Protein Kinase C β Enhances Growth and Expression of Cyclin D1 in Human Breast Cancer Cells

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Abstract

Although alterations in the expressions of protein kinase C (PKC) have been implicated in breast carcinogenesis, the roles of specific isoforms in this process remain elusive. In the present study, we examined the specific roles of PKCβ1 and β2 in growth control in human breast cancer cell lines. The PKCβ-specific inhibitor LY379196 significantly inhibited growth of the breast cancer cell lines MCF-7, MDA-MB-231, and BT474, but not the normal mammary epithelial cell line MCF-10F. Treatment of MCF-7 cells with LY379196 caused an increase in the fraction of cells in the G_1 phase of the cell cycle. To explore the roles of PKCβ1 and β2, we used cDNA expression vectors that encode wild-type and constitutively activated or dominant negative mutants of these two proteins. When compared with vector controls, derivatives of MCF-7 cells that stably overexpress wild-type PKCβ1 or PKCβ2 displayed a slight increase in growth rate; derivatives that stably express the constitutively active mutants of PKCβ1 or PKCβ2 displayed a marked increase in growth rate; and derivatives that stably express a dominant negative mutant of PKCβ1 or β2 displayed inhibition of growth. The derivatives of MCF-7 cells that stably express the constitutively activated mutants of PKCβ1 or β2 were more resistant to growth inhibition by LY379196 than the vector control MCF-7 cells. Immunoblot analysis indicated that MCF-7 cells that stably overexpress wild-type or constitutively activated mutants of PKCβ1 or β2 had higher cellular levels of cyclin D1 than vector control cells, whereas cells that express a dominant negative mutant had decreased levels of cyclin D1. The derivatives that stably express the constitutively activated mutants of PKCβ1 or β2 also displayed increased cyclin D1 promoter activity in transient transfection luciferase reporter assays, and this induction of activity requires activator protein 1. Constitutively activated PKCβ1 and β2 also enhanced the transcription of c-*fos* in transient transfection luciferase reporter assays. Thus, PKCβ1 and β2 may play important positive roles in the growth of at least a subset of human breast cancers. Therefore, inhibitors of these isoforms may be useful in breast cancer chemoprevention or therapy. (Cancer Res 2006; 66(23): 11399-408)

Introduction

Protein kinase Cs (PKC) comprise a family of at least 10 mammalian isozymes that are lipid-dependent serine/threonine protein kinases with a broad range of tissue distribution and differential cellular localizations (1). The PKC family can be divided into three subgroups: conventional PKCs (α, β1, β2, γ), novel PKCs (δ, ε, η, θ), and atypical PKCs (λ/ι and ζ). PKC transduces signals from a plethora of extracellular receptors (2, 3). The role of PKC has repeatedly been implicated in breast cancer (4, 5). It has been shown that the total levels of PKC enzymatic activity are elevated in malignant breast tumors when compared with normal breast tissues (6, 7). Furthermore, estrogen receptor–negative (ER−), hormone-independent breast tumors tend to have higher levels of total PKC activity than estrogen receptor–positive (ER+) tumors (8). This inverse relationship between the levels of ER and PKC was observed both in primary breast tumors and in immortalized breast cancer cell lines (9, 10). Treatment of MCF-7 and other breast cancer cell lines with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate, which is a potent activator of PKC, under conditions that induced down-regulation of PKC activity, resulted in growth inhibition and enhanced expression of variables associated with a more differentiated phenotype (11). These findings suggest that alterations in PKC-mediated signal transduction pathways may be involved in the pathogenesis of breast cancer. However, the precise roles of specific isoforms of PKC in human breast cancer cells have not been elucidated, mainly because these cells contain multiple PKC isoforms, and it has been difficult to distinguish the roles of individual isoforms.

The β isoforms of PKC have been implicated in several types of cancer especially in colon cancer. Increased expression of PKCβ2 is an early event in colon carcinogenesis in mice (12). Increased expression of PKCβ2 in the colon of transgenic mice causes hyperproliferation and increased susceptibility to colon carcinogenesis due, at least in part, to repression of expression of the transforming growth factor-β (TGF-β) type II receptor (13). Overexpression of PKCβ2 also renders transgenic mice more susceptible to carcinogen-induced colonic hyperproliferation, aberrant crypt foci formation, and colon carcinogenesis (13). PKCβ2 also induces the expression of cyclooxygenase type 2 in rat intestinal epithelial cells *in vitro* and in transgenic PKCβ2 mice *in vivo* (14). Overexpression of either PKCβ1 or β2 in human colon carcinoma cell lines causes a 2- to 5-fold increase in the levels of transforming growth factor-α (TGFα) in the growth medium (15). However, previous studies have not specifically addressed the roles of PKCβ1 or PKCβ2 in the proliferation of human breast cancer cells.

Specific isoforms of PKC, including PKCβ1 and β2, regulate the transcriptional control of cyclin D1 (*CCND1*) expression, which plays a critical role in the progression of mammalian cells through the G_1 phase of the cell cycle (16). The G_1 to S transition is a critical step because abnormalities in this step can enhance cell proliferation, genome instability, and tumor progression. The importance of cyclin D1 in breast cancer is evidenced by the fact that overexpression of this cyclin is seen in ~60% of human breast cancers (17, 18). In ~15% of the cases, it is due to gene

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amplification; in the remaining 45%, it is due to increased transcription (19). Furthermore, overexpression of cyclin D1 has been linked with poor prognosis in breast cancer (20, 21). The cyclin D1 promoter is one of the major targets for several growth stimulatory signaling pathways (22, 23). For example, signaling through the Wnt as well as mitogen-activated protein kinase (MAPK) pathways potently induce cyclin D1 gene transcription (22–24). Therefore, in the present study we examined the effects of PKCβ1 and β2 on both cell proliferation and cyclin D1 expression in human breast cancer cells.

Materials and Methods

Chemicals and antibodies. The compound LY379196 was provided by Eli Lilly, Inc. (Indianapolis, IN) and its properties have previously been described (25). PKCβ1 and β2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the PKCδ antibody from PanVera (Madison, WI); the cyclin D1 antibody (06-137) from Upstate Biotechnology, Inc. (Lake Placid, NY); the c-fos and actin antibodies from Sigma (St. Louis, MO); and the hemagglutinin antibody from Covance (Richmond, CA).

Cell lines and growth curves. The MCF-10F normal mammary epithelial cell line and the MCF-7, MDA-MB-231, and BT474 human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Unless indicated otherwise, the cells were grown in MEM with 10% fetal bovine serum (FBS) purchased from Invitrogen Corp. (Carlsbad, CA) in a 100% humidified incubator at 37°C with 5% CO_2.

For growth curve analysis, cells were plated in triplicate at a density of 5×10^4 per well in six-well (35-mm) plates with 2 mL of MEM containing 10% FBS. Geneticin (800 μg/mL; Invitrogen) was added to the growth media for derivatives of MCF-7 cells overexpressing various PKC constructs. The cells were refed with fresh medium every 2 days. Cell numbers were counted using a Coulter counter (Beckman Coulter, Inc., Fullerton, CA) everyday for the subsequent 5 days after suspension with trypsin.

Growth inhibition was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. In brief, cells were plated in triplicate into 96-well plates (5×10^3 per well) with 100 μL MEM containing 10% FBS and cultured overnight to allow for cell attachment. Cells were then treated with 10 nmol/L LY379196 dissolved in DMSO for 48 hours in MEM containing 10% FBS and assayed using a MTT assay kit (Roche Diagnostic Corp., Indianapolis, IN). The number of viable cells was quantified spectrophotometrically using an ELISA reader. All assays were done in triplicate and repeated experiments yielded similar results.

Flow cytometry and cell cycle analysis. Cells were treated with 0.1% v/v DMSO as the solvent control or with LY379196 (10 nmol/L, dissolved in DMSO) for 24 or 48 hours. Both adherent and floating cells were collected, washed with PBS, resuspended in 1 mL of PBS, and fixed with 5 mL of 70% ethanol, centrifuged, resuspended in 1 mL of PBS containing 2 mg/mL RNase (Sigma), and stained with 0.2 mg/mL of propidium iodide (Sigma) in the dark for 30 minutes. The cell suspension was then filtered through a 60-μm Spectra mesh filter (Spectrum Medical Industries, Houston, TX). Samples of 10,000 to 20,000 cells were then analyzed for DNA histograms and cell cycle phase distributions using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and the data were analyzed using CellQuest software (Becton Dickinson) according to the manual provided by the manufacturer.

