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The data presented in this annual report (for award DAMD17-96-1-6022) support our hypothesis for a mechanism of retinoic acid -induced growth arrest of human breast cancer cells. Specifically we believe that retinoic acid induced growth arrest of human breast cancer cells requires protein kinase Ca expression. The constitutive expression of PKCa in hormone independent, MDA-MB-231 cells induced retinoic acid sensitivity to inhibit the uncontrolled proliferation and to alter the proto-oncogene expression. Retinoic acid treatment in PKCa expressing MDA-MB-231 cells reduced 40% of cell proliferation and inhibited serum induction of c-fos expression. Concomitantly, c-jun expression was enhanced. These findings, along with the observation that retinoic acid treatment enhanced PKCa expression, conventional PKC activity and translocation of PKCa to cell membrane and nucleus, raised the possibility that PKCa induced retinoic acid sensitivity in MDA-MB-231 cells is caused by disturbance of mitogenic signaling or trancriptional regulation of target genes. By turning on/off the transcription of PKCa gene, we further demonstrated the PKCa regulation of c-fos and c-jun expression. In total, we demonstrate that the lack of anti-proliferation effect of retinoic acid in MDA-MB-231 cells is caused by the failure of RA induced PKCa expression and subsequent absence of PKCa altered gene expression.

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INTRODUCTION

Retinoic acid (RA) inhibits the proliferation of many, but not all, human breast carcinoma cells lines (Liu et al., 1996; Wilcken et al., 1996). The anti-proliferative action of RA appears to be limited to hormone-dependent breast carcinoma cells, in which RA inhibits estrogen stimulation of proliferation (Liu et al., 1996; Wilcken et al., 1996). Estrogen's mitogenic effects are believed to result from increased expression of autocrine peptide growth factors and surface receptor tyrosine kinases (Daly et al., 1994; Halter et al., 1992). RA inhibits signaling between receptor tyrosine kinases and the nucleus (Leid et al., 1993; Talmage and Listerud, 1994). The components of these signaling pathways that serve as targets for RA have yet to be identified. At least two signaling pathways stimulated by receptor tyrosine kinases utilize Protein Kinase C (PKC) family members. These include phospholipase Cγ derived second messenger activation of the conventional PKCs, PKCα, β and γ, and novel PKCs, PKCδ and ε (Blobe et al, 1994), and activation of the atypical PKC ζ by the phosphatidylinositol 3-kinase product, PtdIns 3,4,5-P_3.

Retinoic acid (RA) induced growth arrest is frequently associated with changes in PKC isozyme expression. In our preliminary data (Cho et al., 1997), RA treatment of hormone dependent T-47D breast cancer cells, but not hormone independent MDA-MB-231 breast cancer cells resulted in increased PKCα and decreased PKCζ expression. Because of the relationships between hormonal dependent mammary cell proliferation, RA growth regulation and protein kinase C, **we have hypothesized that retinoic acid inhibits the uncontrolled proliferation of mammary carcinoma cells, by increasing the overall activity of PKCα and/or decreasing the overall activity of PKCζ.**

Summary of previous findings (Year 1)

Task 1. Proliferation of the hormone dependent, T-47D human breast carcinoma cells was inhibited by retinoids. All-trans retinoic acid (RA), and the RARα-selective synthetic retinoid, Am580 were equally effective at arresting growth of T-47D cells. The similar dose response for growth arrest seen between RA and Am580 argue for a primary role of RARα regulating mammary cell proliferation. In contrast, hormone independent breast carcinoma cells, MDA-MB-231 was insensitive to RA for growth arrest.

Untreated T-47D cells expressed the novel PKC isozymes, PKCδ and PKCε, and the atypical PKCζ, but not conventional PKCs (PKCα, β or γ). When T-47D cells were treated with RA, PKCα expression was induced and concomitantly PKCζ expression decreased. In contrast to T-47D cells, RA treatment of MDA-MB-231 cells had no effect on PKCα or PKCζ expression.

The prompt expression of PKCα following RA treatment coincided with the cessation of proliferation in T-47D cells. When proliferation of T-47D cells was assayed in the presence of Am580, Gö6976, a selective inhibitor of conventional PKCs (cPKCs), or both, addition of Gö6976 prevented the Am580 induced reduction in proliferation. Therefore retinoid arrested proliferation of T-47D cells required the activity of conventional PKCs, presumably the retinoid-induced PKCα.

Task 2. During year 1 of this project, we were establishing T-47D and MDA-MB-231 cells stably expressing PKCα, PKCζ, antisense PKCα and antisense PKCζ. After stable transfection is established, we planned to complete Task 2 in each cell line

including control T-47D and MDA-MB-231 cells. Stable transfection of Sense/Antisense PKCα in MDA-MB231 cells and data from these cell lines are reported below.

Task 3. After stable transfection of PKCα, PKCζ, antisense PKCα and antisense PKCζ, in T-47D and MDA-MB-231 cells, we plan to characterize retinoic acid effect on cell cycle progression in each cell line.

Task 4. In order to determine the functional role of PKCα in mediating the proliferative response of T-47D cells to retinoids, we isolated T-47D cells constitutively expressing PKCα following infection with a recombinant retrovirus (pSVXα). SVXα infected T-47D cells (αT-47D) expressed PKCα in the absence of retinoic acid. No additional alterations in PKCβ, γ, δ, ε and ζ were seen in αT-47D cells. Constitutive expression of PKCα slowed T-47D proliferation more effectively than 10^{-8} M RA treatment. Treatment of αT-47D cells with the selective inhibitor of cPKCs, Gö6976 increased proliferation to near control levels.

By using two human breast carcinoma cells lines; the hormone (estrogen)-dependent T-47D cell line originated from a ductal carcinoma and the hormone (estrogen)-independent MDA-MB-231 cell line, originated from an adenocarcinoma, the data presented in first annual report support our hypothesis for a mechanism of RA-induced growth inhibition of T-47D cells. Induction of PKCα expression and concomitant repression of PKCζ expression following RA treatment are consistent with RA inducing growth arrest of T-47D cells. In contrast, retinoic acid had no effect on growth, or PKC expression in hormone independent, MDA-MB-231 breast cancer cells. By manipulating the expression of PKCα, we have shown that expression and activity of PKCα is sufficient to exert growth inhibitory effects on T-47D cells. The lack of retinoid effect on the hormone-independent MDA-MB-231 cell line could reflect the inability of retinoids to induce PKCα expression in these cells. To address whether this is the result of a defect in the PKCα gene, and to identify in more detail the role of PKCα in retinoid induced growth arrest of human breast carcinoma cells in terms of proto-oncogene expression, during year 2, we characterized the effect of expression of PKCα or antisense PKCα in MDA-MB-231 cells.

BODY FOR ANNUAL REPORT; YEAR 2.

Specific Aims.

Aim1. Determine if retinoic acid arrest of mammary carcinoma cells is associated with changes in the expression of, activation of, or signaling through the protein kinase C family (Task 1, 2 and 3).

Aim2. Determine the importance of PKCα and/or PKCζ in mammary cell proliferation and/or differentiation (Task 4).

Task 1. Characterization of cell lines for expression, translocation and activity of PKC isozymes, month 1-10.

Methods

Cell culture

MDA-MB-231 human breast cancer cells (ATCC# HTB26) were grown on culture dishes in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum. Cells were treated with 10^{-6}M all trans retinoic acid dissolved in ethanol (final ethanol concentration was 0.1%) for up to 5 days or 100 ng/ml phorbol ester (TPA: phorbol 12-myristate 13-acetate) for 30 min prior to harvesting.

Measurement of relative proliferation

MDA-MB-231 cells were plated at 15 x 10^4 cells/60 mm culture dish. Media was changed 24 h later, at which time cells were treated with 10^{-6}M all trans retinoic acid dissolved in ethanol (final ethanol concentration was 0.1%). Media and experimental treatment were renewed every 72h. On the days indicated, cells were harvested, and counted with a hemocytometer. Each sample was counted in duplicate, and each condition was done in triplicate.

Measurement of colony forming

MDA-MB-231 cells were plated at 100 cells/60 mm culture dish. After 24 h all trans retinoic acid was added to a final concentration of 0 or 10^{-6}M from 1,000 x stock solutions dissolved in ethanol (final ethanol concentration was 0.1%). Media and experimental treatment were renewed every 72 h. After 21 days, cells were stained with 0.1 % Giemsa and colonies were quantified. Each sample was counted in triplicate, and each condition was done in six separate experiments.

Western blot analysis for PKC isozyme expression

Cell protein lysates were prepared and separated on 10% SDS-PAGE gel as described previously (Cho et al., 1997). Following electrophoretic transfer to nitrocellulose, membranes were blocked by 5% non fat dry milk in PBS. PKC isozymes were detected by incubating with PKC isozyme specific antibodies (polyclonal PKCα antibody; Transduction Lab., Lexington, KY.)(affinity purified polyclonal PKCβ antibodies; protein G-purified polyclonal δ, ε and ζ antibodies)(Gibco BRL, Inc., Gaithersburg, MD), followed by extensive washing with PBS containing 0.05% Tween 20 (PBST) and subsequent incubation with the horseradish peroxidase coupled anti-rabbit IgG (1:7500 dilution)(Amersham Corp.). Immunoreactive proteins were visualized by enhanced chemiluminescence.

Subcellular fractionation

To fractionate subcellular compartments, cells were homogenized by polytron in ice cold 20 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 0.5 mM EGTA, 250 mM sucrose, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml leupeptin. The cell homogenates were clarified by low speed centrifugation (680g, 5min). Cleared supernatants was subjected to ultracentrifugation at 105,000g for 90 min to obtain the high- speed particulate(membrane) and the soluble (cytoplasmic) fractions. The particulate fraction obtained after ultra-centrifugation was solublized in a homogenization buffer containing 0.2% NP-40 and sonicated for 3 second.

The particulate fraction obtained after low speed centrifugation (680g, 5 min), was further dissolved in 20 mM HEPES buffer (pH 7.0) containing 100 μM vanadate, 100 μM molybdate, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and 0.2% NP-40. After low speed centrifugation (1500 g, 5 min) with 20 % glycerol and 400 mM NaCl, DNA and histone pellet is removed and the cleared supernatant was designated as nuclear fraction. The protein concentration of cytoplasmic, membrane and nuclear fractions was determined by Lowry et al (1951) using bovine serum albumin as a standard.

Assay of PKC activity

To measure total PKC activity, cells were lysed in 20mM Tris-HCl buffer (pH 7.5) containing 10mM EDTA, 1 mM EGTA, 0.5% NP40, and 0.4mM phenylmethylsulfonyl

fluoride for 10 min on ice. The cell lysates were clarified by centrifugation (12,000g, 15 min). The cleared supernatants were applied to DEAE-Sepharose columns (1 ml bed volume) for partial purification of total PKC protein. Columns were washed with 15 column volumes of 20mM Tris, 1 mM EDTA and 0.1 mM EGTA, and then PKC was eluted in 3 column volumes of the same buffer containing 90mM NaCl (preliminary experiments demonstrated that PKC activity eluted between 75 and 100 mM NaCl). The 90 mM NaCl eluates were assayed for PKC activity in 0.1 ml of 20 mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 0.2 mg/ml phosphatidylserine, 4 μM diolein, 50 μg/ml substrate, [γ-^{32}P]ATP (3x 10^5 cpm/nmol, 10 μM), and either 500 μM $CaCl_2$ or 12 mM EGTA at 30°C for 10 min (Murray et al., 1993). Similar results were obtained using either histone H1, a modified pseudosubstrate site peptide, or a peptide corresponding to the myelin basic protein phosphorylation site as substrates. Reactions were stopped by spotting on phosphocellulose paper squares that were washed in 0.5% phosphoric acid before scintillation counting.

Results

In our first annual report, we reported that the inhibition of uncontrolled proliferation following retinoic acid treatment of hormone dependent, T-47D breast cancer cell lines, was consistent with retinoic acid inducing expression of PKCα and concomitant repression of PKCζ expression. In contrast, retinoic acid had no effect on growth or PKC expression in hormone independent, MDA-MB-231 breast cancer cells. By manipulating the expression of PKCα, we further have shown that expression of PKCα is sufficient to exert growth inhibitory effects on T-47D cells. These findings suggest that PKCα plays an active role in mediating the anti-proliferative effect of retinoic acid in human breast cancer cells and also raised the question; Could the lack of retinoid effect on the MDA-MB-231 cells be explained by the inability of retinoids to induce PKCα expression in these cells ? To address whether this is the result of a defect in the PKCα expression, we established MDA-MB-231 cells constitutively expressing PKCα or Antisense PKCα (As-PKCα) by retroviral infection of pMV7 vector encoding bovine PKCα (Borner et al., 1991) or As PKCα as described in methods (task 4).

