Atypical Protein Kinase C-ζ Modulates Clonogenicity, Motility, and Secretion of Proteolytic Enzymes in Murine Mammary Cells

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In this paper, we investigated whether protein kinase C-ζ (PKCζ), a member of the atypical PKC family, induces phenotypic alterations associated with malignant transformation and tumor progression in mammary cells. The stable overexpression of PKCζ in immortalized mammary epithelial cells (NMuMG), activates the mitogenic extracellular signal-regulated kinase (ERK) pathway, enhanced clonal cell growth and exerts profound effects on proteases secretion. The effect on proteases expression seems to be specific for urokinase-type plasminogen activator and metalloproteinase-9 (MMP-9) because no modulation in MMP-2 and MMP-3 production could be detected. In addition, our experiments demonstrated that PKCζ overexpression markedly altered the adhesive, spreading, and migratory abilities of NMuMG cells. The overexpression of this enzyme was not sufficient to confer an anchorage-independent growth capacity. An extensive mutational analysis of PKCζ revealed that the effects observed in NMuMG cells were strictly dependent on the kinase (catalytic) domain of the enzyme. Taken together, these results suggest that in mammary cells PKCζ modulates several of the critical events involved in tumor development and dissemination through the activation of mitogen activated protein kinase (MAPK)/ERK pathway.

Key words: PKCζ; metalloproteinases; urokinase; ERK1/2 MAPK pathway

INTRODUCTION

Mammary epithelial cells undergo several changes during mammary gland growth and involution cycles [1]. These events require the activation of mitogenic and/or apoptotic signals, the expression of extracellular matrix molecules (ECM) and also the degradation of this ECM molecules. ECM degradation is dependent on proteolytic enzymes such as matrix metalloproteinases (MMPs) [2] and urokinase-type plasminogen activator (uPA) [3]. The alteration of the signaling pathways involved in the control of all these molecules may contribute to malignant transformation. An altered expression of some protein kinase C (PKC) isoforms, including PKCα, δ, ε, and ζ, has been associated with an increase in protease levels, found during mammary gland differentiation and involution [4].

PKC comprises a family of at least ten phospholipid-dependent serine-threonine kinases that play central roles in signal tranduction, governing gene expression, proliferation, apoptosis, differentiation, and malignant transformation [5–7]. PKC isozymes are grouped into three subclasses: the ''classical'' (PKCα, β, and γ), which can be stimulated by Ca^{2+} and diacylglycerol or phorbol esters; the ''novel'' (PKCδ, ε, η, and θ), which can be activated by diacylglycerol or phorbol esters but are Ca^{2+} independent, and the ''atypical'' (PKCζ and λ/ι), which are unresponsive to Ca^{2+}, diacylglycerol, and phorbol esters. PKCs possess a regulatory N-terminal domain and a catalytic C-terminal (kinase) domain, presenting conserved regions (C1 to C4) and variable regions (V1 to V5) with specific functional roles. A distinctive feature of the atypical PKCζ is that its regulatory region possesses a single phorbol ester unresponsive C1 domain instead of two. It also lacks a C2 domain, which is involved in calcium and/or phospholipid recognition in classical and novel PKCs. The regulatory regions of PKC isozymes play an essential role in allosteric modulation and intracellular targeting, though in some cases, regulatory regions have been shown to trigger biological

Abbreviations: ECM, extracellular matrix; MMPs, matrix metalloproteinases; uPA, urokinase-type plasminogen activator; PKC, protein kinase C; MEK, mitogen-activated protein kinase/ERK kinase; ERK, extracellular signal-regulated kinase; MEM, minimum essential medium; FCS, fetal calf serum; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CM, conditioned media; PAI, plasminogen activator inhibitor; MAPK, mitogen activated protein kinase.

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responses, as shown for the PKCδ regulatory domain in breast cancer cells [7,8]. Individual PKC isozymes exhibit different tissue distribution, subcellular localization, and biochemical properties, an indication that they play specialized biological roles [9–11].

Deregulation of PKC isozymes has been often associated with malignant transformation and metastatic dissemination [10]. There is strong evidence for the involvement of PKC isozymes in breast cancer progression. Indeed, PKCα overexpression correlates with the lack of estrogen receptor expression in mammary tumor cells, an indicator of poor prognosis [12,13]. In addition, several classical and novel PKC isoforms have been associated with an enhanced expression of MMPs and uPA [14–16], enzymes that play crucial roles in tissue remodeling as well as in cancer initiation and progression [17]. Furthermore, increase of uPA activity functionally correlates with highly aggressive mammary cancers [18]. On the contrary, very little information is available regarding the functionality of the atypical PKCζ in the modulation of proteolyitic enzymes [19].

Upregulation of PKCζ has been detected in various cancer models, including bladder carcinomas [20], hepatocellular carcinomas [21], prostate cancer [22], and melanoma metastasis [23]. Moreover, some studies suggest that atypical PKCs play a functional role in tumor dissemination, and PKCζ has been involved in the control of cell motility in pancreatic adenocarcinoma and melanoma cells [24,25]. Recently, it has been reported that PKCζ is overexpressed in breast carcinomas [26], though the functional implications of this phenomenon are not known.

The main goal of the present work was to analyze whether an aberrantly activated PKCζ pathway facilitates the acquisition of phenotypic characteristics associated with malignancy and invasiveness through the regulation of proteolytic enzymes. As an experimental approach we overexpressed this atypical PKC isozyme in NMuMG cells, a well-established model of normal mouse mammary cells that has been widely used to study mammary carcinogenesis [27–29]. Our experiments revealed that PKCζ activates the mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, leading to profound effects on the ability of NMuMG cells to proliferate, adhere, migrate, and secrete proteases. These findings were substantiated by an extensive mutational analysis of PKCζ which revealed that the kinase domain is essential for such effects.

MATERIALS AND METHODS

Reagents and Antibodies

Media for cell culture (MEM), agarose, geneticin (G418), and the transfection reagent Lipofectamine Plus were from Life Technologies (Rockville, MD). Fetal calf serum (FCS) was from GEN (Buenos Aires, Argentina). Plasminogen was purchased to Chromogenix (Molndal, Sweden). Human urokinase was a gift from Serono (Buenos Aires, Argentina). Triton X-100 was obtained from J.T. Baker (Phillipsburg, NJ). Gelatin, α-casein, PD98059, and acrylamide were from Sigma Co. (St. Louis, MO). All other reagents for polyacrylamide gel electrophoresis and zymography were obtained from Bio-Rad (Richmond, CA). The anti-PKCε (anti-ε-tag) antibody was obtained from Life Technologies. Antitotal-ERK, anti-phospho-ERK, anti-PKCs (α, β, δ, and ζ), and antiactin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase conjugated antirabbit and antimouse antibodies, Hybond-P membranes and chemiluminiscence reagents (ECL) were from Amersham (Aylesbury, UK).

Cell Line

NMuMG cells, a normal immortalized mammary cell line derived from NAMRU mice [30] were used for the stable transfection assays. Cells were maintained in MEM supplemented with 10% FCS and 80 μg/mL gentamycin in humidified atmosphere with 5% CO_2.