Immunoblot analysis. Exponentially growing cells were harvested and sonicated for 10 seconds in modified radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L DTT, 25% glycerol, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L NaF, 0.1 mmol/L Na_3VO_4, 10 mmol/L β-glycerophosphate, and 1 mmol/L phenylmethylsulfonyl fluoride]. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Protein samples, 20 μg per lane, were subjected to SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 5% skim milk and incubated with the indicated primary antibodies for 1 hour at room temperature or overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase–conjugated antimouse immunoglobulin G (IgG) or antirabbit IgG secondary antibodies (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) as indicated. Protein bands were visualized with the enhanced chemiluminescence immunoblotting system (Amersham Pharmacia Biotech). Immunoblotting for actin was done to verify equivalent amounts of loaded protein. Repeat studies gave similar results.

Plasmids. The pcDNA3 plasmid DNA (Invitrogen) was used as a control vector and used as needed to achieve the same total amount of plasmid DNA per transfection. The expression plasmids for wild-type and constitutively activated and dominant negative mutant forms of PKC β1, β2, and δ were previously described (16). The cyclin D1 promoter-luciferase reporters (22), the c-*fos* promoter-luciferase reporter (26), and the c-*fos* serum response element luciferase reporter plasmid pSRE-luc (27) were previously described.

Colony formation assay. MCF-7 and MDA-MB-231 cells were transfected with the control vector pcDNA3 or expression plasmids for various PKC isoforms using the Lipofectin reagent (Invitrogen). After 24 hours, the cells were replated into 100-mm plates at 1×10^5 per plate, and the cells were cultured in the presence of 1 mg/mL Geneticin (Invitrogen) for 3 weeks to select for cells that stably expressed the transfected plasmid. The colonies were fixed with formalin, stained with Giemsa (Sigma), and counted. The results are presented as mean values of triplicate assays.

Generation of cloned derivatives of MCF-7 cells that stably overexpress various PKCβ constructs. MCF-7 cells were transfected with either the control vector pcDNA3 or wild-type or mutant PKCβ constructs and selected as described above. The cells were either pooled and serially passaged, or individual clones were selected. For colony selection, for each transfection, 20 clones were individually selected and serially passaged in MEM containing 10% FBS and 800 μg/mL Geneticin. Two weeks later, immunoblot analysis was done on each clone to detect expression of the hemagglutinin-tagged protein using an anti-hemagglutinin-tag primary antibody. Clones that expressed high levels of the indicated PKCβ constructs were then used in further studies.

Transient transfection reporter assays. Triplicate samples of 1×10^5 MCF-7 cells in 35-mm plates containing MEM plus 10% FBS were cultured overnight to allow for cell attachment. The cells were then transfected using Lipofectin in Opti-MEM (Invitrogen) with 1 μg of the indicated reporter plasmid, 0.5 to 2 μg of various expression vectors, and 0.5 μg of the control reporter plasmid pCMV-β-galactosidase. The pcDNA3 plasmid DNA was added to the transfections to achieve the same total amount of plasmid DNA per transfection. Eighteen hours after transfection, cells were refed with phenol red–free MEM (Invitrogen) with 0.5% charcoal-stripped FBS. Cell extracts were prepared after a 24-hour incubation in the phenol red–free MEM with 0.5% charcoal-stripped FBS. Cell extracts were prepared and luciferase assays were done using the Luciferase Assay System (Promega Corp., Madison, WI). Luciferase activities were normalized with respect to parallel β-galactosidase activities to correct for differences in transfection efficiency. β-Galactosidase assays were done using the β-Galactosidase Enzyme Assay System (Promega). The mean values and SDs were calculated from triplicate samples. Similar experiments were done thrice and representative results from one of these experiments are presented.

Because the cyclin D1-luc reporters, the c-*fos*-luc reporter, and the pSRE-luc can be activated by growth factors that are present in serum, we used a low concentration of 0.5% charcoal-stripped FBS to minimize the stimulatory effects caused by serum. In addition, because phenol red in tissue culture media is a weak estrogen (28), to reduce nonspecific effects on the estrogen-responsive MCF-7 cells, phenol red–free MEM was used in the above transient transfection luciferase reporter assays.

Reverse transcription-PCR analysis. MCF-7 cells grown in 60-mm plates were transfected with Transfectin (Bio-Rad) in phenol red–free MEM with 0.5% charcoal-stripped FBS, with 5 μg of the indicated plasmids encoding wild-type and mutant forms of PKC. Cells were refed with fresh phenol red–free MEM with 0.5% charcoal-stripped FBS and incubated for an additional 48 hours. Total RNA was purified using Trizol reagent (Invitrogen) following the protocol of the manufacturer. cDNA was

synthesized from 1 μg total RNA using the iScript kit (Bio-Rad) and amplified by PCR using Choice Taq DNA Polymerase (Denville, Metuchen, NJ). The primers used for amplification are, for cyclin D1, 5′-CCC-TCGGTGTCCTACTTCAA-3′ and 5′-GGGGATGGTCTCCTTCATCT-3′, and for c-*fos*, 5′-CCAACCTGCTGAAGGAGAAG-3′ and 5′-ATGATGCTGGGA-ACAGGAAG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)–specific PCR products (using primers 5′-CGAGATCCCTCCAAAATCAA-3′ and 5′-TGTGGTCATGAGTCCTTCCA-3′) from the same RNA samples were amplified and served as internal controls. Each amplification cycle consisted of 30 seconds at 94°C for denaturation, 30 seconds at 60°C for primer annealing, and 1 minute at 72°C for extension. After PCR amplification, the fragments were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

Statistical analysis. Data are analyzed using Student's *t* test. A difference between groups with $P < 0.05$ was considered statistically significant.

Results

PKCβ is required for the growth of human breast cancer cells. To study the role of PKCβ1 and β2 in human breast cancer, we first determined their levels of protein expression in exponentially growing human breast cancer cells and normal mammary epithelial cells. We chose the MCF-10F normal mammary epithelial cell line and three human breast cancer cell lines in this assay. The MCF-7 cells are ER+ and do not overexpress Her2/neu or epidermal growth factor receptor (EGFR); the MDA-MB-231 cells are ER− and overexpress EGFR; and the BT474 are ER− and overexpress Her2/neu. Therefore, each of them represents a clinically relevant subtype of human breast cancer. These cells were harvested and protein extracts were examined by immunoblot analysis. We found that PKCβ1 and β2 were expressed at high levels in all three human breast cancer cells, but not in the normal mammary epithelial cells (Fig. 1*A*). The expression levels of PKCβ1 in all three breast cancer cell lines tested were >2-fold higher than that of the MCF-10F normal mammary epithelial cell line (Fig. 1*B*). The expression levels of PKCβ2 in these breast cancer cell lines were 2- to 7-fold higher than that of the MCF-10F normal mammary epithelial cell line (Fig. 1*C*). In contrast, the levels of expression of PKCδ varied among the three human breast cancer cell lines and were not increased in the breast cancer cells when compared with the normal MCF-10F cells (Fig. 1*A*).

To examine whether the breast cancer cell lines require PKCβ for growth, we treated the three breast cancer cell lines, as well as the MCF-10F cells, with the PKCβ-specific inhibitor LY379196, which has >60-fold selectivity for PKCβ versus other PKC isoforms (25). This inhibitor is not, however, selective for PKCβ1 or β2 (25). Although the growth rates of these breast cancer cells were different, treatment with LY379196 for 24 hours significantly inhibited growth of all three breast cancer cell lines ($P < 0.05$), and at 48 and 96 hours caused ~80% and 90% decreases, respectively, in cell viability in the three human breast cancer cells when compared with the control DMSO solvent–treated cells ($P < 0.005$ and $P < 0.002$, respectively). However, this concentration of LY379196 did not cause significant growth inhibition in MCF-10F cells (Fig. 2*A*). Using cell cycle analysis, we found that in MCF-7 cells, treatment with 10 nmol/L LY379196 caused an increase of cells in the G_1 and a decrease of cells in the S phase of the cell cycle (Fig. 2*B* and *C*; $P < 0.05$), suggesting that PKCβ plays an important role in the G_1 to S transition.