Pools of G-418 resistant colonies were isolated and PKC activity was measured in extracts of transfected MDA-MB-231 cells which were treated with either RA (10^{-6} M to induced the growth arrest) or ethanol (as control) (Table 1). In G418 resistant control cells (pMV7 only), 100% of the total PKC activity was calcium independent, suggesting substantial expression of novel and atypical PKC isozymes, which are only diacylglycerol and/or phospholipid dependent. In our previous report (Cho et al., 1997), untreated MDA-MB-231 control cells expressed novel PKC (nPKC), PKCδ and ε, and atypical PKCζ, but not conventional PKC (cPKC), PKCα, β. The absence of calcium dependent PKC activity (i.e. conventional PKC activity which is dependent on phospholipid, diacylglycerol and calcium) in G418 resistant control cells (pMV7 vector) confirmed the lack of expression of cPKC. RA treatment in G418 resistant control cells slightly increased the calcium dependent PKC activity (10.9 %) suggesting a trace of PKC α activity in these cells, which was barely detected by immunoblotting (lane 5 and 6 in Figure 1 and 2A). Anti-sense PKCα transfection abolished the slight increase of calcium dependent PKC activity in these cells. Calcium dependent PKC activity was largely increased (47.6%) as PKCα was transfected and further elevated (53.2%) with

retinoic acid treatment, suggesting the constitutive expression of PKCα in PKCα transfected MDA-MB-231 cells.

Protein extracts from transfected MDA-MB-231 cells were subjected to SDS-PAGE/Western blotting with PKC isozyme-specific antibodies. As expected from the kinase assays, control (C) or G418 resistant control cells (pMV7 vector, V) expressed only nPKC, PKCδ and ε, and atypical PKCζ (Figure 1). No conventional PKC was detected. Retinoic acid (RA) treatment in MDA-MB-231 cells led no change of PKC isozyme expression. PKCα transfection resulted in constitutive expression of PKCα in control cells and allowed enhanced expression of PKCα by retinoic acid (RA) treatment (Figure 1). Despite the barely detectable level of endogenous PKCα protein (lane 5 and 6 in Figure 1 and 2A), this suggests the possibility that RA induces the expression of endogenous PKCα gene in MDA-MB-231 control cells, with a consistent view of PKCα as a transcriptionally response gene of RA via AP-2 promoter region (Haridasse et al., 1998). In fact a trace of immunoreactive PKCα protein was measured (lane 5 in Figure 2A). Endogenous expression of other PKC isozymes such as PKCδ, PKCε and PKCζ was not altered in any of these cells (Figure 1).

To determine the subcellular distribution of PKC isozymes, transfected MDA-MB-231 cells were further fractionated into cytosolic, membrane and nuclear compartments and subjected to SDS-PAGE/Western blotting with PKC isozyme-specific antibodies. In PKCα expressing cells, PKCα proteins were equally distributed in cytosolic and membrane fraction (Figure 2 A and B). The expressed PKCα was also located in nuclear fraction (Figure 2C). Compared to a positive control of translocated PKCα expression in membrane (lane 9 in Figure 2B), RA treatment enhanced the translocation of PKCα into membrane fraction. In human breast cancer cells, PKCα desensitizes the epidermal growth factor receptor, EGFR, to the mitogenic effect of EGF, and as a result downstream signaling pathways are not activated , the AP-1 transcriptional factor is not synthesized or activated, and cells fail to progress through the cell cycle ((Cocekt et al., 1984; Friedman et al., 1984; Saloman, 1981; Lee and Weinstein 1978). Several lines of recent evidence also indicates that PKCα acts in the nucleus as well as the plasma membrane (Hyatt et al., 1990; Zauli et al., 1996; Yoshida et al., 1996; Schmalz et al., 1996). RA enhanced translocation of PKCα to membrane and nuclear fractions in PKCα expressing MDA-MB-231 cells suggests the possibility that PKCα desensitizes the growth factor receptor in membrane and combined with the possible transcriptional regulation of target gene in nucleus, ultimately mediates the regression of cell proliferation in response to RA treatment. The specific target of PKCα in membrane and nucleus is under investigation. Endogenous expression of other PKC isozymes such as PKCδ, ε and ζ was mainly located in cytosolic and membrane fraction (Figure 2A and 2B).

The above data confirmed the successful transfection of PKCα in MDA-MB-231 cells. As a first step toward investigating whether PKCα plays an active role in mediating the anti-proliferative effect of retinoic acid (RA) in human breast cancer cells, and to establish that the lack of RA effect on the MDA-MB-231 cells can be explained by the inability of RA to induce PKCα expression in these cells, we determined the proliferation of transfected MDA-MB-231 cells. Similar to previous reports (Cho et al., 1997), proliferation of control or G418 resistant control MDA-MB-231cells (pMV7 vector) was insensitive to micromolar concentration of RA as shown in Figure 3A. Antisense PKCα transfected MDA-MB-231 cells were also insensitive to RA treatment.

Constitutive expression of PKCα in MDA-MB-231 cells itself did not inhibit proliferation (Figure 3A). However, with addition of 10^{-6} M RA, PKCα expressing MDA-MB-231 cells slightly reduced the proliferation after 3days and was distinct after 5 days (40% reduction)(Figure 3B). To further determine the positive correlation of RA inability to induce PKCα expression and the lack of anti-proliferation effect in MDA-MB-231 cells, we performed the colony forming assay in transfected MDA-MB-231 cells (Table 2). With less cell plating (100 cells), anti-proliferation effect of PKCα was more distinct and resulted 22.67 % inhibition of cell proliferation. Similar to Figure 3B, proliferation of PKCα expressing MDA-MB-231 cells was further reduced (33.83% inhibition) with 10^{-6}M RA treatment for 5 days. Together, these data suggest that the lack of anti-proliferation effect of RA on MDA-MB-231 cells can be explained by the inability of RA to induce PKCα expression in these cells. In consistent view of anti-proliferation effect of retinoids in T-47D cells (Cho et al., 1997), retinoid arrested proliferation of MDA-MB-231 cells required the PKCα, presumably the retinoid-induced PKCα.

Task 2. Characterization of cell lines for proto-oncogene expression, month 11-16.

Methods

RNA isolation and northern blotting

To induce proto-oncogene expression, cells with >80% confluent were serum starved (0.5%) for 24hrs with or without RA (10^{-6}M) and then stimulated with 10% FBS (fetal bovine serum) for 30 min before harvesting. Total RNA was isolated from cells and northern blots were performed as described by Dorsett et al (1989). In every case, filters were hybridized to ^{32}P labeled RNA probes. Probes for L-30, c-fos, c-jun, JunB and JunD have been described previously (Talmage and Lackey, 1992). After washing in 2 x SSC/1% SDS and 0.2 x SSC/1% SDS, the northern blots were exposed to X-ray film at -80°C with intensifying screens.

Results

The above experiments identified that the inability of anti-proliferative effect of retinoic acid (RA) in MDA-MB-231 cells was associated with the lack of RA induced PKCα expression, suggesting that PKCα is an important mediator of the anti-proliferative effect of retinoic acid in human breast cancer cells. The mechanism(s) by which PKCα participates in anti-proliferation of MDA-MB-231 cells remains to be determined. The increase in steady state of PKCα increased the basal level of kinase activity leading to increased phosphorylation of cellular substrates that involves the regression of cell proliferation. Several recent reports demonstrated that PKCα phosphorylates and desensitizes the epidermal growth factor receptor (EGFR)(and/or similar receptor), and as a result downstream signaling pathways are not activated (Cocekt et al., 1984; Friedman et al., 1984; Saloman, 1981; Lee and Weinstein 1978). Alternatively, the effects of PKCα might involve transcriptional regulation of the genes whose products are actively involved in removing MDA-MB-231 cells from the cell cycle. We determined and measured the serum induction of c-fos, c-jun, Jun B and JunD mRNA expression in MDA-MB-231 cells (Figure 4). Consistent with proliferation assays described in Figure 3 and Table 2, a 24 hour RA treatment of PKCα transfected MDA-MB-231 cells repressed the serum induction of c-fos expression and

concomitantly enhanced c-jun immediate early gene (IEG) induction (lane 15 in Figure 4).

Task 3. Characterization of retinoic acid effect on cell cycles progression, month 1-20.

Task 3 was not completed in first and second year. Over 2 years, we have been trying to establish T-47D stably expressing PKCα, PKCζ, antisense PKCα or antisense PKCζ or MDA-MB-231 cells stably expressing PKCζ or antisense-PKCζ. Unfortunately, all of transfected T-47D cells, or sense/antisense PKCζ expressing MDA-MB-231 cells could not be survived . In third year, we are trying again to establish these transfected cell lines and plan to characterize intensively retinoic acid effect on cell cycle progression in each cell line including control T-47D and MDA-MB-231 cells.

Task 4. Manipulation of individual PKC isozyme gene expression, month 18-36.

Methods

Plasmids

The anti- sense PKCα plasmid (pMV7-As PKCα) was constructed by ligating the 2.44kb EcoR I fragment of PKCα cDNA from pβ-actin SP72-PKCα (Cho et al., 1997) into the unique EcoR I site of the pMV 7 vector (Talmage and Lackey, 1992). The antisense-orientation was confirmed by BamH1 restriction digestion.

In Stratagene's LacSwitch™ II inducible mammalian expression system, sense or antisense PKCα cDNA (pOPRSVI-PKCα; pOPRSVI-As PKCα) were subcloned by ligating the 2.46-kb Sma I and Kpn I fragment of PKCα cDNA from pβ-actin SP72-PKCα into Sma I and Kpn I multicloning site of pOPRSVI/MCS. The sense or antisense orientation was confirmed by EcoR I.

Stable transfection

To stably express PKCα or antisense PKCα (As-PKCα), pSVXα encoding bovine PKCα (Borner et al., 1991) or pMV7-As PKCα was transfected into the NIH3T3 GP + env Am12 packaging cell line (Markowitz et al., 1988) and colonies stably producing recombinant virus were isolated following selection for G418 resistance (250 μg/ml). MDA-MB-231 cells were infected with freshly conditioned medium derived from subconfluent NIH3T3 GP + env Am12 cultures. Following infections, cultures were subjected to G418 (250 μg/ml) selection. Resistant cells were maintained in 100 $\mu g.ml^{-1}$ G418.

For Stratagene's LacSwitch™ II inducible mammalian expression system, both pCMVLac I repressor and pOPRSVI/MCS encoding PKCα were transfected sequentially into MDA-MB-231 cells by lipofectAMINE reagent (Gibco BRL, Inc., Gaithersburg, MD)(Bebok et al., 1996). Following transfection, cells were subjected to hygromycin B (200 μg/ml) and G418 (800 μg/ml) selection over 4 weeks. Expanded colonies was isolated and treated with IPTG (isopropyl β-D-thiogalactopyranoside; 5 mM) for 6, 12, 18 and 24 hr followed by the IPTG removal for 48 or 72 hours to examine expression levels of PKCα induction and Lac repressor by western blotting with PKC antibody (polyclonal PKCα antibody; Transduction Lab., Lexington, KY.) and polyclonal antiserum to Lac repressor (Stratagene, Inc.)

Results

To delineate further the role of PKCα in RA induced growth arrest of human breast cancer cells, we used the Stratagene's Lac Switch™ II inducible mammalian expression system, in which gene transcription can be reversibly turned on or off. In the escherichia coli lactose (lac) operon, the Lac repressor binds as a homotetramer to the lac operator, blocking transcription of the lacZ gene. Physiological or synthetic inducers, such as allolactose or isopropyl β-D-thiogalactopyranoside (IPTG), respectively, bind to the Lac repressor, causing a conformational change and effectively decreasing the affinity of the repressor for the operator. When the repressor is removed from the operator, transcription of genes from the lac operon resumes. In Stratagene's Lac Switch™ II inducible mammalian expression system, sense/anti-sense PKCα cDNA was subcloned to pOPRSVI/MCS, in which RSV (Rous sarcoma virus)-LTR promoter drives expression of gene, and ideal operator sequences for the Lac repressor binding (op) are present in the RSV promoter and in the intron.

After sequential transfection of pLac and pOPR-PKCα in MDA-MB-231 cells, we examined the induction of PKCα expression by IPTG treatment in these cells. As shown in Figure 5A, the Lac repressor was constantly expressed as a dimer or monomer in all cells regardless of IPTG treatment. Compared to the positive control of PKCα expression in pOPR-PKCα single transfected MDA-MB-231 cells (lane 2 in Figure 5B), PKCα was not expressed initially in pLac + pOPR-PKCα transfected MDA-MB-231 cells (lane 4 in Figure 5B). By 6 hr IPTG treatment, PKCα expression was gradually induced and was maximal after 24 hr (Figure 5B). 48 hr after removing IPTG, PKCα had decreased to undetectable levels, confirming the reversibility of PKCα induction. For future experiments using pLac + pOPR-PKCα MDA-MB-231 cells, 24 hour IPTG treatment and 72 hour removal of IPTG was used for turning on/off of PKCα gene transcription.

