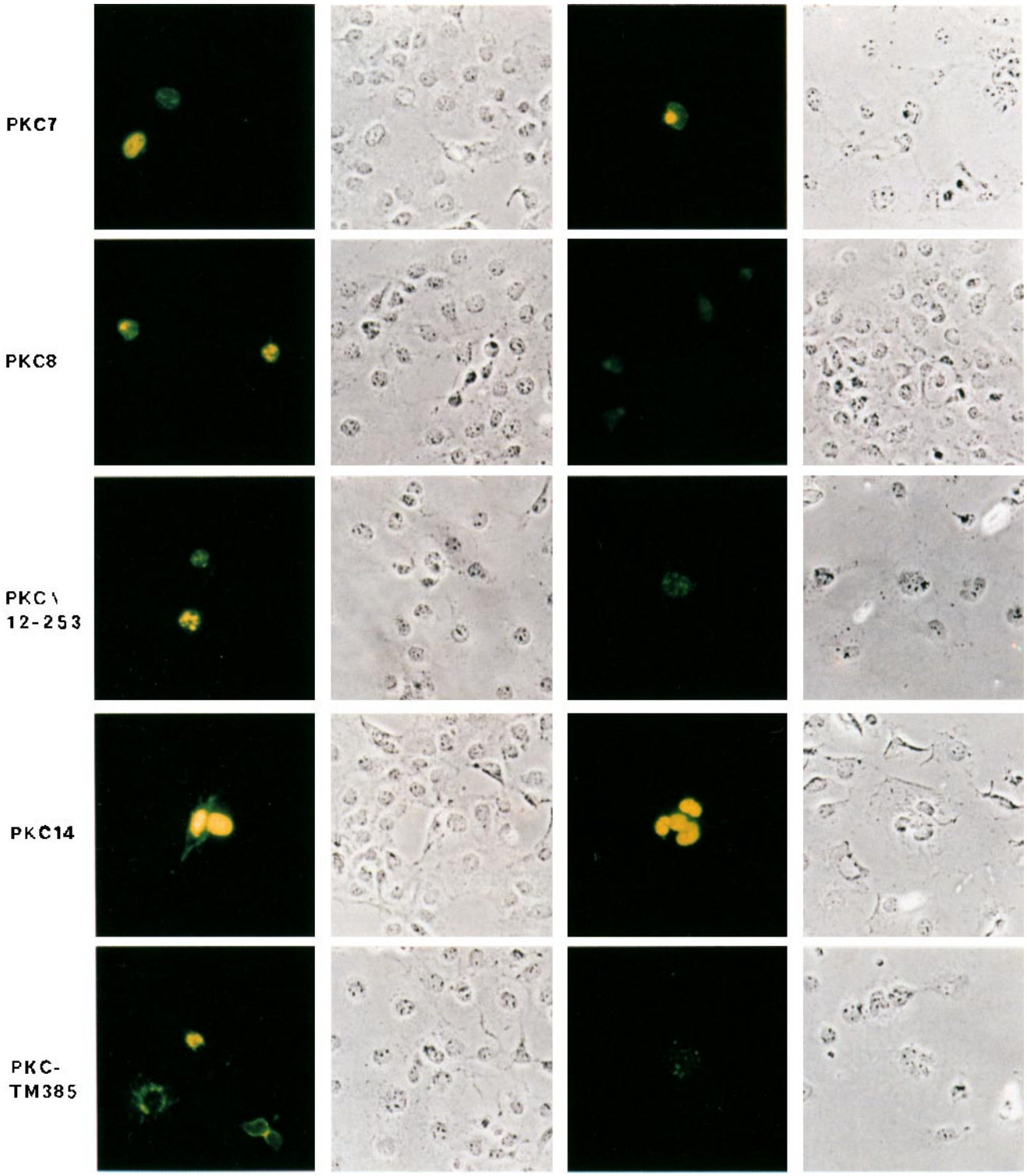

Figure 5. Immunostaining of COS cells expressing nuclear envelope-localized PKC mutants. COS cells in 35-mm dishes were transiently transfected with 5 μg each of the desired plasmids, as indicated at left. Cells were fixed and stained using the MC5 (*PKC7, PKC8, PKCΔ12-253,* and *PKC-TM385*) or anti-catalytic domain (*PKC14*) antibodies as described in Materials and Methods. Two representative fields for each mutant are shown, with the corresponding phase contrast photograph shown at right of each fluorescent image. Immunostaining of each mutant was performed at least three times, and in some cases up to eight times, with identical results.

