Title: Tumor Suppressing Effects of Protein Kinase C Delta

By

Desmond N Jackson

A dissertation submitted to the Graduate Faculty in Biology in Partial fulfillment of the requirements for the degree of Doctor of Philosophy
The City University of New York

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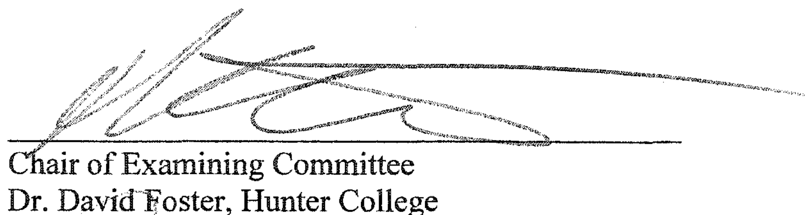

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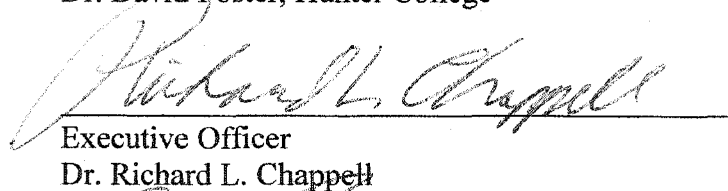

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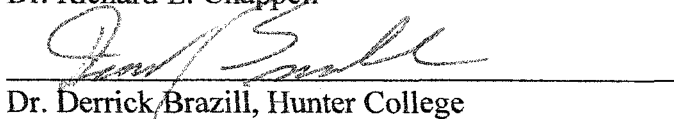

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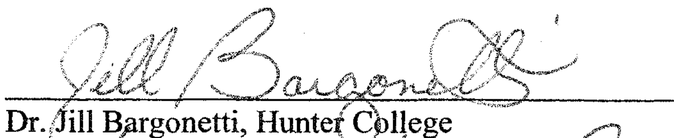

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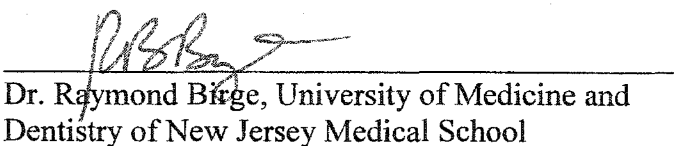

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Abstract

Tumor Suppressing effects of Protein Kinase C Delta
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Protein Kinase Cδ (PKCδ) has been implicated both as a tumor suppressor and as a positive regulator of cell cycle progression. PKCδ has also been shown to both negatively and positively regulate apoptotic programs. To further complicate the picture, PKCδ has been shown to be involved in the promotion of migration of some cells. These studies have led to conflicting hypotheses regarding the role of PKCδ in tumorigenesis. In an attempt to clarify the mechanism by which PKC δ might influence such diverse processes, we employed phorbol esters, which have been shown to down regulate PKC isoforms upon prolonged exposure. Using this approach, we show that treatment of MCF-7 cells with PMA depleted these cells of PKCδ. Further, PMA induced multiple effects including secretion of Matrix Metalloproteinase-9 (MMP-9), up-regulation of Extracellular Matrix Metalloproteinase Inhibitor (EMMPRIN), activation of c-Src and up-regulation of the level of the Epidermal Growth Factor Receptor (EGFR). Treatment of MCF-7 cells with PMA also induced migration in a Boyden Chamber Assay, with

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kinetics that was consistent with depletion of PKCδ. Further, Bryostatin-1 a compound shown to retain PKCδ in cells blocked the PMA induced migration. Co-treatment of MCF-7 cells with PMA and Bryostatin-1 blocked the PMA induced activation of c-Src and induction of EGFR protein level. Mouse Embryo Fibroblasts from PKCδ null mice, migrate at a rate that is three fold higher than that of their wild type counterparts. These data taken together suggest a role for PKCδ in the inhibition of migration and metastasis in breast cancer cell lines.