The serum induction of c-fos and c-jun expression in PKCα expressed MDA-MB-231 cells by IPTG treatment was determined as shown in Figure 6. As a control, we also measured c-fos expression in control, and pLac or pOPR-PKCα single transfected MDA-MB-231 cells (Figure 6A). As shown earlier in cells constitutively expressing PKCα (Figure 4) or transfected pOPR-PKCα only (lane 12 in Figure 6A), concomitant 24 hr RA and IPTG treatment of pLac and pOPR-PKCα transfected cells repressed c-fos expression (lane 8 in Figure 6B). This effect was lost 72 hour after removing out the IPTG (lane 12 in Figure 6B). As soon above, c-jun expression was enhanced and then repressed. In Figure 4 and 6, PKCα expressing MDA-MB-231 cells showed a response to retinoic acid in terms of modulating c-fos and c-jun expression. These cells also showed the regression of cell proliferation in the presence of RA (Figure 3 and Table 2).

CONCLUSION

The data presented in this annual report support our hypothesis for a mechanism of retinoic acid (RA)-induced growth inhibition of human breast cancer cells. The induction of PKCα expression following RA treatment coincided with the cessation of proliferation of hormone dependent, T-47D breast cancer cells. In contrast, RA had no effect on growth or PKC expression in hormone-independent MDA-MB-231 cells. To determine whether the lack of RA effect on MDA-MB-231 cells is caused by the failure

of PKCα expression, we established MDA-MB-231 cell lines stably expressing PKCα or antisense PKCα RNAs. MDA-MB-231 cells transfected with PKCα, constitutively expressed PKCα in cell cytosol, membrane and nucleus. RA treatment enhanced cPKC activity and the translocation of PKCα into the cellular membrane and nucleus. Although constitutive expression of PKCα had no effect by itself, on MDA-MB-231 proliferation, it allowed RA to inhibit both serum induction of c-fos expression and cell proliferation (by ~40%). By turning on/off the transcription of PKCα gene, we further have shown the PKCα regulation of c-fos and c-jun expression as a downstream target. In total, our data demonstrate that the lack of anti-proliferation effect of RA in MDA-MB-231 cells is partially caused by the failure of RA to induce PKCα expression and consequently the absence of PKCα altered gene expression.

It is not clear how PKCα expression changed MDA-MB-231 cell sensitivity to RA. Several observations strongly favor RARα as the mediator of RA growth regulation and PKCα expression in T-47D cells (Cho et al., 1997; Sheikh et al., 1994; Valette et al., 1987; Kennedy et al., 1992). For example, RARα is the major RAR subtype expressed in T-47D cells and the synthetic retinoid Am580 arrests T-47D proliferation at concentration ($<10^{-8}$ M), at which it demonstrates greatest selectivity for RARα (Delescluse et al., 1991). RARα is present at very low levels, and RARγ is the major RAR subtype in MDA-MB-231 cells. (Sheikh et al., 1994). The lack of RA effect on MDA-MB-231 cell proliferation or PKCα expression could reflect the relative lack of RARα. Constitutive expression of PKCα repaired the RA inability for RARα dependent induction of PKCα expression (Figure 1 and 2) and RA treatment can increase the level of RARγ expression (Sheikh et al., 1994). By prolonged treatment (5 days) of micromolar concentration of RA, constitutively expressed PKCα in MDA-MB-231 cells can phosphorylate RARγ which expression is increased with RA treatment. Although activated RA-RARγ and PKCα synergistically affect transcriptional regulation of c-fos and c-jun and ultimately slowed MDA-MB-231 cell progress through cell cycle, the effect was not equivalent to the strong and rapid growth suppression of T-47D cells by retinoic acid. MDA-MB-231 cells expressing a transfected RARα gene also are considerably less responsive to retinoic acid than T-47D cells (Sheikh et al., 1994). Based on our findings, cotransfection of PKCα and RARα in MDA-MB231 cells might result to increase the RA sensitivity comparable to hormone dependent T-47D cells.

Future work: we have described an important role of PKCα in mediating the anti-proliferative action of retinoic acid in human breast cancer cells. Our goal is to identifying in more detail the role of PKCα in retinoid induced growth arrest of human breast cancer cells in terms of proto- oncogene and cell cycle progression. During first and second year of this project, we only established the stable transfection of sense/ antisense PKCα in MDA-MB-231 cells. After stable transfection of sense/antisense PKCα in T-47D and sense/antisense PKCζ in T-47D and MDA-MB-231 cells, we plan to complete Task 2 in transfected T-47D cells, and Task 3 in transfected T-47D and MDA-MB-231 cells.

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Appendix

Table 1-2, Figure 1-6, Attached.
At the end of Figures, publication and meeting abstract produced during September 1997-September 1998 was attached.

Figure legends

Figure 1. Protein Kinase C isozyme expression in transfected MDA-MB-231 cells.
Whole Cell extracts (30 μg protein/lane) from control (C; lanes 1, 5), G418 resistant control (pMV7 vector only, V; lanes 2, 6) , PKCα (α; lanes 3, 7) or antisense PKCα (Asα; lanes 4,8) transfected MDA-MB-231 cells were subjected to SDS-PAGE and western blotting with PKC isozyme specific antibodies (polyclonal PKCα antibody; Transduction Lab., Inc., Lexington, KY.)(affinity purified polyclonal PKCβ antibody; protein G-purified polyclonal PKCδ, ε and ζ antibodies)(Gibco BRL, Inc., Gaithersburg, MD). MDA-MB-231 cells in lanes 5-8 were treated with retinoic acid (RA)(10^{-6}M) for 5 days. The region of each gel shown was between the Mr 67,000 and 93,000 prestained molecular weight markers (New England Biolabs, Beverly, MA) run in adjacent lanes.

Figure 2. Subcellular distribution of PKC isozyme in transfected MDA-MB-231 cells.
To determine the subcellular distribution of PKC isozyme, cytosolic, membrane or nuclear protein extracts (15 μg protein /lane) from control (C; lanes 1,5), G418 resistant control (pMV7 vector only, V; lanes 2, 6) , PKCα (α; lanes 3, 7) or antisense PKCα (Asα; lanes 4, 8) transfected MDA-MB-231 cells were subjected to SDS-PAGE followed by western blotting with PKC isozyme specific antibodies as described under methods. MDA-MB-231 cells in Lanes 5-8 were treated with retinoic acid (RA)(10^{-6} M) for 5 days. As a positive control of translocated PKCα expression in membrane fraction, cytosolic, membrane or nuclear protein extracts (7.5 μg protein /lane) from PKCα transfected MDA-MB-231 cell followed by 30 min stimulation of 100ng/ml phorbol ester(+TPA) was loaded in lane 9. The region of each gel shown was between the Mr 67,000 and 93,000 prestained molecular weight markers (New England Biolabs, Beverly, MA) run in adjacent lanes.

Figure 3. Retinoic acid becomes to inhibit proliferation of MDA-MB-231 cells with constitutive expression of PKCα.
Hormone independent control, G418 resistant control (pMV7 vector only), PKCα or antisense PKCα transfected MDA-MB-231 cells were plated at 15 x 10^4 cells/dish. After 24hr retinoic acid was added to a final concentration of 0 or 10^{-6} M from 1,000x stock solutions in ethanol. Media and experimental treatment were renewed every 72 hrs. Cells were trypsinized, stained with trypan blue, and counted after indicated intervals. Data shown (± SE) are from three independent experiments in which treatments were done in duplicate. Day 1 represents the time of retinoid or solvent addition.

Figure 4. Stable expression of PKCα alters proto-oncogene expression.
Control (C), G418 resistant control (pMV7 vector only, V) , PKCα (α) or antisense PKCα (Asα) transfected MDA-MB-231 cells were maintained for 24 hrs in either 10% fetal bovine serum (FBS), 0.5% FBS (Serum starvation) or 0.5% FBS + retinoic acid (RA;10^{-6}

M). For induction of proto-oncogene expression, 0.5% FBS or 0.5% FBS + 10^{-6}M RA treated MDA-MB-231 cells were stimulated with 10% FBS for 30 min before harvesting. Total RNA was isolated and analyzed by northern blotting for expression of L-30, c-fos, c-jun, JunB and JunD.

Figure 5. IPTG Induced PKCα expression in Lac repressor and PKCα cotransfected MDA-MB-231 cells.
The expanded clone #4 of pCMVLac 1 repressor (pLac) and pOPRSVI/MCS-PKCα (pOPRα) cotransfected MDA-MB-231 cells were treated with IPTG for 6, 12, 18 or 24 hrs followed by IPTG removal for 48 hrs or 72 hrs. Whole cell extracts (15 μg protein /lane) were subjected to SDS-PAGE and western blotting with polyclonal antiserum to Lac repressor (Stratagene, Inc.)**(A)** or PKCα antibody (polyclonal PKCα antibody; Transduction Lab., Inc., Lexington, KY)**(B)**. For a positive control of either Lac repressor expression or PKCα expression, whole cell extracts (15 μg protein /lane) of single transfected MDA-MB-231 cells with pLac I repressor or pOPRα was loaded in lane 1 or 2. Also, whole cell extracts (15 μg protein /lane) from OPRSVI/MCS-AntisensePKCα (pOPR-Asα) transfected MDA-MB-231 cells was loaded in lane 3.

Figure 6. PKCα expressed MDA-MB-231 cells by IPTG have altered c-fos and c-jun expression.
A. Control (C), pCMVLac I repressor (pLac) or pOPRSVI/MCS-PKCα (pOPRα) single transfected MDA-MB-231 cells were maintained for 24 hrs in either 10% fetal bovine serum (FBS), 0.5% FBS (Serum starvation) or 0.5% FBS + retinoic acid (RA;10^{-6}M). For induction of proto-oncogene expression, 0.5% FBS or 0.5% FBS + 10^{-6}M RA treated MDA-MB-231 cells were stimulated with 10% FBS for 30 min before harvesting. Total RNA was isolated and analyzed by northern blotting for expression of L-30, c-fos and c-jun. **B.** In parallel with serum starvation/stimulation treatment in Figure 6A, the expanded clone #4 of pLac and pOPRα cotransfected MDA-MB-231 cells were further treated with IPTG for 24hr followed by IPTG removal for 72 hrs for turning on/off of PKCα transcription. Total RNA was isolated and analyzed by northern blotting for expression of L-30, c-fos and c-jun.

Table 1. Protein Kinase C activity in transfected MDA-MB231 cells

Transfected MDA-MB-231 cells	PKC activity (10^6 cpm/min/mg protein) Cofactors in Assay DAG/PS/Ca^{++}	DAG/PS	PS	% Calcium dependency
pMV7	1.1 ± 0.11	1.14 ± 0.11	0.37 ± 0.05	-
PKCα	2.29 ± 0.30	1.2 ± 0.14	ND	47.6
AsPKCα	1.18 ± 0.16	1.6 ± 0.31	0.34 ± 0.16	-
pMV7 + RA	1.92 ± 0.16	1.71 ± 0.55	0.25 ± 0.10	10.9
PKCα + RA	3.55 ± 0.65	1.66 ± 0.45	ND	53.2
AsPKCα +RA	1.86 ± 0.61	1.8 ± 0.26	0.27 ± 0.13	-

G418 resistant control (pMV7 vector only), PKCα (PKCα) or antisense PKCα (As-PKCα) transfected MDA-MB-231 cells were further treated with 10^{-6} M retinoic acid (RA) for 5 days. The detergent extract were absorbed to DEAE-Sepharose, and the fraction eluting with 90 mM NaCl was assayed for PKC activity in the presence of diacylglycerol (DAG)/phosphatidylserine (PS)/Ca^{++}, DAG/PS/EGTA (-calcium) or PS only. Activity (±SE) is shown as ^{32}P transferred from ATP to peptide substrate/min/mg extract protein at 30°C. The increase in calcium dependent activity with PKCα transfection and 5 day RA treatment were significant at $p < 0.05$ (as determined using Student's t-test).

Table 2. Inhibited colony formation of MDA-MB231 cells with constitutive expression of PKCα and Retinoic Acid (RA) treatment.

