

Protein Kinase C δ Enhances Proliferation and Survival of Murine Mammary Cells

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Protein kinase C (PKC) δ , a member of the novel family of PKC serine-threonine kinases, has been implicated in negative regulation of proliferation and apoptosis in a large number of cell types, including breast cancer cell lines, and postulated as a tumor suppressor gene. In this study we show that in murine NMuMG mammary cells PKC δ promotes a mitogenic response. Overexpression of PKC δ in NMuMG cells leads to a significant increase in [³H]-thymidine incorporation and cell proliferation, as well as enhanced extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) activation. Activation of PKC δ with a phorbol ester leads to elevated cyclin D1 expression and an hyperphosphorylated Rb state. Surprisingly, ectopic expression of PKC δ conferred anchorage-independent growth capacity to NMuMG cells. PKC δ overexpressors showed enhanced resistance to apoptotic stimuli, such as serum deprivation or doxorubicin treatment, an effect that correlates with hyperactivation of the Akt survival pathway. Our results provide evidence for a role of PKC δ as a positive modulator of proliferative and survival signals in immortalized mammary cells. The fact that PKC δ exerts differential responses depending on the cell context not only highlights the necessity to carefully understand the signaling events controlled by this PKC in each cell type but also suggests that we should be cautious in considering this kinase a target for cancer therapy. © 2007 Wiley-Liss, Inc.

Key words: PKC δ ; ERK1/2; Akt; proliferation; survival

INTRODUCTION

Carcinogenesis is a multistep process that involves mutations (initiation) and the selected amplification of mutated cells (promotion). It is widely accepted that deregulation of growth-promoting signals contributes to abnormal cell proliferation. Protein kinase C (PKC) isozymes represent key components of signal transduction pathways that control proliferation, apoptosis, and malignant transformation [1–3]. On the basis of their structural similarities and biochemical properties PKC isoforms have been grouped into three families [4,5]: *classical* (α , β I, β II and γ), which are activated by calcium and diacylglycerol (DAG); *novel* (δ , ϵ , η , and θ), which require DAG but are calcium-insensitive; and *atypical* (ζ and λ /i), which are not responsive to either DAG or calcium [1,3,6]. The classical and novel PKCs are targets for the phorbol esters, widely studied tumor promoters that mimic the actions of DAG [7]. Changes in the expression of PKC isozymes have been reported in numerous human cancers including lung, colon, and breast [2,8–11]. In some instances a correlation between elevated PKC protein levels and more aggressive cancers has been reported [12–14]. Based on the current knowledge it is clear that PKC isozymes have distinct roles in different cell types. Intriguingly, PKC isozymes that mediate proliferative responses in some cell lines could behave as growth inhibitory in others, as it was

described for the classical PKC β in lung and colon cancer cell lines, respectively. It is believed that the differential effects of PKC isozymes in each cell type relate to their unique localization and/or access to substrates upon activation [15].

PKC δ is a widely expressed member of the novel PKC subfamily [16]. This isoform has been described as growth inhibitory in a large number of cell types. For example, NIH3T3 cells overexpressing PKC δ show an important reduction in their proliferation rate [17,18]. This effect has also been reported in other cell types, including glial [19–21], vascular smooth muscle [22], and endothelial cells [23]. Moreover, PKC δ impinges negatively on both G₁/S and G₂/M cell-cycle transitions. Early studies determined that PKC δ overexpression in CHO cells results

Abbreviations: PKC, protein kinase C; MEM, minimum essential medium; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; FCS, fetal calf serum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; pRb, phosphorylated retinoblastoma protein; MEK, mitogen-activated protein kinase/ERK kinase.

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in cell-cycle arrest in G₂/M [24]. In other cell types, such as lung adenocarcinoma cells, activation of PKC δ leads to G₁ arrest, an effect that involves a PKC δ -specific upregulation of the cell-cycle inhibitor p21 [15]. Several reports also showed that activation of PKC δ promotes apoptosis of colon [25] and prostate [26] tumor-derived cell lines. Moreover, activation or overexpression of PKC δ enhances cisplatin-induced apoptosis in gastric [27] and lung [28] cancer cell lines. Several studies have postulated a tumor suppressor role for this PKC. Indeed, overexpression of PKC δ in mice skin inhibits the tumor promoting activity of phorbol 12-myristate 13-acetate (PMA) [29]. Moreover, inhibition of PKC δ function confers a malignant phenotype to cells with c-Src amplification by providing a PI3K/Akt-independent survival capacity [30].

There has been considerable interest in understanding the role of PKC δ in breast cancer cells, as studies have shown that this PKC mediates anti-proliferative responses in these cells. For example, the anti-mitogenic effect of inositol hexaphosphate in MCF-7 human breast cancer cells, which involves inhibition of extracellular signal-regulated kinase (ERK) and Akt as well as phosphorylated retinoblastoma protein (pRb) hypophosphorylation, is mediated by PKC δ [31]. A small molecule PKC δ inhibitor or a dominant-negative PKC δ mutant impaired phorbol ester-induced arrest in G₁ in SKRB-3 breast cancer cells [32]. Conversely, a few studies suggested that PKC δ could act as a positive regulator of growth in tumor-derived mammary cells [2,33], probably through the activation of the mitogenic Ras/ERK1/2 pathway [2,34]. A pro-survival role for PKC δ in breast cancer cells has also been reported [9,35]. Despite such functional heterogeneity in PKC δ responses in breast tumor cells, a question that still remains unanswered is whether PKC δ modulates proliferation or survival in normal mammary cells. To-date there are no studies assessing the impact of this novel PKC on signaling cascades that modulate proliferation and survival in mammary cells, such as the MEK/ERK or the PI3K/Akt pathways.

