The Catalytic Domain of Protein Kinase C Chimeras Modulates the Affinity and Targeting of Phorbol Ester-induced Translocation*

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Emerging evidence suggests important differences among protein kinase C (PKC) isozymes in terms of their regulation and biological functions. PKC is regulated by multiple interdependent mechanisms, including enzymatic activation, translocation of the enzyme in response to activation, phosphorylation, and proteolysis. As part of our ongoing studies to define the factors contributing to the specificity of PKC isozymes, we prepared chimeras between the catalytic and regulatory domains of PKCα, -δ, and -ε. These chimeras, which preserve the overall structure of the native PKC enzymes, were stably expressed in NIH 3T3 fibroblasts. Their intracellular distribution was similar to that of the endogenous enzymes, and they responded with translocation upon treatment with phorbol 12-myristate 13-acetate (PMA). We found that the potency of PMA for translocation of the PKCα/x chimeras from the soluble fraction was influenced by the catalytic domain. The ED_{50} for translocation of PKCα/α was 26 nM, in marked contrast to the ED_{50} of 0.9 nM in the case of the PKCα/ε chimera. In addition to this increase in potency, the site of translocation was also changed; the PKCα/ε chimera translocated mainly into the cytoskeletal fraction. PKCx/ε chimeras displayed twin isoforms with different mobilities on Western blots. PMA treatment increased the proportion of the higher mobility isoform. The two PKCx/ε isoforms differed in their localization; moreover, their localization pattern depended on the regulatory domain. Our results emphasize the complex contributions of the regulatory and catalytic domains to the overall behavior of PKC.

Protein kinase C (PKC)[1] is a major family of serine/threonine kinases that plays a crucial role in cell signal transduction, regulating cell growth and differentiation (1). Emerging evidence suggests important differences among PKC isozymes both in their regulation and in their biological roles. Thus, in K-562 erythroleukemia cells, PKCα was implicated in mediating PMA-induced cytostasis, whereas PKCβII was involved in proliferation (2). In RBL-2H3 basophilic leukemia cells, the PKCα and -ε isoforms preferentially inhibited phospholipase C activity (3), whereas the PKCβ and -ε isoforms linked the mast cell high affinity receptor for IgE to the expression of c-*fos* and c-*jun* (4). In PKCβ knockout mice, signaling through the antigen receptor-dependent signaling pathway was markedly impaired (5). Not only may some PKC isoforms be active whereas others not for a given response, but the actions of different isoforms may even be antagonistic. In NIH 3T3 cells, for example, PKCδ arrested cell growth, whereas PKCε stimulated it (6, 7). As part of our ongoing studies to explore the basis of specificity of PKC isozymes, we have prepared chimeras between the regulatory and the catalytic domains of PKCα, -δ, and -ε and investigated their behavior in intact cells.

Protein kinase C consists of an N-terminal regulatory domain and a C-terminal catalytic domain. The catalytic domain acts as a serine/threonine-specific protein kinase, and the regulatory domain is thought to inhibit this catalytic activity through a so-called pseudosubstrate region near its N terminus. Immediately C-terminal to this pseudosubstrate region is a pair of highly conserved zinc finger structures termed the C1 domains that are the sites of phorbol ester binding on the molecule and contribute to the association with anionic phospholipid (8, 9). In the classic isozymes, α, βI, βII, and γ, a second domain in the regulatory region, the C2 domain, bestows Ca^{2+} dependence. The novel isozymes, δ, ε, η, and θ, lack this region and correspondingly lack Ca dependence, although they have a modified C2 homolog N-terminal to the C1 domain (10). The individual C1 domains of PKC bind phorbol esters with similar affinity to the intact PKC. X-ray crystallography of the PKCδ C1b domain revealed that the phorbol ester inserts into a hydrophilic cleft in an otherwise hydrophobic surface, promoting interaction of the C1 domain with the membrane (11). It thus functions as a hydrophobic switch.

In the intact unstimulated cell, PKC is largely cytosolic with some proportion depending on the isoform and the cell type present in the membrane and cytoskeletal fractions (12, 13). Phorbol ester addition leads to translocation of PKC from the cytosol to the membranes, presumably reflecting enhanced membrane affinity of the C1-phorbol ester complex. This translocation provides one measure of the response of specific PKC isoforms in the context of the intact cell. Emerging understanding suggests that translocation should not only depend on the strength of the association between the C1 domain and the membrane but should also be coupled to other factors contributing to the membrane association and the energetics of conformational changes in the enzyme upon activation. Receptors for activated protein kinase C, the binding proteins for the regulatory domain of PKC, have been described (14), which stabilize the activated conformation of the enzyme (15). Specific substrates likewise can drive association, as elegantly

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[1] The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; PDBu, phorbol 12,13-dibutyrate.

shown by Jaken and co-workers (16). The state of phosphorylation of PKC is another important regulator that influences both activity and localization (17, 18). Finally, the ability of the pseudosubstrate domain to interact with the catalytic domain is central to its function.