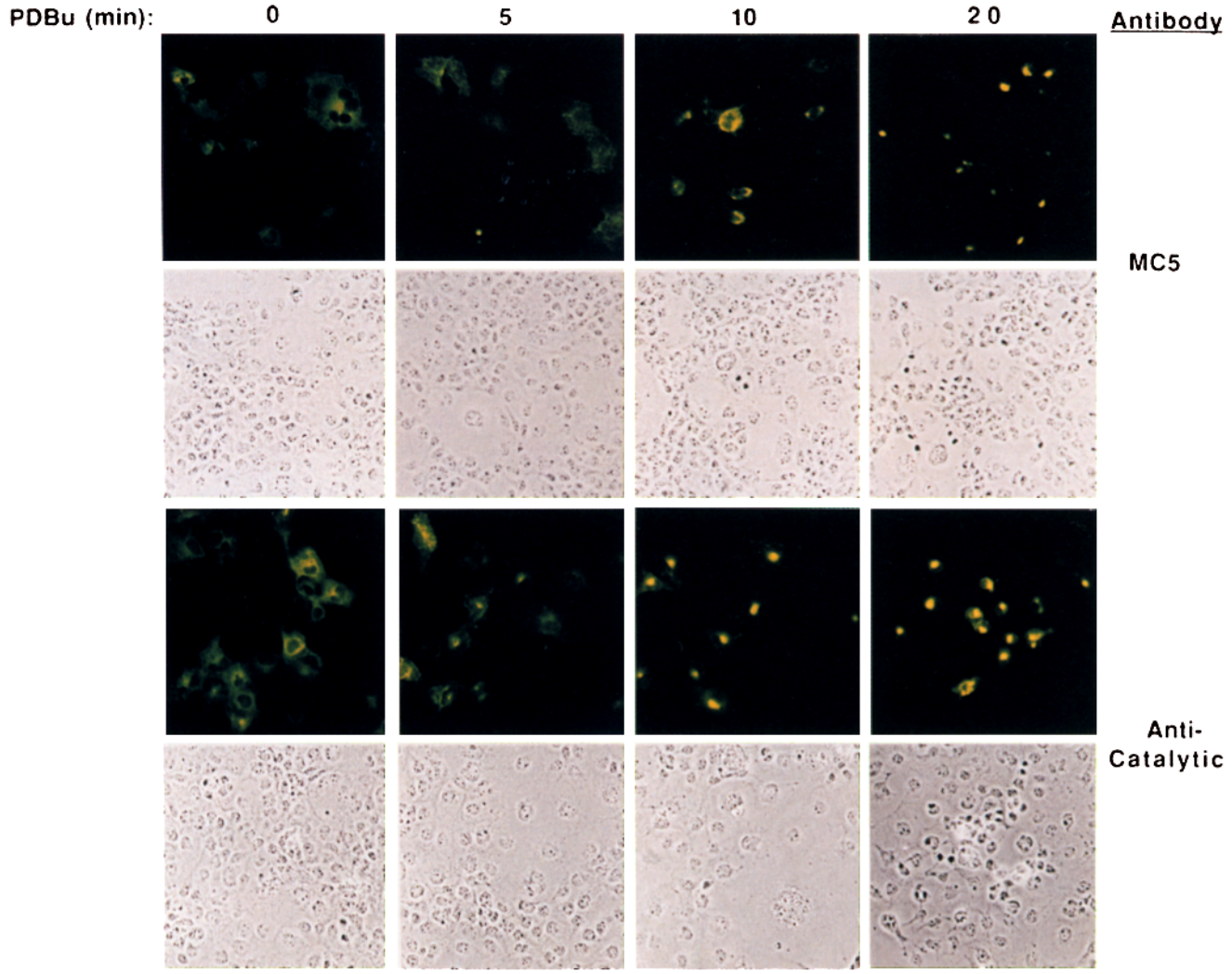

Figure 6. Wild-type PKCα is translocated to the nucleus following PDBu treatment. COS cells in 35-mm dishes were transiently transfected with 5 μg each of plasmid encoding PKCα. 48 h after transfection, PDBu was added for the indicated lengths of time at a final concentration of 1 μg/ml. Duplicate dishes were prepared for each time point and immunostaining was performed as described in Materials and Methods using either MC5 (*top*) or anti-catalytic domain (*bottom*) antibodies. The corresponding phase contrast photograph is shown below each fluorescent image. This experiment was performed twice with identical results.

phorbol ester–independent kinase activity (Kaibuchi et al., 1989).

Discussion

The data presented here reveal that the subcellular distribution of PKCα may be mediated by regions outside the regulatory domain. These results indicate that activation of the enzyme by deletion of the regulatory domain induces a conformational change in the hinge and catalytic domains, exposing at least two separate regions of the primary sequence that have high affinity for one or more binding sites present in the nuclear envelope.

The subcellular distributions of PKC-TM385 and PKC-TM310 indicate that the sequence between amino acids 310 and 385 encompasses a region that allows interaction of PKC with the nuclear envelope. This segment consists of the last 26 amino acids of the hinge region and the first 49 amino acids of the catalytic domain (Parker et al., 1986). Since the consensus ATP binding site lies between residues 346 and 368, we feel that the region responsible for localization to the nuclear envelope is probably between amino acids 310 and