Expression Vectors, Transfection, and Selection

For the generation of stable transfectants, NMuMG cells were transfected using Lipofectamine Plus with 5 μg of expression vectors encoding for wild-type PKCζ or various PKCζ deletion mutants (PS-cat, Zn-cat, V3-cat, and REG) in pCR3ε vectors. The generation of these PKCζ mutants is described elsewhere [25]. All the expression constructs possess an ε-tag insert sequence that encodes for a KGFSYFGEDLMP peptide, derived from the last 12 amino acids of PKCε [31]. The ε-tagged proteins can be readily detected with a commercially available antibody specific for the ε-peptide. NMuMG cells transfected with the empty vector (pCR3ε) were used as controls (NMuMG-vector). Forty-eight hours after transfection, cells were selected by adding 500 μg/mL of G418. After selection, approximately forty G418-resistant clones corresponding to each transfectant were pooled to avoid clonal variations. Transfection assays were carried out in at least two independent experiments for PKCζ mutants and three times for PKCζ wt and the vector alone. Transfected cell lines were maintained for 10–12 passages before use.

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 9% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Gels

were blotted to Hybond-P membranes. Membranes were incubated for 1 h with PBS containing 5% skim-milk and 0.1% Tween-20. Membranes were incubated with the first antibodies overnight at 4°C, and then with a secondary antibody coupled to horseradish peroxidase. Detection was performed by chemiluminiscence. Bands were quantified by scanning on a digital GS-700 densitometer and the resulting data processed with Molecular Analyst software (Bio-Rad).

Cell Proliferation

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 60 mm Petri dishes in MEM supplemented with 10% FCS, 80 μg/mL gentamycin, and 250 μg/mL G418. At different times after seeding, cells from triplicate wells were washed twice with PBS, trypsinized, and counted using an hemocytometer and trypan blue exclusion.

Clonogenic Assay

Clonal cell growth was studied as previously described [32]. Briefly, single-cell suspensions containing 500–1000 cells were seeded onto 60 mm plastic dishes in MEM with 10% of FCS either in the presence or absence of 50 μM PD98059. Medium was changed every 72 h. After 10 d of culture, cells were washed, fixed with methanol, and stained with hematoxylin. The number of colonies was determined using an inverted microscope. Plating efficiency was defined as the percentage of cells able to grow as colonies having more than ten cells.

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 (0.4% agar) containing log phase growing monodispersed cells (10^5 cells/dish). Fifteen days after seeding, cultures were fixed by adding 10% formaldehyde in PBS, and the number of colonies was determined using an inverted microscope. Colony efficiency was determined as the percentage of cells able to form colonies in soft agar.

Apoptosis Susceptibility

Subconfluent monolayers growing in 96-well plates were extensively washed with PBS and incubated with MEM without FCS for 48 h or treated with 0.5–2 μM of doxorubicin for 2 h. In this last case, the drug was removed, and the cells were further incubated in MEM plus 10% FCS for an additional 24 h. Cell viability was evaluated with an MTS assay (Celltiter 96™ Nonradioactive Proliferation Assay, Promega) as described by the manufacturer.

Adhesion Assay

Suspensions of 4×10^5 cells were seeded onto 35 mm Petri dishes in MEM supplemented with 10% FCS and incubated at 37°C. After 90 minutes the medium was removed and adherent cells were washed twice with PBS, trypsinized, and counted using an hemocytometer. Determinations were carried out by triplicate. Adhesion efficiency was expressed as the percentage of the total number of cells seeded.

Spreading Assay

Suspensions containing 4×10^5 cells were seeded onto 35 mm Petri dishes in MEM supplemented with 10% FCS. Cells were allowed to adhere, and after 60 min nonadherent cells were removed by washing with PBS. The remaining cells were fixed with 4% formaldehyde. The percentage of spread cells was determined by scoring 200–300 cells under a phase-contrast microscope (Nikon, Tokyo, Japan). Data was collected by random observation in triplicate, as described previously by Aguirre Ghiso et al. [33].

Wound Migration Assay

Migration was studied as previously described [32]. Briefly, wounds of approximately 400 μm width were made in subconfluent monolayers of the different transfectant cultures. Cells were then allowed to migrate into the cell-free area for a period of 12 h. The same spot was photographed at different times and the migratory area was analyzed using a digital densitometer GS-700 (Bio-Rad). Cell migration was expressed as the percentage of the area occupied by the migratory cells in the original cell-free wounded area.

Preparation of Conditioned Media (CM)

Secreted uPA and MMP activities were evaluated in CM. Briefly, semiconfluent cell monolayers growing in 35-mm plastic Petri dishes were extensively washed with PBS. Serum-free medium (1 mL) was then added for 24 h. CM were individually harvested, the remaining monolayers were lysed with 1% Triton-X100-PBS, and cell protein content was determined (Bio-Rad Protein Assay). CM samples were centrifuged (600*g*, 10 min), aliquoted, and stored at −40°C. Samples were used only once after thawing.

Quantification of uPA Activity by Radial Caseinolysis

To determine uPA activity, a radial caseinolysis assay was used, as previously described [34]. Briefly, 4 mm wells were punched in the plasminogen-rich casein-agarose gels and 10 μL of CM were seeded. Gels were incubated for 24 h at 37°C in a humidified atmosphere. The diameter of lytic zones was measured, and the areas of degradation were referenced to a standard curve of purified urokinase (0.1–50 IU/mL) and normalized to the original cell culture protein

content [35]. Specificity of uPA activity was determined by blocking its activity with B428 (18 μM), a uPA-specific catalytic inhibitor [36].

Determination of Plasminogen Activator Inhibitor (PAI) Activity

PAI activity was determined by reverse zymography, as described by Mackay et al. [37]. Briefly, samples of CM were electrophoresed in 10% SDS/PAGE gel co-polymerized with 30 mg/mL of skim dry milk and 2 μg/mL plasminogen. Gels were washed with 1% Triton X-100-PBS, rinsed with water and incubated for 2 h in a buffer containing 20 mM Tris-HCl, pH 8.3, 15 mM EDTA, and 1 IU/mL purified urokinase. Gels were stained with Coomassie Brilliant Blue G-250 (Bio-Rad) and PAI activity was visualized as blue bands in a lighter background.

Zymography for MMPs

MMPs enzymatic activities were determined on substrate-impregnated gels, as previously described by Aguirre-Ghiso et al. [38] and Simian et al. [39]. Briefly, samples were collected and run on 9% SDS polyacrylamide slab gels containing 1 mg/mL of gelatin (to detect MMP-9 and MMP-2) or α-casein (to detect MMP-3), under nonreducing conditions. After electrophoresis, gels were washed for 30 min using 2.5% Triton X-100 and subsequently incubated for 48 h at 37°C in a buffer containing 0.25 M Tris-HCl pH 7.4, 1 M NaCl, and 25 mM $CaCl_2$. Nonspecific activity was detected with gels incubated in the same buffer solution but supplemented with 40 mM EDTA. After incubation, gels were fixed and stained with 0.5% Coomassie Brilliant Blue G-250 in methanol/acetic acid/H_2O (30:10:60). Gelatinolytic bands were measured using a digital densitometer GS-700. Data were expressed as arbitrary units and normalized to the values observed in control cells.