We report here that the catalytic domain of PKC influences both the potency of phorbol ester for PKC translocation and the compartment to which PKC is translocated in response to phorbol ester.

EXPERIMENTAL PROCEDURES

Construction of PKC Chimeras—Protein kinase C chimeras were generated by swapping the regulatory and the catalytic domains of PKCα, PKCδ, and PKCε. The regulatory domain of PKCε was amplified by polymerase chain reaction (PCR) employing high fidelity thermostable vent DNA polymerase using the following primers: 5'-CTC-GAGATGGTAGTGTTCAATGGCCTTCTTA-3' and 5'-GCTGCCTTT-GACTAGTACCTTGAT-3'. To amplify the catalytic domain of PKCε, the primers we used were: 5'-GTACTAGTCAAAGGCAGCTTTGGCAA-3' and 5'-GACGCGTTCAGGGCATCAGGTCTTCACCAAA-3'. The regulatory and catalytic domains of PKCδ were amplified by utilizing the primers below, respectively: 5'-GGGCTCGAGATGGCACCCTTCCT-TCGCATTT-3', 5'-GCTGCCTTTGACTAGTACTTTT-3', 5'-GTAC-TAGTCAAAGGCAGCTTTGGCAA-3', and 5'-CCACGCGTAATGTC-CAGGAATTGCTCAAACTT-3'. To amplify the regulatory and catalytic domains of PKCα, we employed the following primers, respectively: 5'-CGCTCGAGATGGCTGACGTCTTCC-3', 5'-GCTGCCTTTGACTAG-TACCATGAGGAA-3', 5'-GTACTAGTCAAAGGCAGCTTTGGCAA-3', and 5'-CGACGCGTTACCGCGCTCTGCAGGATGG-3'. To reduce the chance of introducing mutations, we not only used high fidelity enzymes but we also kept the number of PCR cycles low (8 cycles). To facilitate subsequent cloning steps, into the inner PCR primers we introduced a unique restriction site (*Spe*I). After 8 cycles of polymerase chain reaction, we added an adenine overhang to the constructs with *Taq* polymerase at 72 °C after removing the primers, and we then ligated them into the pGEM-T vector. From this point on we employed only classical cloning techniques to further reduce the possibility of mutations in our constructs. Using the pGEM-T vector as a shuttle vector we amplified the different PKC domains separately by transforming them into bacteria; we then subcloned the catalytic domains into the vectors containing the regulatory domains using *Spe*I and *Mlu*I restriction enzymes. An important advantage of this approach is that we could reconstruct the wild type PKCα, -δ, and -ε isoforms using the same inserts as for the chimeras, providing us with wild type controls constructed the same way as the chimeras. Next we performed site-directed mutagenesis to mutate the *Spe*I site back to the original sequence using the following mutagenesis primers: for chimeras with PKCα regulatory domain, 5'-TCCTCATGGTGCTGGGCAAAGGCAGC-3'; for chimeras with PKCδ regulatory domain, 5'-CCAAAAAGTACTTGGCAAAGGCAGC-3'; and for those with PKCε regulatory domain, 5'-CTTCATCAAGGTGTTAG-GCAAAGGCAGC-3'. We used the *Spe*I site for the selection. The chimeras along with the wild type PKC isozymes were then subcloned into an epitope-tagging mammalian expression vector described in detail by Olah *et al.* (19). The *Xho*I and *Mlu*I sites ensure unidirectionality, and the vector attaches a C-terminal 12-amino acid tag to the end of the proteins, originally derived from the C terminus sequence of PKCε. Finally, our constructs were sequenced by Paragon Biotech Inc. (Baltimore, MD) to assure that no mutations had been introduced. The chimeras were designated as PKCx/y, where x and y refer to the regulatory and the catalytic domains, respectively. Thus, PKCα/δ, for example, refers to the chimera between the α regulatory domain and the δ catalytic domain.