Table I. PKC7 and PKC8 Are Catalytically Active in the Absence of Cofactors

Construct	Kinase activity	Relative activity
	(pmol/min/mg)	
pCDM8	29	1.0
PKC7	234	8.1
PKC8	312	10.8

COS cells in 10-cm dishes were transfected in quadruplicate with pCDM8, PKC7, or PKC8. The mutant proteins were partially purified from lysates as described in Materials and Methods, and fractions were assayed for kinase activity in the absence of calcium, phosphatidylserine, diacylglycerol, and PDBu. Activities are expressed as pmol of phosphate transferred to the peptide substrate per min per mg of protein. The experiment was performed twice with similar results.

346. However, we cannot at present rule out involvement of residues lying in close proximity to the ATP binding site.

The sequence between amino acids 310 and 346 is of particular interest, since it is part of the largest region of nonconserved sequence among the different PKC subtypes (Coussens et al., 1986). The site for proteolysis by calpain of the α, β, and γ subtypes of PKC has been mapped to this region in vitro (Kishimoto et al., 1989). Also, in cell types that co-express two or more PKC subtypes, downregulation of the various isoforms in response to chronic phorbol ester treatment occurs at different rates, suggesting the possibility of subtype-specific proteases in vivo (Ase et al., 1988; Huang et al., 1989; Isakov et al., 1990). The presence of receptors that interact specifically with the hinge regions of different PKC subtypes would allow targeting of the activated enzymes to precise locations within the cell, which would in turn provide a means of regulating their substrate specificities, susceptibilities to proteolysis, or both.

