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Both the Catalytic and Regulatory Domains of Protein Kinase C Chimeras Modulate the Proliferative Properties of NIH 3T3 Cells*

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Protein kinase C (PKC) isozymes exhibit important differences in terms of their regulation and biological functions. Not only may some PKC isoforms be active and others not for a given response, but the actions of different isoforms may even be antagonistic. In NIH 3T3 cells, for example, PKCδ arrests cell growth whereas PKCϵ stimulates it. To probe the contribution of the regulatory and the catalytic domains of PKC isozymes to isozyme-specific responses, we prepared chimeras between the regulatory and the catalytic domains of PKCα, -δ, and -ϵ. These chimeras, which preserve the overall structure of the native PKC enzymes, were stably expressed in mouse fibroblasts. A major objective was to characterize the growth properties of the cells that overexpress the various PKC constructs. Our data demonstrate that both the regulatory and the catalytic domains play roles in cell proliferation. The regulatory domain of PKCϵ enhanced cell growth in the absence or presence of phorbol 12-myristate 13-acetate (PMA), and, in the presence of PMA, all chimeras with the PKCϵ regulatory domain also gave rise to colonies in soft agar; the role of the catalytic domain of PKCϵ was evident in the PMA-treated cells that overexpressed the PKC chimera containing the δ regulatory and the ϵ catalytic domains (PKCδ/ϵ). The important contribution of the PKCϵ catalytic domain to the growth of PKCδ/ϵ-expressing cells was also evident in terms of a significantly increased saturation density in the presence of PMA, their formation of foci upon PMA treatment, and the induction of anchorage-independent growth. Aside from the growth-promoting effect of PKCϵ, we have shown that most chimeras with PKCα and -δ regulatory domains inhibit cell growth. These results underscore the complex contributions of the regulatory and catalytic domains to the overall behavior of PKC.

Protein kinase C (PKC)[1] comprises a family of serine/threonine kinases that play crucial roles in signal transduction and in the regulation of cell growth and differentiation (1, 2). The complexity of the PKC pathway is clearly shown by the fact that PKC represents at least 11 isozymes with different patterns of tissue expression, subcellular localization and cofactor requirements as well as functional diversity (3, 4).

In all cases, the PKC isozymes consist of an N-terminal regulatory domain and a C-terminal catalytic domain. The catalytic domain has serine/threonine-specific protein kinase activity. In the "classical" (α, βI, βII, γ) and "novel" (δ, ϵ, η, θ) PKC isozymes, the regulatory domain is thought to inhibit this catalytic activity through a so-called pseudosubstrate region near its N terminus. Adjacent to this pseudosubstrate region is a pair of highly conserved zinc finger structures, which represent the phorbol ester binding domain and which contribute to the interaction with an essential cofactor, anionic phospholipid (5). Diacylglycerol, the endogenous activator, and its ultrapotent analogs, the phorbol esters, act as hydrophobic switches upon binding (6), helping to recruit protein kinase C to the membrane (7), a process referred to as translocation.

Not only do the various isozymes show considerable diversity in their structures and regulatory properties, but they have been shown to exhibit different biological effects (1, 8). PKCα and -ϵ were shown to inhibit phospholipase C activity (9), and PKCβ and -ϵ were proven to link the mast cell high affinity receptor for IgE to the expression of c-*fos* and c-*jun* (10). PKCδ and -α participate in phorbol 12-myristate 13-acetate (PMA)-induced myeloid differentiation in 32D cells (11). Of particular relevance to the current study, PKCδ and -ϵ have been shown by several groups to be involved in arresting or stimulating cell growth, respectively. PKCδ was shown to decrease cell growth, reduce cell density at confluence and not to permit anchorage-independent growth in NIH 3T3 fibroblasts (12), and to cause growth arrest in G_2/M phase of the cell cycle in Chinese hamster ovary cells (13). A recent study showed that, in rat fibroblasts that overexpress the c-*src* proto-oncogene, the tumor-promoting effect of phorbol esters is due to depletion of PKCδ, further supporting its tumor suppressor function (14). In contrast, overexpression of PKCϵ leads to increased cell growth, increased cell density at confluence, and induction of anchorage-independent growth in NIH 3T3 and rat fibroblasts (12, 15). Overexpression of PKCϵ in these cells also renders them tumorigenic in nude mice (12, 15). Against this background, it is crucial to emphasize that the effect of PKC isozymes is greatly influenced by the host cell; PKCδ enhances tumorigenicity of breast cancer cells and may thus promote tumor progression in that system (16). This cell type-specific function also holds for the PKCα and -βII isotypes, which play distinct roles in the transduction of proliferative and differentiating signals in K-562 erythroleukemia cells; PKCα inhibits whereas PKCβII promotes cell growth (17). Similar results were obtained using the R6 rat embryo fibroblast cell line (18). On the other hand, PKCα, like PKCδ, enhances the growth rate of breast cancer cells making them display a more aggressive

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[1] The abbreviations used are: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco's modified Eagle's medium; PDGF, platelet-derived growth factor; PAGE, polyacrylamide gel electrophoresis.

neoplastic phenotype (19).