PKC δ has been implicated in proliferation and survival of breast cancer cells, often with conflicting results; we investigated the role of PKCδ in these processes in MDA-MB-231 cells. We found that MDA-MB-231 cells subjected to growth restrictive conditions and Rapamycin underwent apoptosis. Transforming Growth Factor Beta-1 (TGFβ1) but not Insulin- like Growth factor-1 (IGF-1) inhibited rapamycin induced apoptosis in MDA-MB-231 cells. To implicate PKCδ , we used siRNA mediated knockdown of PKCδ. Transfection of MDA-MB-231 cells with PKCδ siRNA restored the rapamycin induced cell death in the presence of TGF- β1. Therefore, PKCδ mediated TGF-β1 blockade of apoptosis by a Smad dependent induction of p21.

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Chapter I

1.1 General Introduction

The American Cancer Society estimates that in 2004, approximately 215,990 women in the United States will be diagnosed with invasive breast cancer. Another 59,309 women will be diagnosed with carcinoma in situ. Of these, approximately 40,000 will die from the disease. Various early detection methods have dramatically lowered the mortality associated with development of breast cancer; however, an understanding of the molecular mechanisms underlying the development of breast cancer is essential if we are to eradicate this disease. Cancer development is progressive, requiring the stepwise accumulation of genetic lesions. The cancer cell must acquire the ability to circumvent tightly controlled restrictions to deregulated growth and survival observed by their normal counterparts. Involved in cancer development is the complex interplay of signal transduction pathways consisting of oncogenes and tumor suppressor genes. It is the deregulation of these pathways that allow the cancer cell to circumvent normal constraints. Alterations in PKC expression have been associated with changes in tumorigenicity and metastatic potential (Blobe et al., 1993). O'Brian et al showed increased expression of PKC in breast tumors compared to normal breast tissue (O'Brian et al., 1989). Further, human breast cancer cell lines show an inverse correlation between PKC expression and the estrogen receptor (Wyss et al., 1987). The current thesis is a contribution to the understanding of the mechanism whereby Protein Kinase Cδ (PKCδ) signaling is involved in the growth and survival of breast cancer.

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1.2 PKC Family

Protein Kinase C (PKC) refers to a multigene family of serine-threonine kinases consisting of approximately 11 isoforms (Nishizuka, 1988). Differences in structural and enzymatic properties of PKC isoforms allow classification into three main groups that share common requirements for phospholipids, but differ in their dependency on other cofactors (Ohno et al., 1988). All PKC isoforms consist of single polypeptide chains containing conserved (c), variable (v) domains, a regulatory and a catalytic domain. Conventional PKCs (cPKC,α, β, γ) are responsive to Ca^{2+} and diacylglycerol (DAG). The C2 region of cPKC mediates the dependence on Ca^{2+}. Novel PKCs (nPKC,δ,ε,η, θ) do not contain the C2 region, and are therefore Ca^{2+} independent. Atypical PKCs (aPKCζ,ι,λ) are both Ca^{2+} and DAG independent, further, aPKCs do not bind phorbol esters (Goodnight et al., 1995) . PKC is regulated by two sequential and equally important mechanisms: 1) interaction of hormones, growth factors, and mitogens with their cell surface receptors, leading to the hydrolysis of inositol phospholipids into diacylglycerol (DAG) and inositol triphosphate by phospholipase C (Newton, 2003). DAG and Ca^{2+} are second messengers that together with phosphotydylserine (PS) activate PKC, and 2) phosphorylation triggered by upstream kinase(s) including PDK-1 and mTOR (Parekh et al., 1999; Sonnenburg et al., 2001). Upon activation, PKC phosphorylates proteins on serine and threonine residues thereby eliciting a wide variety of responses including cell growth, differentiation, tumor promotion and apoptosis. PKC generated immense interest when it was realized that they were the major high affinity intracellular receptor for tumor promoting phorbol esters (Kikkawa et al.,

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1983). This finding showed that PKC played an essential role in the regulation of proliferation in cells.