Cell Culture and Transfection of Cells—NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 4500 mg/liter glucose, 4 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (Advanced Biotechnologies Inc., Columbia, MD) and 10% fetal calf serum (Life Technologies, Inc.). The cells were transfected with either the empty vector or the different PKC expression vectors using Lipofectamine (Life Technologies, Inc.) following the procedure recommended by the manufacturer. The transfected cells were subsequently grown in selection medium containing 750 μg/ml G418 (Life Technologies, Inc.). After 12–18 days in selection medium, single colonies were picked and subsequently screened for the presence of different PKC chimeras by Western blot analysis. Where indicated, cells were treated with different concentrations (0.01 nM–10 μM) of PMA (LC Laboratories, Woburn, MA) for 1 h, 3 h, and 6 h at 37 °C; dimethyl sulfoxide was

added to the control cells. Analyses were routinely carried out on pools of transfected cells, but all results were confirmed on individual clones.

Cell Lysis, Subcellular Fractionation, and Western Blot Analysis—The cells were harvested into 20 mM Tris-Cl (pH 7.4) containing 5 mM EGTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 20 μM leupeptin and lysed by sonication. The cytosolic fraction represents the supernatant following centrifugation at $100,000 \times g$ for 1 h at 4 °C. The Triton X-100 soluble particulate fraction was prepared by a 2-h extraction of the pellet with the same buffer containing 1% Triton X-100 and a subsequent centrifugation for 1 h at $100,000 \times g$. The remaining pellet is the Triton X-100 insoluble fraction. The samples were subjected to SDS-polyacrylamide gel electrophoresis according to Laemmli (20) and transferred to nitrocellulose membranes. The protein content of individual samples was determined (12) by staining the Western blots with 0.1% Ponceau S solution in 5% acetic acid (Sigma). The protein staining was found to be linear up to 30 μg of protein/lane. The Ponceau S staining was removed by several washes with phosphate-buffered saline (pH 7.4); the membranes were blocked with 5% milk in phosphate-buffered saline and subsequently immunostained with polyclonal antibodies generated against a polypeptide corresponding to amino acids 726–737 of PKCε (Life Technologies, Inc.). In some cases the chimeras containing the α and the δ catalytic domains were detected with the corresponding anti-catalytic domain antibodies from Upstate Biotechnology (Lake Placid, NY) and Research and Diagnostic Antibodies (Berkeley, CA), respectively. Secondary antibodies were goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad), and the immunoreactive bands were visualized by the ECL Western blotting detection kit purchased from Amersham Corp. The densitometric analysis of the immunoblots and the normalization to the protein content of each individual lane were performed as described (12).

Protein Kinase C Assay—Protein kinase C activity was assayed by measuring the incorporation of ^{32}P from $[\gamma^{-32}P]ATP$ (Amersham Corp.) into substrates (as described previously (21)) in the presence of 100 μg/ml phosphatidylserine and 1 μM PMA. Cell lysates were partially purified on a HiTrap Q column (Pharmacia Biotech Inc., Uppsala, Sweden) and 10 μl of the partially purified cell lysates were incubated in assay buffer containing 20 mM HEPES, pH 7.5, 10 mM MgCl$_2$, 0.5 mM CaCl$_2$, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μM leupeptin, 10 μg/ml aprotinin (all purchased from Sigma), 50 μM ATP, 1 μCi of $[\gamma^{-32}P]ATP$, and 50 μM/assay myelin basic protein (from bovine brain) (Sigma) as substrate at 30 °C for 10 min. The reaction was stopped by adding trichloroacetic acid at 10% final concentration. After centrifugation for 5 min at $15\,000 \times g$ a 25-μl aliquot of the supernatant was spotted onto phosphocellulose disks (Life Technologies, Inc.). The disks were washed three times in 0.5% phosphoric acid and three times in distilled water. The bound radioactivity was measured by liquid scintillation counting. The kinase assay was linear with time over this incubation period, and at 50 μM substrate was linear with the amount of protein over the range of cell lysates used in the assays.

[³H]PDBu Binding—[³H]PDBu binding was measured by using the polyethylene glycol precipitation assay (21). Briefly, cell lysates (40–60 μg of protein/assay) were incubated with 20 nM [³H]PDBu in the presence of 100 μg/ml phosphatidylserine. Nonspecific binding, determined in the presence of 30 μM nonradioactive PDBu, was subtracted to give specific binding. Data presented represent triplicate determinations in each experiment.

RESULTS

We have constructed protein kinase Cα, -δ, and -ε chimeras to study the relative contributions of the regulatory and the catalytic subunits of these isozymes to their behavior.