The fact that PKCα, PKCδ, and PKCε cause opposite effects in stimulating and inhibiting cell growth makes them attractive targets for antitumor drug development. To probe the contribution of the regulatory and the catalytic domains of PKC isozymes to isozyme-specific responses, we prepared reciprocal chimeras between the regulatory and the catalytic domains of PKCα, -δ, and -ε, combined at the highly conserved hinge region. We describe here the effects caused by overexpression of these chimeric enzymes on cell growth of NIH 3T3 fibroblasts. We found that both the regulatory and the catalytic domains of PKC contributed to the isotype-specific effects on cell growth.

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 construction of the chimeric proteins is detailed elsewhere (20). Briefly, the regulatory and the catalytic domains of PKCα, -δ, and -ε were amplified separately by polymerase chain reaction employing high fidelity thermostable Vent™ DNA polymerase (New England Biolabs Inc., Beverly, MA). To avoid mutations, in addition to using high fidelity enzymes, we kept the number of polymerase chain reaction cycles low (eight cycles). Into the inner primers we introduced a unique restriction site (*Spe*I) for subsequent cloning steps. Using the pGEM-T vector as a shuttle vector, we amplified the different PKC domains separately by transforming them into bacteria, then subcloning the regulatory domains into the vectors that contained the catalytic 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 using the same inserts as for the chimeras providing us with wild type controls constructed the same way as the chimeras. The chimeras along with the wild type PKC isozymes were subcloned into an epitope tagging mammalian expression vector described in detail by Olah *et al.* (21). The *Xho*I and *Mlu*I sites ensure unidirectionality, and the vector attaches to the end of the proteins a C-terminal 12-amino acid tag, originally derived from the C-terminal sequence of PKCε. Our constructs were sequenced by Paragon Biotech Inc. (Baltimore, MD) to verify that no mutations had been introduced. The chimeras were designated as PKC*x*/*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.) (complete DMEM). 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, expanded, and screened for the presence of different PKC chimeras by Western blot analysis. Where indicated, cells were treated with 30 nM PMA (LC Laboratories, Woburn, MA) every other day for the duration of the experiment; likewise, where indicated, PDGF (Upstate Biotechnology Inc., Lake Placid, NY) was added to a 100 ng/ml final concentration after overnight serum starvation.

Cell Lysis 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 samples were subjected to SDS-PAGE according to Laemmli (22) and transferred to nitrocellulose membranes. Western blots were stained with 0.1% Ponceau S solution in 5% acetic acid (Sigma) for determining the protein content of individual lanes. The protein staining was found to be linear up to 30 μg of protein/lane. The Ponceau S staining was removed by several washes of phosphate-buffered saline (pH 7.4); the membranes were blocked with 5% dry milk in phosphate-buffered saline and subsequently immunostained with antibodies generated against the C terminus (PKCδ amino acids 662–673) of PKCδ (Research & Diagnostics Antibodies, Berkeley, CA), the C terminus of PKCα (Upstate Biotechnology Inc.) or an affinity-purified polyclonal antibody against a polypeptide corresponding to amino acids 726–737 of PKCε (Life Technologies, Inc.). Secondary antibodies were goat anti-rabbit or goat anti-mouse IgG coupled to horseradish peroxidase (Bio-Rad), and the immunoreactive bands were visualized by the ECL Western blotting detection kit (Amersham).

Growth Curves, Determination of Doubling Times, and Maximal Cell Numbers—10^4 cells/well were plated in 12-well plates in triplicate in complete DMEM in the presence or absence of 30 nM PMA (LC Laboratories, Woburn, MA) and increasing concentrations (0.1 nM-1000 nM) of the catalytic site-directed PKC inhibitor GF 109203X (bisindolylmaleimide I) (LC Laboratories). Fresh medium was added every other day, and cells in triplicate wells were harvested every second day by trypsinization and counted in a Coulter counter. To determine the average doubling time, cells were plated the same way, but cells were trypsinized and counted every 24 h for 7 consecutive days. The average doubling time was calculated by using the 24-h time point as the starting point to avoid artifacts due to the initial lag period after plating (12). To determine the maximal cell density, cells were grown in 12-well plates to confluence and kept postconfluent for 3 additional days with daily medium changes. Cells were counted as described above.

Soft Agar Assays—10^5 cells were resuspended in complete DMEM containing 0.4% Noble agar (Sigma) in the presence or absence of 30 nM PMA and were overlaid above a layer of 0.6% Noble agar in complete DMEM. The cells were fed every fifth day by overlaying the agar with 2 ml of complete medium containing the appropriate amount of PMA. The presence or absence of colonies was scored after 14 days.