To further examine the specific the roles of PKCβ1 and β2 in the growth of human breast cancer cells, we employed expression plasmids that encode wild-type and mutant forms of PKCβ1 or β2. Because the NH_2-terminal domain of PKC contains a pseudosubstrate region that binds to the COOH-terminal catalytic domain and inhibits its activity, truncation mutants that contain only the COOH-terminal catalytic domain are constitutively activated (CAT; ref. 16). The dominant negative (KR) mutants encode a full-length PKC with a point mutation (K371R) that abolishes the ATP-binding ability (16). We transfected MCF-7 and MDA-MB-231 cells with the control vector pcDNA3 or various expression plasmids that encode the wild-type (WT) or constitutively activated (CAT) or dominant negative (KR) mutants of PKCβ1 or β2, and then did colony formation assays. We found that both MCF-7 and MDA-MB-231 cells transfected with PKCβ1-WT or PKCβ2-WT formed more colonies than cells transfected with the control vector pcDNA3 ($P < 0.05$). With both cell lines, cells transfected with PKCβ1-CAT or PKCβ2-CAT formed even a larger number of colonies than cells transfected with the control vector ($P < 0.02$ or $P < 0.01$). Cells transfected with PKCβ1-KR or PKCβ2-KR formed fewer colonies than the control cells, but the difference was not statistically significant, possibly because the levels of the expression of these dominant negative mutants were not high enough to inhibit the endogenous proteins (Fig. 3*A*). In contrast to the results obtained with the PKCβ isoforms, MCF-7 cells transfected with the constitutively activated PKCδ plasmid formed fewer colonies when compared with cells transfected with the control vector ($P < 0.01$). Similar results were also seen in BT474 breast cancer cells (data not shown). These findings are consistent with evidence that PKCδ often exerts growth inhibitory and/or apoptotic effects (29) and confirm the isoform specificity of our PKC constructs.

In view of the above results, it was of interest to isolate individual clones of MCF-7 cells that stably overexpress wild-type or mutant forms of PKCβ1 and β2 and further examine their properties. After transfection with the respective plasmids and selection with Geneticin, 20 individual clones were isolated and serially passaged. Extracts of these clones were then examined by immunoblot analysis for stable expression of the related proteins using an antibody to the hemagglutinin tag (Fig. 3*B*). Two clones (designated MCF-7-PKCβ1-CAT-1 and MCF-7-PKCβ1-CAT-2) were identified that stably express high levels of PKCβ1-CAT. One clone (designated MCF-7-PKCβ2-WT-7) was identified that stably expresses a high level of PKCβ2-WT. Three clones (designated MCF-7-PKCβ2-CAT-2, MCF-7-PKCβ2-CAT-3, and MCF-7-PKCβ2-CAT-4) were identified that stably express high levels of PKCβ2-CAT. One clone (designated MCF-7-PKCβ2-KR-9) was identified that stably expresses a high level of PKCβ2-KR.

To investigate the effects of overexpression of these forms of PKCβ1 or β2 on the growth of MCF-7 cells, we did growth curve analysis. When compared with vector controls, the two clones of MCF-7 cells that stably express a constitutively active mutant of PKCβ1 (CAT) displayed a marked increase in growth rate (Fig. 3*C*; $P < 0.01$). The clone that stably overexpresses wild-type PKCβ2 displayed a slight increase in growth rate (Fig. 3*D*; $P < 0.02$). The three clones that express a constitutively active mutant of PKCβ2 (CAT) also displayed a marked increase in growth rate (Fig. 3*D*; $P < 0.01$). It is of interest that the clone that expresses a dominant negative mutant (KR) of PKCβ2 displayed inhibition of growth (Fig. 3*D*; $P < 0.01$).

We also attempted to overexpress wild-type and constitutively activated mutants of PKCδ in MCF-7 cells to compare the effects of overexpression of different PKC isoforms. However, after Geneticin selection, none of the clones that were picked and examined by immunoblot analysis expressed the corresponding PKCδ protein.

Presumably this reflects the cytotoxicity of PKCδ to MCF-7 cells, which is consistent with previous studies on PKCδ (29).

Because the control MCF-7 cells were very sensitive to growth inhibition by the PKCβ-specific inhibitor LY379196, we examined the effects of this compound on the growth of derivatives of MCF-7 cells that stably overexpress various constructs of PKCβ1 or β2.

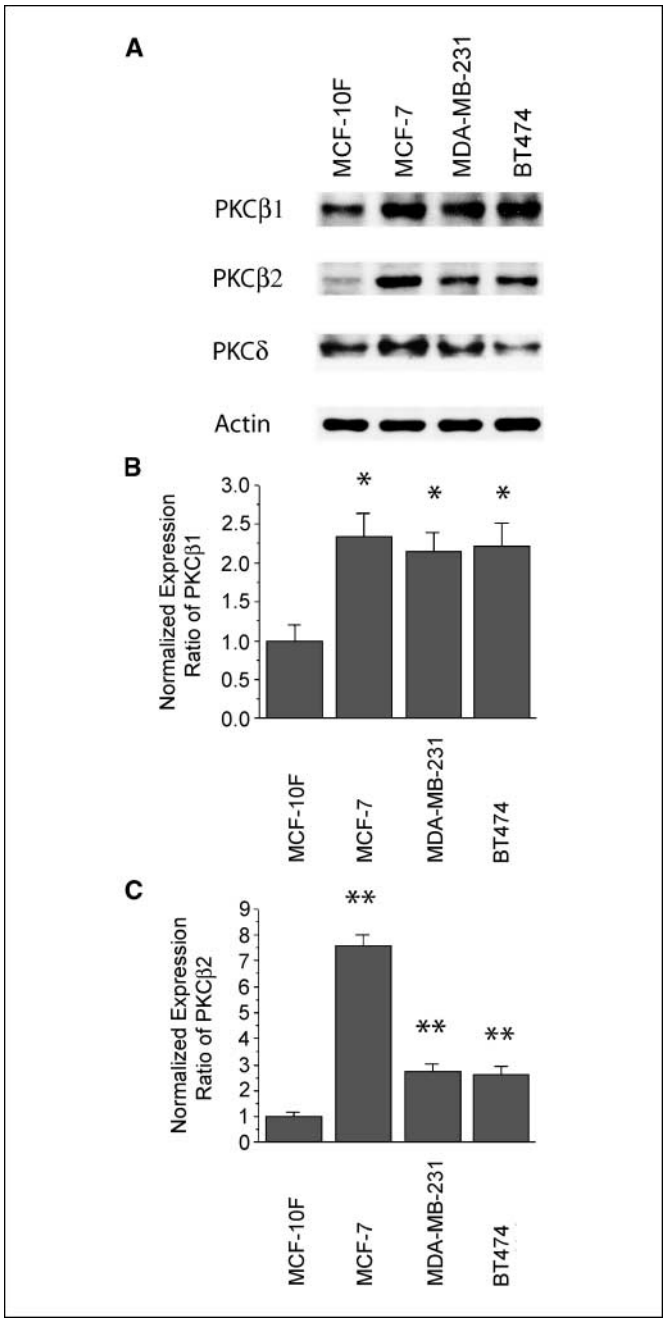

Figure 1. *A,* immunoblot analysis of the expression of endogenous PKCs β1, β2, and δ in exponential cultures of MCF-10F, MCF-7, MDA-MB-231, and BT474 cells. *B,* densitometry analysis of the levels of PKCβ1 protein in exponential cultures of MCF-10F, MCF-7, MDA-MB-231, and BT474 cells. The expression ratios were normalized with respect to levels of actin. Three independent analyses were done and SDs were calculated. *, $P < 0.05$, comparing the normalized ratios to those in MCF-10F cells. *C,* similar densitometry analysis of the protein expression levels of PKCβ2 in exponential cultures of MCF-10F, MCF-7, MDA-MB-231, and BT474 cells. **, $P < 0.01$, compared with normalized ratios of MCF-10F cells.