The localization of PKC14 to the nuclear envelope was unexpected, and suggested that a second region present in the catalytic domain also interacts tightly with a component of this fraction. Previous studies that concluded that proteolysis in the hinge region resulted in a constitutively active, cytoplasmic catalytic domain would argue against any portion of the catalytic domain–mediating association with the particulate fraction (Lee and Bell, 1986; Melloni et al., 1985; Mizuta et al., 1985). However, the possibility existed that a second proteolytic cleavage might occur simultaneously near the COOH terminus, resulting in removal of a small COOH-terminal fragment that has not previously been detected. This possibility led us to construct PKC16, which was similar to PKC14 but lacked the last 62 amino acids at the COOH terminus. Although PKC16 yielded a protein of the expected molecular mass when transcribed and translated in vitro, we were unable to detect it following transfection into COS cells, suggesting the possibility that it is indeed soluble and rapidly degraded. This interpretation is consistent with the inability of numerous investigators to detect a soluble catalytic fragment by Western blotting (Kikkawa et al., 1989; Ways et al., 1991), as well as with the apparent instability of PKC-TM310, suggesting that the individual domains of PKC are targeted for rapid degradation once they are released into the cytosol.

Although little is known about the involvement of particular regions of the catalytic domain of PKC in catalysis, Hanks et al. (1988) have identified highly conserved individual amino acids within the catalytic domains of both serine/threonine and tyrosine protein kinases, suggesting that they play fundamental roles in catalytic activity. The last conserved residue in protein kinase catalytic domains is an arginine, found at position 580 in PKCα. In many protein kinases this residue lies very close to the COOH terminus of the molecule (Hanks et al., 1988), but members of the PKC family have from 85 to 92 additional amino acids between the arginine and the end of the protein, suggesting that the extreme COOH terminus is involved in functions other than catalysis (Coussens et al., 1986; Knopf et al., 1986; Ono et al., 1988). Thus, it is possible that this region interacts with a receptor in the nuclear envelope. In contrast to the hinge region, the COOH terminus is largely conserved among the different PKC isoforms (Coussens et al., 1986; Knopf et al., 1986; Ono et al., 1988), suggesting the possibility of both common and subtype-specific binding sites. The presence of two separate regions in PKCα that are capable of interacting with one or more receptors would provide the cell with an additional mechanism by which to regulate the enzyme's subcellular distribution, substrate specificity, or both.

The existence of receptors for activated PKC was suggested by a recent study in which purified PKC was demonstrated to bind in a saturable and specific manner to proteins present in the TI fraction (Mochly-Rosen et al., 1991). Binding was dependent upon the presence of phosphatidylserine, calcium, and diacylglycerol and was not inhibited by preincubation with a peptide substrate. The authors concluded that activation of PKC resulted in exposure of regions outside the active site that bind via protein–protein interactions to receptor molecules. Our results are consistent with this model and reveal that a portion of the hinge region and at least one other segment of the catalytic domain, possibly the extreme COOH terminus, are responsible for this interaction. Furthermore, the subcellular distribution of the constitutive mutants indicates that the receptor(s) resides in the nuclear envelope. Indeed, using a PKC8 bacterial fusion protein as a probe in a blotting assay similar to that described by Mochly-Rosen et al. (1991), we have detected binding to nuclear proteins of 30, 32, 55, and 100 kD.[2] Experiments designed to map the regions of PKC required for interaction with the various candidate receptors are in progress.

Despite original beliefs that PKC was primarily a plasma membrane–localized enzyme, it has become clear in recent years that translocation of these molecules to the nucleus also occurs under certain conditions. In HL-60 cells PKCβ_{II}, but not PKCα, was translocated to the nucleus after treatment with bryostatin (Hocevar and Fields, 1991). This translocation resulted in increased phosphorylation of several nuclear envelope proteins, including lamin B. PKCβ has also been detected in nuclei from rat liver (Rogue et al., 1990), and phorbol ester treatment of NIH 3T3 fibroblasts resulted in translocation of PKCα to the nucleus (Leach et al., 1989). In the latter study, it was demonstrated that PKCα co-fractionated with lamin B, indicating that the enzyme was localized to the nuclear envelope. Localization of constitutively active mutants of PKC to the nuclear envelope is consistent with translocation of wild-type PKC to the nuclear envelope in response to known activators of the enzyme, and suggests a common mechanism.