In this study we focused on how PKC δ regulates cell-cycle progression and signaling events in mammary cells. As an experimental approach we overexpressed PKC δ in NMuMG cells, an established model of immortalized mouse mammary cells that has been widely used in mammary carcinogenesis studies [36–39]. We found that PKC δ overexpression promotes NMuMG cell proliferation through the activation of the MEK/ERK pathway and the elevation of cyclin D1 levels. In addition, PKC δ activation causes a significant change in Rb phosphorylation status in NMuMG-PKC δ cells. Strikingly, PKC δ overexpression in NMuMG cells confers anchorage-independent growth and enhances survival via the activation of the Akt signaling pathway.

MATERIALS AND METHODS

Reagents and Antibodies

Medium for cell culture, agarose, Geneticin (G418), Lipofectamine Plus, and monoclonal anti-PKC ϵ antibody were obtained from Life Technologies, Inc. (Rockville, MD). Fetal calf serum (FCS) was from GEN (Buenos Aires, Argentina). Acrylamide, PD98059 and LY294002 were from Sigma (St. Louis, MO). All other reagents for polyacrylamide gel electrophoresis (PAGE) were obtained from Bio-Rad (Richmond, CA). Insulin was from Beta, Buenos Aires (Argentina). Monoclonal anti-PKC α , β , δ and ζ , and anti-pRb antibodies were purchased from BD Biosciences (San Diego, CA). Monoclonal antibodies for ERK and phospho-ERK (pERK), and polyclonal antibodies for actin and cyclin D1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies for Akt and phospho-Akt (pAkt, Ser 473) were purchased from Cell Signaling Technology (Beverly, MA). RNAi duplexes were obtained from Dharmacon (Dallas, TX). Horseradish peroxidase conjugated anti-rabbit or anti-mouse antibodies and PMA were obtained from Sigma. Hybond-P membranes for blotting and chemiluminescence reagents (ECL) were from Amersham (Aylesbury, UK).

Cell Line

Normal murine mammary gland (NMuMG) cells, an immortalized mammary cell line derived from NAMRU mice [40], were cultured at 37°C in minimum essential medium (MEM) supplemented with FCS and 80 μ g/mL gentamicin in a humidified air atmosphere with 5% CO₂.

Expression Vectors, Transfection, and Selection

NMuMG cells were stably transfected with 5 μ g of pMTH-PKC δ , a mammalian expression vector encoding for murine PKC δ , with Lipofectamine Plus. This plasmid has an ϵ -tag to facilitate detection with a commercial anti- ϵ -tag antibody [41]. NMuMG cells transfected with the empty vector (pMTH) were used as control. Forty-eight hours after transfection, cells were selected with 500 μ g/mL of G418. After selection, approximately 40 resistant clones were pooled to avoid clonal variations.

Knock-Down of Endogenous PKC δ by RNAi

The following target sequence was used to knock-down PKC δ : AACCACGAGTTTATCGCCACC. Scramble RNAi duplex that was not homologous to any mammalian gene was utilized as control. Both RNAi duplexes (50 nM) were transfected into NMuMG-vector cells with Oligofectamine, and cell extracts were prepared 48 h later.

Western Blot

Semiconfluent monolayers were washed twice with ice-cold phosphate-buffered saline (PBS) and

then lysed with 1% Triton X-100 in PBS by scrapping with a teflon scrapper. Samples were denatured by boiling in sample buffer with 5% β -mercaptoethanol and run in 10% sodium dodecyl sulfate (SDS)-PAGE (or 6% for pRb detection). Fifty μ g of protein were loaded in each lane. Gels were blotted to Hybond-P membranes. After incubation for 1 h in PBS containing 5% skim milk with 0.1% Tween-20, membranes were incubated with the first antibody overnight at 4°C, and then for 1 h with a secondary antibody coupled to horseradish peroxidase. Detection was performed by chemiluminescence. The intensity of the bands was quantified with a digital GS-700 densitometer and the Molecular Analyst software (Bio-Rad).

Subcellular Fractionation

Semiconfluent monolayers of NMuMG cells were washed twice with ice-cold PBS, and lysed with 1% Triton X-100 in PBS. The cytosolic (soluble) fraction was obtained by collecting the supernatant after centrifugation of the cell lysate (1 h at 100 000g). The remaining pellet represents the particulate fraction. Protein concentration of the total lysate and fractions was determined and equal amounts of protein for each fraction (40 μ g) were subjected to SDS-PAGE and transferred to Hybond-P membranes. Membranes were blocked with PBS containing 5% skim milk and 0.1% Tween-20 and subsequently immunostained with anti-PKC δ antibodies, as described above.