RESULTS

Overexpression of PKCζ in NMuMG Cells

In order to investigate whether the overexpression of the atypical PKCζ isozyme modulated mammary cellular properties associated with malignant transformation and/or metastatic dissemination, we used a stable transfection approach, as previously described [25,40]. Overexpression of PKCζ was achieved upon transfection of NMuMG cells with the expression vector pCR3ε-PKCζ [25] followed by selection with G418. To avoid clonal variations, G418-resistant clones were pooled 2 wk after transfection. Western blot analysis with the anti-ε-tag antibody revealed a 75 kDa band corresponding to the ε-tagged PKCζ in PKCζ-transfected NMuMG cells but not in cells transfected with the vector alone (Figure 1A). PKCζ was expressed in a high percentage of cells (>80%), as determined by immuno-staining assays, and expression levels were moderate but similar for all analyzed

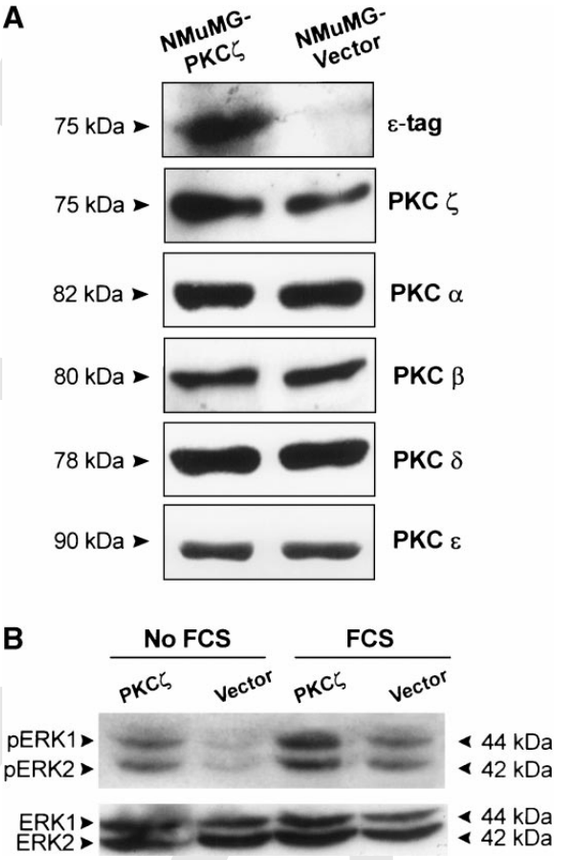

Figure 1. (Panel A) Expression of ε-tagged protein kinase C-ζ (PKCζ) in NMuMG cells. Whole cell lysates prepared from NMuMG cells transfected with either pCR3ε-PKCζ or pCR3ε constructs were resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and blotted with an anti-ε-tag antibody or with antibodies against individual PKC isozymes (50 μg/lane). (Panel B) Activation of extracellular signal-regulated kinase (ERK)1/2 MAPK by PKCζ overexpression in NMuMG cells. Overnight starved cells were treated with 10% FCS (20 min) and subjected to Western blot analysis (40 μg/lane) with specific phospho-42/44 ERK or total anti-ERK antibodies. Results are representative of three independent experiments.

cells (data not shown). Using this stable transfection approach, we achieved a 2.8-fold increase in PKCζ levels, relative to control transfected cells (Figure 1A, second panel). Overexpression of PKCζ did not alter significantly the expression of other PKC isozymes, present in NMuMG cells, as previously described in other cell models [40] (Figure 1A). All pooled cell lines obtained after separated transfection experiments showed similar expression levels and biological properties described below.

Evaluation of p42/p44 Mitogen Activated Protein Kinase (MAPK) Pathway Activation

Atypical PKCs activate various intracellular signaling cascades controlling mitogenesis, including the Raf-1/MEK/ERK cascade [41–44]. In order to analyze whether the ERK pathway was indeed activated in

the NMuMG-PKCζ stable transfectants, we determined the levels of phosphorylated (active) and total p44/p42 ERK MAPK by Western blot. As shown in Figure 1B, PKCζ-expressing cells show increased basal phospho-ERK levels relative to control cells (Figure 1B). Short treatments (20 min) with FCS caused a marked increase in phospho-ERK levels in PKCζ cells. The same treatment induced only a moderate activation of ERK in control cells. No changes were observed in total ERK levels (Figure 1B).

Effect of PKCζ on Proliferation and Survival of NMuMG Cells

Cell counting at early times after plating (5–24 h) revealed a significantly higher cell number in PKCζ transfectants compared to control (vector-transfected) cells (Figure 2A). The increase in cell number was blocked by PD98059, a MEK inhibitor (data not shown). Despite the higher number of cells in PKCζ transfectants, analysis of growth rate between 48 and 96 h did not show a significant difference in the population doubling time (17.8 ± 3.7 vs. 19.1 ± 2.9 h, for PKCζ and control cells, respectively) (Figure 2B). Then, we explored whether PKCζ overexpression conferred a growth advantage under more stringent conditions such as clonal cell growth. As shown in Figure 2C, PKCζ transfectants show a higher clonogenic ability than control cells. Again, this capacity was reverted by PD98059 (Figure 2C).

The ability to grow independently of substrate attachment represents a hallmark of malignant cell transformation. Thus, we next assayed whether PKCζ overexpression conferred NMuMG cells with anchorage-independent growth capacity in soft agar assay. It was found that neither NMuMG-PKCζ nor the vector-transfected control cells were able to form colonies in soft agar.

Transformed cell populations tend to expand in number not only by increasing proliferation rate but also by evading apoptosis [45]. The overexpression of PKCζ did not confer any survival advantage when cells were deprived of serum or exposed to the cytotoxic drug doxorubicin. In this regard, NMuMG-PKCζ cells presented a similar doxorubicin inhibitory concentration 50 than control transfectants (1.4 ± 0.3 vs. 1.8 ± 0.4 μM, respectively).

Role of PKCζ in Adhesion, Migration, and Spreading Ability

The increase in cell number in NMuMG-PKCζ transfectants observed at early times after plating prompted us to examine whether this could be a consequence of changes in adhesion. Indeed, it was found that PKCζ transfectants presented an enhanced adhesive ability relative to control (vector-transfected) cells. A quantitative analysis revealed that 90 min after plating a significant higher number of PKCζ-transfected cells adhered to the plastic surface, as compared to control (Figure 3A). The rate