MDA-MB231 cells	1	2	3	4	5	6	mean (±SE)	p value	% inhibition
control	37	39	51	50	50	53	46.7(2.8)		0
pMV7	40	36	52	52	51	50	46.8(2.9)		0
PKCα	29	28	44	40	38	38	36.2(2.6)	.003	22.67
As-PKCα	33	38	48	53	60	52	47.3(4.1)		0
control + RA	40	ND	ND	50	50	54	48.5(3.0)		0
pMV7 + RA	37	ND	ND	51	50	50	47.0(3.3)		0
PKCα + RA	23	25	36	31	37	34	31.0(2.4)	.005	33.83
As-PKCα + RA	43	39	50	48	52	55	47.8(7.4)		0

Control, G418 resistant control (pMV7 vector only) MDA-MB-231 cells, PKCα (PKCα) or antisense PKCα (As-PKCα) transfected MDA-MB-231 cells were plated at 100 cells/dish. After 24hr retinoic acid was added to a final concentration of 0 or 10^{-6} M from 1,000x stock solutions in ethanol. Media and experimental treatment were renewed every 72 hrs. After 21 days, cells were stained with 0.1% Giemsa and colonies were counted. The results from six separate experiments with triplicate are shown as average colonies per 60-mm dish. The percent of inhibition was determined by using the formula: 1-(colony# in each cell line/ colony # in control cell line). The inhibited colony formation with constitutive expression of PKCalpha (P= 0.0003) and constitutive expression of PKCalpha + retinoic acid (RA) treatment (P= 0.005) were significant at $P<0.05$ vs. control (as determined using student's t-test). The average percent of inhibition by PKCα transfection or PKCα transfection + 10^{-6} M RA is shown in the last column. ND, not determined.

Figure 1

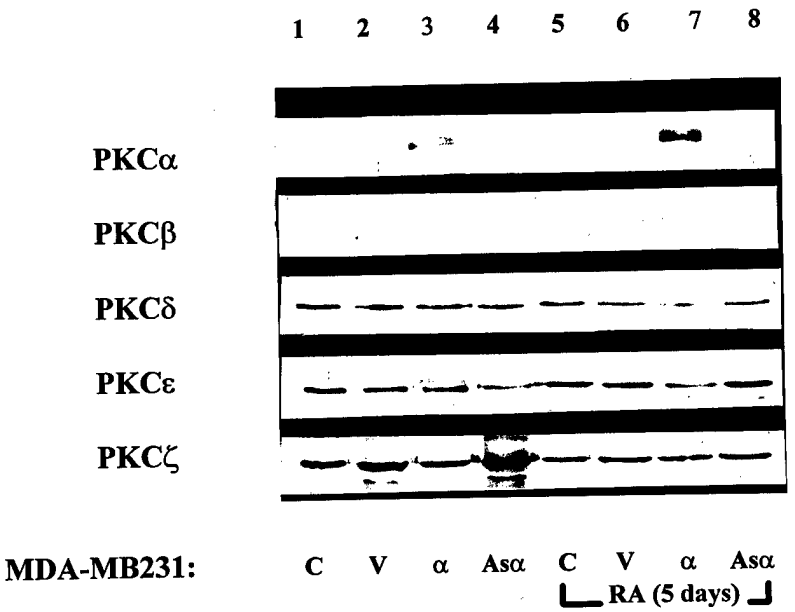

1 2 3 4 5 6 7 8
PKCα
PKCβ
PKCδ
PKCε
PKCζ
MDA-MB231:
C V α Asα C V α Asα
RA (5 days)

Figure 2.

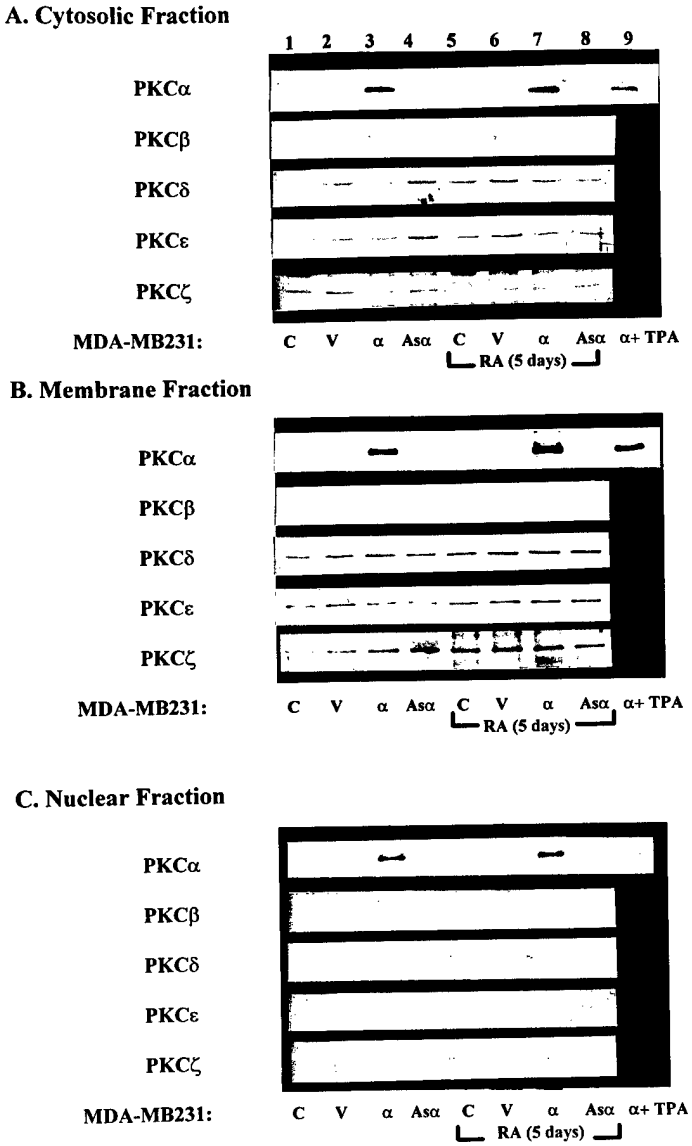
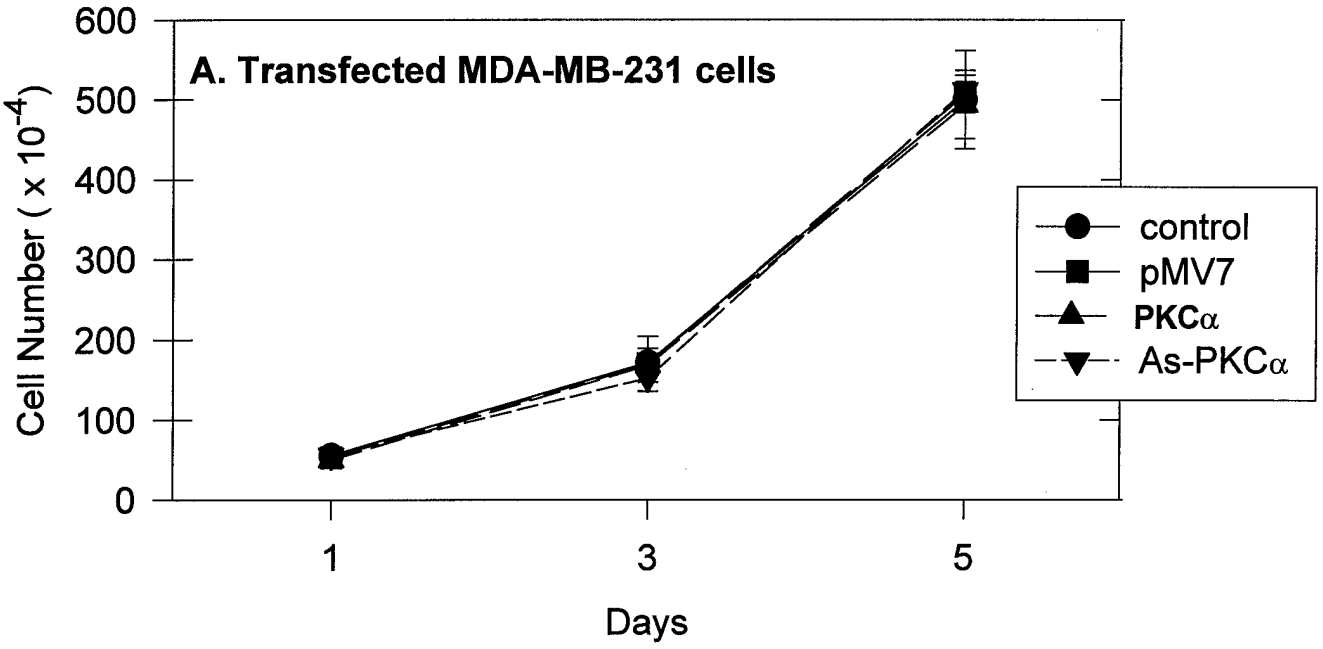

A. Cytosolic Fraction
1 2 3 4 5 6 7 8 9
PKCα
PKCβ
PKCδ
PKCε
PKCζ
MDA-MB231:
C V α Asα C V α Asα α+ TPA
RA (5 days)
B. Membrane Fraction
PKCα
PKCβ
PKCδ
PKCε
PKCζ
MDA-MB231:
C V α Asα C V α Asα α+ TPA
RA (5 days)
C. Nuclear Fraction
PKCα
PKCβ
PKCδ
PKCε
PKCζ
MDA-MB231:
C V α Asα C V α Asα α+ TPA
RA (5 days)

Figure 3


A. Transfected MDA-MB-231 cells
Cell Number (x 10-4)
600
500
400
300
200
100
0
1
3
5
Days
control
pMV7
PKCα
As-PKCα

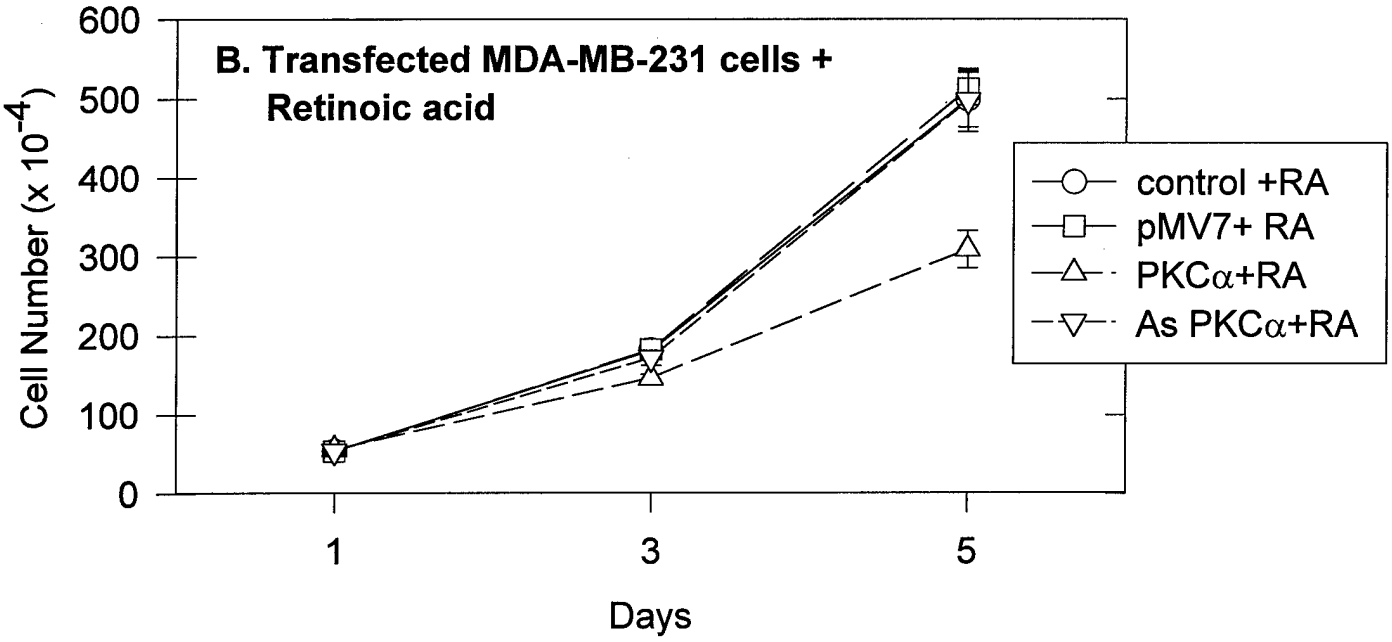

B. Transfected MDA-MB-231 cells +
Retinoic acid
Cell Number (x 10-4)
600
500
400
300
200
100
0
1
3
5
Days
control +RA
pMV7+ RA
PKCα+RA
As PKCα+RA

Figure 4.