1.3 Protein Kinase Cδ

PKCδ, a member of the nPKC group, is ubiquitously expressed in many cells and tissues, suggesting a universal function (Livneh and Fishman, 1997). PKCδ is regulated by signaling inputs from PDK-1 and mTOR although the functional significance of these inputs is unclear. An increasing number of reports have implicated PKCδ in growth suppression, tumor promotion and apoptosis (for review see (Jackson and Foster, 2004; Kikkawa et al., 2002). Emerging data from several studies implicate PKCδ in either negative or positive regulation of the G1/S transition. In vascular endothelial cells, treatment with TPA late in G1 inhibited DNA synthesis, while it stimulated passage through G1/S when added early in G1. This regulation occurs via alterations in expression of cyclins and/or cyclin dependent kinase inhibitors, and modulation of the activity of specific cyclin-CDK complex (Fukumoto et al., 1997). For example, PKCδ was found to inhibit the G1/S transition in capillary endothelial cells by inducing the CDK inhibitor $p27^{kip1}$ (Harrington et al., 1997). This picture was further complicated by the finding that PKCδ activity may also regulate the cell cycle at the G2/M transition. Watanabe et al. showed that over-expression of PKCδ in CHO cells resulted in the accumulation of cells in G2/M, in response to phorbol ester treatment (Watanabe et al., 1992). Although the vast majority of studies reported associate PKCδ in negative regulation of proliferation; there are reports that PKCδ activity is correlated with increased proliferation. For instance, PKCδ was required for insulin-like growth factor-1

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(IGF-1) receptor mediated transformation (Li et al., 1998). Consistent with a role for PKCδ in IGF-1 signaling, the von-Hippel-Lindau tumor suppressor pVHL interacted directly with PKCδ in renal cancer cell lines and inhibited a required association between the IGF-1 receptor and PKCδ for downstream signaling (Pal et al., 1997). A positive role for PKCδ in cell transformation was also demonstrated by (Liao et al., 1994), who showed increased levels of PKCδ in progressively transformed rat embryo fibroblasts. Further, a dominant negative mutant of PKCδ inhibited anchorage independent growth of these cells. A positive correlation was also noted between PKCδ expression and metastatic potential. In a series of experiments, (Kiley et al., 1999a) correlated increased expression of PKCδ with low metastatic potential with progression to a more malignant phenotype. More recent work by several groups has linked PKCδ signaling to the secretion of Matrix Metalloproteinase (MMPs) from human cancer cell lines (Liu et al., 2002). MMPs are thought enhance metastatic progression by the degradation of basement membranes thereby facilitating migration and invasion of tumor cells.

In contrast to the above mentioned studies, we have previously demonstrated that in 3Y1 rat fibroblasts over-expressing either c-src (Lu et al., 1997) or the EGFR (Hornia et al., 1999), dominant negative PKCδ or the PKCδ specific inhibitor rottlerin promoted anchorage independent growth. These results are consistent with the notion that the transforming effect of phorbol esters was due to down-regulation of PKCδ.

Preliminary data generated in our laboratory suggest a model in which over-expression of c-Src or the EGF receptor sensitizes the cell for division, however cell division is prevented by PKCδ. Since tyrosine kinase over-expression is not sufficient to transform normal cells, one implication of this model is that compounds capable of

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stimulating cell division in partially transformed cells may do so by down-regulating PKCδ. That is, down-regulation of PKCδ could play a significant role in the promotion phase of tumor progression in cells over-expressing a tyrosine kinase. In a model proposed several years ago (Land et al., 1983), signaling oncogenes such as Ras or Src cooperate with Myc or large T antigen to transform primary cells. Studies into the tumor promoting effects of phorbol esters showed that TPA complimented the signaling oncogenes, but not Myc (Dotto et al., 1985). Hence, down-regulation of PKCδ could substitute for either Myc or large T antigen in this model. Cells in the resting stage of the cell cycle require "competence" and "progression" factors in order to exit this stage and traverse G1 (Pardee, 1974). In our model system, c-Src is postulated to be the competence factor that prevents entry into, or exit from G_o, and PKCδ is the progression factor that allows passage through G_1. Hence we propose that PKCδ is a tumor suppressor that is capable of inhibiting passage through the G_1/S checkpoint. This hypothesis is strongly supported by our earlier observation that down-regulation of PKCδ inhibited the basal transcription of the tumor suppressor p53 in a variety of human cancer cell lines that expressed wild type p53, but not in cells lacking p53(Abbas et al., 2004). These findings attest to a tumor suppressive role for PKCδ in human cancer.

The following thesis describes experiments that sought to investigate the role of PKCδ in tumor progression and test the hypothesis that PKCδ is a tumor suppressor that contributes to tumor promotion when down-regulated.