Immunoprecipitation—NIH 3T3 fibroblasts that overexpressed the PKC chimeras were washed three times with ice-cold phosphate-buffered saline. The cells were scraped from a 60-mm dish into 1 ml of lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 1 mM EGTA. After mixing, the samples were incubated on ice for 30 min and then centrifuged in a microcentrifuge at 4 °C for 5 min. The supernatant was removed and preabsorbed with 25 μl of Protein A/G-Sepharose (50%) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 10 min; the samples were then spun at 4 °C for 3 min at 15,000 × *g*, and the supernatants were taken for immunoprecipitation. Immunoprecipitation was performed by rotating the samples overnight with 30 μl of Protein A/G-Sepharose (50%) and 4 μg/ml anti-PKCε antibody (Life Technologies, Inc.) at 4 °C. The samples were spun at 15,000 × *g* at 4 °C for 3 min and washed three times with radioimmune precipitation buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate. The pellets were resuspended in 25 μl of SDS sample buffer and boiled for 5 min. Before SDS-PAGE, samples were centrifuged again as described above and the entire supernatants were subjected to Western blotting and probed with anti-phosphotyrosine (monoclonal $IgG2b_k$) (Upstate Biotechnology Inc.) and anti-PKCε antibodies (Life Technologies, Inc.).

RESULTS

We constructed reciprocal chimeras from N-terminal and C-terminal halves of PKCα, PKCδ, and PKCε to study the relative contributions of the regulatory and catalytic subunits of these isozymes. We chose the first part of the highly conserved C3 region for the exchange, since the function of this region is well known and the sequence is identical in all the different isozymes (23, 24). We engineered the constructs in a way that allowed us to make wild type PKC isozymes the same way as the chimeric constructs, thus providing us with proper positive controls. Having the PKCε tag on our chimeras allowed better detection in Western blotting and a good epitope for immunoprecipitation.

First, we determined whether our isozymes can be stably expressed in NIH 3T3 fibroblasts. For this purpose, cells were stably transfected with "wild type" PKCα/α, -δ/δ, -ε/ε and the chimeras PKCα/δ, -α/ε, -δ/α, -δ/ε, -ε/α, and -ε/δ (Fig. 1). To assess the level of protein production, total cellular protein was collected from five individual antibiotic resistant clones of cells that overexpressed each isozyme and also from five vector-only control cell lines, and they were examined by Western blot analysis using monoclonal anti-PKCα antibodies or polyclonal anti-PKCδ or anti-PKCε antibodies. The antibodies recognized the two previously described PKCε-specific bands at 90 and 93 kDa (15) of the overexpressed PKCε. As we have described (20), PKCα/ε and PKCδ/ε chimeras also showed double bands on

Western blots, suggesting that the PKCε catalytic domain underwent posttranslational phosphorylation similar to the wild type. At the same time, PKCε/δ and -ε/α chimeras showed a single band on Western blots, suggesting that this posttranslational modification of PKCε occurs only on the catalytic domain. These data are consistent with the PKC chimeras being expressed and processed in a fashion similar to the parental PKC isoforms.

To establish that the overexpressed PKC chimeras were functionally intact, *i.e.* display kinase activity and bind phorbol ester, we assayed *in vitro* kinase activity and phorbol 12,13-dibutyrate binding on partially purified cell lysates. All overexpresser cells exhibited increased phorbol 12,13-dibutyrate binding and kinase activity as compared with the control cells transfected with the empty vector, as reported elsewhere (20).

The localization and translocation patterns of the various chimeras are reported separately (20). All the chimeras are found predominantly in the soluble fraction, and they all translocate upon PMA treatment. PMA-induced translocation and targeting of the enzymes are influenced by the catalytic domain as well as by the regulatory domain (20).

A major objective was to characterize the growth properties of the cells that overexpressed the various PKC constructs. Consistent with previous reports (12, 15, 17), our cells that overexpressed PKCα/α and PKCδ/δ proliferated significantly more slowly than the control cells, whereas cells that overexpressed PKCε/ε had a significantly higher rate of growth. Table I shows the values for the doubling time both in the presence and absence of PMA ($n = 3$ experiments). In the absence of PMA chimeras containing the regulatory domain of PKCα grew significantly more slowly than the control cells (cells transfected with the empty vector), whereas those that contained the regulatory domain of PKCε had a higher growth rate. It is important to note, however, that the catalytic domain also influenced the growth behavior of the chimeras. Thus, cells that expressed PKCα/ε grew fastest of those that expressed PKCα/γ chimeras and, reciprocally, cells that expressed PKCε/α grew most slowly of the PKCε/γ chimeras. Treatment with 30 nM PMA enhanced the difference between cells expressing the different isozymes. This is evident both upon microscopic examination (Fig. 2) and from growth curves (Fig. 3). This concentration of PMA activates PKC but does not cause