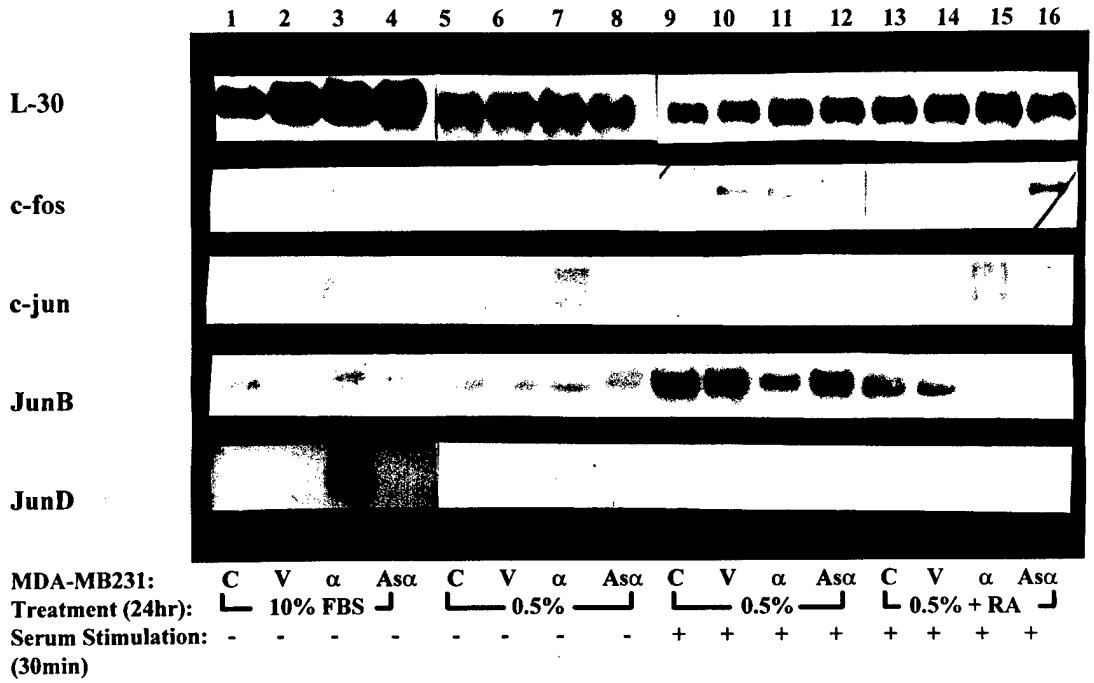

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
L-30
c-fos
c-jun
JunB
JunD
MDA-MB231: C V α Asα C V α Asα C V α Asα C V α Asα
Treatment (24hr): 10% FBS 0.5% 0.5% 0.5% + RA
Serum Stimulation: - - - - - - - - + + + + + + + +
(30min)

Figure 5.

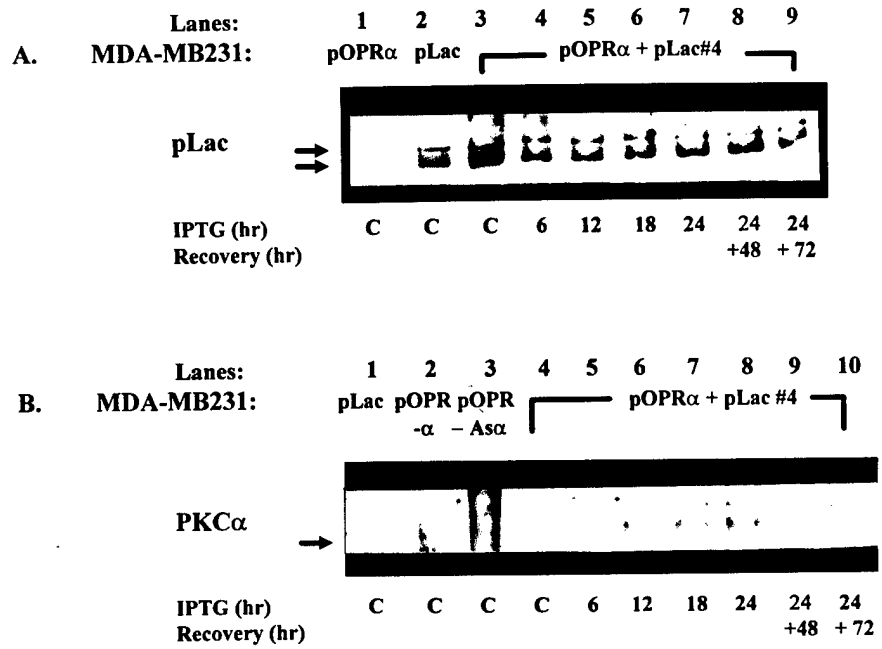
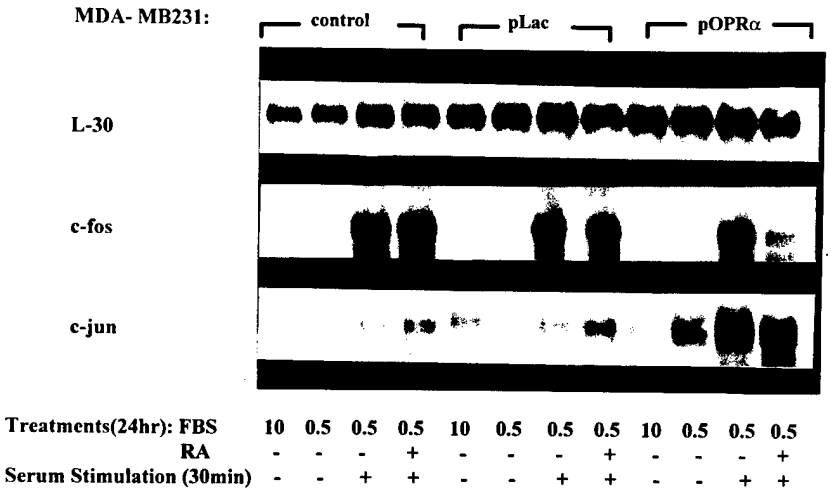
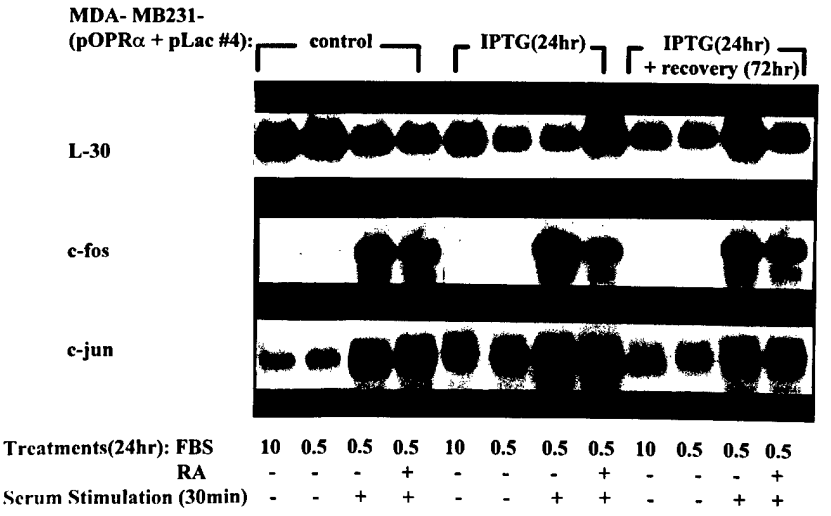

A.
Lanes: 1 2 3 4 5 6 7 8 9
MDA-MB231:
pOPRα pLac pOPRα + pLac#4
pLac
IPTG (hr) C C C 6 12 18 24 24 24
Recovery (hr) +48 + 72
B.
Lanes: 1 2 3 4 5 6 7 8 9 10
MDA-MB231:
pLac pOPR -α pOPR – Asα pOPRα + pLac #4
PKCα
IPTG (hr) C C C C 6 12 18 24 24 24
Recovery (hr) +48 + 72

Figure 6

A.


MDA- MB231:
control
pLac
pOPRα
L-30
c-fos
c-jun
Treatments(24hr): FBS 10 0.5 0.5 0.5 10 0.5 0.5 0.5 10 0.5 0.5 0.5
RA - - - + - - - + - - - +
Serum Stimulation (30min) - - + + - - + + - - + +

B.


MDA- MB231-
(pOPRα + pLac #4):
control
IPTG(24hr)
IPTG(24hr)
+ recovery (72hr)
L-30
c-fos
c-jun
Treatments(24hr): FBS 10 0.5 0.5 0.5 10 0.5 0.5 0.5 10 0.5 0.5 0.5
RA - - - + - - - + - - - +
Serum Stimulation (30min) - - + + - - + + - - + +

Bibliography

Meeting Abstract

Retinoids'97; European Retinoid Research Group
(September 28 – October 1, 1997; Nice, France)

Abstract # A099
Retinoic acid –induced cell cycle arrest of a human breast cancer cell line is PKCα-dependent.
Ann P. Tighe, Yunhi Cho and David A. Talmage Institute of Human Nutrition, Columbia University, new York, NY 10032.

We hypothesize that retinoids inhibit hormone-dependent human breast cancer cell proliferation by inducing a state of functional mitogen deprivation. Retinoic acid reversibly arrested proliferation of hormone-dependent breast cancer cells. Retinoic acid treatment repressed epidermal growth factor induction of genes involved in progression through the G_1 phase of the cell cycle, leading to accumulation of cells in G_0/G_1. Retinoic acid treatment inhibited all measures of epidermal growth factor signaling including: (1) epidermal growth factor receptor kinase activity, (2) activation of $p70^{s6k}$, Jun-N-terminal kinase, p38 kinase and mitogen activated protein kinase, and (3) immediate early gene expression. We have previously shown retinoic acid-induced expression of protein kinase Cα. The antagonistic effect of retinoic acid on epidermal growth factor signaling is prevented by inhibiting protein kinase Cα activity. We conclude that retinoic acid arrests cell cycle progression by first inducing protein kinase Cα expression. Subsequent activation of protein kinase Cα inhibits mitogenic signal transduction from the epidermal growth factor receptor.

Distinct Functions of Protein Kinase Cα and Protein Kinase Cβ during Retinoic Acid-induced Differentiation of F9 Cells[1]

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Abstract

As F9 embryonal carcinoma cells differentiate into parietal endoderm-like cells, expression of conventional protein kinase C (PKC) changes. Undifferentiated stem cells express PKCβ but not PKCα, whereas differentiated parietal endoderm cells express PKCα but not PKCβ. To determine whether changes in PKCα and/or PKCβ expression control retinoic acid (RA)- and dibutyryl cyclic AMP-induced F9 cell differentiation, we established cell lines stably expressing PKCα, PKCβ, antisense PKCα, or antisense PKCβ RNAs. Constitutive expression of PKCα or inhibition of PKCβ expression in F9 stem cells enhanced RA induced differentiation, both by increasing total expression and accelerating RA-induced expression of laminins A, B1, B2, and type IV collagen. In addition, expressing PKCβ in a parietal endoderm cell line caused these cells to retrodifferentiate into stem cells. Based on these results, we conclude that PKCβ and PKCα are key targets for RA-regulated gene expression, that PKCα plays an important, active role in inducing and maintaining the parietal endoderm phenotype, and that PKCβ activity is incompatible with maintaining the differentiated state of these cells.

Introduction

F9 mouse embryonal carcinoma cells have limited developmental potential and exhibit little spontaneous differentiation. When treated with RA,[3] F9 cells differentiate into primitive endoderm-like cells (1, 2), which have the ability to respond to secondary differentiation factors that determine the ultimate differentiated phenotype. Exposure of these primitive endoderm-like cells to agents that elevate intracellular cAMP induces formation of parietal endoderm (1, 2). In the absence of cAMP, aggregates of RA-treated F9 cells differentiate into a cell type resembling the visceral endoderm of the mouse embryo (3, 4).

During F9 differentiation, RA profoundly alters the expression of intracellular signaling pathways. Notable among the reported changes are increases in the AP-1 transcription factor (5–9), increased total PKC activity (10–12), and increased levels of phorbol ester receptors (13). RA also increases the amount and alters the subcellular distribution of protein kinase A, changes that are believed to contribute to the ability of cAMP to induce parietal endoderm differentiation (14).

PKC is a calcium-, diacylglycerol-, and phospholipid-dependent enzyme involved in regulating differentiation and proliferation (15, 16). There are at least 11 distinct genes constituting the PKC family that can be grouped into three subfamilies based on sequence homology and cofactor requirement: the conventional PKCs (α, β1, β2, and γ) are calcium, diacylglycerol, and phospholipid dependent; the novel PKCs (δ, ε, θ, η, and μ) are diacylglycerol and phospholipid dependent but calcium independent, whereas the atypical PKCs (λ and ζ) are phospholipid dependent but diacylglycerol and calcium independent (16). Tumor-promoting phorbol esters are potent diacylglycerol agonists that bind to and activate both conventional PKCs and novel PKCs but not atypical PKCs (17, 18). In addition to activating these enzymes, chronic treatment of cells with phorbol esters down-regulates conventional PKCs and novel PKCs (16).