Cell Proliferation

Population-doubling time was determined by assessing cell number during the exponential growth phase of unsynchronized monolayer cultures. Briefly, 1×10^5 cells were seeded onto 35-mm multiplates in MEM supplemented with 10% FCS, 80 μ g/mL gentamicin, and 250 μ g/mL G418. At different times after seeding, cells from triplicate wells were washed twice with PBS, trypsinized, and counted with a hemocytometer and trypan blue exclusion. To assess the effect of PMA on proliferation, 2×10^4 cells were seeded onto 24-well plates. After 24 h cells were treated with 50 nM PMA for 1 h. Twenty-four hours later cells from triplicate wells were washed with PBS, trypsinized, and counted with a hemocytometer and trypan blue exclusion. Alternatively, [3 H]-thymidine incorporation was determined. Briefly, 1×10^4 cells were seeded in 96-well multiplates and 36 h later PMA (50 nM, 1 h) was added. Each well was then pulsed with 1 μ Ci [3 H]-thymidine (specific activity 20 Ci/mmol) for 4 h. Six hours later, cells were washed twice with ice-cold PBS, treated with 5% trichloroacetic acid for 30 min at 4°C and solubilized with 0.5 N NaOH. [3 H]-Thymidine incorporation was determined on a Triathler liquid scintillation counter (Hidex, Turku, Finland). Data points for all

assays were obtained in triplicate, and expressed as $\text{cpm} \times 10^3/\mu\text{g}$ protein. Background radioactivity from cell-free wells was determined and subtracted from all data points.

Analysis of Cell-Cycle Distribution by Flow Cytometry

Cells were serum starved overnight and then treated with PMA (50 nM) in the presence of 2% FCS up to 16 h. After washing with PBS, cells were detached, fixed with 75% ice-cold ethanol and stained with 50 μ g/mL propidium iodide. DNA content was analyzed by flow cytometry with an Epics Elite ESP coulter cytometer (Beckman coulter, Fullerton, CA).

Anchorage-Independent Growth

For soft agar assays, 60-mm Petri dishes were prepared with 2 mL base feeder layer of 0.6% agar in complete medium and a semisolid top layer (0.3% agar in complete medium) containing 10^5 mono-dispersed cells detached from log phase monolayers. Fifteen days after seeding, cultures were fixed with 10% formaldehyde in PBS, and the number of colonies with more than 20 cells was determined with an inverted microscope. To test the effect of PKC δ activation on anchorage-independent growth, 1 mL of complete medium containing 50 nM PMA was added to each Petri dish. After 1 h, PMA was removed and agar layers were extensively washed with PBS. PMA treatment was repeated every 48 h.

Effect of Serum Starvation and Treatment With Insulin and Cytotoxic Drugs

Subconfluent monolayers growing in 96-well plates were extensively washed with PBS and subject to serum starvation (48 h in serum-free MEM) in the presence or absence of insulin (5 μ g/mL), or treated with 0.5-2 μ M doxorubicin (Dox) for 2 h, washed twice with PBS, and subsequently incubated in MEM-10% FCS for 48 h. To analyze the involvement of different signaling pathways, cells were treated with or without Dox (1 μ M) in the presence of the MEK1 inhibitor PD98059 (50 μ M) or the PI3K inhibitor LY294002 (30 μ M) for 2 h. After Dox was washed out, cells were incubated for 48 h in complete medium supplemented with the corresponding inhibitor. Cell viability was evaluated with the MTS assay (Celltiter 96TM Non-Radioactive Proliferation Assay, Promega, Madison, WI), as described by the manufacturer.

RESULTS

Generation of a PKC δ -Overexpressing Cell Line (NMuMG-PKC δ)

In order to study the role of the novel PKC δ isozyme in the modulation of proliferation and survival of murine mammary cells, we used a stable

transfection approach. Stable overexpression of PKC δ was achieved by transfecting NMuMG cells with pMTH-PKC δ followed by G418 selection. To avoid clonal variations, G418-resistant clones were pooled 2 wk after transfection. Western blot analysis with the anti- ϵ -tag antibody revealed a 78 kDa band corresponding to PKC δ that was present in NMuMG-PKC δ cells but not in cells transfected with the vector alone (pMTH) (Figure 1A). The level of the overexpression of PKC δ in the transfected cells was \sim 8-fold. Similar expression levels of PKC δ were obtained in two separate transfections. PKC δ overexpression did not alter the expression of other PKC isoforms present in NMuMG cells, namely PKC α , β and ζ . As shown in Figure 1B, PMA induced the translocation of PKC δ from the cytosolic (soluble) to the particulate fraction. From these data, we conclude that the transfection of PKC δ into NMuMG cells results in an efficient expression of a phorbol ester-responsive PKC δ .