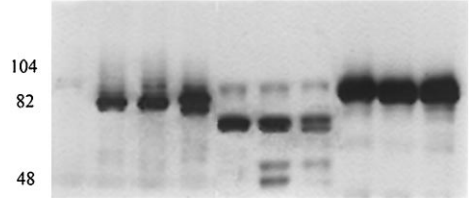

FIG. 1. **The expression of PKC chimeras in NIH 3T3 cells.** NIH 3T3 fibroblasts that had been stably transfected with overexpressing vectors were harvested and subjected to SDS-PAGE and Western immunoblotting as described under "Experimental Procedures." The figure illustrates one representative experiment of five similar experiments. The membrane was probed with anti-PKCε antibody that also stains the endogenous PKCε. Due to the level of overexpression of the PKCε/ε chimera, the higher and lower mobility bands of PKCε/ε mentioned in the text do not separate in this figure, but the two bands in the case of the PKCα/ε and PKCδ/ε chimeras can be easily observed.

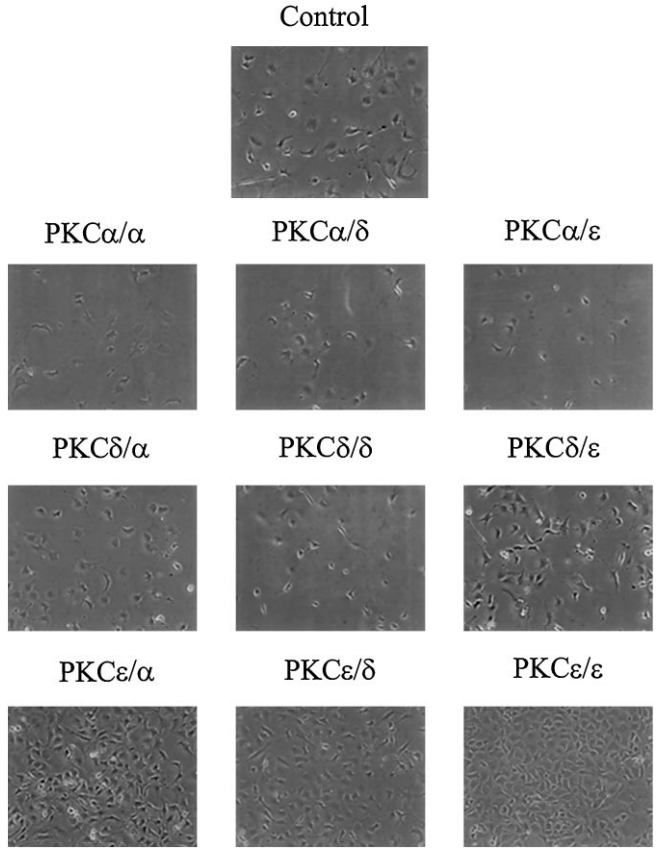

FIG. 2. **Cultures of NIH 3T3 cells that overexpress the PKC chimeras, after 7 days of PMA treatment.** Overexpressers were seeded in 12-well plates at 10^4 cells/well. Cells were treated with 30 nM PMA as described under "Experimental Procedures." The figure shows representative areas of the cell cultures after 7 days of treatment.

TABLE I

Proliferation characteristics of NIH 3T3 cells that overexpress PKC constructs

Control (only bearing the empty vector) NIH 3T3 cells and overexpressers were treated with 30 nM PMA, and the doubling time and the maximal cell number at saturation were calculated as described under "Experimental Procedures." Values represent the mean ± S.E. of three independent experiments with six determinations of the values in each experiment. Growth in soft agar was determined in the presence and absence of 30 nM PMA as described under "Experimental Procedures." *, $p < 0.05$; **, $p < 0.01$.

	Doubling time		Saturation density		Growth in soft agar	
	−PMA	+PMA	−PMA	+PMA	−PMA	+PMA
	h		× 10^5		~*colonies*	
Control	19.8 ± 1.2	25.4 ± 0.8	12.4 ± 0.4	11.2 ± 0.8	0	0
PKCα/α	26.2 ± 2.0*	42.7 ± 2.8**	10.1 ± 1.2	4.1 ± 0.6**	0	0
PKCα/δ	28.5 ± 1.6*	40.8 ± 5.2*	11.3 ± 0.7	4.3 ± 0.8**	0	0
PKCα/ε	25.5 ± 3.2	34.9 ± 4.7	12.2 ± 1.2	7.3 ± 1.2*	0	0
PKCδ/α	26.4 ± 2.3*	37.6 ± 3.1*	11.8 ± 0.6	7.4 ± 0.5*	0	0
PKCδ/δ	25.4 ± 1.8	39.4 ± 2.8**	12.2 ± 0.4	7.0 ± 0.6**	0	0
PKCδ/ε	21.3 ± 2.3	23.6 ± 1.4	12.8 ± 1.3	14.7 ± 1.4	0	250
PKCε/α	18.1 ± 1.4	22.2 ± 1.8	12.6 ± 0.6	10.3 ± 1.8	0	210
PKCε/δ	16.8 ± 1.1	21.8 ± 0.9*	12.4 ± 0.8	12.9 ± 1.1	0	300
PKCε/ε	14.6 ± 1.0*	18.1 ± 1.4**	12.8 ± 0.6	20.1 ± 1.4**	0	520