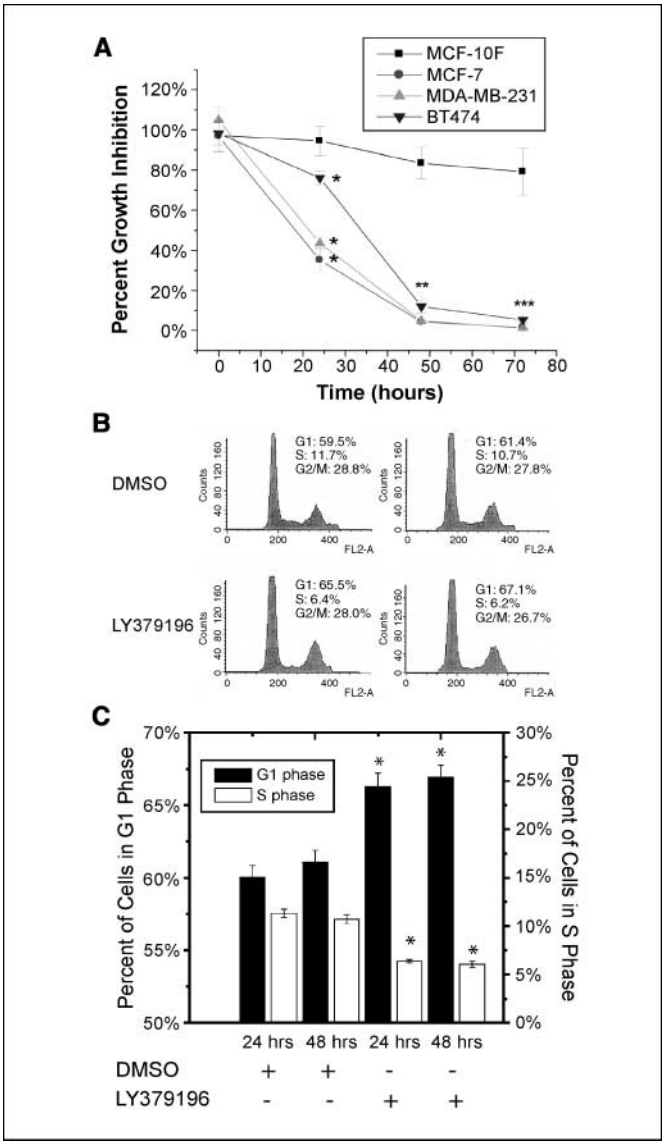

Figure 2. *A,* growth inhibition caused by 10 nmol/L LY379196 in MCF-10F, MCF-7, MDA-MB-231, and BT474 cells. Percent growth inhibition was calculated by comparing LY379196-treated cells with control DMSO solvent–treated cells. *B,* cell cycle analysis of MCF-7 cells after treatment with 10 nmol/L LY379196. The analyses were done at least thrice and yielded similar results. *C,* statistical analysis of the cell cycle distribution of MCF-7 cells after treatment with LY379196. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$, compared with the control DMSO solvent–treated cells.

The derivatives that stably express the constitutively activated mutants of PKCβ1 or β2 were ~3-fold more resistant to growth inhibition by LY379196 than the vector control cells or cells that expressed the wild-type or dominant negative (KR) forms of PKCβ2 (Fig. 3*E*; $P < 0.05$).

Pooled populations, rather than individual clones of MCF-7 derivatives that stably overexpress wild-type PKCβ1 or β2, or express the constitutively activated mutant of PKCβ1 or β2, also displayed greater tolerance to serum starvation because there was reduced growth inhibition when these cells were serum starved when compared with the vector control cells (Fig. 3*F*; $P < 0.01$).

PKCβ1 and β2 activate the expression of cyclin D1. Because in MCF-7 cells the PKCβ-specific inhibitor LY379196 caused cell cycle arrest at the G_1 phase, we investigated whether PKCβ1 or β2

plays a role in regulating expression of cyclin D1 because it plays a critical role in the G_1-S transition (20). Using immunoblot analysis, we found that MCF-7 derivatives that overexpress constitutively activated mutants of PKCβ1 or β2 had higher cellular levels of cyclin D1 than vector control cells, whereas cells that expressed a dominant negative mutant of PKCβ2 had decreased levels of cyclin D1 (Fig. 4*A*). Using transient transfection cyclin D1 promoter-luciferase reporter assays, we found that the derivatives that stably express the constitutively activated mutants of PKCβ1 or β2 also displayed increased cyclin D1 promoter activity, and in these derivatives the cyclin D1 promoter could be activated in the absence of serum (Fig. 4*B*; $P < 0.05$ or $P < 0.01$).

In view of the above findings indicating that PKCβ1 and β2 stimulate the transcriptional activity of the cyclin D1 promoter

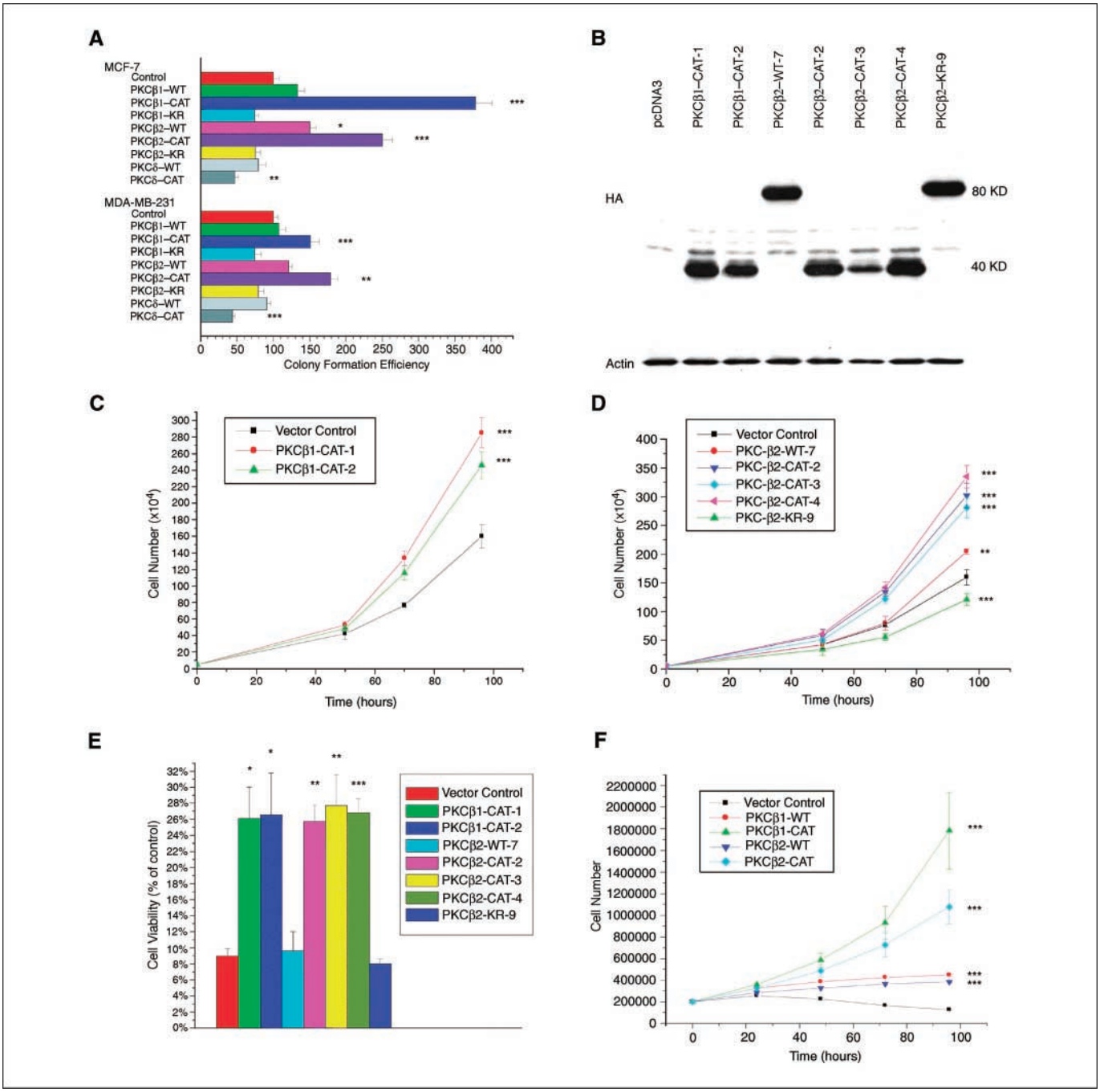

Figure 3. *A,* colony formation efficiency of MCF-7 or MDA-MB-231 cells after transfection with the control vector pcDNA3 or the indicated expression plasmids of PKCs. All assays were done in triplicate and the results were expressed as percent of control (i.e., the number of colonies obtained compared with the vector control plasmid). *B,* immunoblot analysis of the expression of PKCβ wild-type and mutant proteins in clones of MCF-7 derivatives. Actin was used as a loading control. *C,* growth curves of two clones of MCF-7 derivatives that stably expressed a constitutively activated (*CAT*) mutant of PKCβ1. *D,* growth curves of clones of MCF-7 derivatives that stably expressed the indicated PKCβ2 constructs. *E,* growth curves of MCF-7 derivatives treated with 10 nmol/L LY379196. Cell viability was determined with MTT assays and expressed as percent of the control DMSO solvent–treated cells. *F,* growth curves of MCF-7 derivatives done in the absence of serum. *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$, compared with vector control cells.