Cell migration plays a central role in a wide variety of processes including embryogenesis and breast development. Migration of fibroblasts and vascular endothelial cells is also essential for wound healing. During metastasis, tumor cells

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migrate from the primary mass into the circulation and subsequently into a new site. Cell migration is thought to be influenced by several signal transduction pathways including the MAP-Kinase, the Ras/Raf and the PI3K/Akt pathways. These pathways are also involved in survival of cancer cells, and an interesting correlation has been shown between migration and survival signaling. Migration involves the continual remodeling of the actin cytoskeleton, in a complex interplay of adhesion and release, over and over again.

PKCδ has been shown to play a role in cell migration, albeit a controversial one. The phorbol ester TPA has been shown to induce migration in several cancer cell lines. In the breast cancer line MCF-7, TPA induced a PKC dependent migration. Bryostatin-1, a weak tumor promoter, is able to antagonize the TPA induced migration.

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Chapter II

Materials and Methods

2.1. Materials

[^{3}H] myristate was obtained from New England Nuclear (NEN). The PKC inhibitors Rottlerin and Go6976 were obtained from Calbiochem. Bryostatin-1, Bistratene A and Rapamycin were obtained from Sigma. TGF-β1 was obtained from R&D systems. Blocking antibodies to TGF-β1 was purchased from R&D systems. All antibodies used in this work were obtained from Santa Cruz Biotechnology except phospho-specific antibodies which were obtained from Cell Signaling.

2.2. Cells and cell culture conditions

All cell lines used in this study were obtained from the American Tissue Type Culture Collection. MCF-7 and MDA-MD-231 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Bovine Calf Serum (Hyclone). BT-549 cells were maintained in RPMI 1640(Gibco) supplemented with 10% Fetal Bovine Serum (FBS). Mouse Embryo Fibroblasts (MEFs) from wild type and PKCδ knockout mice were prepared from 14-17 day old embryos. MEFs were maintained in DMEM supplemented with 10% Fetal Bovine Serum (FBS). MEFs were used for experiment prior to passage five.

2.3. Transfections

Cells were plated at a density of 10^5 cells/60mm plate 24hr prior to transfection. All transfections were performed using lipofectamine 2000 (Gibco) according to the

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manufacturer's instructions. MCF-7δDN cells were made by transfecting MCF-7 cells with a plasmid containing a dominant negative PKCδ. Cultures were selected with neomycin (G418) at 400μg/ml for 14 days at 37°C. Antibiotic resistant colonies were picked and expanded for further analysis under selective conditions.

siRNA against PKC δ was obtained from Qiagen. Cells were transfected with 2μg of siRNA using RNAfect (Qiagen), according to the manufacturer's instructions.

2.4. Immunoassays

Proteins were extracted from cultured cells in modified RIPA buffer (Upstate). Equal amounts of proteins were subjected to SDS-PAGE on poly acrylamide separating gels. Electrophoresed proteins were then transferred to nitrocellulose. After transfer, membranes were blocked in an isotonic solution containing 5% non-fat dry milk in Phosphate Buffered Saline (PBS). The membranes were then incubated with primary antibodies as described in the text. Depending on the origin of the primary antibody, either anti-mouse or anti-rabbit HRP conjugated IgG was used for detection using ECL system (Pierce).

2.5. Zymography

Cells were plated in complete growth media in 60mM plates and allowed to reach 90% confluence. The cells were then shifted to serum free medium containing 1% Bovine Serum Albumin and drug treated overnight. Conditioned media was then collected and an amount normalized to cell number was combined with loading buffer under non-reducing conditions and electrophoresed on a 10% gel containing 1% gelatin (Invitrogen). After the run, the gel was incubated in zymogen renaturation buffer for 30

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minutes at room temperature. The gel was then placed in developing buffer and incubated overnight at 37°C. Following the incubation the gel was stained in commassie blue solution for 30 minutes and then distained.

2.6. Cell Viability and Apoptosis assays

Cell viability was determined by trypan blue exclusion. Cells were placed in serum free medium and treated as described in the figure legends. Twenty four hours later, cells were harvested, washed and treated with trypan blue at a concentration of 0.4% v/v. After 10 min, trypan blue uptake (dead cells), was scored using a hemocytometer. The percentage of apoptotic cells was determined by FACS analysis of cells stained with propidium iodide.