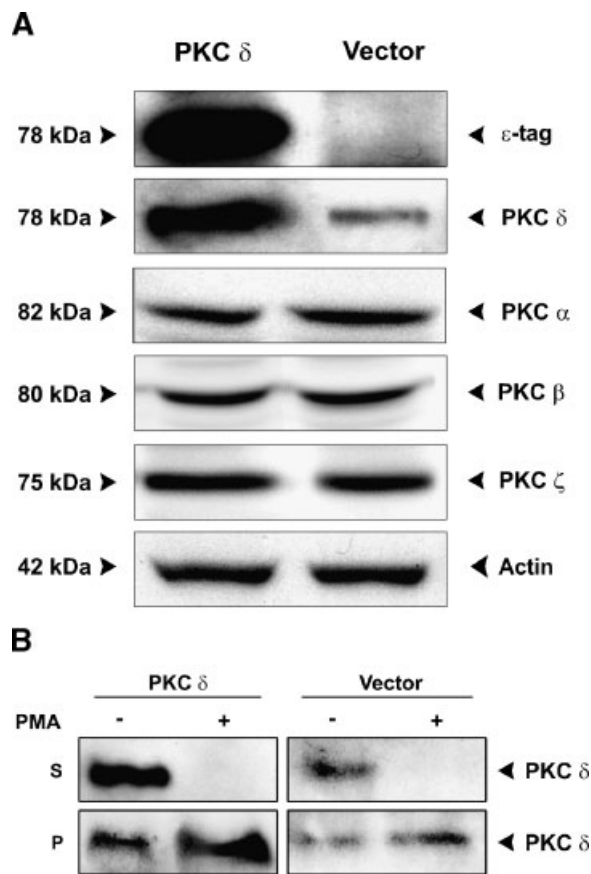


Figure 1. (Panel A) Expression of ϵ -tagged PKC δ in NMuMG cells. Whole cell lysates prepared from NMuMG cells transfected with either pMTH-PKC δ or pMTH (vector alone) were resolved on 10% SDS-PAGE and blotted with anti- ϵ -tag antibodies or with antibodies against individual PKC isoforms (50 μ g protein/lane). (Panel B) Translocation of PKC δ isozyme in NMuMG transfected cells. NMuMG cells transfected with either pMTH-PKC δ or pMTH vector were incubated with PMA (50 nM) for 15 min. Soluble (S) and particulate (P) fractions were then separated by ultracentrifugation and subjected to Western blot analysis with anti-PKC δ antibodies.

Effect of PKC δ on Anchorage-Independent Growth

The ability to grow independently of substrate attachment represents an important indicator of cell transformation. It has been reported that PKC δ inhibits malignant transformation of skin and prostate cells [26,29]. While control (vector-transfected) cells were unable to form colonies in soft agar, surprisingly, PKC δ overexpression conferred to NMuMG cells anchorage-independent growth capacity (Figure 2A and B). Moreover, upon phorbol ester activation (PMA, 50 nM, 1 h) a significant increase in the number and size of colonies was observed in NMuMG-PKC δ cells (Figure 2A and B). These results suggest that PKC δ overexpression confers a transformed phenotype *in vitro*.

Effect of PKC δ on Cell Proliferation

Analysis of cell proliferation revealed a small but significant increase in the number of NMuMG-PKC δ

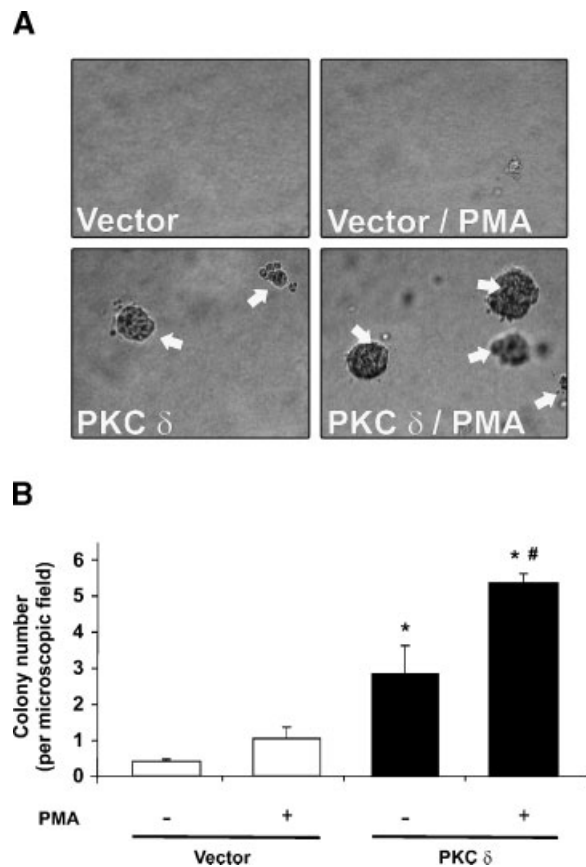


Figure 2. PKC δ confers anchorage-independent growth to NMuMG cells. (Panel A) NMuMG cells were seeded in agar in complete medium in the presence or absence of 50 nM PMA. Representative microscopic photos are shown. (Panel B) Colony number was assessed with an inverted microscope 15 d after seeding. Results were expressed as mean \pm SD, and they are representative of three independent experiments. * P < 0.05 versus NMuMG-vector cells that received the same treatment.

cells compared to control cells at all the analyzed times (Figure 3A). However, growth rate analysis revealed only a slight change in the population-doubling time (17.7 ± 3.6 h in control vs. 15.5 ± 2.8 h in NMuMG-PKC δ cells). In order to determine whether phorbol esters affect proliferation, unsynchronized monolayers of control and PKC δ transfectants were treated with PMA (50 nM, 1 h). PMA

induced a significant increase both in [3 H]-thymidine incorporation (Figure 3B) and in cell number (Figure 3C) only in PKC δ -overexpressing cells, thus indicating that the activation of the overexpressed PKC δ constitutes an important feature in the modulation of the proliferative capacity of this cell line. The distribution of cells in the cell cycle after serum starvation was also analyzed. While both NMuMG-vector and PKC δ cells remained arrested in G $_1$ up to 10 h after PMA treatment, at 16 h about 39% of PKC δ -overexpressing cells were found in the S-G $_2$ /M phases versus only 31.4% of vector transfected cells (data not shown).

PKC δ Overexpression Leads to ERK Hyperactivation

Next, we analyzed whether PKC δ overexpression or its activation with PMA modulates ERK mitogen-activated protein kinase (MAPK), a crucial mitogenic signaling pathway. Compared to vector transfected cells, PKC δ -overexpressing cells showed an increase in the basal level of phosphorylated (active) ERK. PMA (50 nM, 20 min) caused a marked increase in the levels of pERK in NMuMG-PKC δ cells, while activation was smaller in control cells (Figure 4A). A time-course analysis revealed that PMA caused a significant elevation in pERK levels, both in vector- and PKC δ -transfected cell lines, which was detected as early as 5 min after the addition of PMA. However, control cells showed lower and less sustained ERK activation than NMuMG-PKC δ cells. Furthermore, pERK remained elevated in PKC δ overexpressing cells for more than 90 min, while it decreased to a basal level after 60 min in control cells (Figure 4B).