(Fig. 4*B*) and the fact that LY379196 specifically inhibits the activity of PKCβ1 and β2, we examined the effects of LY379196 on cyclin D1 promoter activity. The various derivatives of MCF-7 cells described above were transiently transfected with a cyclin D1 promoter-luciferase reporter plasmid for 18 hours, together with a β-gal reporter plasmid. Then the cells were grown for 24 hours in phenol red–free MEM with 0.5% charcoal-stripped serum, with or without 30 nmol/L of LY379196. Treatment with LY379196 caused inhibition of cyclin D1 promoter activity in the vector control cells and in the derivatives. It is of interest that the PKCβ2-WT-7 cells were significantly more resistant to the inhibitory effects of the drug, but this was not seen with the CAT mutant–expressing cells

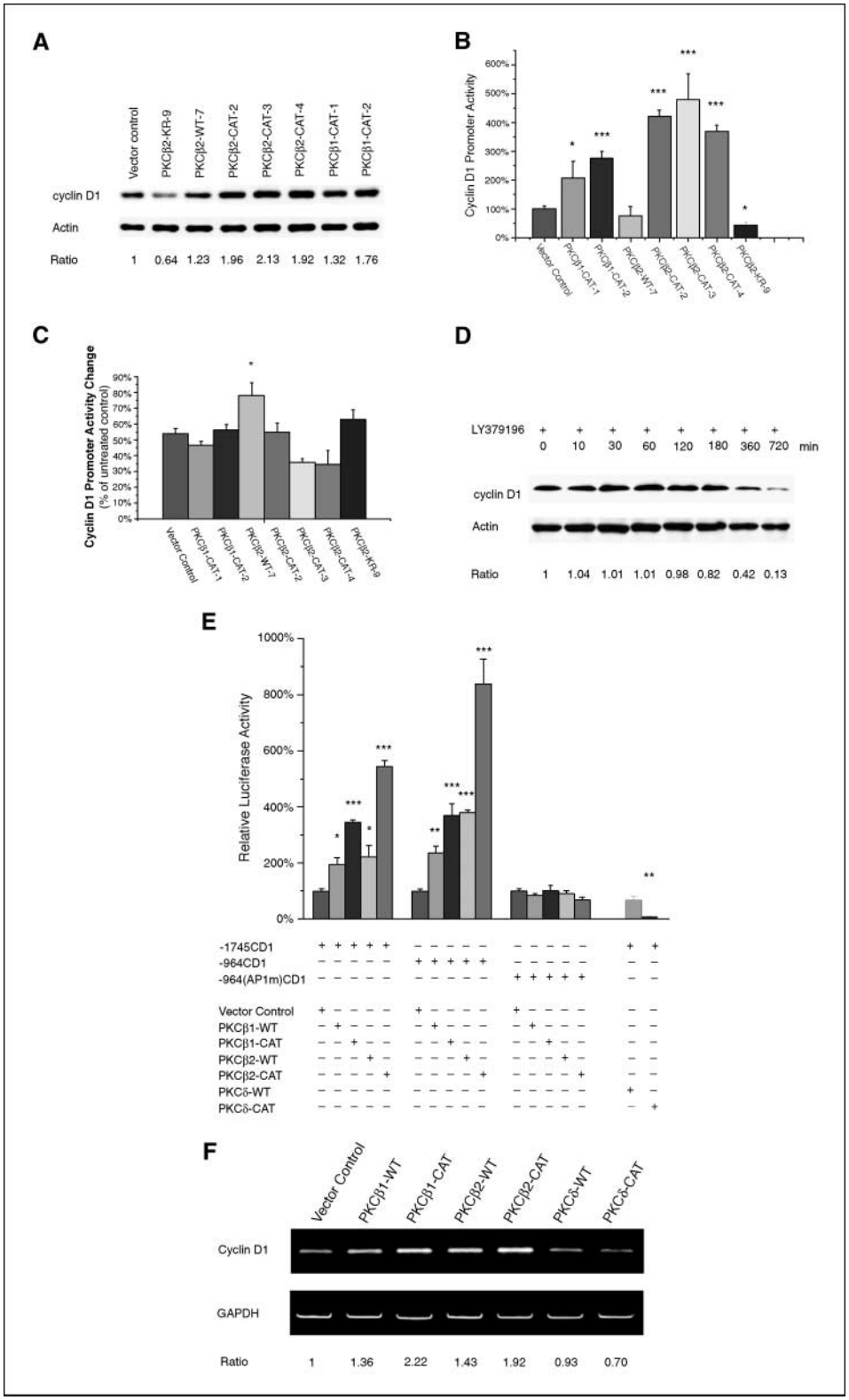

Figure 4. *A,* cell extracts were prepared from exponentially dividing cultures of the clones of MCF-7 cells that stably express constitutively activated PKCβ1 or β2 and examined by immunoblot analysis with a cyclin D1 antibody. Actin was used as a loading control. *B,* transient transfection reporter assays. Each of the indicated clones was transiently transfected with a cyclin D1 promoter-luciferase reporter plasmid for 18 hours, together with a β-gal reporter plasmid. Then the cells were grown for 24 hours in phenol red–free MEM with 0.5% charcoal-stripped serum. Extracts were prepared and luciferase activity was determined and normalized with respect to β-gal activity. *C,* each of the indicated clones was transiently transfected with a cyclin D1 promoter-luciferase reporter plasmid for 18 hours, together with a β-gal reporter plasmid. Then the cells were grown for 24 hours in phenol red–free MEM with 0.5% charcoal-stripped serum, with or without 30 nmol/L LY379196. Luciferase activity was determined and normalized with respect to β-gal activity. Results are expressed as percent of promoter activity obtained in the drug-treated cells when compared with the same cell line treated with DMSO solvent (control), which was assigned a value of 100%. *D,* MCF-7 cells were treated with 10 nmol/L LY379196 and cell lysates were prepared after the indicated time and examined by immunoblot analysis with a cyclin D1 antibody. Actin was used as a loading control. *E,* MCF-7 cells were transiently cotransfected with a cyclin D1 promoter-luciferase reporter plasmid and the indicated PKC expression plasmid for 18 hours, together with a β-gal reporter plasmid. Then the cells were grown for 24 hours in phenol red–free MEM with 0.5% charcoal-stripped serum. Luciferase activity was determined and normalized with respect to β-gal activity. *F,* analysis of cyclin D1 mRNA levels by reverse transcription-PCR (RT-PCR) after MCF-7 cells were transiently transfected with the indicated PKC constructs. GAPDH was used as an internal control. *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$.

(Fig. 4*C*; $P < 0.05$). This may reflect the different properties of the intact versus the mutant proteins. We further analyzed the cyclin D1 protein level after treating cells with LY379196 and found that LY379196 caused a decrease in cyclin D1 protein level after 6 hours of treatment (Fig. 4*D*).