The PKC mutants found in the nuclear envelope remained there following extraction with buffer containing 1.6 M NaCl, suggesting a high-affinity interaction. This is also suggested by the absence of PKC7 in the TS fraction. The majority of the MARCKS protein is bound to the plasma membrane (James and Olson, 1989*a*), and this association is resistant to extraction with 0.5 M NaCl (Albert et al., 1986). A nonmyristoylated mutant of the MARCKs protein is primarily cytoplasmic (Graff et al., 1989), with a small amount loosely associated with the membrane fraction.[2] Thus, the myristoylated amino terminus of this protein confers high-affinity binding to a component of the plasma membrane. The lack of PKC7 in the TS fraction, where the wild-type MARCKS protein is found, suggests that the PKCα nuclear targeting signals, when exposed, are sufficient to override the normal targeting information present in the amino terminus of the MARCKS protein. The nuclear-localized mutants described in this report, with their high affinity

for the nuclear envelope, will be valuable tools in the isolation and characterization of the putative nuclear PKC receptor(s).

It has recently been demonstrated that deletion of portions of the regulatory domains of PKCα and PKCβ is sufficient to induce phorbol ester-independent kinase activity (Kaibuchi et al., 1989). However, the authors stated that their mutant proteins were found mainly in the cytoplasmic fraction. Two possible explanations may account for this apparent discrepancy between their results and ours. First, the data supporting the cytoplasmic localization of their mutants was not shown and it is not clear whether the nuclear fraction was analyzed. It is therefore possible that a substantial amount of these mutants were present in the nuclear fraction and went undetected. In addition, their mutants were missing only selected portions of the regulatory domain, as opposed to PKC7 and PKC8, in which the entire regulatory domain was deleted. We have found that a PKCα mutant that is missing the first 186 amino acids, therefore resembling a number of the previously described mutants, resides in both the nuclear envelope (~60%) and TS (~40%) fractions (data not shown). This suggests that localization to the nucleus is dependent on the amount of regulatory domain deleted, and that portions of the regulatory domain, when present in a particular conformation, might exert a negative influence on the interaction with the putative receptor(s). We believe that the near complete localization of mutants such as PKC8 and PKC14 to the nuclear envelope reflects the "permanent" exposure of their nuclear targeting signals. In contrast, these signals in the intact enzyme are probably masked by the regulatory domain and are only exposed following a stimulus-induced conformational change. In the holoenzyme, this conformational change could be subject to reversal, mediated by the regulatory domain, resulting in recovering of the targeting signals and release from the nucleus.

The targeting of activated PKC mutants to the nucleus, along with this and other reports demonstrating nuclear translocation of wild-type PKC (Hocevar and Fields, 1991; Leach et al., 1989; Rogue et al., 1990), suggest that the enzyme may exert its effects on nuclear events in a more direct manner than was originally believed. Traditional models depicted PKC as a component of a signaling cascade that culminated, at least in part, in altered transcription of target genes (Bell, 1986; Kikkawa et al., 1989). However, our results and those of others raise the possibility of direct modification of nuclear regulatory proteins by PKC. In this regard, we have found that PKC7 and PKC8 phosphorylate the nuclear transcription factor myogenin on a threonine residue in its DNA-binding domain in vivo, suppressing by ~95% its ability to activate transcription from the muscle creatine kinase enhancer (Li, L., G. James, R. Heller-Harrison, M. Czech, and E. Olson, manuscript submitted for publication). This same residue is phosphorylated by wild-type PKCα in vitro, indicating that deletion of the regulatory domain does not alter the substrate specificity of PKC.

The existence of numerous PKC isoforms, many of which are co-expressed in several cell types, has suggested that subtle differences might exist in their subcellular distribution and/or substrate specificities (Kikkawa et al., 1989). Indeed, these expectations have been borne out in recent studies (Hocevar and Fields, 1991; Kariya et al., 1991). The findings presented in this report provide the basis for a model by which these differences may be explained and suggest that the effects of PKC activation on cellular processes might be more complex than has previously been believed.

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