To further assess the role of PKC δ on ERK activation, we used a RNAi approach. A significant reduction in PKC δ levels ($\sim 70\%$) was observed upon delivery of a PKC δ RNAi into NMuMG-vector cells compared to the levels observed in the same cells transfected with a scramble RNAi. Importantly, pERK levels were significantly reduced in PKC δ -depleted cells (Figure 4C), thus supporting a role for PKC δ in ERK activation in NMuMG cells.

In order to determine whether PMA treatment affects cell-cycle progression, cells were synchronized in G $_0$ by serum starvation and the expression of G $_1$ markers was determined 6 h after serum release, either in the absence or presence of PMA (50 nM, 1 h). As shown in Figures 5A and B, PMA treatment significantly enhanced cyclin D1 expression, but the effect was markedly stronger in PKC δ overexpressing cells. Moreover, PMA treatment induced a marked Rb hyperphosphorylated state in NMuMG-PKC δ cells (Figures 5A and B). Taken together these results are consistent with the concept that PKC δ overexpression and its activation with PMA promotes proliferation by activating the MEK/ERK pathway and enhancing cyclin D1 expression.

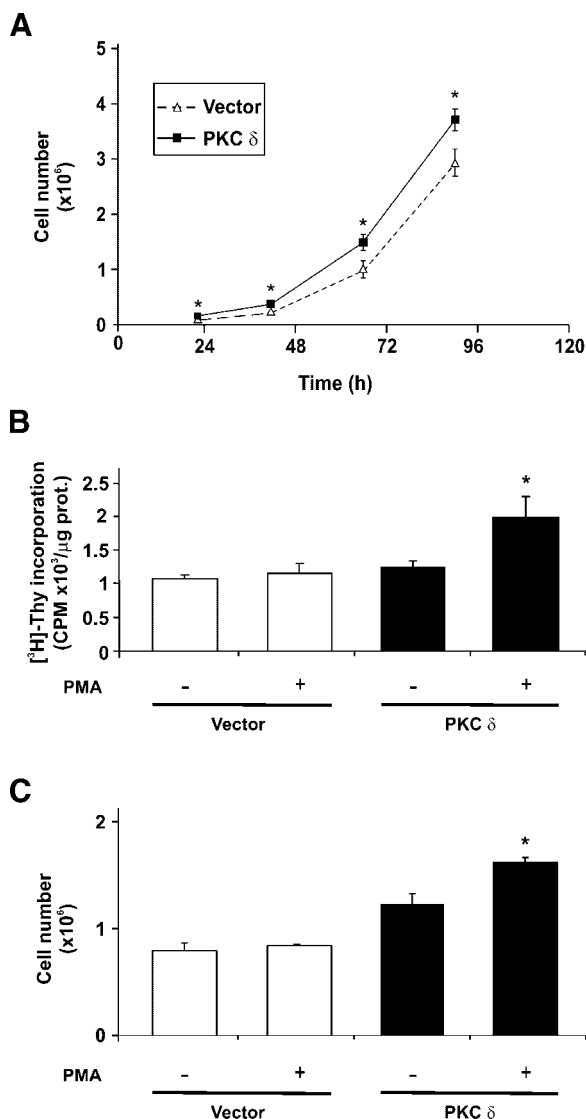


Figure 3. (Panel A) Effect of PKC δ on cell population-doubling time. Cell number was assessed at different times during the exponential growth phase of unsynchronized NMuMG cultures. Each data point represents the mean \pm SD of triplicate determinations. * $P < 0.05$ versus NMuMG-vector cells (Student's t -test). At least three independent experiments were performed with similar results. (Panels B and C) Effect of PKC activation on cell proliferation. Cells were treated with PMA (50 nM) as described in Materials and Methods, and the effect on cell proliferation was determined by measuring [3 H]-thymidine incorporation (Panel B) or cell number (Panel C) on exponentially growing monolayers. Results were expressed as mean \pm SD, and they are representative of three independent experiments. * $P < 0.05$ versus NMuMG-PKC δ cells without treatment (Student's t -test).