To extend the evidence that PKCβ1 and β2 stimulate cyclin D1 promoter activity in MCF-7 cells, and to further elucidate which element in the cyclin D1 promoter PKCβ1 and β2 might activate, we carried out transient transfection reporter assays in which we cotransfected pcDNA3, PKCβ2-WT, PKCβ2-CAT, PKCδ-WT, or PKCδ-CAT plasmids together with a series of cyclin D1 promoter-luciferase reporter plasmids. These cyclin D1 promoter constructs included the full-length promoter −1745CD1, a truncated −964CD1 promoter that includes an activator protein 1 (AP-1) site, and a truncated −964(AP1m)CD1 promoter with a mutated AP1 site (22). We found that transient transfection of the wild-type PKCβ1 or β2 stimulated the activity of both the full-length cyclin D1 promoter and the truncated −964CD1, but not the activity of the AP-1 site mutated promoter (Fig. 4*E*). Transient transfection of the constitutively activated mutants of PKCβ1 or β2 also stimulated the activity of both the full-length cyclin D1 promoter and the truncated −964CD1, but did not stimulate the activity of the AP1 site mutated promoter (Fig. 4*E*). These findings provide evidence that the AP1 site in the cyclin D1 promoter is required for the stimulation of transcription of cyclin D1 by PKCβ1 or β2. In contrast, transient transfection of the wild-type or the constitutively activated mutant of PKCδ inhibited cyclin D1 promoter activity (Fig. 4*E*), again indicating the isoform specificity of our constructs. In addition, we also examined the mRNA levels of cyclin D1 after transfection with the above-described PKC constructs. We confirmed that the mRNA levels were increased after transient transfection with the wild-type or constitutively activated forms of PKCβ1 or β2 (Fig. 4*F*). In contrast, transfection with the constitutively activated forms of PKCδ decreased the mRNA levels in MCF-7 cells (Fig. 4*F*).

PKCβ1 and β2 activate the serum responsive element in the c-*fos* gene. Because the above findings suggest that the AP-1 site in the cyclin D1 promoter is required for the induction of transcription mediated by PKCβ1 and β2, and because induction of the early response gene c-*fos* has been shown to be enhanced by PKCβ (30, 31), it was of interest to determine whether overexpression of PKCβ1 or β2 activates c-*fos* transcription in our MCF-7 derivatives. Therefore, MCF-7 derivatives that express wild-type or mutant forms of PKCβ were transfected with the c-*fos* promoter-luciferase reporter plasmid. Eighteen hours after growing the transfected cells in serum-free medium, cell extracts were prepared and assayed for luciferase activity. We found that the derivatives that express the constitutively activated PKCβ1 or β2 displayed increased transcriptional activity of the c-*fos* promoter, and this effect was seen even in the absence of serum (Fig. 5*A*). The serum responsive element (SRE) transcriptional control element in the c-*fos* promoter region plays an important role in the transcription of c-*fos*. Using a pSRE-luc reporter plasmid (32), we found that in the MCF-7 derivatives that express constitutively activated PKCβ1 or β2, there was an increase in the activity of the SRE transcriptional control element (Fig. 5*B*). The derivative that overexpressed wild-type PKCβ2 also displayed increased SRE activity (Fig. 5*B*), and this activity was more resistant to inhibition by LY379196 than the vector control MCF-7 cells (Fig. 5*C*). Consistent with the activation of the c-*fos* promoter in the MCF-7 derivatives expressing the constitutively activated PKCβ1 or β2, the c-Fos protein levels were also increased in these cells (Fig. 5*D*). We also carried out transient transfection reporter assays in which we cotransfected pcDNA3, PKCβ2-WT, PKCβ2-CAT, PKCδ-WT, or PKCδ-CAT plasmids together with the pSRE-luc plasmid. We found that transient transfection of the wild-type PKCβ1 or β2 stimulated the activity of SRE, and transient transfection of the constitutively activated PKCβ1 or β2 further stimulated SRE activity (Fig. 5*E*). When we examined the mRNA levels of c-*fos* after transfection with the above-described PKC constructs, we found that the mRNA levels were increased after transient transfection with the wild-type or constitutively activated forms of PKCβ1 or β2 (Fig. 5*F*). In contrast, transfection with the constitutively activated forms of PKCδ decreased the mRNA levels of c-*fos* in MCF-7 cells (Fig. 5*F*). These findings, with respect to increased cyclin D1, c-*fos*, and SRE promoter activity, suggest that in MCF-7 cells, PKCβ1 and β2 enhance and possibly integrate multiple pathways of signal transduction and gene expression that stimulate cell proliferation.

Discussion

The precise roles of specific isoforms of PKC in growth control and cell cycle regulation in human breast cancer cells have not previously been elucidated, in part because these cells contain multiple isoforms of PKC and it has been difficult to distinguish the roles of individual isoforms. We focused on the β isoforms of PKC because of their ubiquitous expression in various cell types and the previous evidence that they might play important roles in other types of cancer (12, 33–36). We used the drug LY379196 that specifically inhibits the activities of PKCβ1 and β2 to address this question. In addition, as a separate approach, we constructed a series of mammalian expression vectors that encode wild-type or mutant forms of PKCβ1 and β2. These constructs were then used to generate and characterize derivatives of the MCF7 human breast cancer cell line that stably overexpress the wild-type or constitutively activated or dominant negative mutants of these two isoforms of PKC. PKCβ1 and β2 are splice variants of the same gene, which differ only by amino acid sequences in the last 50 residues in the COOH end of catalytic domain. Therefore, it is likely that our constitutively activated (CAT) mutants retain specificity between PKCβ1 and β2.

We found that endogenous PKCβ1 and β2 are expressed at relatively high levels in the human breast cancer cell lines MCF-7, MDA-MB-231, and BT474 when compared with normal MCF-10F mammary epithelial cells (Fig. 1). The PKCβ-specific inhibitor LY379196 markedly inhibited the growth of MCF-7 (ER+, HER2−), MDA-MB-231 (ER−, HER2+, EGFR overexpressing), and BT474 (ER+, HER2 overexpressing) cells (Fig. 2*A*), and caused slight arrest of the cells at the G_1 phase of the cell cycle (Fig. 2*B* and *C*). This suggests that the β isoforms of PKC may be indispensable for the growth of both estrogen-dependent and estrogen-independent breast cancer cells. Stable overexpression of wild-type PKCβ1 or β2 or expression of the constitutively activated mutants of PKCβ1 or β2 stimulated the growth of MCF-7 cells (Fig. 3*C* and *D*) and increased the protein levels of cyclin D1 and c-Fos (Figs. 4*A* and 5*D*), whereas stable expression of a dominant negative mutant of PKCβ2 inhibited growth (Fig. 3*D*) and decreased the protein levels of cyclin D1 and c-Fos (Figs. 4*A* and 5*D*). In addition, transient transfection of parental MCF-7 cells with wild-type or constitutively activated mutants of PKCβ1 or β2 stimulated cyclin D1 and c-*fos* promoter activity (Figs. 4*E* and 5*E*) and increased the