The increased level of calcium-dependent PKC activity reported during RA- and cAMP-induced differentiation of F9 cells into parietal endoderm results from increased expression of PKCα (11, 12); concurrently, PKCβ expression declines. F9 stem cells and visceral endoderm cells express PKCβ but not PKCα, whereas F9 cells differentiating into parietal endoderm express PKCα but not PKCβ. The transition from PKCβ to PKCα is accompanied by changes in phorbol ester-induced gene expression. Specifically, phorbol ester induces c-fos expression in PKCβ-expressing F9 stem cells but not in PKCα-expressing parietal endoderm cells. In contrast, phorbol ester induces type IV collagen, a parietal endoderm marker gene, in PKCα-expressing parietal endoderm cells.

To establish a functional role for PKCα and PKCβ in mediating parietal endoderm differentiation, we established F9-derived cell lines constitutively expressing PKCα, PKCβ, antisense PKCα, or antisense PKCβ RNAs. Constitutive expression of PKCα in stem cells was sufficient to allow phorbol ester induction of type IV collagen even in the absence of

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[3] The abbreviations used are: RA, retinoic acid; cAMP, cyclic AMP; PKC, protein kinase C; TTR, transthyretin; TPA, 12-O-tetradecanoylphorbol-13-acetate; dbcAMP, dibutyryl cAMP.

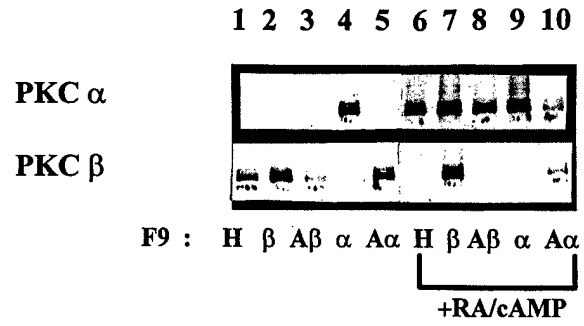

Fig. 1. PKCα and PKCβ expression in transfected F9 cells. Cell extracts (30 μg protein/lane) from hygromycin-resistant control F9 cells (*H, Lanes 1* and *6*), PKCβ (*β, Lanes 2* and *7*), antisense PKCβ (*Aβ, Lanes 3* and *8*), PKCα (*α, Lanes 4* and *9*), and antisense PKCα (*Aα, Lanes 5* and *10*) transfected F9 cells were subjected to SDS-PAGE and Western blotting with either PKCα- or PKCβ-specific antibodies (affinity-purified polyclonal PKCα and PKCβ antibodies; Life Technologies, Inc.). F9 cells in *Lanes 6–10* were treated with RA (2×10^{-7} M) and dbcAMP (5×10^{-4} M) for 5 days. The region of each gel shown was between the M_r 67,000 and 93,000 prestained molecular weight markers run in adjacent lanes.

RA and cAMP. Furthermore, PKCα expression both accelerated and increased the total expression of laminin A/B1/B2 and type IV collagen in response to RA and cAMP. Antisense PKCα or PKCβ expression decreased RA and cAMP induction of these parietal endoderm markers and alternatively enhanced the expression of visceral endoderm marker genes. In addition, expressing PKCβ in a parietal endoderm cell line gradually caused these cells to retrodifferentiate into stem cells. These results indicate that PKCα is critical in establishing and maintaining the parietal endoderm phenotype, and furthermore, that PKCβ is incompatible with maintaining the differentiated state of these cells. PKCβ expression is associated with maintaining stem cell proliferation and inducing visceral endoderm formation.

Results

Constitutive Expression of PKCβ and PKCα after Stable Transfection of F9 Cells. F9 cells were cotransfected with a plasmid encoding hygromycin resistance and expression plasmids encoding either PKCβ, antisense PKCβ, PKCα, or antisense PKCα cDNA. Expression in each case was controlled by the β-actin promoter, which is expressed in both differentiated and undifferentiated F9 cells (11). Pools of hygromycin B-resistant colonies were isolated. As shown in Fig. 1 and Table 1, when compared with F9 stem cells expressing only the *hph* gene (H; conferring hygromycin B resistance), expression of PKCβ from the β-actin promoter elevated total PKCβ levels in stem cells by 2.2-fold and allowed continued PKCβ expression following RA + cAMP treatment. Expression of antisense PKCβ RNA decreased steady-state PKCβ levels in F9 stem cells by 50% (Fig. 1, *Lane 3*). Transfection with the PKCα-expressing plasmid resulted in the constitutive expression of PKCα in stem cells. Induction of the endogenous *PKCα* gene by RA + cAMP was unaffected in cells transfected with PKCβ, antisense PKCβ, or PKCα. Constitutive expression of antisense PKCα RNA partially prevented RA + cAMP-induced PKCα expression

Table 1 Relative levels of PKCα and PKCβ expression in transfected F9 cells

Films from Fig. 1 were scanned with a densitometer, and the area of the resulting peaks were integrated. For PKCα, the fold induction was calculated over the area value found in RA + cAMP-treated, hygromycin-resistant F9 cells (*Lane 6*). For PKCβ, the fold induction was calculated over the area value of hygromycin resistant F9 control cells (H; *Lane 1*). Each value is indicated as fold unit.

	H	β	Aβ	α	Aα	RA+ cAMP				
						H	β	Aβ	α	Aα
PKCα	ND[a]	ND	ND	1.4	ND	1.0	1.7	0.9	1.8	0.3
PKCβ	1.0	2.2	0.5	ND	1.8	ND	2.7	ND	ND	0.5

[a] ND, not detected.

(by 70%). Endogenous expression of other PKC isozymes such as PKCγ, δ, ε, and ζ was not altered in any of these cells (data not shown). As we reported previously (11), differentiation-induced changes in PKCβ and PKCα reflect altered mRNA levels (data not shown). Experimental manipulation of PKCβ (either up or down) had little effect on PKCα expression (mRNA or protein). In contrast, expression of PKCα in F9 stem cells decreased PKCβ expression. F9 stem cells transfected with the PKCα plasmid had no detectable PKCβ (Fig. 1, *Lane 4*). The negative effect of PKCα on PKCβ expression was confirmed in cells expressing antisense PKCα RNA. When these cells were induced to differentiate with RA + cAMP, PKCα expression remained low, and the loss of PKCβ expression was incomplete (Fig. 1, *Lane 10*). In contrast, constitutive expression of PKCβ did not prevent PKCα expression in differentiating F9 cells (Fig. 1, *Lane 7*).

PKCβ Enhanced c-fos Expression and PKCα Allowed Phorbol Ester-activated Expression of Collagen IV in F9 Stem Cells. The transition from PKCβ to PKCα in differentiating F9 cells is accompanied by changes in phorbol ester stimulation of target gene expression (11). Specifically, phorbol ester induced c-fos expression in PKCβ-expressing stem cells but not in PKCα-expressing parietal endoderm cells. In contrast, phorbol ester induced type IV collagen, a parietal endoderm marker gene, in F9 stem cells transfected with and expressing PKCα. The causal relationship between PKCβ activity and c-fos expression and between PKCα activity and collagen IV expression was supported by altering expression of PKCβ or PKCα in F9 cells independently of RA + cAMP treatment (Fig. 2 and Table 2). PKCβ expression increased basal expression of both c-fos and c-jun to levels equal to (c-fos) or greater than (c-jun) seen following phorbol ester treatment of F9 stem cells (Table 2; hybridization intensities in each sample were normalized to the L-30 ribosomal protein mRNA control). Decreasing PKCβ by expressing antisense PKCβ RNA had the opposite effect; decreasing both basal and phorbol ester induced expression of c-fos and c-jun. Changes in c-fos/c-jun expression resulted specifically from changes in PKCβ and did not simply reflect increased total PKC activity (11). Expression of comparable levels of PKCα caused little or no alteration of basal c-fos and c-jun expression in F9 stem cells (Fig. 2, *Lane 4*). Although these results support a link between PKCβ activity and c-fos expression in stem cells, constitutive expression of

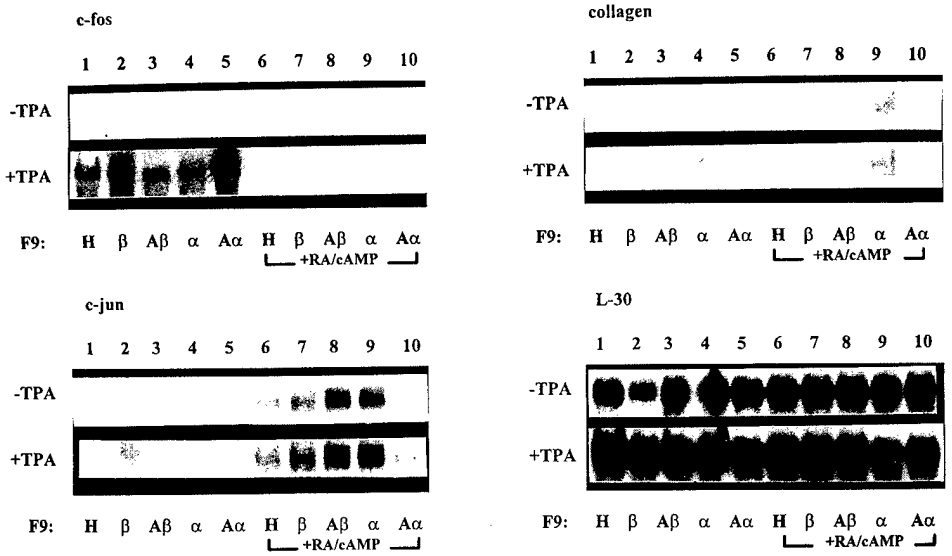

Fig. 2. PKC α/β-expressing F9 cells have altered basal and phorbol ester-induced c-fos, c-jun, and type IV collagen expression. Total RNA (20 μg/lane) from hygromycin-resistant control (*H, Lanes 1* and *6*), PKCβ (*β, Lanes 2* and *7*), antisense PKCβ (*Aβ, Lanes 3* and *8*), PKCα (*α, Lanes 4* and *9*), and antisense PKCα (*Aα, Lanes 5* and *10*) transfected F9 cells was analyzed using Northern blotting for expression of c-fos, type IV collagen, c-jun, and L-30. Cells in *Lanes 6–10* were treated with RA (2×10^{-7} M) and dbcAMP (5×10^{-4} M) for 5 days. One h before harvesting, one-half of the cells were stimulated with 100 ng/ml phorbol ester (+*TPA, lower panel*). The Northern blots were exposed to X-ray film for 3 days (c-fos, c-jun, and collagen) or overnight (L-30) at −80°C. L-30 is mRNA expression control.

Table 2 Relative levels of c-fos, c-jun, and type IV collagen mRNA expression in transfected F9 cells

Films from Fig. 2 were scanned with a densitometer, and the integrated area of the resulting peaks were normalized to the integrated area of the corresponding L-30 peak. For c-fos and c-jun mRNA levels, the fold induction of each treated cells was calculated over the area value found in hygromycin-resistant F9 control cells (H; *Lane 1*). For type IV collagen mRNA levels, the fold induction was calculated over the area value of RA + cAMP-treated, hygromycin-resistant F9 control cells (*Lane 6*).

	H	β	Aβ	α	Aα	RA + cAMP H	RA + cAMP β	RA + cAMP Aβ	RA + cAMP α	RA + cAMP Aα
A. c-fos										
c-fos										
−TPA	1.0	4.2	ND[a]	ND	ND	ND	ND	ND	ND	ND
+TPA	4.3	8.0	2.8	2.3	7.4	ND	ND	ND	ND	ND
B. c-jun										
c-jun										
−TPA	1.0	6.7	0.2	1.0	0.4	4.8	13.8	25.8	20.7	1.9
+TPA	1.7	8.2	0.6	2.7	2.6	5.7	17.0	27.6	34.3	4.8
C. collagen										
Type IV collagen										
−TPA	ND	ND	ND	ND	ND	1.0	0.3	2.4	3.4	0.2
+TPA	ND	ND	ND	1.1	ND	0.4	0.2	0.5	2.9	0.2

[a] ND, not detected.

PKCβ in RA + cAMP-treated F9 cells did not restore c-fos expression in response to phorbol ester(Fig. 2, *Lane 7*).