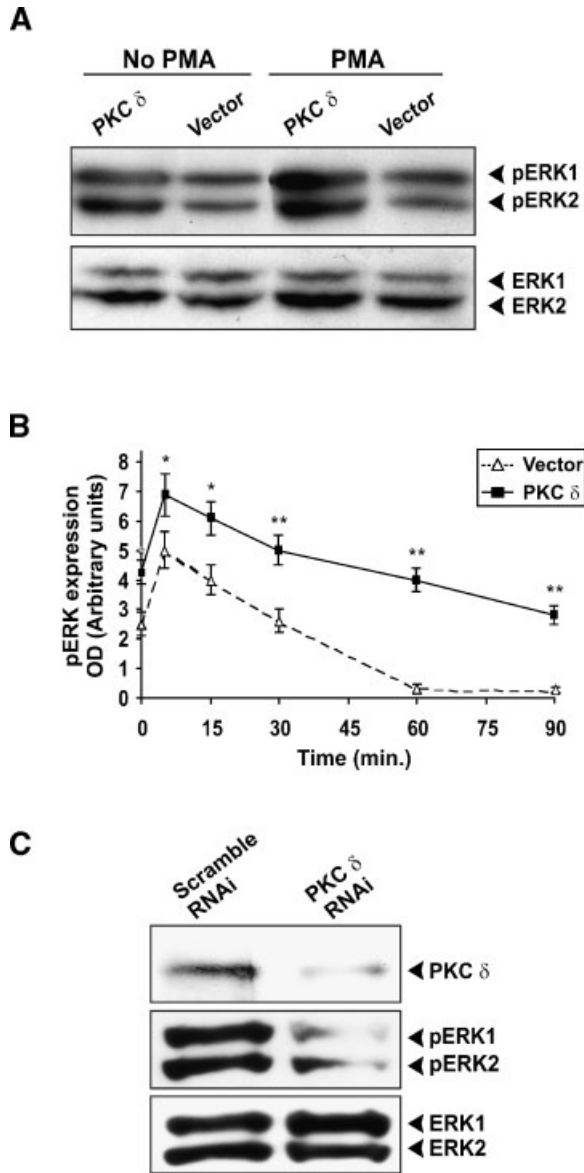


Figure 4. (Panel A) Activation of ERK1/2 MAPK by PKC δ overexpression in NMuMG cells. Serum starved NMuMG cells were treated with 50 nM PMA (20 min), lysed, and subjected to Western blot analysis with specific pERK or total ERK antibodies. Total ERK1/2 level was used as protein-loading control. Results are representative of three independent experiments. (Panel B) Time course ERK1/2 MAPK activation. Serum starved NMuMG cells were treated with 50 nM PMA for different times (0–90 min), and subjected to Western blot analysis with the anti-pERK antibody. A densitometric analysis is shown. Data were normalized to total ERK1/2 levels and expressed as arbitrary units. * $P < 0.05$, ** $P < 0.01$ versus NMuMG-vector cells (Student's t -test). Data were expressed as the mean \pm SD ($n = 3$). (Panel C) Effect of endogenous PKC δ inhibition by RNAi on PKC δ expression and ERK1/2 activation. NMuMG-vector cells were transfected with PKC δ - or scramble-RNAi. Forty-eight hours after transfection cells were harvested and PKC δ and p-ERK1/2 expression were analyzed by Western blot. Total ERK1/2 level was used as protein loading control. Results are representative of three independent experiments.

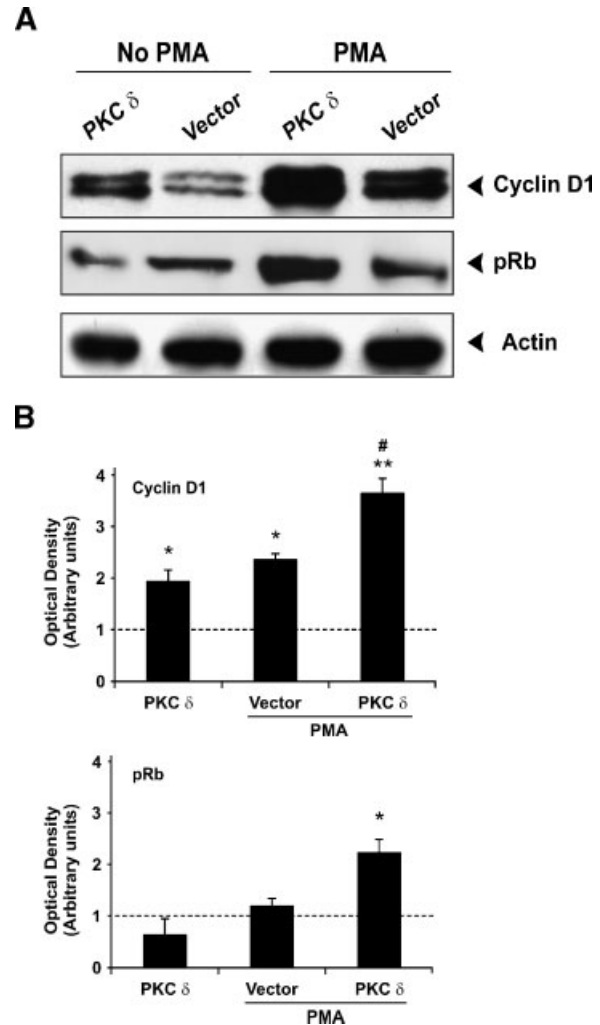


Figure 5. Regulation of cyclin D1 levels and Rb phosphorylation by PKC δ overexpression. (Panel A) Cell lysates prepared from NMuMG-PKC δ or vector transfected cells, treated or not with 50 nM PMA (1 h), were subject to Western blot analysis with antibodies against cyclin D1 and pRb. A representative experiment is shown. (Panel B) A densitometric analysis from three independent experiments is shown. Results were expressed in arbitrary units as relative to vector-transfected cells without treatment (dotted line). Data were expressed as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$ versus NMuMG-vector cells without treatment; # $P < 0.05$ versus NMuMG-PKC δ cells without treatment (Student's t -test).