We have determined that these PKC chimeras can be stably expressed in NIH 3T3 cells, bind [³H]PDBu, and exhibit cofactor-dependent kinase activity as do the wild type PKC isozymes (Table I, Fig. 1). Using a previously described tagging system (19) we could readily distinguish the endogenous and overexpressed isozymes of PKCα/x and PKCδ/x (sizes are determined by the regulatory domain). In the case of PKCε/α and PKCε/δ we confirmed our findings by using anti-PKCα and anti-PKCδ anti-catalytic antibodies. Because the levels of these overexpressed enzymes as well as the levels of the overexpressed PKCε/ε were much higher than the endogenous PKCε, interference by the endogenous PKCε was not a problem. The antibodies recognized the two previously described PKCε-specific bands at 90 and 93 K_d (7) of the overexpressed PKCε. PKCδ/ε

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	[³H]PDBu bound
	pmol/mg protein
Control	1.12 ± 0.01
PKCα/α	6.34 ± 0.07
PKCα/δ	4.1 ± 0.1
PKCα/ε	7.75 ± 0.03
PKCδ/α	4.18 ± 0.07
PKCδ/δ	4.27 ± 0.09
PKCδ/ε	5.53 ± 0.05
PKCε/α	4.55 ± 0.08
PKCε/δ	4.17 ± 0.06
PKCε/ε	4.32 ± 0.05

and PKCα/ε chimeras also showed double bands on Western blots, suggesting that the posttranslational phosphorylation of the PKCε catalytic domain was similar to that of the wild type. In contrast, the PKCε/α and PKCε/δ chimeras show a single band suggesting that the posttranslational modification of PKCε occurs only on the catalytic domain.

To explore the role of the catalytic domains in phorbol ester response, we determined the translocation and subcellular localization patterns of the different chimeras after PMA treatment (Fig. 2). Based on the kinetics of translocation of the endogenous PKCα, -δ, and -ε isoforms in these cells (12) (Table II), we examined the effects on the chimeras of treatment with varying doses of PMA for 1, 3, and 6 h. The translocation dose-response curves were stable after 1 h of exposure to PMA, and at this time point down-regulation was not yet detectable (data not shown). Fig. 2 illustrates one representative dose-response experiment using pooled transfected cells (at least three experiments were performed at each time point). The tagged overexpressed wild type PKC isozymes (PKCα/α, PKCδ/δ, and PKCε/ε) translocated with time courses and dose-response curves similar to those of the endogenous enzymes reported earlier (12), arguing against artifacts caused either by the overexpression or by the ε-epitope tag (Table II). Experiments were also performed with at least one clone of each chimera, yielding similar results to the pooled cultures.

The subcellular distribution of PKC chimeras in the untreated cells is summarized in Table III. The distribution of the overexpressed wild type enzymes was similar to that reported previously for the endogenous enzymes (12) except that 10% of PKCδ/δ was found in the insoluble fraction. The control of distribution in the case of PKCα/x (PKCα regulatory domain) chimeras was dominated by the α regulatory domain. A role for the catalytic domain was evident in the case of PKCε/α chimera; moreover, the catalytic domain of PKCε seemed to have some effect in bringing PKCα/ε and PKCδ/ε into the insoluble fraction (PKC ε/ε was also higher there as expected). The PKCδ/α distribution differed from that of both parent isozymes with a much higher proportion in the Triton X-100 insoluble fraction. The cofactor-dependent stimulation of kinase activity was somewhat lower for PKCδ/α than that of the other chimeras (see Fig. 1); on the other hand, the PKCδ/α chimera showed good [³H]PDBu binding activity (see Table I). How these differences may be related to the higher portion in the Triton X-100 insoluble fraction remains to be determined.

In the case of the PKCα regulatory chimeras, the catalytic

domain markedly influenced the apparent affinity of PMA for the enzyme. PMA was less potent in translocating the wild type PKCα/α than the PKCα/δ and α/ε chimeras. The dose-response curves for the decrease in PKC in the soluble fraction were quantitated and fitted to the Hill equation (Fig. 3) In the case of wild type PKCα/α, the ED_{50} for translocation was 26 ± 1 nM ($n =$ five experiments) (similar to that reported earlier for the endogenous PKCα (12)) (Table II). It decreased to 9.1 ± 0.3 nM in the case of the PKCα/δ chimera ($n =$ five experiments) and was yet an order of magnitude more sensitive in the case of the PKCα/ε chimera (ED_{50}, 0.91 ± 0.05 nM ($n =$ five experiments)). For the chimeras involving the δ and ε regulatory domains the ED_{50} values for PMA-induced translocation were similar independent of the specific regulatory or catalytic domain (PKCδ/α, 9.3 ± 0.1 nM; PKCδ/δ, 12.4 ± 0.3 nM; PKCδ/ε, 8 ± 1 nM; PKCε/α, 12 ± 1 nM; PKCε/δ, 7.4 ± 0.2 nM; PKCε/ε, 7.0 ± 0.4 nM; $n =$ three experiments for all values) (Fig. 4).