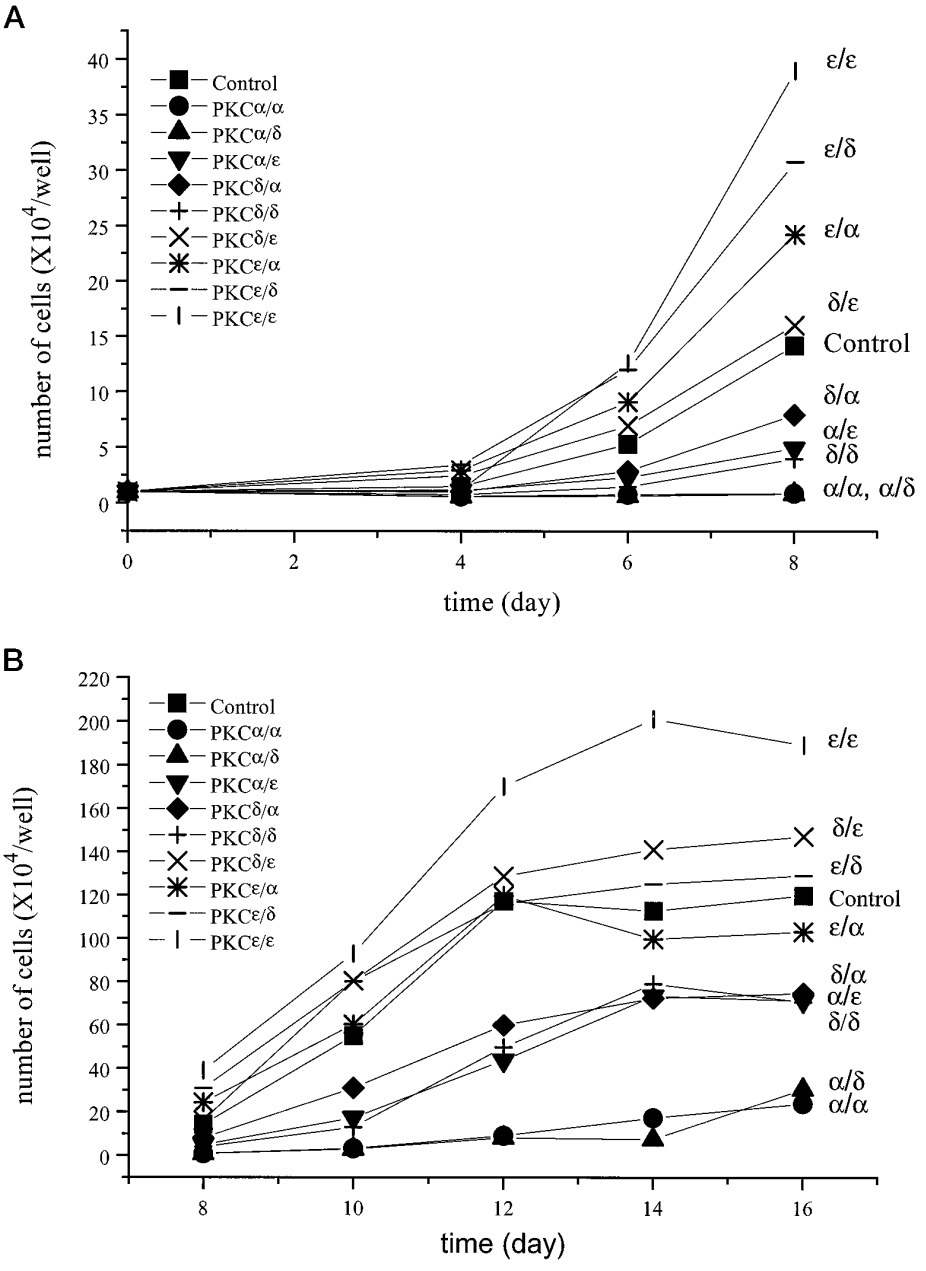

FIG. 3. **Growth curves of NIH 3T3 cells overexpressing PKC chimeras in the presence of phorbol ester.** Overexpressers were seeded in 12-well plates at 10^4 cells/well. Cells were chronically treated with 30 nM PMA as described under "Experimental Procedures," and the cell number was counted every other day. *A* shows the cell numbers during the first 8 days, and *B* shows the period between the 8th and 16th days of the experiment. Values represent the mean of three independent experiments. (Error bars were omitted for clarity; the S.E. was less than 10%.)