Effect of PKC δ on Cell Survival

The ability of tumor cell populations to expand in number is determined by the balance between proliferation and death. The acquisition of resistance toward cell death constitutes an essential feature in malignant transformation and represents a hallmark for most types of cancer. This prompted us to explore whether PKC δ could modulate the survival capacity of NMuMG cells in response to either serum-starvation or the cytotoxic agent Dox. As shown in Figure 6A, PKC δ -overexpressing cells were more resistant to cell death induced either by Dox

exposure or by FCS deprivation. This last feature also indicates that PKC δ overexpression is associated with a lower dependence on FCS factors. Similar results were obtained when NMuMG-PKC δ cells were incubated with insulin in serum-free medium (Figure 6A). Next we investigated the signaling pathways involved in the increased survival conferred by PKC δ with pharmacological inhibitors. While the specific MEK-1 inhibitor PD98059 did not influence the effect of Dox, the PI3K inhibitor LY294002 reverted the protective effect conferred by PKC δ overexpression

in NMuMG cells (Figure 6B). No cytotoxic effects were induced by treatment with either LY294002 alone (Figure 6B) or PD98059 alone (data not shown). As the PI3K effector Akt is known to be a crucial mediator of survival, we assessed the levels of pAkt by Western blot. Basal pAkt levels were significantly higher in NMuMG-PKC δ cells relative to control cells (Figures 6C and D). No significant changes were observed in total Akt levels. Changes in pAkt levels were reverted by LY294002 but not by PD98059, suggesting a prominent role for the PI3K-Akt pathway in the effect.

DISCUSSION

Our studies underscore an unexpected role for PKC δ in proliferation and survival of murine mammary cells. Here we show that elevated PKC δ levels in NMuMG cells leads to increased proliferation and resistance to death in response to apoptotic stimuli, as well as the acquisition of anchorage-independent growth in soft agar.

The effect of PKC δ on cell proliferation was investigated after treatment with PMA, one of the most studied tumor promoter PKC activators. This treatment resulted in a sustained increase in ERK activation, an enhancement in [³H]-thymidine incorporation, and an increase in cell number in PKC δ overexpressing NMuMG cells. Moreover, the mitogenic effect of PMA was associated with elevations in cyclin D1 levels and Rb phosphorylation. These results are consistent with previous reports showing that phorbol esters induce cyclin D1 expression and trigger cell-cycle progression in nontransformed cells, such as NIH 3T3 and MEFs [42,43]. In addition, Yan et al. reported that the activation of a novel PKC (PKC ϵ) is required for the activation of MEK/ERK pathway during PMA stimulation of cell-cycle progression [42]. The role of the MEK/ERK pathway in cell proliferation is well established. Mitogenesis in cultured cell lines correlates with p42/44 ERK-MAPK activation, and