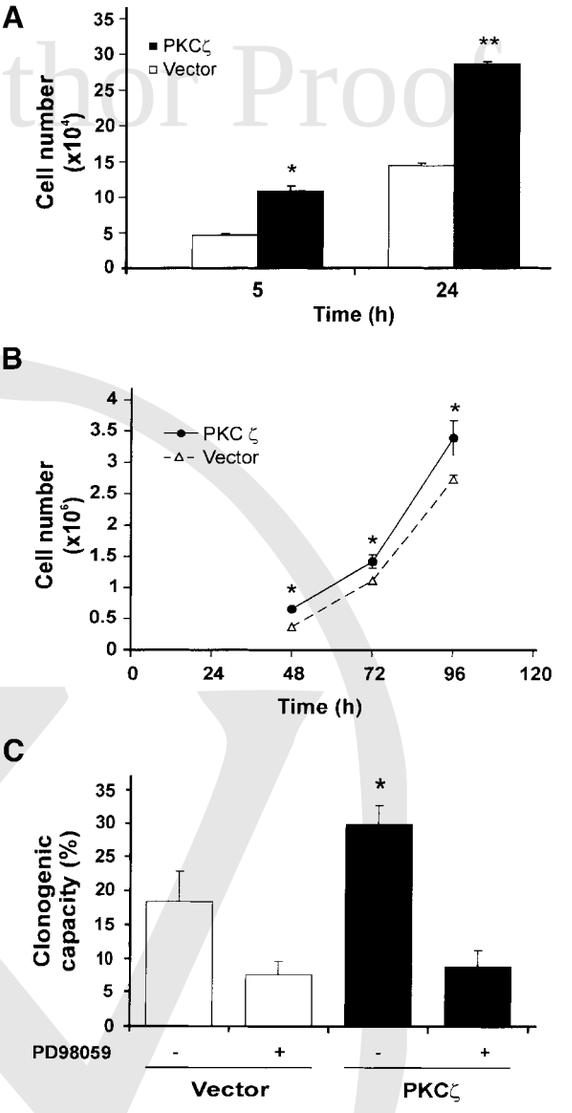

Figure 2. (Panel A) Effect of PKCζ on cell growth. Cell number was assessed in unsynchronized NMuMG cultures at different times. At least three independent experiments with similar results were performed. *$P < 0.05$, **$P < 0.01$ versus NMuMG-vector cells (Student's *t*-test). Each data point represents the mean ± SD of triplicate determinations. (Panel B) Effect of PKCζ on population doubling time. Cell number was assessed at different times during the exponential growth phase of unsynchronized NMuMG cultures. Results are representative of three independent experiments. *$P < 0.05$ versus NMuMG-vector cells (Student's *t*-test). (Panel C) Clonogenic capacity. Clonal cell growth was studied as previously described in ''Materials and Methods'' in the presence or absence of PD98059 (50 μM). Results are representative of three independent experiments. *$P < 0.05$ versus NMuMG-vector cells (Student's *t*-test).

of adhesion was time-dependent, and 100% of NMuMG-PKCζ cells were adhered at 5 h incubation while vector-transfected cells did so only after 10 h incubation (data not shown). The spreading behavior was also markedly enhanced in PKCζ overexpressing cells (Figure 3B).

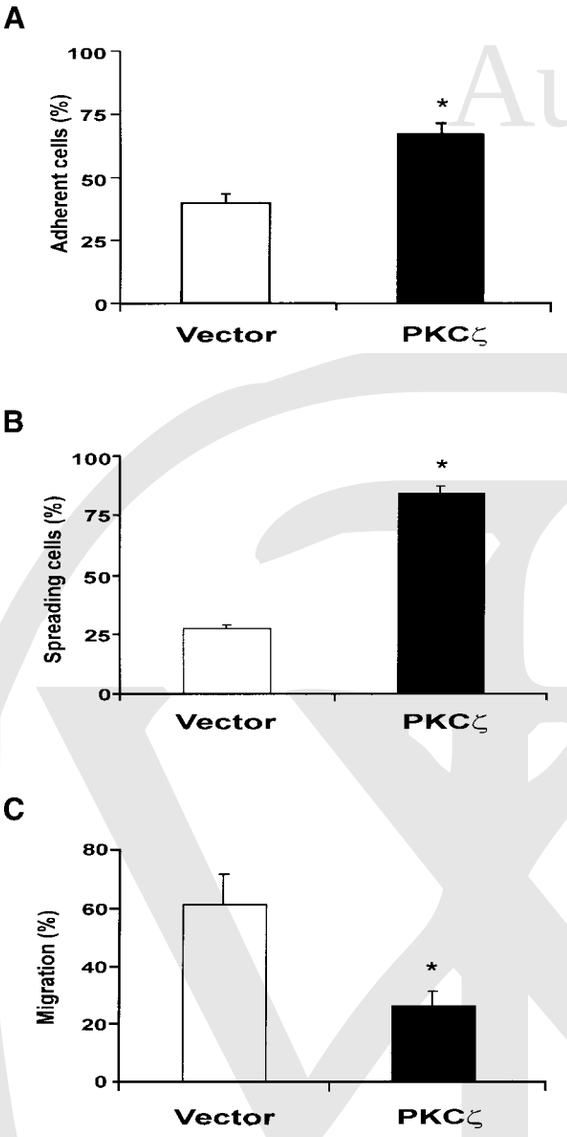

Figure 3. Effect of PKCζ on adhesion, spreading, and migration. (Panel A) Adhesion studies. Control and PKCζ transfectants were assayed for their adhesive capacity as described in ''Materials and Methods.'' The rate of adhesion was expressed as the percentage of the total number of cells seeded. Data expressed as the mean ± SD are representative of three independent experiments. $*P < 0.05$ versus NMuMG-vector cells (Student's *t*-test). (Panel B) Spreading ability. Control and PKCζ transfectants were assayed for their spreading ability, as described in ''Materials and Methods.'' The percentage of spreading cells was determined by scoring cells under a phase-contrast microscope. Data were collected by random observation. Data expressed as the mean ± SD are representative of three independent experiments $*P < 0.01$ versus NMuMG-vector cells (Student's *t*-test). (Panel C) Migration assay. Subconfluent monolayers were ''wounded'' at time 0 and cells were allowed to migrate into the cell free area for 12 h. The same spot was photographed at different times and cell migration was quantified by calculating the percentage of the area occupied by cells that migrated into the original cell-free wounded area. Data expressed as the mean ± SD are representative of three independent experiments $*P < 0.01$ versus NMuMG-vector cells. Student's *t*-test.

In order to analyze whether PKCζ overexpression modulates the migratory properties of NMuMG cells, we performed a ''wound'' assay. Figure 3C shows that, PKCζ-transfected cells showed a significantly lower migratory rate than control cells.

Role of PKCζ in Proteases Secretion

We next analyzed whether PKCζ affects the secretion of proteases. Previous reports from our laboratory have shown that phorbol ester-responsive PKC isoforms positively regulate both uPA and MMPs expression in normal and tumor cells [16,29]. Interestingly, we found that overexpression of PKCζ in NMuMG mammary cells also induced a significant increase in uPA and MMP-9 secreted activities (Figure 4A and B). In contrast, no changes in MMP-2 and MMP-3 (stromelysin) activities were observed (see Figure 7C below). PAI activity was also determined by reverse zymography assay in the CM of both control and PKCζ transfectants. As shown in

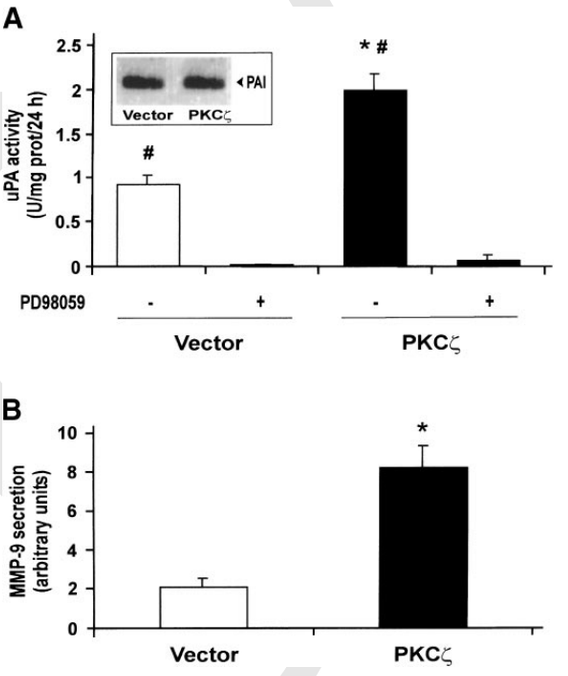

Figure 4. Effect of PKCζ on the secretion of proteolytic enzymes. (Panel A) Quantification of urokinase-type plasminogen activator (uPA) activity by radial caseinolysis. uPA secreted activity was quantified by radial caseinolysis in conditioned media (CM) from PKCζ transfected or control cell cultures, either in the presence or absence of PD98059 (50 μM). $*P < 0.01$ versus NMuMG-vector cells, $^{\#}P < 0.01$ versus same cell treated with PD98059 (Student's *t*-test). Data are expressed as the mean ± SD of triplicate determinations and are representative of at least three independent experiments. Inset: Secreted plasminogen activator inhibitor (PAI) activities, detected by reverse zymography in the same CM. Two additional experiments gave similar results. (Panel B) Quantification of MMP-9 activity by zymography. MMP-9 activity was analyzed as described in ''Materials and Methods.'' Proteolytic activity bands were measured with a digital densitometer, and data were expressed as arbitrary units. $*P < 0.05$ versus NMuMG-vector cells (Student's *t*-test). Data are expressed as the mean ± SD of triplicate determinations and are representative of at least three independent experiments.