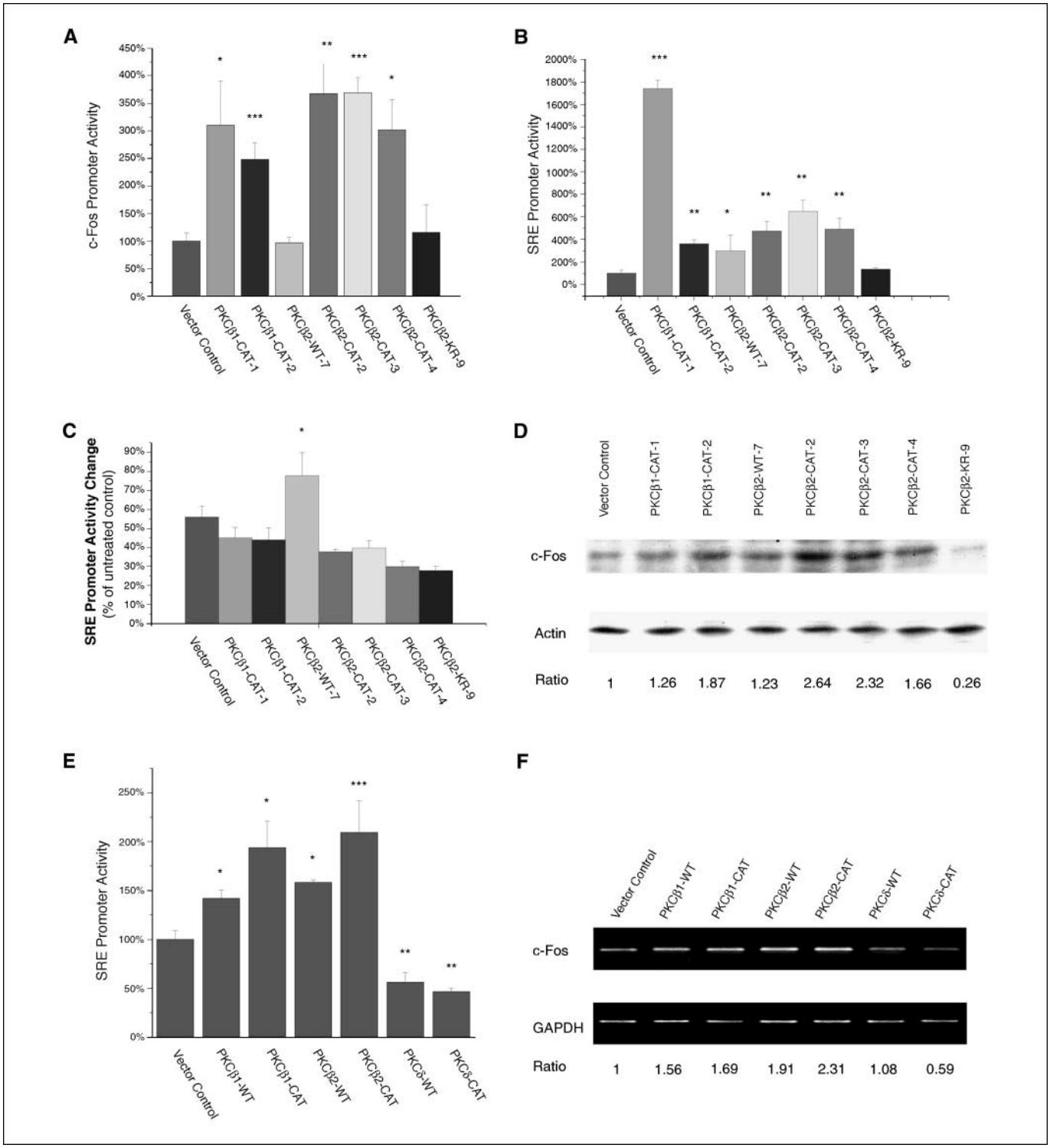

Figure 5. *A,* each of the indicated clones was transiently transfected with a c-*fos* promoter-luciferase reporter plasmid for 18 hours, together with a β-gal reporter plasmid. Then the cells were grown for 24 hours in phenol red–free MEM with 0.5% charcoal-stripped serum. Luciferase activity was determined and normalized with respect to β-gal activity. *B,* each of the indicated clones was transiently transfected with a SRE promoter-luciferase reporter plasmid for 18 hours, together with a β-gal reporter plasmid. Then cells were grown for 24 hours in phenol red–free MEM with 0.5% charcoal-stripped serum. Luciferase activity was determined and normalized with respect to β-gal activity. *C,* each of the indicated clones was transiently transfected with a SRE promoter-luciferase reporter plasmid for 18 hours, together with a β-gal reporter plasmid. Then the cells were grown for 24 hours in phenol red–free MEM with 0.5% charcoal-stripped serum, with or without 30 nmol/L LY379196. Luciferase activity was determined and normalized with respect to β-gal activity. Results are expressed as percent of promoter activity obtained in the drug-treated cells when compared with the same cell line treated with DMSO solvent (control), which was assigned a value of 100%. *D,* c-Fos protein levels in PKCβ clones. *E,* MCF-7 cells were transiently cotransfected with a SRE promoter-luciferase reporter plasmid and the indicated PKC expression plasmid for 18 hours, together with a β-gal reporter plasmid. Then the cells were grown for 24 hours in phenol red–free MEM with 0.5% charcoal-stripped serum. Luciferase activity was determined and normalized with respect to β-gal activity. *F,* analysis of c-*fos* mRNA levels by RT-PCR after MCF-7 cells were transiently transfected with the indicated PKC constructs. GAPDH was used as an internal control. *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$, compared with the vector control cells.

mRNA levels of these two genes (Figs. 4*F* and 5*F*), whereas transient transfection with wild-type or constitutively activated mutants of PKCδ exerted the opposite effect (Figs. 4*E-F* and 5*E-F*). Furthermore, in parental MCF-7 cells, the PKCβ-specific inhibitor LY379196 inhibited transcriptional activation of the cyclin D1 promoter and the SRE element in the c-*fos* promoter (Figs. 4*C* and 5*C*).

Cyclin D1 plays a critical role in regulating the progression of cells through the G_1 phase of the cell cycle by binding to and activating cyclin-dependent kinase (CDK)-4 or CDK6. This results in phosphorylation of the pRb protein, leading to activation of the E2F family of transcription factors, which, in turn, enhances the progression of cells into the S phase. Thus, increased expression of cyclin D1 can enhance cell cycle progression and cell proliferation (37). Therefore, it is of interest that increased expression of cyclin D1 occurs in ~60% of human breast cancers (17, 18). And in some studies, this is associated with a poor prognosis (20, 21). We found that the AP-1 enhancer element in the cyclin D1 promoter is required for activation of the cyclin D1 promoter by PKCβ1 or β2. Furthermore, constitutively activated mutants of PKCβ1 or β2 activated the SRE element, which is present in the promoter region of the c-*fos* gene (Figs. 4*E* and 5*B*). AP-1 activity is regulated by Fos, Jun, and the activating transcription factor subfamily of transcription factors, which in turn are activated by upstream MAPKs. These findings suggest that PKCβ1 and β2 might exert their stimulatory effects at the level of MAPKs, which is consistent with previous findings from our laboratory (32) and those of other investigators (38, 39). However, the exact site(s) at which PKCβ1 and β2 exert their primary effects in breast cancer cells remains to be determined. We should also emphasize that although some of our studies focused on cyclin D1 (Fig. 4), it is likely that the growth-stimulating effects of PKCβ1 and β2 on breast cancer cells are also exerted via other pathways. Furthermore, our results do not exclude the possibility that other isoforms of PKC might also play a role in enhancing the proliferation of breast cancer cells, although this does not seem to be the case for PKCδ (Figs. 1*A*, 3*A*, 4*E-F*, and 5*E-F*).

Although PKCβ1 and β2 are derived from a single gene by alternative splicing, there is evidence that in some cell systems they may play different roles in cell growth, apoptosis, and cell transformation (40, 41). Thus, in a human gastric cancer cell line, transfection with a PKCβ1 antisense oligonucleotide resulted in aberrant cell morphology, suppression of cell growth, and decreased colony-forming ability *in vitro* (42). In addition, this antisense oligonucleotide inhibited the growth of gastric tumor xenografts in nude mice (42). However, in cells transfected PKCβ2 antisense oligonucleotide, there were no significant changes in cell morphology, cell growth, or tumorigenecity (42). Another example is that increased expression of PKCβ2 in the colon of transgenic mice leads to hyperproliferation and increased susceptibility to colon carcinogenesis (12), and in gastric cancer cells PKCβ1 acts as a survival factor in response to chemotherapeutic agents that induce apoptosis (43, 44). However, in our studies, we did not observe major differences between PKCβ1 and β2 with respect to their *in vitro* effects on human breast cancer cells. On the other hand, our findings do not exclude the possibility that these two isoforms might exert other differential effects on breast cancers *in vivo*.

In view of our results, we searched the Oncomine DNA microarray database for relative levels of expression of PKCβ mRNA in human breast cancers (45). In one study (46), the levels of this RNA were significantly higher in breast carcinomas than in breast fibroadenomas and normal breast tissues. In another study (47), PKCβ expression was significantly higher in lobular carcinoma than in fibroadenoma or human mammary epithelial cells growing in culture. In both studies (46, 47), the data suggest that lobular carcinoma had higher expression of PKCβ than did ductal carcinoma or other histologic subtypes of breast cancer. There was, however, no difference in terms of PKCβ expression between ER+ and ER− subtypes of breast cancer. However, BRCA1 mutation–positive breast cancers had significantly higher PKCβ expression levels than BRCA2 mutation–positive or sporadic breast cancers (48). Thus, BRCA1 mutations may lead to overexpression of PKCβ, and this may warrant further studies.

In summary, our findings provide evidence that increased expression of PKCβ1 or β2 in human breast cancer cells may play an important role in enhancing cell cycle progression and cell proliferation. The fact that PKCβ1 and PKCβ2 stimulated the growth of both ER+ MCF-7 cells and ER− MDA-MB-231 cells suggests that the stimulation of breast cancer cell growth by these isoforms of PKC is independent of ER status. Therefore, inhibitors that target PKCβ1 and/or PKCβ2 might be useful in the treatment of both ER+ and ER− breast cancers.