down-regulation of the enzyme, as revealed by Western blotting (data not shown). The doubling times of the cells that overexpressed chimeras containing the regulatory domain of PKCε were shorter than that of the control cells (demonstrated in Fig. 3*A*). We obtained similar data by measuring [^{3}H]thymidine incorporation into these cells with and without PMA treatment (data not shown). The effects of the chimeras on cell density at confluence were most evident in the presence of PMA (Table I). Cells expressing PKCε/ε and -δ/ε chimeras grew to the highest cell density, with the former achieving nearly twice the saturation density of the control. At the same time cells expressing PKCε/α and -ε/δ chimeras saturated at levels closer to the control cells (demonstrated in Fig. 3*B*). Overexpression of all three chimeras that contained the PKCε regulatory domain as well as the PKCδ/ε chimera enabled the cells to grow and form colonies in soft agar. We observed this anchorage-independent growth only in the presence of 30 nM PMA (Table I).

To confirm that the growth alterations observed in these cells were due to the presence of overexpressed PKC isozymes, we examined the effect of an inhibitor of PKC catalytic activity, GF 109203X. Administering increasing concentrations of GF 109203X, we could reverse the effect of the overexpressed proteins in the presence of PMA, consistent with the central role of PKC in these processes (Fig. 4). Furthermore, cells that overexpressed PKCε/ε and the PKCδ/ε chimera formed dense foci and were not contact inhibited. This effect was also blocked by GF 109203X (data not shown).

PKCδ has been reported to be tyrosine-phosphorylated upon stimulation with PDGF (25). To determine which domain is involved in this process, we starved the PKC-overexpressing cells overnight and then treated them with 100 ng/ml PDGF. After immunoprecipitation and Western blotting we probed the membranes with anti-phosphotyrosine antibody. PKCδ/δ, PKCδ/α, and PKCδ/ε were tyrosine-phosphorylated (Fig. 5), whereas PKCα/α, -ε/ε, -ε/δ, and -α/δ were not (data not shown). This suggests that tyrosine phosphorylation of PKCδ upon PDGF treatment occurred exclusively when the PKCδ regulatory domain is present, and it is independent of the catalytic domain.