Not only did the PKCα/ε chimera have a dose-response curve that was shifted to the left, but the destination of translocation changed; *i.e.* the chimera translocated mostly to the Triton X-100 insoluble fraction. This shift in distribution was parallel to a changing proportion of the PKCα/ε chimera in the lower band as compared with the upper band (easiest to observe in the total PKC fraction). The lower band of PKCα/ε was present just in the Triton X-100 insoluble fraction, whereas the upper band was predominantly in the cytosolic and particulate fractions (Fig. 2).

The PKCδ/ε and PKCε/ε chimeras also revealed a PMA dependent shift in the proportions of the two bands with an increase in the lower band at higher PMA concentrations. The subcellular distribution of the two bands depended on the identity of the regulatory domain, whereas the presence of the two bands depended on the ε catalytic domain. Compared with PKCε/ε, PKCδ/ε and PKCα/ε showed a reduced proportion of the upper band present in the Triton X-100 insoluble fraction. Unlike PKCε/ε or PKCα/ε, PKCδ/ε maintained large amounts of the higher mobility isoform (lower band) in the cytosolic and membrane fractions.

We determined the PMA dependence of the increase in the lower band for the PKCx/ε chimeras. The ED_{50} values were similar to those observed for the decrease in the soluble fraction (1.5 *versus* 0.91 nM for PKCα/ε, 11.1 *versus* 8 nM for PKCδ/ε, and 9.1 *versus* 7.0 nM for PKCε/ε). We conclude that the two processes occur in parallel.

DISCUSSION

A major objective is to dissect the factors regulating the flow of information through the families of PKC isoforms present in specific cell types. For therapeutic intervention, isoform selective ligands would greatly enhance specificity. Unfortunately it is becoming clear that current *in vitro* binding assays to recombinant PKC isozymes neglect major contributions to selectivity in the intact cells. Thus, phorbol esters have a 4-fold weaker affinity for PKCε compared with PKCα *in vitro* (21), whereas PMA is 160-fold more potent for translocation of PKCε than of PKCα in mouse keratinocytes (13). Selectivity depends, moreover, on the specific cell type. In NIH 3T3 cells, the selectivity of PMA for PKCε compared with PKCα is only 3.5-fold *versus* the 160-fold in the keratinocytes (12, 13). Our current results demonstrate that the factors controlling the phorbol ester interactions depend on the catalytic domain of PKC as well as on the phorbol ester binding C1 domains. Identification of the specific mechanisms by which the catalytic domain contributes to the translocation remains to be determined.

One possible mechanism by which the catalytic domain could influence protein kinase C unfolding, and indirectly phorbol ester binding, would be through the strength of the interaction