Figure 4A (inset), we did not observe measurable differences in PAI activity. These results suggest that PKCζ modulation of uPA activity cannot be attributed to a regulation of its inhibitory molecule. Remarkably, the inhibition of MEK with PD98059 impaired both basal uPA secretion as well as the enhanced activity observed in PKCζ overexpressors (Figure 4A) indicating that active signaling through PKCζ and the MEK/ERK pathway is required for uPA production. Similar results were also observed for MMP-9 (data not shown).

Structure-Function Analysis of PKCζ in NMuMG Mammary Cells

An important issue in PKC-mediated function is the elucidation of the roles of individual domains in triggering biological effects. This is important because it has been reported that PKC isozymes can signal independently of their catalytic (kinase) activity [25,46–48]. We used a series of deletion mutants comprising different regions of the molecule (Figure 5A). The N-terminal deletion mutants would allow for the assessment of the potential roles of the V1, pseudosubstrate and C1 domains. The constructs, which were epitope-tagged (ε-tag) to facilitate the comparison of the expression levels, were transfected into NMuMG cells followed by G418 selection. The mobilities of the truncated mutants in SDS-PAGE were consistent with their predicted molecular weights and comparable levels of expression were observed for all mutants (Figure 5B). Only those cell lines expressing an intact PKCζ catalytic (kinase) domain showed increased phospho-ERK1/2 levels. Expression of the regulatory domain alone did not affect phospho-ERK1/2 levels, as they were similar to those observed in control (vector-transfected) cells (Figure 5C). Total ERK was similar in all cases (Figure 5C).

In the next set of experiments, we analyzed the effect of the PKCζ mutants on adhesion, spreading, and migration. As shown in Figure 6, only those transfectants expressing an intact catalytic (kinase) present similar phenotypes as those observed in wild-type (full-length) PKCζ transfectants. Indeed, the adhesion and spreading assays showed that the presence of an intact catalytic domain was required to enhance the adhesive properties of NMuMG cells (Figure 6A and B). Likewise, only those mutants having an intact catalytic domain were capable of inhibiting migration in the wound assay (Figure 6C).

Effect of PKCζ Mutants on the Production of uPA and MMPs

Finally, we tested whether the PKCζ mutants induce changes in the production of proteases. NMuMG cells expressing PKCζ mutants with an intact kinase domain secreted more uPA than control cells (twofold to fourfold increase) (Figure 7A). This increase was more evident in ZN-cat expressing cells,

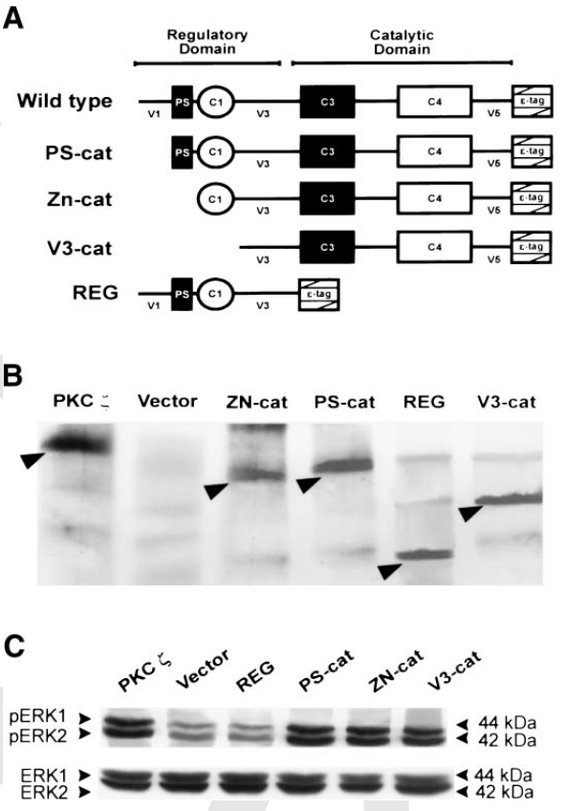

Figure 5. Expression of PKCζ mutants in NMuMG cells. (Panel A) Schematic representation of PKCζ mutants. The pseudosubstrate region (PS), cysteine-rich (Zn-finger) domain (C1), variable regions V1, V3, and V5, and the conserved region C3 are indicated. (Panel B) Expression of ε-tagged PKCζ mutants in NMuMG cells. Western blot analysis was performed with lysates of NMuMG cells stably transfected with each mutant (60 μg/lane). The arrows indicate the position of each mutant protein. (Panel C) Modulation of ERK1/2 MAPKs activation by PKCζ mutants. Transfectants were lysed and subjected to a Western blot analysis (40 μg/lane) with specific antibodies for phospho-p42/44 ERK or total-ERK. Three independent experiments gave similar results.

although the reasons for these differences are not known. Again, the mutant without the catalytic domain (REG), behaved like control cells. In all cases uPA activity could be inhibited by B428 and was not detected in plasminogen-free substrates (data not shown). Gelatin zymograms revealed that MMP-9 was significantly increased in cells overexpressing either wild-type PKCζ or the kinase active PKCζ mutants (Figure 7B and C). The effect was specific for MMP-9, as no changes were observed in MMP-2 and MMP-3 secreted activity (Figure 7C). No evidence for gelatinolytic or caseinolytic activity was detected in gels incubated in EDTA-containing buffers, confirming that the observed activities corresponded to metalloproteinases (data not shown).

DISCUSSION

In the present study, we showed that the overexpression of the atypical PKCζ isoform modulates