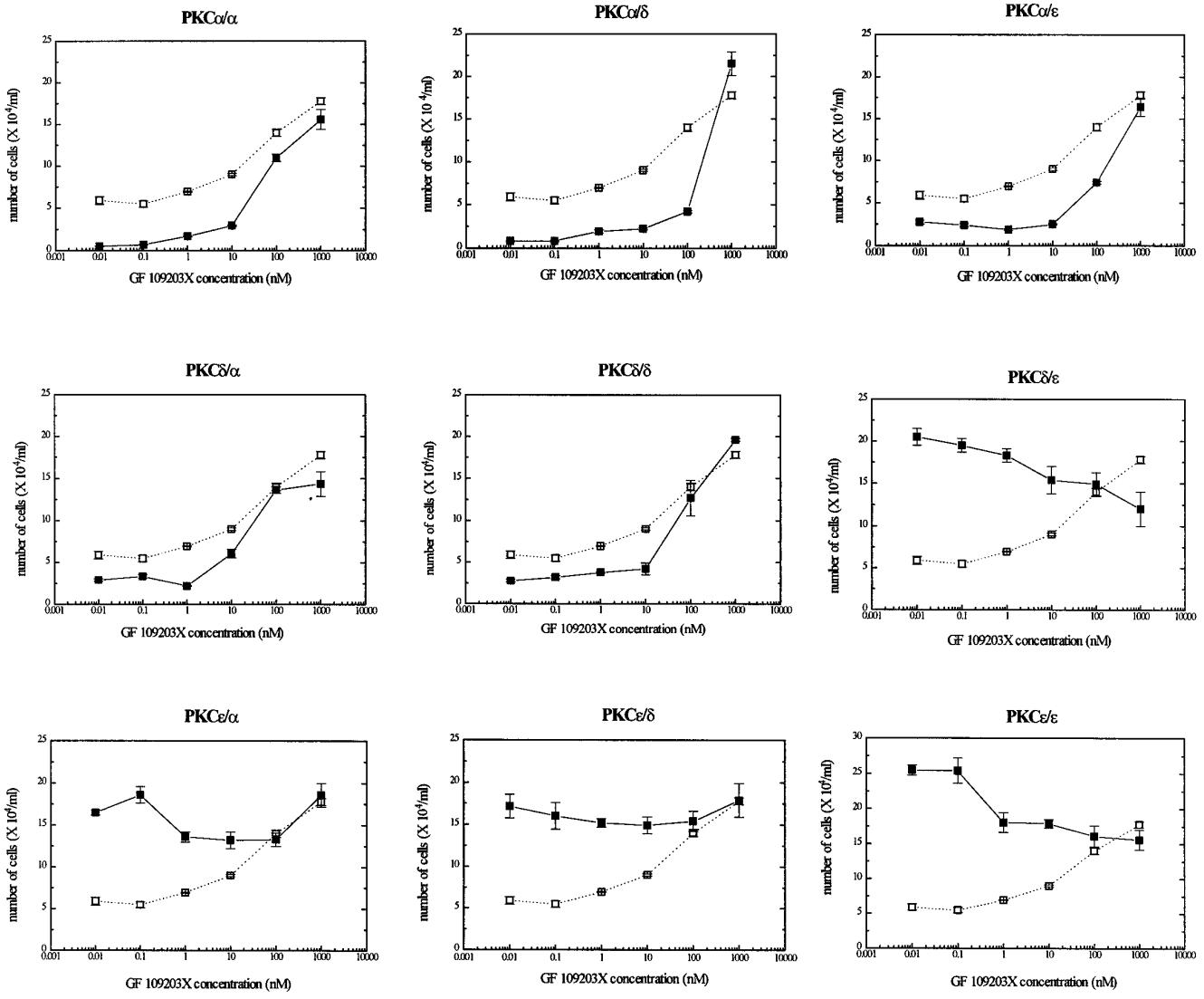

FIG. 4. **Effect of PMA and PKC inhibitor GF 109203X on the growth of NIH 3T3 cells that overexpress PKC constructs.** NIH 3T3 fibroblasts transfected with PKC chimeras (■) were treated with different concentrations of GF 109203X in the presence of 30 nM PMA. The number of cells was determined after 7 days of treatment. Points represent the average of three independent experiments ± S.E. Note that the same control curve is shown in each panel to show the behavior of the cells that overexpress the empty vector (□).

DISCUSSION

The protein kinase C molecule consists of two functional domains; an N-terminal regulatory domain that binds phosphatidylserine, diacylglycerol and, in the case of the classic isozymes, calcium; and a C-terminal catalytic domain that contains the catalytic center of the enzyme (1, 8).

Individual domains of protein kinase C or mutants that are truncated or deleted have been used to examine the functions of different regions of PKC (26, 27), but these mutants have been of limited value for analysis of PKC isotype function in intact cells because of their unstable expression, aberrant intracellular localization, and disregulated enzymatic activity (28–31). To identify structural determinants of protein kinase C activity and isozyme-specific functions, we chose a different approach. We designed PKC chimeras that preserved the overall structure of native PKC, had inducible kinase activity and could be found in the soluble cytosolic fraction as well.

PKCγ/ε chimeras have previously been used *in vitro* to investigate the basis of substrate specificity (32). In addition, PKCα/βII and PKCδ/ε chimeras were employed to study isozyme-specific functions in intact cells (33, 34). The catalytic domain of PKCβII was shown to be responsible for promoting cell growth in K-562 cells, and the catalytic domain of PKCδ is necessary for PMA-induced differentiation of 32D cells (33, 34).

In the present study, our aim was to define the functional domains of PKCα, -δ, and -ε for promoting and inhibiting growth in NIH 3T3 cells. PKC function is regulated by various mechanisms. These include activation by diacylglycerol or phorbol esters, phosphorylation, positional control, and proteolysis (2). These mechanisms are tightly connected, and thus it requires intact isozymes to obtain relevant data on these processes. Our chimeras fulfill this requirement, since they can be stably expressed, display phorbol ester inducible kinase activity, and show translocation upon PMA treatment. Isotype-specific effects may arise from each of the above mentioned mechanisms. Diacylglycerol or phorbol esters bind to PKC, causing conformational changes that reveal important sites of interaction on the molecule. These sites include receptor for activated protein kinase C (RACK)-binding sites that were described in the regulatory domain (35), but the role of the catalytic domain in the targeting of PMA-induced translocation has also been shown[2] (20, 33). Other localization signals have been identified in the regulatory domain of PKCε that appear to determine

[2] Wang, Q. J., Ács, P., Goodnight, J., Blumberg, P. M., Mischak, H., and Mushinski, J. F. (1997) *Oncogene*, in press.