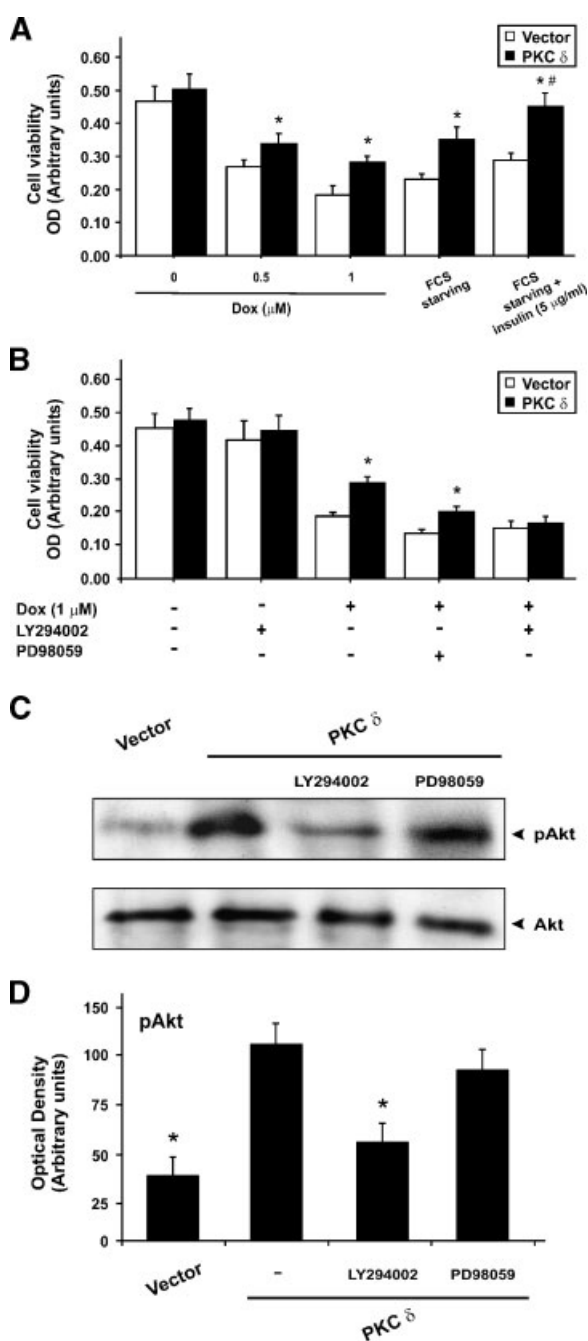


Figure 6. (Panel A) Cell death susceptibility. Cells were deprived of serum, and treated with insulin or with different concentrations of Dox for 2 h. Cell viability was evaluated 48 h later with the MTS assay. Data were expressed as the mean \pm SD of three independent experiments. * P < 0.05 versus NMuMG-vector cells that received the same treatment (Student's t -test); # P < 0.05 versus PKC δ cells deprived of serum (Student's t -test). (Panel B) PI3K is involved in cell death resistance induced by PKC δ . Cells were pretreated with PD98059 (50 μ M) or LY294002 (30 μ M) for 2 h and then incubated with Dox (1 μ M, 2 h). Cell viability was evaluated 48 h later with the MTS assay. Data were expressed as the mean \pm SD of three independent experiments. * P < 0.05 versus NMuMG-vector cells that received the same treatment (Student's t -test). (Panel C) Elevated phospho-Akt levels in PKC δ overexpressors. Cell lysates prepared from control and PKC δ overexpressing cells, untreated or pretreated with either PD98059 (50 μ M) or LY294002 (30 μ M), were subject to Western blot analysis with anti-pAkt and anti-Akt antibodies. Total Akt level was used as protein loading control. Results are representative of three independent experiments. (Panel D) Densitometric analysis of bands in panel C. * P < 0.05 versus NMuMG-PKC δ cells without treatment (Student's t -test). Data were expressed as the mean \pm SD (n = 3).

inhibition of the ERK pathway results in most cases in reduced proliferation [44]. The link between enhanced ERK1/2 signaling and the induction of cyclin D1 expression was previously described by several authors [38,44]. Our studies in NMuMG cells show that PKC δ is responsible of ERK activation followed by a significant increase in cyclin D1 expression. Cyclin D1 is a critical component of the cell-cycle machinery required, and the amplification and/or overexpression of the cyclin D1 gene is often detected in human cancers [45]. Consistent with the elevations in cyclin D1 levels, PKC δ overexpressors show Rb hyperphosphorylation in response to PMA. The Rb protein is a critical regulator of cell-cycle progression, and its phosphorylation coincides with the passage of the cell through a restriction point in G₁ phase. Rb undergoes continued phosphorylation throughout the S-phase, and Rb cyclin D1-dependent phosphorylation is required for cyclin D1 to promote cell-cycle progression. It is then clear that in NMuMG cells PKC δ activation exerts an effect on G₁/S transition by modulating cyclin D1 expression levels and the phosphorylation status of Rb. These results markedly differ from results in lung cancer cells, in which we found that PKC δ negatively regulates G₁/S transition via upregulation of p21 and Rb dephosphorylation [15].

Signaling through PI3K and the downstream kinase Akt has been widely implicated in cell survival [30]. In our studies we observed that PKC δ overexpression conferred resistance against cytotoxic drugs or serum starvation, an effect that is prevented by the PI3K inhibitor LY940002. Furthermore, a substantial elevation in the levels of activated (phosphorylated) Akt was detected in PKC δ overexpressors, and this hyperactivation returns to normal upon PI3K inhibition. On the other hand, the MEK inhibitor PD98059 did not revert cell survival or alter Akt activation in PKC δ overexpressors, suggesting that the MAPK pathway is not essential in this context. The relationship between PKC activation and Akt function has been the subject of intense investigation by several laboratories. It is clear that there is a strict isozyme- and cell type-dependency. For example, studies have shown that PKCs activate Akt in endothelial and myeloid cells, an effect that has been attributed in most cases to the classical PKC isozymes [46,47]. Other studies have shown that PKC negatively regulates Akt [48]. Our studies in prostate cancer cells revealed that PKC α negatively regulates Akt via the activation of a PP2A phosphatase [26]. A similar paradigm has been recently described in keratinocytes, but in this case Akt inactivation in response to PMA involves PKC δ and PKC ϵ [49]. Our results are the first to report that Akt can be activated through a PKC δ -dependent pathway. While the identification of the PKC δ effectors that confer such differential responses is beyond the scope of this paper, one may predict that

the differential involvement of PKC isozymes in each case relates to unique patterns of intracellular localization, as demonstrated for several other PKC-mediated signaling events, including for PKC δ . A differential access of PKC isozymes to substrates may well explain the differences in selective coupling of PKC δ to proliferative, apoptotic and survival pathways in normal versus cancer cells. Overexpression of PKC δ may even confer an apoptotic response or protect from cell death depending on the nature of the stimuli, as described in glial cells [21,50], thus suggesting additional levels of complexity. It has been postulated that tyrosine phosphorylation of PKC δ dictates the nature of the functional outcome, and this could certainly be a relevant factor in breast cells [51].

The ability of cells to grow independently of substrate attachment represents a hallmark of malignant cell transformation, because it is commonly associated with an *in vitro* transformed phenotype. The fact that PKC δ overexpression confers to NMuMG mammary cells the ability to form colonies in soft agar was an unexpected finding, and it attests for the heterogeneity in PKC δ responses.

The main relevance of our study resides in the demonstration that PKC δ induced, in an immortalized mammary cell line, an increase in proliferative and survival capacities together with the acquisition of anchorage-independent growth ability. Although it is still premature to ascribe a contribution of PKC δ to mammary carcinogenesis, these changes support the concept that PKC δ overexpression could favor the malignant transformation process in mammary cells. These results agree with studies by Kiley et al. [2], who reported a correlation between elevated PKC δ levels and aggressiveness in breast cancer, and McCracken et al. [9], who proposed a pro-survival role for PKC δ in mammary tumor-derived cell lines.

An important lesson derived from these studies is that the paradoxical PKC δ stimulatory versus inhibitory responses may greatly impact in the rationale design of isozyme-specific PKC modulators as therapeutic agents. Indeed, substantial heterogeneity in the responses to bryostatin 1, a PKC δ ligand that has been in clinical trials as an antineoplastic agent, has been observed in cellular models [52,53]. Our studies argue that a thorough understanding of the mechanisms that confer distinctive responsiveness by PKCs is needed, particularly the PKC δ isozyme.

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