FIG. 1. **Kinase activity of protein kinase C chimeras.** PKC chimeras were partially purified from cell lysates, and protein kinase activity was assessed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into substrates in the presence and absence of 100 μg/ml phosphatidylserine (*PS*) and 1 μM PMA as described under "Experimental Procedures." Data represent the mean $\pm$ S.E. of three independent experiments. The values for the control and the PKCα/α chimera are expressed as pmol/min/mg of protein, and the values for the other chimeras were further normalized based on their level of expression relative to the PKCα/α chimera. For the experiments shown in the figure we used myelin basic protein as substrate.
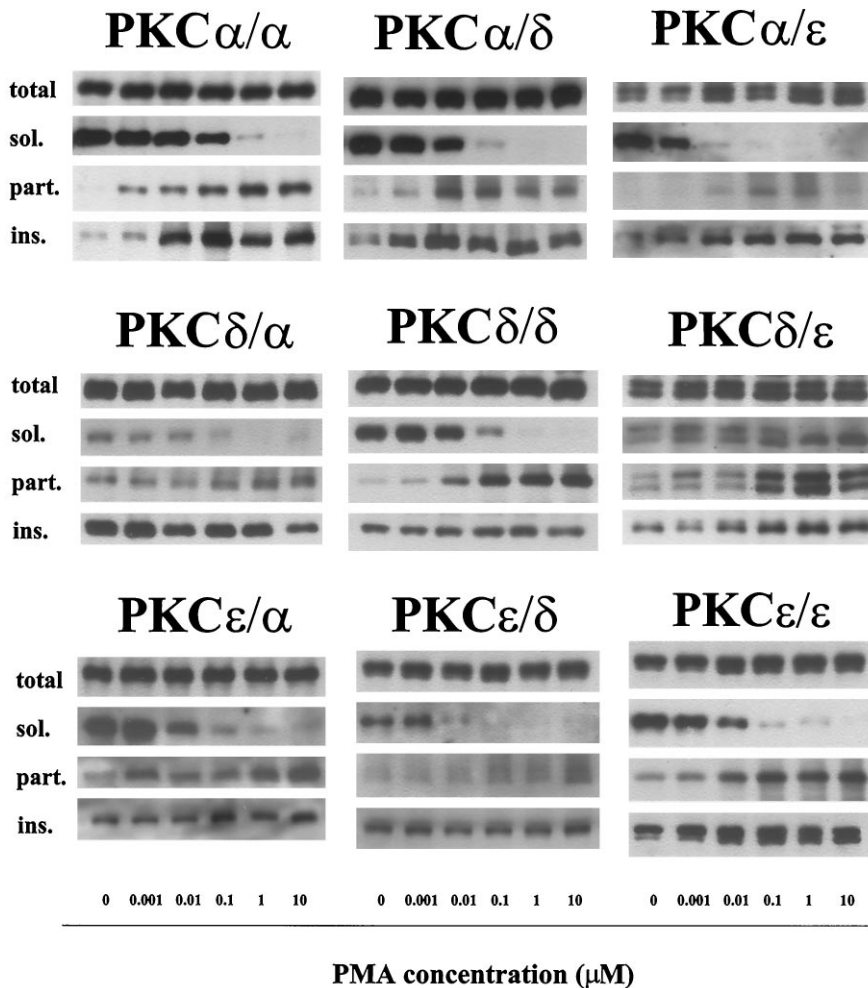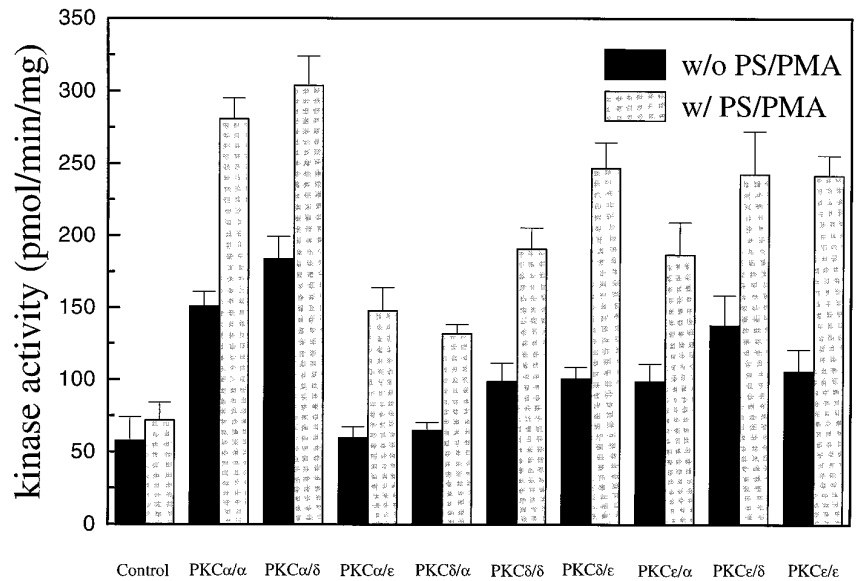

FIG. 2. **Translocation and localization patterns of PKC chimeras induced by PMA treatment.** NIH 3T3 fibroblasts overexpressing various PKC chimeras were treated with increasing concentrations of PMA. Samples for SDS-polyacrylamide gel electrophoresis were prepared and Western immunoblotting was performed as described under "Experimental Procedures." The figure illustrates one representative experiment. The fractions were labeled in the figure as follows: *total*, total fraction; *sol*, soluble fraction; *part*, Triton X-100 soluble fraction; and *ins*, Triton X-100-insoluble fraction. Similar results were obtained in three to five sets of independent experiments at each time point.