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References

1. Saito N, Kikkawa U, Nishizuka Y. The family of protein kinase C and membrane lipid mediators. J Diabetes Complications 2002;16:4–8.
2. Dekker LV, Parker PJ. Protein kinase C—a question of specificity. Trends Biochem Sci 1994;19:73–7.
3. Newton AC. Protein kinase C: structure, function, and regulation. J Biol Chem 1995;270:28495–8.
4. Mansour SJ, Matten WT, Hermann AS, et al. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 1994;265:966–70.
5. Amundadottir LT, Leder P. Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes. Oncogene 1998;16:737–46.
6. O'Brian C, Vogel VG, Singletary SE, Ward NE. Elevated protein kinase C expression in human breast tumor biopsies relative to normal breast tissue. Cancer Res 1989;49:3215–7.
7. Gordge PC, Hulme MJ, Clegg RA, Miller WR. Elevation of protein kinase A and protein kinase C activities in malignant as compared with normal human breast tissue. Eur J Cancer 1996;32A:2120–6.
8. Borner C, Wyss R, Regazzi R, Eppenberger U, Fabbro D. Immunological quantitation of phospholipid/Ca^{2+}-dependent protein kinase of human mammary carcinoma cells: inverse relationship to estrogen receptors. Int J Cancer 1987;40:344–8.
9. Fabbro D, Kung W, Costa SD, Borner C, Regenass U, Eppenberger U. Involvement of protein kinase C in the growth regulation of human breast cancer cells. Cancer Treat Res 1992;61:229–48.
10. Bignon E, Ogita K, Kishimoto A, Nishizuka Y. Protein kinase C subspecies in estrogen receptor-positive and -negative human breast cancer cell lines. Biochem Biophys Res Commun 1990;171:1071–8.
11. Davidson NE, Kennedy MJ. Protein kinase C and breast cancer. Cancer Treat Res 1996;83:91–105.
12. Gokmen-Polar Y, Murray NR, Velasco MA, Gatalica Z, Fields AP. Elevated protein kinase C βII is an early promotive event in colon carcinogenesis. Cancer Res 2001;61:1375–81.
13. Murray NR, Davidson LA, Chapkin RS, Clay Gustafson W, Schattenberg DG, Fields AP. Overexpression of protein kinase C βII induces colonic hyperproliferation and increased sensitivity to colon carcinogenesis. J Cell Biol 1999;145:699–711.
14. Yu W, Murray NR, Weems C, et al. Role of cyclooxygenase 2 in protein kinase C βII-mediated colon carcinogenesis. J Biol Chem 2003;278:11167–74.

15. Maruvada P, Levine AE. Increased transforming growth factor-α levels in human colon carcinoma cell lines over-expressing protein kinase C. Int J Cancer 1999;80:72–7.
16. Soh JW, Weinstein IB. Roles of specific isoforms of protein kinase C in the transcriptional control of cyclin D1 and related genes. J Biol Chem 2003;278:34709–16.
17. Keyomarsi K, Pardee AB. Redundant cyclin overexpression and gene amplification in breast cancer cells. Proc Natl Acad Sci U S A 1993;90:1112–6.
18. Gillett C, Fantl V, Smith R, et al. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res 1994;54:1812–7.
19. Arnold A. The cyclin D1/PRAD1 oncogene in human neoplasia. J Investig Med 1995;43:543–9.
20. Hunter T, Pines J. Cyclins and cancer. II: cyclin D and CDK inhibitors come of age. Cell 1994;79:573–82.
21. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G_1-phase progression. Genes Dev 1999;13:1501–12.
22. Albanese C, Johnson J, Watanabe G, et al. Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J Biol Chem 1995;270:23589–97.
23. Shtutman M, Zhurinsky J, Simcha I, et al. The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A 1999;96:5522–7.
24. Tetsu O, McCormick F. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–6.
25. Jirousek MR, Gillig JR, Gonzalez CM, et al. (*S*)-13-[(Dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16, 21-dimetheno-1*H*, 13*H*-dibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-d ione (LY333531) and related analogues: isozyme selective inhibitors of protein kinase C β. J Med Chem 1996;39:2664–71.
26. Han TH, Lamph WW, Prywes R. Mapping of epidermal growth factor-, serum-, and phorbol ester-responsive sequence elements in the c-jun promoter. Mol Cell Biol 1992;12:4472–7.
27. Johansen FE, Prywes R. Two pathways for serum regulation of the c-fos serum response element require specific sequence elements and a minimal domain of serum response factor. Mol Cell Biol 1994;14:5920–8.
28. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci U S A 1986;83:2496–500.
29. Ghayur T, Hugunin M, Talanian RV, et al. Proteolytic activation of protein kinase C δ by an ICE/CED 3-like protease induces characteristics of apoptosis. J Exp Med 1996;184:2399–404.
30. Messina JL, Standaert ML, Ishizuka T, Weinstock RS, Farese RV. Role of protein kinase C in insulin's regulation of c-fos transcription. J Biol Chem 1992;267:9223–8.
31. Zhang J, Wang L, Petrin J, Bishop WR, Bond RW. Characterization of site-specific mutants altered at protein kinase C β1 isozyme autophosphorylation sites. Proc Natl Acad Sci U S A 1993;90:6130–4.
32. Soh JW, Lee EH, Prywes R, Weinstein IB. Novel roles of specific isoforms of protein kinase C in activation of the c-fos serum response element. Mol Cell Biol 1999;19:1313–24.
33. Choi PM, Tchou-Wong KM, Weinstein IB. Overexpression of protein kinase C in HT29 colon cancer cells causes growth inhibition and tumor suppression. Mol Cell Biol 1990;10:4650–7.
34. Goldstein DR, Cacace AM, Weinstein IB. Overexpression of protein kinase C β1 in the SW480 colon cancer cell line causes growth suppression. Carcinogenesis 1995;16:1121–6.
35. Svensson K, Zeidman R, Troller U, Schultz A, Larsson C. Protein kinase C β1 is implicated in the regulation of neuroblastoma cell growth and proliferation. Cell Growth Differ 2000;11:641–8.
36. Zhang J, Anastasiadis PZ, Liu Y, Thompson EA, Fields AP. Protein kinase C (PKC) βII induces cell invasion through a Ras/Mek-, PKCι/Rac1-dependent signaling pathway. J Biol Chem 2004;279:22118–23.
37. Sherr CJ. D-type cyclins. Trends Biochem Sci 1995;20:187–90.
38. Cai H, Smola U, Wixler V, et al. Role of diacylglycerol-regulated protein kinase C isotypes in growth factor activation of the Raf-1 protein kinase. Mol Cell Biol 1997;17:732–41.
39. Marais R, Light Y, Mason C, Paterson H, Olson MF, Marshall CJ. Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. Science 1998;280:109–12.
40. Ono Y, Kikkawa U, Ogita K, et al. Expression and properties of two types of protein kinase C: alternative splicing from a single gene. Science 1987;236:1116–20.
41. Deacon EM, Pongracz J, Griffiths G, Lord JM. Isoenzymes of protein kinase C: differential involvement in apoptosis and pathogenesis. Mol Pathol 1997;50:124–31.
42. Jiang XH, Tu SP, Cui JT, et al. Antisense targeting protein kinase C α and β1 inhibits gastric carcinogenesis. Cancer Res 2004;64:5787–94.
43. Zhu GH, Wong BC, Slosberg ED, et al. Overexpression of protein kinase C-β1 isoenzyme suppresses indomethacin-induced apoptosis in gastric epithelial cells. Gastroenterology 2000;118:507–14.
44. Jiang XH, Lam SK, Lin MC, et al. Novel target for induction of apoptosis by cyclo-oxygenase-2 inhibitor SC-236 through a protein kinase C-β(1)-dependent pathway. Oncogene 2002;21:6113–22.
45. Rhodes DR, Kalyana-Sundaram S, Mahavisno V, Barrette TR, Ghosh D, Chinnaiyan AM. Mining for regulatory programs in the cancer transcriptome. Nat Genet 2005;37:579–83.
46. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98:10869–74.
47. Perou CM, Jeffrey SS, van de Rijn M, et al. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proc Natl Acad Sci U S A 1999;96:9212–7.
48. van't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530–6.

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