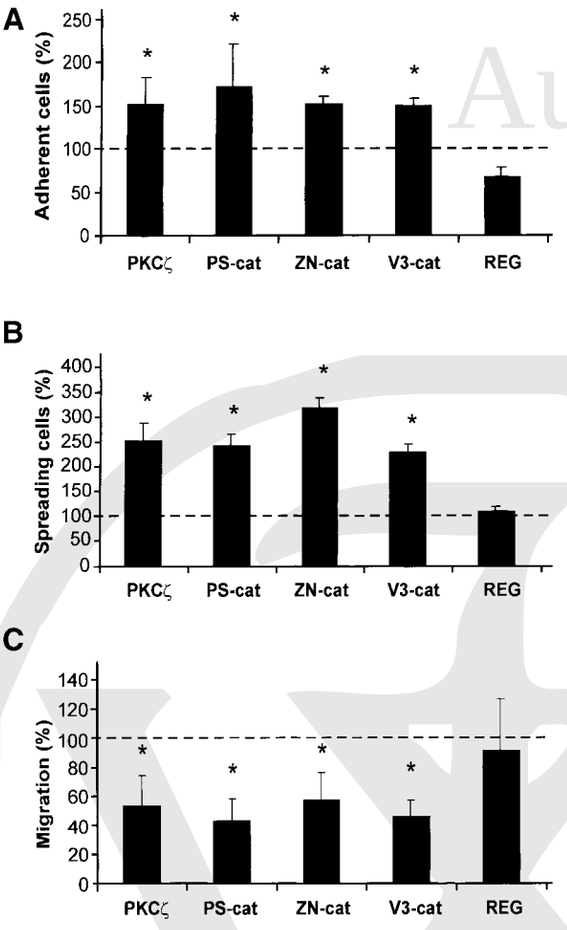

Figure 6. Effect of truncated PKCζ mutants on adhesion, spreading, and migration. (Panel A) Adhesion studies. PKCζ mutants were assayed for their adhesive capacity as described in ''Materials and Methods.'' Values were compared to those obtained with the vector-transfected cells, which were arbitrarily set as 100% (dotted line). *$P < 0.05$ versus NMuMG-vector cells (ANOVA test). Data expressed as the mean ± SD are representative of three independent experiments. (Panel B) Spreading ability. PKCζ mutants were assayed for their spreading ability as described in ''Materials and Methods.'' Values were compared to those obtained with the vector-transfected cells, which were arbitrarily set as 100% (dotted line). *$P < 0.01$ versus NMuMG-vector cells (ANOVA test). Data expressed as the mean ± SD are representative of three independent experiments. (Panel C) Migration assay. Wound migration was studied as previously described in ''Materials and Methods.'' Values were compared to those obtained with the vector-transfected cells, which were arbitrarily set as 100% (dotted line). *$P < 0.05$ versus NMuMG-vector cells (ANOVA test). Data expressed as the mean ± SD are representative three independent experiments.

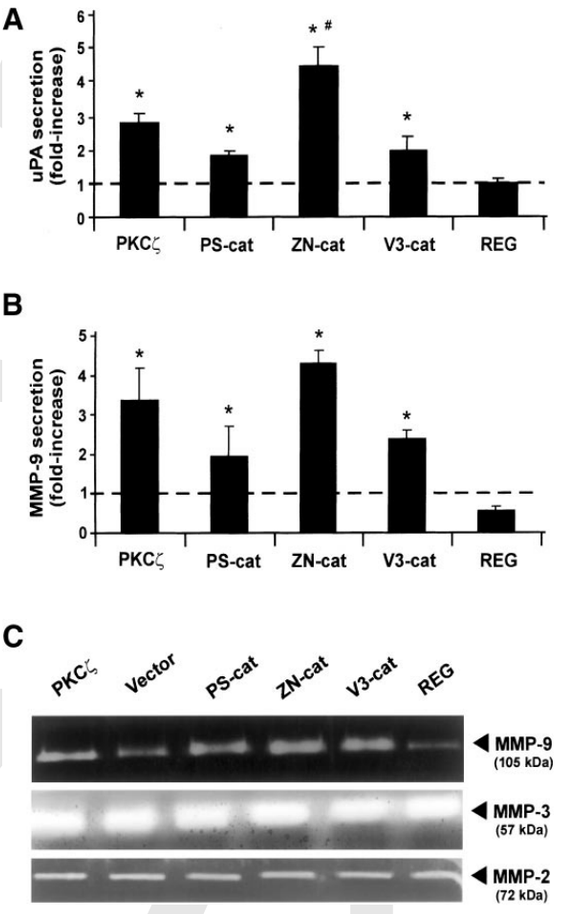

Figure 7. Effect of truncated PKCζ mutants on the secretion of proteolytic enzymes. (Panel A) Quantification uPA activity by radial caseinolysis. uPA secreted activity was quantified by radial caseinolysis as described in ''Materials and Methods.'' Values were referred to those secreted by vector-transfected cells (dotted line). *$P < 0.05$ versus NMuMG-vector cells (ANOVA test). #$P < 0.05$ versus PKCζ cells (Student's *t*-test). Data are expressed as the mean ± SD of three determinations and are representative of at least four independent experiments. (Panel B) Quantification MMP-9 activity by zymography. MMP-9 activity was analyzed as described in ''Materials and Methods.'' Proteolytic activity bands were measured with a digital densitometer. Activities were expressed as arbitrary units and normalized to that of vector-transfected cells (dotted line). *$P < 0.05$ versus NMuMG-vector cells (ANOVA test). Data are expressed as the mean ± SD of three determinations and are representative of at least four independent experiments. (Panel C) Zymography for MMP-2, MMP-3, and MMP-9 detection. MMP-9 and MMP-2 activities were assessed by gelatin zymography. MMP-3 activity was analyzed by casein zymography. Results are representative of at least three independent experiments.

clonogenicity, cell adhesiveness, motility, and proteases secretion of the immortalized normal murine mammary gland cell line NMuMG through the activation of the ERK/MAPK pathway. In addition, we demonstrated that a functional kinase domain is necessary for these effects. While it has been reported that the overexpression of some classical and novel PKCs confers characteristics associated with malignant transformation to fibroblasts [49] or to mammary epithelial cells [8,29], there have been no studies addressing the role of the atypical PKCζ isoform and the functionality of its structural domains as it pertains to mammary cancer initiation and progression.

PKC enzymes are key elements of the mitogenic signaling pathway that modulate the MEK/ERK phosphorylation cascade, culminating with the activation of transcription factors associated with cell cycle regulation [16,29,43]. Contrarily to the classical and novel PKCs, it has been shown that

PKCζ activates this cascade by a mechanism independent of c-Raf1 that probably involves a direct interaction with MEK [43,50]. Although NMuMG cells overexpressing PKCζ showed a constitutive activation of the ERK/MAPK pathway, we did not find any significant shortening in the population doubling time. In contrast, other studies have reported enhanced proliferation in response to PKCζ overexpression in thyroid gland cells [40], suggesting that the effects of atypical PKCs may be cell-type dependent. Nevertheless, it is important to note that when NMuMG cells were grown under more stringent conditions such as clonal cell growth, overexpression of PKCζ conferred a significantly higher clonogenic capacity. In agreement with experimentsin other cell model [51,52], the overexpression of PKCζ was not sufficient to confer anchorage-independent growth capacity.

It is known that proteases expression, ECM remodeling, and adhesive and migratory properties play fundamental roles in the normal physiology as well as in the malignant transformation and tumor progression of the mammary gland [17,53]. Furthermore, an altered expression of proteases, a crucial event for cell invasiveness [54,55], has been associated with poor prognosis in breast cancer [18,56]. In this paper, we report that PKCζ overexpression enhanced the secretion of uPA and MMP-9, but not MMP-2 and MMP-3, by NMuMG mammary cells. In the same model, we have previously observed an increase of uPA, MMP-2, and MMP-9 secretion as consequence of a classical PKC overexpression [29]. In support of our conclusions, it has been recently reported that PMA-insensitive (atypical) PKC isozymes are key regulators of MMP-9 expression in rat glioma cells [19]. Conversely, our unpublished results show that PKCδ overexpression plays the opposite role in NMuMG cells, inhibiting the secretion of both uPA and MMP-9. Taken together, these data are consistent with the idea that individual PKC isozymes have different and even opposing functions within the same cell context [9–11].

To further analyze some of the mechanism implicated in the changes induced by PKCζ overexpression in adhesion, spreading, and migration, we have studied the expression of some cell-adhesion molecules and ECM components. Our preliminary results indicate that CD44, β1 integrin, and fibronectin seem not to be modulated by PKCζ, contrarily to our own findings in the same cell model overexpressing a classical PKC [29].