between the pseudosubstrate region and the catalytic site. Cantley and co-workers (22) have examined in depth the substrate selectivities of the PKC isozymes. The α pseudosubstrate peptide shows similar K_m values for the α and δ catalytic activities (ϵ was not reported) (22). Also, we had observed similar relative activities of the PKCα and PKCϵ pseudosubstrates for PKCϵ (21). Although the regulation of PKC isozymes by second messengers and membrane components has been

extensively studied (1, 23), the mechanisms by which PKCs can separately modulate signals from distinct receptor pathways remain under active investigation. In cells stimulated with hormones or phorbol esters, most of the cellular PKC translocates to new subcellular sites, including the plasma membrane (24), cytoskeleton (15), nucleus (15, 25), and elsewhere (15). Furthermore, within the same cell various isozymes may each be localized to different subcellular sites after cell stimulation

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TABLE II

Comparison of the affinity of PMA to induce translocation of the endogenous PKC isozymes and the overexpressed PKC isozymes with ϵ-epitope tag

NIH 3T3 fibroblasts transfected with PKC chimeras were treated by 0.01 nM–1 μM PMA, the soluble fraction was prepared, and Western immunoblotting was performed as described under "Experimental Procedures." The amount of the enzyme was quantitated by densitometry, and dose-response curves and ED_{50} values were calculated from the Hill equation. Points represent the mean $\pm$ S.E. of at least three independent experiments. The ED_{50} values for the endogenous PKCs are from Ref. 12.

	ED_{50} for the endogenous PKCs (12)	ED_{50} for the ϵ-epitope tagged PKCs
	nM	*nM*
PKCα	21 ± 1.2	26 ± 1
PKCδ	11 ± 0.3	12.4 ± 0.3
PKCϵ	6 ± 0.8	7.0 ± 0.4

TABLE III

Subcellular localization of various PKC chimeras in untreated cells

The distribution of the individual PKC isozymes among the fractions obtained by centrifugation was determined based on the protein levels measured in these fractions. (The soluble fraction contains about 45–50%, the Triton X-100 soluble particulate fraction contains about 5%, and the Triton X-100 insoluble fraction contains about 45–50% of the total protein.) Similar results were obtained in a second set of independent experiments.

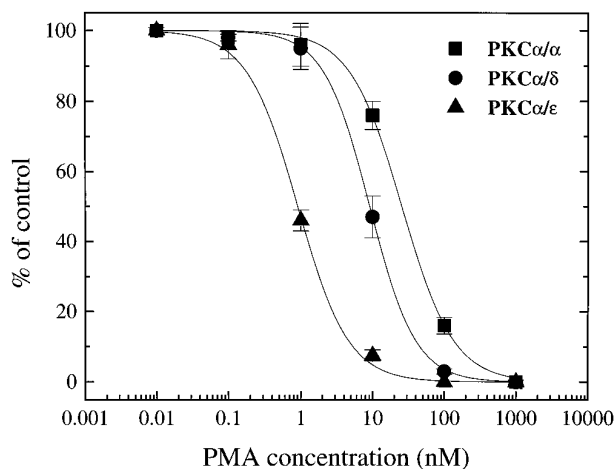|	Soluble fraction	Particulate fraction	Triton X-100 insoluble fraction
	% of total		
PKCα/α	80	10	10
PKCα/δ	80	10	10
PKCα/ϵ	80	5	15
PKCδ/α	30	15–20	50
PKCδ/δ	65–75	15–25	10
PKCδ/ϵ	50–55	15–20	30
PKCϵ/α	80	10	10
PKCϵ/δ	50–55	10–15	30–35
PKCϵ/ϵ	50–55	10–15	30–35

FIG. 3. **PMA-induced changes in the level of PKCα/x chimeras in the cytosolic fraction of NIH 3T3 fibroblasts.** NIH 3T3 fibroblasts transfected with PKCα/α, -α/δ, and -α/ϵ chimeras were treated by the indicated doses of PMA. The soluble fraction was prepared, and Western immunoblotting was performed as described under "Experimental Procedures." The amount of the enzyme was quantitated by densitometry and expressed as the percentage of the amount of isozyme present in the soluble fraction in the untreated cells. The illustrated dose-response curves were calculated from the Hill equation. Points represent the average of five independent experiments $\pm$ S.E.

(25, 26). Translocation of protein kinases to new sites necessarily alters their access to substrates (15).