Steady-state c-jun mRNA levels were ~5-fold higher in RA + cAMP-treated, hygromycin-resistant cells than in stem cells (Fig. 2, *Lane 1 versus Lane 6*; Table 2). Although phorbol ester treatment had only a modest effect on c-jun expression, increasing PKCβ expression resulted in elevated c-jun mRNA, both in stem cells and in differentiated cells (Fig. 2, *Lanes 2* and *7*; Table 2, 8.2- and 17-fold). PKCα expression in stem cells had no effect on c-jun expression but was associated with substantially increased c-jun expression in RA + cAMP-treated cells (Table 2, 20.7-fold), an effect reversed by antisense PKCα RNA (Table 2, 1.9-fold). Paradoxically, whereas antisense PKCβ RNA expression lowered c-jun levels in stem cells, it actually increased expression in differentiating cells (Table 2, 25.8-fold; an additional 5-fold over the effect of RA + cAMP).

The most plausible explanation for this result is that decreasing PKCβ (and increasing PKCα) enhances the differentiation of F9 cells in response to RA + cAMP. This conclusion was supported by measuring type IV collagen mRNA as a marker of parietal endoderm differentiation. Previously, we demonstrated that expressing PKCα in F9 stem cells resulted in phorbol ester-induced collagen IV expression in the absence of RA-induced differentiation (11). A similar effect of PKCα expression was seen in these experiments (Fig. 2, *Lane 4*; Table 2, 1.1-fold over basal expression of RA + cAMP-treated F9 cells), and antisense PKCα expression prevented RA + cAMP induction of collagen IV expression (Fig. 2, *Lane 10*). Further treatment of phorbol ester in differentiated cells with RA + cAMP down-regulated collagen expression in general. This experiment also established a striking antagonistic effect of PKCβ on collagen IV expression. Collagen IV mRNA was induced only slightly by RA + cAMP in cells constitutively expressing PKCβ (Table 2, 0.3-fold), whereas antisense PKCβ enhanced (Table 2, 2.4-fold) the response to RA + cAMP. In this respect, decreasing PKCβ levels (Table 2, 2.4-fold) or increasing PKCα (Table 2, 3.4-fold) had the similar effects on collagen expression.

PKCα Enhanced RA and cAMP-induced Differentiation of F9 Cells into Parietal Endoderm. To determine whether the antagonistic interaction between PKCβ and PKCα was limited to the collagen IV promoter or was an important component of parietal endoderm differentiation, we measured the expression of other differentiation markers in these cell lines. We used metabolic labeling and SDS-PAGE to examine the expression of basement membrane proteins, laminin A, B1/B2, and type IV collagen (Fig. 3) in the cell lines expressing various levels of PKCα and PKCβ. In addition, we measured the induction of the visceral endoderm marker, TTR (20), by RNase protection (Fig. 4).

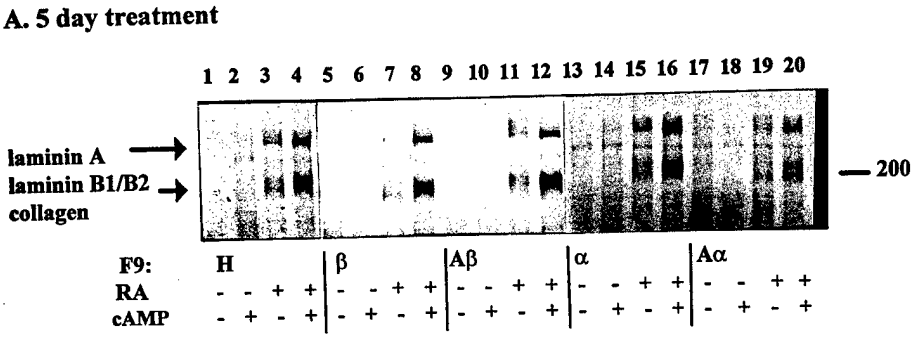

Fig. 3. PKCα or antisense PKCβ expression enhances extracellular basement membrane protein synthesis in differentiated F9 cells. Basement membrane synthesis in hygromycin-resistant control (*H*), PKCβ (*β*), antisense PKCβ (*Aβ*), PKCα (*α*), and antisense PKCα (*Aα*) transfected F9 cells was analyzed by resolving conditioned media (2.2×10^6 trichloroacetic acid insoluble cpm/lane) from radiolabeled cells on SDS-PAGE (5%). F9 cells were treated with either ethanol solvent (*−RA −cAMP*), 5×10^{-4} M dbcAMP (*+cAMP*), 2×10^{-7} M all-*trans* RA (*+RA*), or both (*+RA +cAMP*) for 5 days (*A*) or 3 days (*B*). Cells were labeled with [^{35}S]methionine (50 μCi/ml) for the final 16–20 h. *Right,* relative migration of molecular weight standards (in thousands). Parietal endoderm differentiation is characterized by the expression of laminin A (M_r ~400,000), laminins B1 and B2, and type IV collagen (which all run at about M_r 200,000).

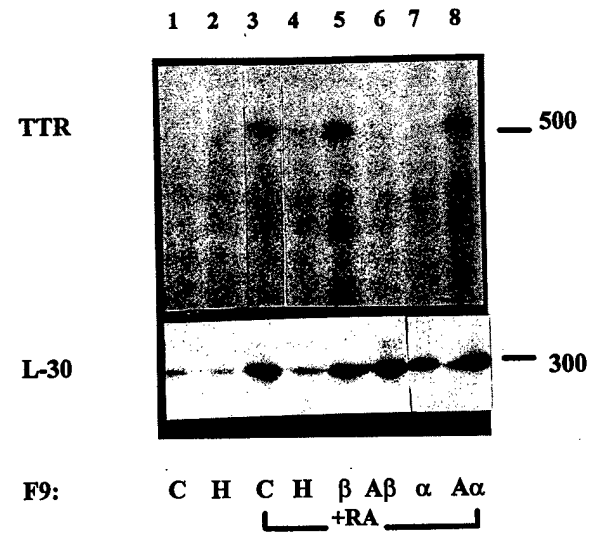

Fig. 4. PKCα and antisense PKCβ expression inhibit expression of TTR, a visceral endoderm marker. F9 cells were grown as aggregates in suspension for 8 days. Total RNA was isolated, and the expression of the visceral endoderm markers TTR, retinol binding protein (data not shown; results same as TTR), and L-30 (as internal control) was measured in RNase protection assays. *Lane 1,* parental F9 control cells (*C*); *Lane 2,* hygromycin-resistant control cells (*H*); *Lanes 3–8,* parental F9 control (*C*), hygromycin-resistant control (*H*), PKCβ (*β*), antisense PKCβ (*Aβ*), PKCα (*α*), and antisense PKCα (*Aα*) transfected cells treated with 2×10^{-8} M all-*trans* RA (*+RA*). *Right,* relative migration of molecular weight standards (nucleotides).

The overall pattern of basement membrane protein synthesis (Fig. 3) paralleled that reported above for the type IV collagen mRNA (Fig. 2). After 5 days of treatment with either RA or RA and dbcAMP, in cells constitutively expressing PKCα, laminin and collagen expression were elevated relative to control cells (Fig. 3*A, Lanes 3* and *4* compared with *Lanes 15* and *16*). This was more apparent at 3 days (Fig. 3*B,* compare *Lanes 2* and *6*). Similar results were seen in cells in which PKCβ expression was reduced by antisense RNA expression (Fig. 3*A, Lanes 11* and *12*; Fig. 3*B, Lanes 3* and *4*) but not as great as increasing PKCα expression. Although overexpression of PKCβ or reduced PKCα did not prevent basement membrane protein synthesis, in both cell lines the response to RA and cAMP was reduced relative to the control cells. Therefore, PKCα expression is an integral component for parietal endoderm differentiation in general and not only for the expression of type IV collagen.

F9 cells are bipotential. When aggregated and grown in the presence of RA, F9 cells acquire a visceral endoderm phenotype. The change in PKC expression seen during parietal endoderm formation does not occur during visceral endoderm formation (3, 11). By measuring TTR mRNA levels, we determined the ability of RA-treated aggregates of F9 cells transfected with PKCβ, antisense PKCβ, PKCα, or antisense PKCα to acquire a visceral endoderm phenotype (20). Increasing PKCα or decreasing PKCβ levels inhibited the RA-induced synthesis of TTR (Fig. 4, *Lanes 6* and *7*). In contrast, increased expression of PKCβ enhanced the expression of TTR in RA-treated F9 aggregates (Fig. 4, *Lane 5*).

PKCβ Induced the Gradual Retrodifferentiation of the Parietal Endoderm Cell Line F9RA5. The above results strongly support a role of PKCα signaling in F9 parietal endoderm differentiation that is distinct from, and most likely antagonistic to, the role of PKCβ signaling. To provide more direct evidence for a negative role of PKCβ in parietal

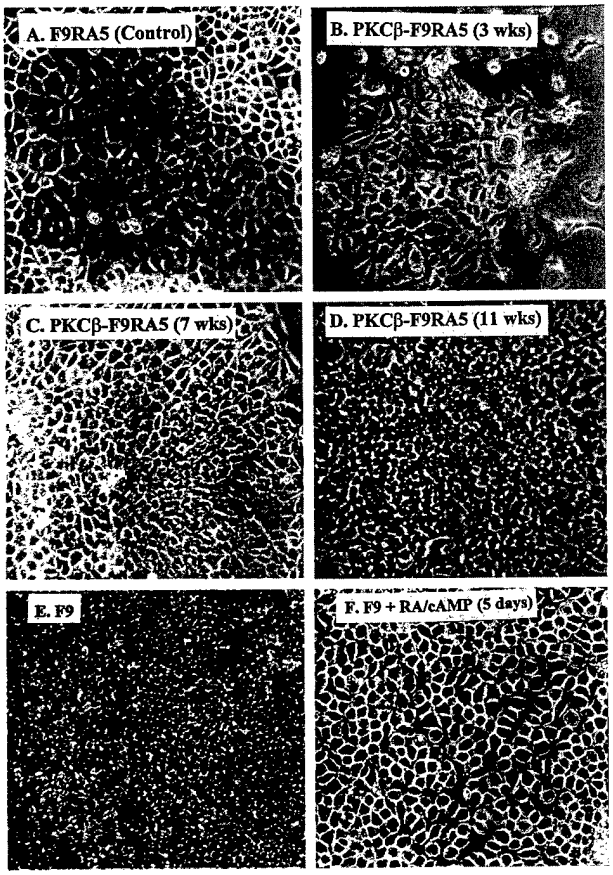

Fig. 5. PKCβ induces parietal endoderm cells to retrodifferentiate. Exponentially growing F9RA5 control (*A*), PKCβ-transfected F9RA5 in 3rd passage (*B*), 7th passage (*C*), and 11th passage (*D*), F9 control (*E*), and F9 cells treated with RA + cAMP for 5 days (*F*) were photographed using an inverted microscope (×40).

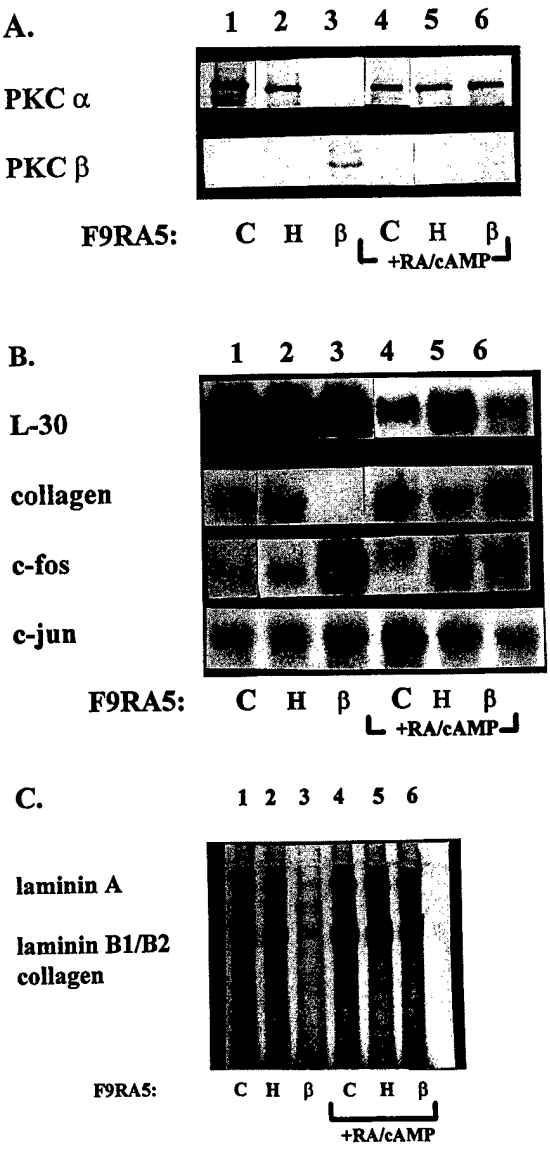

Fig. 6. Parietal endoderm marker gene expression is lost in PKCβ-expressing F9RA5 cells. *A*, PKCα and PKCβ expression in PKCβ-transfected F9RA5 cells. Whole-cell protein extract (30 μg/lane) from control (*C*), hygromycin-resistant control (*H*), and PKCβ-transfected F9RA5 cells (*β*; in 11th passage) was analyzed for PKCα and PKCβ expression using immunoblotting as described in "Materials and Methods." In *Lanes 4–6*, cells were treated with 2×10^{-7} M all-*trans* RA and 5×10^{-4} M dbcAMP (*+RA/cAMP*) for 7 days. *B*, total RNA (20 μg/lane) from control (*C*), hygromycin-resistant control (*H*), and PKCβ-transfected F9RA5 cells (*β*; in 11th passage) was analyzed for L-30, collagen IV, c-fos, and c-jun expression using Northern blotting. In *Lanes 4–6*, cells were treated with 2×10^{-7} M all-*trans* RA and 5×10^{-4} M dbcAMP (*+RA/cAMP*) for 7 days. L-30 is mRNA expression control. *C*, basement membrane synthesis in control (*C*), hygromycin-resistant control (*H*), and PKCβ-transfected F9RA5 cells (*β*; in 11th passage) was analyzed for protein expression of laminin A, B1, B2, and type IV collagen as described in "Materials and Methods." In *Lanes 4–6*, cells were treated with 2×10^{-7} M all-*trans* RA and 5×10^{-4} M dbcAMP (*+RA/cAMP*) for 7 days.