Though, at first sight, the reduced ability to migrate of NMuMG-PKCζ cells could be interpreted as a contradictory finding with the increased production of specific proteases observed in the same cells, this effect must be analyzed in the context of the complexity of molecular events and cell-cell and cell-environment interactions necessary for the expression of a fully malignant phenotype in vivo, which are probably not included in the usual, but limited, in vitro assays. In fact, preliminary experiments showed that NMuMG-PKCζ transfectants presented an increase in their capacity to invade a collagen matrix, assay that integrates both migratory and invasive capacities. Wolf et al. [17,57] have also reported apparent contradictions between protease secretion and migration, as they found that even in the absence of pericellular proteolysis, malignant mammary cells could still invade a collagen matrix due to migratory compensation strategies.

Next, an extensive mutational analysis was performed to address the function of the different PKCζ structural domains in proteases secretion. We found that the presence of an intact kinase domain was necessary and sufficient to increase uPA and MMP-9 secretion. The modulation of these two different proteolytic enzymes by PKCζ and mutants showed almost identical pattern, and only minor changes could be observed. These subtle differences may be related to unknown functional roles of PKCζ domains, as described for other PKCs, even though more biochemical and functional analysis are required to explain them.

Although the PS-cat and V3-cat mutants secreted significantly more uPA and MMP-9 than control cells, their protease secretion level was somehow lower than that of the wild-type PKCζ transfectants. This suggested that the V1 region may be relevant in the modulation of protease secretion. When this domain is absent (ZN-cat mutants) proteases secretion level is even higher than that shown by wild-type PKCζ cells. Neither MMP-2 nor MMP-3 (stromelysin 1) activities displayed any modulation in wild-type or mutant PKCζ transfectants, demonstrating the specificity of this atypical isoform on uPA and MMP-9 regulation. We speculate that these differences may be related to issues of localization due to the important roles that discrete domains in the regulatory region of PKCs play in intracellular targeting.

Our results are coincident with those of Sanz-Navarez [25] in the sense that PKCζ overexpression inhibits the migration of murine B16 melanoma cells. However, a more detailed analysis of their results revealed that, although PKCζ mutants with a functional catalytic domain were also able to inhibit migration, the highest response was attributed to theregulatory domain devoid of enzymatic activity. This discrepancy with our results, clearly showing that the PKCζ regulatory domain did not modulate migration, can be attributed to differences in the tissue origin of the cell model employed, as well as to the fact that NMuMG is a normal, though immortalized, cell line.

Our findings unambiguously revealed that the kinase domain of this atypical PKCζ is essential for the analyzed biological effects, probably through the activation of the MEK/ERK cascade. However, the activation and/or inhibition of other pathways by

PKCζ signaling is not addressed yet and our results do not rule out this possibility.

In summary, the overexpression of PKCζ or its mutants with kinase activity in mammary cells induced an enhanced expression of specific proteases, together with alterations in the adhesive, spreading, and migratory potential, and an increased capability to grow under very stringent conditions. Our results suggest that a PKCζ-dependent pathway could be critical for late events leading to an enhanced invasive and metastatic phenotype, thus revealing a novel rationale for targeting this atypical PKC for the control of breast cancer cell dissemination.

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REFERENCES

1. Lukashev ME, Werb Z. ECM signalling: Orchestrating cell behaviour and misbehaviour. Trends Cell Biol 1998;8:437–441.
2. Thomasset N, Lochter A, Sympson CJ, et al. Expression of autoactivated stromelysin-1 in mammary glands of transgenic mice leads to a reactive stroma during early development. Am J Pathol 1998;153:457–467.
3. Ossowski L, Biegel D, Reich E. Mammary plasminogen activator: Correlation with involution, hormonal modulation, and comparison between normal and neoplastic tissue. Cell 1979;16:929–940.
4. Masso-Welch PA, Verstovsek G, Ip MM. Alterations in the expression and localization of protein kinase C isoforms during mammary gland differentiation. Eur J Cell Biol 1999;78:497–510.
5. Dekker LV, Parker PJ. Protein kinase C—a question of specificity. Trends Biochem Sci 1994;19:73–77.
6. Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 1995;9:484–496.
7. Jaken S, Kiley SC. PKC: Interactions and consequences. Trends Cell Biol 1994;4:223–227.
8. Kiley SC, Clark KJ, Duddy SK, Welch DR, Jaken S. Increased protein kinase C delta in mammary tumor cells: Relationship to transformtion and metastatic progression. Oncogene 1999;18:6748–6757.
9. Blobe GC, Obeid LM, Hannun YA. Regulation of protein kinase C and role in cancer biology. Cancer Metastasis Rev 1994;13:411–431.
10. Gomez DE, Skilton G, Alonso DF, Kazanietz MG. The role of protein kinase C and novel phorbol ester receptors in tumor cell invasion and metastasis (review). Oncol Rep 1999;6: 1363–1370.
11. Ron D, Kazanietz MG. New insights into the regulation of protein kinase C and novel phorbol ester receptors. FASEB J 1999;13:1658–1676.
12. Ways DK, Kukoly CA, deVente J, et al. MCF-7 breast cancer cells transfected with protein kinase C-alpha exhibit altered expression of other protein kinase C isoforms and display a more aggressive neoplastic phenotype. J Clin Invest 1995; 95:1906–1915.
13. Tonetti DA, Chisamore MJ, Grdina W, Schurz H, Jordan VC. Stable transfection of protein kinase C alpha cDNA in hormone-dependent breast cancer cell lines. Br J Cancer 2000;83:782–791.
14. Juarez J, Clayman G, Nakajima M, et al. Role and regulation of expression of 92-kDa type-IV collagenase (MMP-9) in 2 invasive squamous-cell-carcinoma cell lines of the oral cavity. Int J Cancer 1993;55:10–18.
15. Aguirre-Ghiso JA, Alonso DF, Farias EF, Bal de Kier Joffé ED. Overproduction of urokinase-type plasminogen activator is regulated by phospholipase D and protein kinase C-dependent pathways in murine mammary adenocarcinoma cells. BBA 1997;1356:171–184.
16. Aguirre Ghiso JA, Alonso DF, Farias EF, Gomez DE, Bal de Kier Joffé ED. Deregulation of the signaling pathways controlling urokinase production. Its relationship with the invasive phenotype. Eur J Biochem 1999;263:295–304.
17. Egeblad M, Werb Z. New functions for matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002;2: 161–174.
18. Andreasen PA, Kjoller L, Christensen L, Duffy MJ. The urokinase-type plasminogen activator system in cancer metastasis: A review. Int J Cancer 1997;72:1–22.
19. Esteve PO, Chicoine E, Robledo O, et al. Protein kinase C-zeta regulates transcription of the matrix metalloproteinase-9 gene induced by IL-1 and TNF-alpha in glioma cells via NF-kappa B. J Biol Chem 2002;277:35150–35155.
20. Langzam L, Koren R, Gal R, et al. Patterns of protein kinase C isoenzyme expression in transitional cell carcinoma of bladder. Relation to degree of malignancy. Am J Clin Pathol 2001;116:377–385.
21. Tsai JH, Hsieh YS, Kuo SJ, et al. Alteration in the expression of protein kinase C isoforms in human hepatocellular carcinoma. Cancer Lett 2000;161:171–175.
22. Cornford P, Evans J, Dodson A, et al. Protein kinase C isoenzyme patterns characteristically modulated in early prostate cancer. Am J Pathol 1999;154:137–144.
23. Selzer E, Okamoto I, Lucas T, Kodym R, Pehamberger H, Jansen B. Protein kinase C isoforms in normal and transformed cells of the melanocytic lineage. Melanoma Res 2002;12:201–209.
24. Laudanna C, Sorio C, Tecchio C, et al. Motility analysis of pancreatic adenocarcinoma cells reveals a role for the atypical zeta isoform of protein kinase C in cancer cell movement. Lab Invest 2003;83:1155–1163.
25. Sanz-Navares E, Fernandez N, Kazanietz MG, Rotenberg SA. Atypical protein kinase Czeta suppresses migration of mouse melanoma cells. Cell Growth Differ 2001;12:517–524.
26. Schondorf T, Kurbacher CM, Becker M, Warm M, Kolhagen H, Gohring UJ. Heterogeneity of proteinkinase C activity and PKC-zeta expression in clinical breast carcinomas. Clin Exp Med 2001;1:1–8.
27. Van den BC, Vleminckx K, De Bruyne G, et al. Morphotypic plasticity in vitro and in nude mice of epithelial mouse mammary cells (NMuMG) displaying an epithelioid (e) or a fibroblastic (f) morphotype in culture. Clin Exp Metastasis 1996;14:282–296.
28. Bandyopadhyay A, Cibull ML, Sun LZ. Isolation and characterization of a spontaneously transformed malignant mouse mammary epithelial cell line in culture. Carcinogenesis 1998; 19:1907–1911.
29. Mazzoni E, Adam A, Bal de Kier Joffé ED, Aguirre-Ghiso JA. Immortalized mammary epithelial cells overexpressing protein kinase C gamma acquire a malignant phenotype and become tumorigenic in vivo. Mol Cancer Res 2003;1: 776–787.
30. Owens RB. Glandular epithelial cells from mice: A method for selective cultivation. J Natl Cancer Inst 1974;52:1375–1378.
31. Olah Z, Lehel C, Jakab G, Anderson WB. A cloning and epsilon-epitope-tagging insert for the expression of polymerase chain reaction-generated cDNA fragments in *Escherichia coli* and mammalian cells. Anal Biochem 1994; 221:94–102.