Increasing evidence implicates both the regulatory and the catalytic domains in modulating PKC translocation. It has
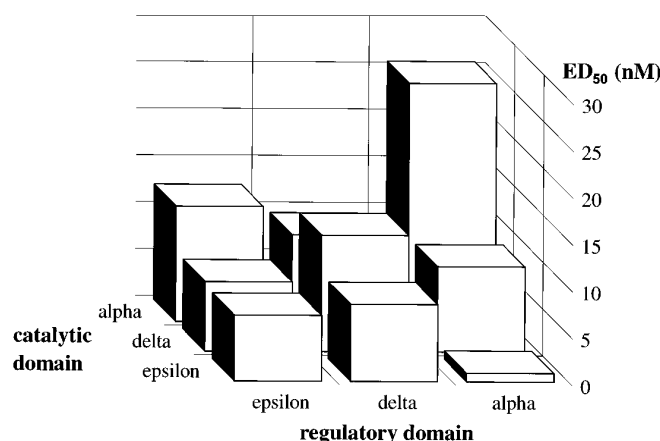

FIG. 4. **ED_{50} values of PMA-induced translocation of PKC chimeras in NIH 3T3 cells.** NIH 3T3 fibroblasts transfected with PKC chimeras were treated with 0.01 nM–1 μM PMA; the soluble fraction was prepared, and Western immunoblotting was performed as described under "Experimental Procedures." The amount of the enzyme was quantitated by densitometry and expressed as the percentage of the amount of isozyme present in the soluble fraction in the untreated cells. Dose-response curves were calculated from the Hill equation, and the calculated ED_{50} values are shown in this diagram. Points represent the average of at least three independent experiments.

been previously proposed that membrane binding of PKC *in vivo* reflects the binding of the activated enzyme to the anchored receptors for activated protein kinase C (27). This occurs via the regulatory domain of PKC (14), stabilizing the active conformation of the enzyme (15). Protein kinase Cϵ was also reported to bind to actin through a binding site located within the regulatory domain (28). Conversely, there is strong evidence for the role of the catalytic domain in isozyme-specific localization. Although PKCβI and -βII differ only at the C terminus, they localize differently (29), strongly arguing that unique C-terminal sequences may target these isoforms to different subcellular locations (30). Also, using PKCα and -βII chimeras, a region within the catalytic domain of βII PKC was shown to be responsible for its isotype-specific translocation to the nucleus (31). Targeting may also occur by binding to cellular proteins that function as substrates. Examples include myristoylated alanine-rich C kinase substrate, γ-adducin, and kinesin light chain (29, 32). Furthermore, the constitutive membrane association of the truncated PKC regulatory domain, in contrast to the cytosolic localization of the holoenzyme, argues for a role of the catalytic domain (33). Finally, immunohistochemical studies reveal a role for the catalytic domain in the pattern of localization and translocation of PKCδ and PKCϵ chimeras.[2] Complementing these other studies, the findings described here show that the catalytic domain can control the potency of PMA for driving translocation.

Not surprisingly, phosphorylation has emerged as an important mechanism of PKC regulation (17). Phosphorylation provides negative charges on the so-called activation loop of PKC that is necessary for enzymatic activity (34, 35). This transphosphorylation is followed by two autophosphorylation steps. Both occur on the C terminus of the enzyme, further stabilizing the catalytically active conformation and also making the enzyme soluble (18). Our studies support a role for phosphorylation in determining the localization of PKCϵ, with PMA changing the proportion of the higher mobility isoform of PKCx/ϵ chimeras in the Triton X-100 insoluble fraction. At the same time the localization patterns of these chimeras depend

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[2] Q. J. Wang, P. Acs, J. Goodnight, P. M. Blumberg, H. Mischak, J. F. Mushinski, submitted for publication.

on the regulatory domain. The fact that the phosphorylation state of PKCs as well as PKC chimeras can change after PMA treatment emphasizes that protein phosphatases may play a cell-specific role in targeting different PKCs during translocation.

PMA translocates chimeras that have the same regulatory domains but different catalytic domains with potencies that differ by an order of magnitude. Phorbol esters bind to the C1 domains of PKC providing a hydrophobic cap over a hydrophilic cleft (11). At the same time their side chains contribute to stabilizing the enzyme at the membrane. Our results suggest that binding of PMA reveals other site(s) that facilitate(s) translocation (shift in ED_{50}) and that play(s) a role in targeting PKC to separate subcellular sites (translocation of PKCx/ϵ chimeras to the insoluble fraction).

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