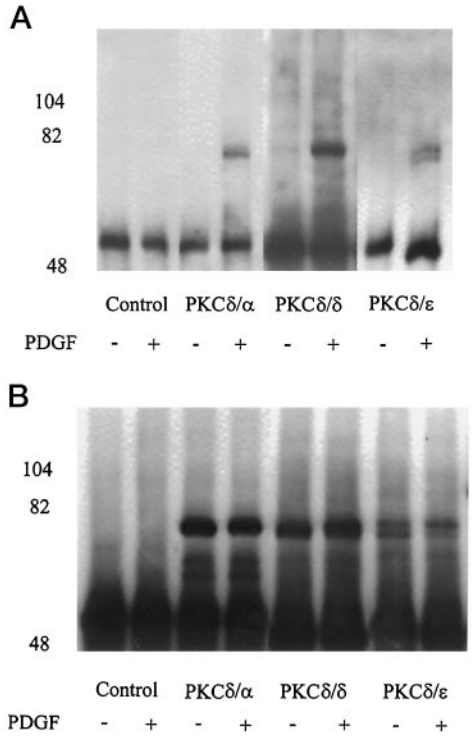

FIG. 5. **Tyrosine phosphorylation of PKCδ/γ chimeras upon PDGF treatment.** NIH 3T3 fibroblasts transfected with PKC chimeras were treated with 100 ng/ml PDGF for 30 min following overnight serum starvation. Cells were harvested, and immunoprecipitation of PKC chimeras was performed as described under "Experimental Procedures." After SDS-PAGE, we probed the blots with anti-phosphotyrosine antibody (*A*) and anti-PKCε antibody (*B*) to confirm the effectiveness of the immunoprecipitation. The figure represents one of three independent experiments with identical results.

whether this isoform associates with the plasma membrane, the cytoskeleton, or the Golgi apparatus (31). The PKCε/γ chimera was likewise shown to display the substrate specificity of PKCε (32). Phosphorylation occurs on the catalytic domain rendering the enzymes active and soluble (2).

Our data demonstrate that both the regulatory and the catalytic subunits play roles in cell proliferation. The regulatory domain of PKCε enhanced cell growth in the absence or presence of PMA, whereas the role of the catalytic domain of PKCε was evident in the saturation density of PMA-treated cells that overexpressed PKCδ/ε. The important contribution of the PKCε catalytic domain was also evident in terms of significantly increased saturation density in the presence of PMA and in formation of foci upon PMA treatment as well. At the same time, all chimeras with the PKCε regulatory domain and the PKCδ/ε chimera gave rise to colonies in soft agar. Extensive studies on the tumorigenicity of PKCδ and -ε chimeras demonstrate that NIH 3T3 cells that overexpress chimeric PKCδ/ε, which contains the PKCε catalytic domain, form tumors in nude mice.[2] These results show that both the regulatory and catalytic domains of PKC may be responsible for certain isozyme-specific functions, presumably depending on localization or substrate specificity.

Our studies confirmed that overexpressed PKCε is a powerful growth stimulus, but we have also shown that chimeras that substitute the PKCε regulatory domain with that of PKCα or -δ inhibit growth. Cells that overexpress PKCδ/ε showed cell growth markedly reduced from cells that overexpress PKCε/ε. PKCδ has been generally found to inhibit fibroblast growth (12, 13), but reports on PKCα are equivocal (17, 36–38). In our studies, overexpressed PKCα inhibited fibroblast growth, and chimeras that contained either the PKCα, or the PKCδ regulatory domain inhibited fibroblast growth as well. The translocation pattern and the destination of PKCα/ε have been shown to be chiefly influenced by the catalytic domain of PKCε (20). Moreover, this isozyme translocates principally into the cytoskeletal fraction, appearing in a band showing a higher mobility isoform. This may account for the difference in the effect on cell growth when compared with PKCδ/ε. Another possible mechanism might be a dominant negative effect of PKCα/ε on the endogenous PKCε.

Although tyrosine phosphorylation of PKCδ has been demonstrated to occur in response to a variety of stimuli, the phosphorylation sites and the effect of this phosphorylation on the activity of the kinase are still unclear (25, 39–41). *In vitro* phosphorylation studies revealed three tyrosine phosphorylation sites in PKCδ: two in the regulatory (Tyr-52 and Tyr-155) and one in the catalytic domain (Tyr-565) (39). We determined that tyrosine phosphorylation of PKC chimeras upon PDGF treatment occurred only in chimeras that contained the PKCδ regulatory domain. Assuming that the PKCδ is the only tyrosine-phosphorylated isozyme, this must be the site of tyrosine phosphorylation.

Protein kinases Cα, -δ, and -ε are nearly ubiquitously expressed and display opposite effects on cell growth. We constructed chimeras to determine the domain responsible for isozyme-specific effects. The regulatory domains proved to influence cell growth, but the catalytic domain of PKCε combined with the PKCδ regulatory domain was able to cause a significant increase in saturation density and to form colonies in soft agar. It has been reported that the PKCα regulatory domain can activate phospholipase D *in vitro* through a mechanism that is independent of the kinase activity (42). Studies with PKCδ and -ε chimeras on 32D cells also revealed functions that can be linked to the regulatory domain (34). These data, along with our data presented here, emphasize that PKC should not be treated as a simple string of independent domains; rather, interdomain influences can be important. We can conclude that both domains are necessary for adequate isozyme-specific functioning of PKC, some of which can be triggered by the regulatory, others by the catalytic domain. PKC chimeras thus remain important tools to obtain more profound insights into cellular processes that are brought about by isozyme-specific PKC activity.

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