32. Urtreger AJ, Diament MJ, Ranuncolo SM, et al. New murine cell line derived from a spontaneous lung tumor induces paraneoplastic syndromes. Int J Oncol 2001;18:639–647.
33. Aguirre Ghiso JA, Farias EF, Alonso DF, Arregui C, Bal de Kier Joffé ED. A phospholipase D and protein kinase C inhibitor blocks the spreading of murine mammary adenocarcinoma cells altering f-actin and beta1-integrin point contact distribution. Int J Cancer 1997;71:881–890.
34. Urtreger AJ, Aguirre Ghiso JA, Werbajh SE, Puricelli LI, Muro AF, Bal de Kier Joffé ED. Involvement of fibronectin in the regulation of urokinase production and binding in murine mammary tumor cells. Int J Cancer 1999;82:748–753.
35. Alonso DF, Farias EF, Bal de Kier Joffé ED. Impairment of fibrinolysis during the growth of two murine mammary adenocarcinomas. Cancer Lett 1993;70:181–187.
36. Alonso DF, Farias EF, Ladeda VE, Davel L, Puricelli LI, Bal de Kier Joffé ED. Effects of synthetic urokinase inhibitors on local invasion and metastasis in a murine mammary tumor model. Breast Cancer Res Treat 1996;40:209–223.
37. Mackay AR, Ballin M, Pelina MD, et al. Effect of phorbol ester and cytokines on matrix metalloproteinase and tissue inhibitor of metalloproteinase expression in tumor and normal cell lines. Invasion Metastasis 1992;12:168–184.
38. Aguirre Ghiso JA, Kovalski K, Ossowski L. Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. J Cell Biol 1999;147:89–104.
39. Simian M, Hirai Y, Navre M, Werb Z, Lochter A, Bissell MJ. The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. Development 2001;128:3117–3131.
40. Fernandez N, Caloca MJ, Prendergast GV, Meinkoth JL, Kazanietz MG. Atypical protein kinase C-zeta stimulates thyrotropin-independent proliferation in rat thyroid cells. Endocrinology 2000;141:146–152.
41. Berra E, Diaz-Meco MT, Dominguez I, et al. Protein kinase C zeta isoform is critical for mitogenic signal transduction. Cell 1993;74:555–563.
42. Burgering BM, Bos JL. Regulation of Ras-mediated signalling: More than one way to skin a cat. Trends Biochem Sci 1995; 20:18–22.
43. Schonwasser DC, Marais RM, Marshall CJ, Parker PJ. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Mol Cell Biol 1998;18:790–798.
44. Miranti CK, Ohno S, Brugge JS. Protein kinase C regulates integrin-induced activation of the extracellular regulated kinase pathway upstream of Shc. J Biol Chem 1999;274: 10571–10581.
45. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
46. Acs P, Wang QJ, Bogi K, et al. Both the catalytic and regulatory domains of protein kinase C chimeras modulate the proliferative properties of NIH 3T3 cells. J Biol Chem 1997;272:28793–28799.
47. Wang QJ, Acs P, Goodnight J, Blumberg PM, Mischak H, Mushinski JF. The catalytic domain of PKC-epsilon, in reciprocal PKC-delta and -epsilon chimeras, is responsible for conferring tumorgenicity to NIH3T3 cells, whereas both regulatory and catalytic domains of PKC-epsilon contribute to in vitro transformation. Oncogene 1998;16:53–60.
48. Kahn SM, O'Driscoll KR, Jiang W, et al. Suppression of mitogenic activity by stable expression of the regulatory domain of PKC beta. Carcinogenesis 1994;15:2919–2925.
49. Persons DA, Owen RD, Ostrowski MC, Finn OJ. Protein kinase C gamma expression mimics phorbol ester-induced transcriptional activation of a murine VL30 enhancer element. Cell Growth Differ 1991;2:7–14.
50. Monick MM, Carter AB, Flaherty DM, Peterson MW, Hunninghake GW. Protein kinase C zeta plays a central role in activation of the p42/44 mitogen-activated protein kinase by endotoxin in alveolar macrophages. J Immunol 2000;165: 4632–4639.
51. Crespo P, Mischak H, Gutkind JS. Overexpression of mammalian protein kinase C-zeta does not affect the growth characteristics of NIH 3T3 cells. Biochem Biophys Res Commun 1995;213:266–272.
52. Montaner S, Ramos A, Perona R, Esteve P, Carnero A, Lacal JC. Overexpression of PKC zeta in NIH3T3 cells does not induce cell transformation nor tumorigenicity and does not alter NF kappa B activity. Oncogene 1995;10:2213–2220.
53. Witty JP, Wright JH, Matrisian LM. Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. Mol Biol Cell 1995;6:1287–1303.
54. Ossowski L, Reich E. Antibodies to plasminogen activator inhibit human tumor metastasis. Cell 1983;35:611–619.
55. Westermarck J, Kahari VM. Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J 1999;13: 781–792.
56. Ranuncolo SM, Armanasco E, Cresta C, Bal de Kier Joffé ED, Puricelli L. Plasma MMP-9 (92 kDa-MMP) activity is useful in the follow-up and in the assessment of prognosis in breast cancer patients. Int J Cancer 2003;20:745–751.
57. Wolf K, Mazo I, Leung H, et al. Compensation mechanism in tumor cell migration: Mesenchymal-amoeboid transition after blocking of pericellular proteolysis. J Cell Biol 2003;160: 267–277.

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