endoderm differentiation, we transfected the parietal endoderm cell line, F9RA5, with the PKCβ expression plasmid. With the exception of their ability to proliferate indefinitely, the F9RA5 cells retain all morphological and biochemical characteristics of RA + cAMP-treated F9 cells, including the appropriate PKC expression pattern (Ref. 11; morphological similarity in Fig. 5, *A* and *F*). F9RA5 cells were co-transfected with plasmids encoding hygromycin (hph) resistance and either expression plasmids encoding PKCβ and PKCα. Pools of hygromycin B-resistant colonies were expanded and frozen. After thawing, transfected F9RA5 cells were grown in media containing 50 μg/ml hygromycin. F9RA5 cells expressing only the *hph* gene or *hph* gene + PKCα together retained the morphology of parental F9RA5 cells (data not shown) at all passages. However, F9RA5 cells expressing PKCβ underwent a morphological transition. These cells maintained their parietal endoderm phenotype for passages 1–4 (Fig. 5*B*). Between passages 5 and 10, these cells underwent a morphological transition, such that around the 11th passage, the entire culture had reacquired a stem cell phenotype (Fig. 5, *C* and *D*; morphological similarity in Fig. 5, *D* and *E*). By all measurable criteria, the transition was complete. After the 11th passage, PKCβ-expressing F9RA5 cells no longer expressed PKCα (Fig. 6*A*, *Lane 3*), type IV collagen (Fig. 6*B*, *Lane 3*), or laminins A/B1/B2 (Fig. 6*C*, *Lane 3*). In contrast, PKCβ and c-fos were highly expressed. When the phenotypically reverted cells (PKCβ ex-

pressing F9RA5 after 11th passage) were treated with RA and cAMP, PKCβ expression was suppressed, and the expression of PKCα, type IV collagen, and the parietal endoderm phenotype was regained (Fig. 6, *Lanes* 6). Although PKCα expression was lost in PKCβ-expressing F9RA5 cells, PKCα expression was promptly induced by RA and cAMP treatment (Fig. 6*A*, *Lane 6*). c-jun was constantly expressed in F9RA5 cells and was slightly elevated after RA + cAMP treatment, suggesting that PKCβ expression in F9RA5 cells does not affect c-jun expression (Fig. 6*B*).

Discussion

In this study, we demonstrated that PKCα expression enhanced RA- and cAMP-induced differentiation of F9 mouse embryonal carcinoma cells into parietal endoderm. F9 cells that constitutively express PKCα showed an accelerated response to RA and cAMP in terms of synthesis and secretion of the basement membrane components, laminin A, B1/B2, and type IV collagen. These cells also failed to express TTR after aggregation in the presence of RA. Blocking the RA-induced expression of PKCα with antisense PKCα RNA substantially reduced induction of the parietal endoderm but not the visceral endoderm phenotype. Therefore, PKCα is critical for F9 cell differentiation and for determining which developmental pathway is used by these cells.

PKC represents a family of 11 distinct genes that show tissue and developmental stage-specific expression (16). The complexity of the PKC gene family and the specific expression patterns of members of this family led to the prediction that different PKC isozymes serve distinct, nonredundant functions. In general, PKC regulates growth and differentiation of a diverse group of cell types. In a number of these cases, induction of differentiation correlates with the differential activation of a specific PKC isozyme. Induction of PKCα expression by RA is not unique to F9 cells but also has been reported in vascular smooth muscle cells (21), B16 melanoma cells (22), NT-2 human teratocarcinoma cells (23), and human breast cancer cells (24). PKCα induction by RA (11, 21–24) appears to be a central component of differentiation in multiple cell types.

The mechanism(s) by which PKCα participates in F9 cell differentiation remains to be determined. The increase in steady-state PKCα should increase the basal level of kinase activity leading to increased phosphorylation of cellular substrates that induce the expression of parietal endoderm differentiation markers. Maciaszek *et al.* (25) and Tahayato *et al.* (26) demonstrated that phorbol esters and retinoids synergistically activate transcription of multiple promoters, possibly as a result of RA receptor phosphorylation by PKC. Phosphorylation of RA receptors increases their DNA binding activity and transcriptional activation (27, 28). The alteration of the equilibrium between phosphorylation and dephosphorylation of steroid/thyroid hormone receptor family members is proposed to be a major regulator of the balance between cell growth and differentiation (27, 29).

An alternative mechanism for the PKCα induction of F9 cell differentiation involves transcriptional regulation of parietal endoderm marker genes through phorbol ester response elements. Phorbol ester, by activating either conventional or novel PKCs, increases the expression of genes containing TPA-responsive DNA elements in their promoter region (30). TPA-responsive DNA elements are bound by AP-1 transcription factors consisting of either homodimers of Jun family members or heterodimers of Jun and Fos family members (30). The RA- and cAMP-induced differentiation of F9 cells is accompanied by a large increase in AP-1 activity (9) and an accumulation of c-jun mRNA (Fig. 2; Ref. 11). Phorbol esters and AP-1 activity are implicated in the activation of c-jun and *laminin B2* gene transcription (9, 31). The transcriptional activation of parietal endoderm marker genes such as the laminins and type IV collagen by RA is indirect, requiring prior new protein synthesis (1, 2, 4, 32). The identity of the new RA-induced gene products that subsequently mediate parietal endoderm gene expression remains unclear. Based on the data presented here, PKCα is a strong candidate for one of these RA target genes.

The second major observation in this study is that PKCβ expression is incompatible with maintenance of the parietal endoderm state. This is supported by three results: (*a*) PKCβ expression is specifically repressed during parietal endoderm but not during visceral endoderm differentiation (11); (*b*) reduction of PKCβ with antisense RNA accelerates the response of F9 cells to RA and cAMP (Figs. 2 and 3); and (*c*) reexpression of PKCβ in the parietal endoderm cell line F9RA5 results in these cells reacquiring a stem cell phenotype (Figs. 5 and 6).

How PKCβ accomplishes this is not clear. Several studies have linked PKCβ positively with cell proliferation (19, 33). PKCβ is expressed in both F9 stem cells and visceral endoderm cells that continue to proliferate but not in parietal endoderm cells that exit the cell cycle. F9RA5 cells, although they morphologically and biochemically resemble parietal endoderm cells, have retained the ability to proliferate. F9RA5 cells do not show the same degree of uncontrolled proliferation as the F9 stem cell, and appear to have distinctly different regulation of cell cycle progression (including altered cyclin D expression and Rb phosphorylation).[4] Overexpression of PKCβ in rat fibroblasts decreases their sensitivity to growth-inhibitory signals and increases their sensitivity to oncogenes (19). We have shown that PKCβ is required for phorbol ester induction of c-fos in F9 cells, and that increasing PKCβ levels increases c-fos expression, even in the absence of phorbol ester induction. This was especially apparent in the retrodifferentiated F9RA5 cells expressing PKCβ. These data support the hypothesis that PKCβ, possibly by deregulating c-fos expression, is critical in maintaining the stem cell phenotype including the characteristic uncontrolled proliferation.

Materials and Methods

Plasmid. The antisense PKCβ plasmid (pβ-actin SP72-As-PKCβ) was constructed by ligating the 2.3-kb *Eco*RI fragment of PKCβ cDNA from pJ6-PKCβ (19) into the unique *Eco*RI sites of the pβ-actin SP72 vector. The antisense orientation was confirmed by *Sma*I restriction digestion. The

[4] Y. Cho *et al.*, unpublished observations.

sense and antisense PKCα plasmids (pβ-actin SP72-PKCα and pβ-actin SP72-As-PKCα) were constructed by ligating the 2.4-kb *Eco*RI fragment of PKCα cDNA from pMV7-PKCα into the unique *Eco*RI site of the pβ-actin SP72 vector. The orientation was confirmed by *Bgl*II restriction digestion.

Cell Culture. The F9 embryonal carcinoma cells (ATCC CRL 1720) were grown on gelatin-coated tissue culture dishes in DMEM supplemented with 10% fetal bovine serum (Upstate Biotechnology, Inc., Saranac Lake, NY). Differentiation was induced by treating monolayers with 2×10^{-7} M RA and 5×10^{-4} M dbcAMP (to induce parietal endoderm) or treating aggregates plated on bacteriological Petri dishes with 2×10^{-8} M RA (to induce visceral endoderm formation). The cell line displaying stable parietal endoderm morphology and biochemical properties, F9RA5, was isolated as described previously (11).

Stable Transfection. F9 embryonal carcinoma cells were grown in DMEM containing 10% fetal bovine serum, and subconfluent cultures were cotransfected with a hygromycin resistance plasmid (pβhygro having hph cDNA) and expression plasmids encoding either PKCβ (19), antisense PKCβ (As PKCβ), PKCα, or antisense PKCα (As PKCα) by DNA-calcium phosphate coprecipitation (34). Following transfection, cells were subjected to hygromycin B selection (150 μg/ml) over 4 weeks. Resistant cells were maintained in DMEM, 10% fetal bovine serum, and 50 μg/ml hygromycin B.

Western Blot Analysis for PKC Isozyme Expression. Whole-cell protein extracts were prepared with lysis buffer [20 mM Tris (pH 8), 150 mM NaCl, 10 mM sodium phosphate, 100 mM sodium vanadate, 100 mM ammonium molybdate, 10% glycerol, 1% NP40, and 0.1% SDS] as described previously (24). The protein extracts (30 μg/lane) were separated by SDS-PAGE (10%). Following electrophoretic transfer to Hybond-C extra nitrocellulose, membranes were blocked with 5% nonfat dry milk in PBS. PKC was detected by incubating the membrane with anti-PKC isozyme antibodies (0.5 μg/ml; affinity-purified polyclonal PKCα and PKCβ antibodies; Life Technologies, Inc.), followed by extensive washing with PBST (0.05% Tween 20 in PBS) and subsequent incubation with peroxidase-conjugated secondary antibody (1:7500 dilution). PKC bands were visualized by enhanced chemiluminescence (Amersham Corp.).

RNA Isolation and Northern Blotting. The levels of specific RNAs were determined using Northern blots of total RNA isolated as described previously (24). In every case, filters were hybridized to ^{32}P-labeled RNA probes. Probes for L-30, c-fos, c-jun, collagen, and TTR have been described (11, 35). PKCα and PKCβ RNAs were detected with riboprobes generated from human cDNA cloned into pSP72 vector. After washing in 2× SSC/1% SDS and 0.2× SSC/1% SDS, the Northern blots were exposed to X-ray film at −80°C with intensifying screens, and the band intensity was quantified by densitometry.

Analysis of Basement Membrane Protein Synthesis. Cells were labeled overnight with [^{35}S]methionine (50 μCi/ml; DuPont NEN). Synthesis of type IV collagen and laminin A, B1, and B2 were determined by separating ^{35}S-labeled extracellular proteins on 5% acrylamide gels (SDS-PAGE). Gels were fixed, dried, and exposed to X-ray film at room temperature for 1 day.

RNase Protection Assay. TTR RNA levels were determined using an RNase protection assay (36). A ^{32}P-labeled TTR RNA probe was synthesized from TTR cDNA clone. Accurately measured amounts (10 μg/lane) of total RNA were hybridized to the cRNA probe for 16 h at 45°C. Nonhybridized RNA and probe were removed by digestion with RNases A and T1. The hybridized RNA was subjected to separation on a denaturing 10% acrylamide gel. After fixing and drying, the gel was exposed to X-ray film with intensifying screen at −80°C.

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