2.7. Migration Assays

Cell migration assays were performed using the modified Boyden chambers, consisting of cell culture inserts containing .8μm pores (BD Biosciences). Cells growing in complete growth media were trypsinized and counted using a hemocytometer. Cells were resuspended in migration medium (DMEN supplemented with .5% BSA), in the presence or absence of drug as indicated in figure legends. 15,000 cells were added to the upper chamber, in .5mL of migration medium. Complete growth medium was added to the lower chamber as a chemoattractant. Treated plates were returned to a 37°C incubator for 16hr. After incubation, cells remaining in the upper chamber were removed using a cotton swab. Those cells that migrated to the underside of the insert were fixed and stained using the Diff-Quik system (Dade Behring cat# B4132-1A), and nuclei photographed and counted using light microscope.

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Chapter III

Enhancement of Metastasis Associated Phenotypes by Phorbol Ester

Introduction

PKC δ has been implicated in cell growth, differentiation and apoptosis, but its role in migration and metastasis remains controversial. The tumor promoting phorbol ester PMA has been shown to enhance metastasis associated phenotypes in some cell types (Park et al., 2003); (Park et al., 2000). Results generated in our laboratory, (Hornia et al., 1999; Lu et al., 1997; Zhong et al., 2002), suggested that the tumor promoting effects of phorbol esters was as a consequence of the down-regulation of PKCδ. These studies implied that phorbol esters mediated transformation by eliminating a tumor suppressing effect of PKCδ. This conclusion was supported by subsequent studies in mice engineered to express elevated PKC δ in their skin. Such mice were resistant to the tumor promoting effects of phorbol esters (Reddig et al., 1999). Other studies have also suggested negative effects for PKCδ on cell proliferation (Acs et al., 1997; Mischak et al., 1993), and PKCδ was recently reported to negatively regulate B-cell proliferation in a transgenic mouse model system (Miyamoto et al., 2002). PKCδ was recently reported to respond to DNA damage by activating the SAPK/JNK signaling pathway (Kim et al., 2002), which negatively regulates cell proliferation. Collectively, these data are consistent with PKCδ having a tumor suppressing effect that involves the suppression of cell proliferation.

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Down-regulation of PKCδ also prevented apoptosis in cells deprived of growth factors (Zhong et al., 2002), indicating that down-regulating PKCδ can lead to the generation of survival signals. Interestingly, there is a correlation between survival signaling and cell migration (Di Cristofano and Pandolfi, 2000). Inhibition of the PI3K/Akt survival pathway blocks cell migration (Silva et al., 2002; Sugatani et al., 2003). Since down-regulating PKCδ provides survival signals, it is possible that down-regulating PKCδ enhances cell migration and invasion. In this report, we have investigated the effect of PKCδ on cell migration in the human breast cancer cell line MCF-7.

Results

3.1. Treatment of MCF-7 cells with PMA elicits secretion of MMP-9

Elevated Matrix Metalloproteinase-9 (MMP-9) expression has been correlated with increased metastatic potential utilizing in vivo models of melanoma (Chakraborti et al., 2003) and prostate cancers (Itoh et al., 1999). Further, mice lacking expression of MMP-9 show suppression of experimental metastasis (Biswas et al., 1995). Previous studies indicated a role for PKC in the secretion of MMP-9 in several systems, including breast and ovarian cells (Ellerbroek et al., 2001; Johnson et al., 1999) (Esteve et al., 2002). To begin to dissect the role of PKCδ in the secretion of MMP-9, we treated MCF-7 breast cancer cells with the phorbol ester PMA. Conditioned medium was collected and assayed by zymography. As demonstrated in figure 1A, treatment of MCF-7 cells with PMA induced secretion of MMP-9 from these cells. Purified MMP-9 was used as a positive control (A). Kinetic analysis of the PMA-induced release of MMP-9 shows that

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this proteinase was detectable in the medium at approximately eight hours post treatment figure 1B.

A

←MMP-9

TPA - + Purified MMP-9

B

Time (hr)

TPA (100nM) **0 2 4 8 12 24**

←MMP-9

Figure 1. PMA induces secretion of MMP-9 from MCF-7 cells.

(A) MCF-7 cells were seeded in DMEM supplemented with 10% FCS. 24 hours later, the cells were shifted to DMEM supplemented with .5% BSA, in the presence or absence of 100nM PMA. A volume of conditioned medium normalized to cell number was loaded into a 10 % gel containing .1% gelatin, and assayed by zymography as detailed in materials and methods. (B), kinetic analysis of PMA induced MMP-9 secretion from MCF-7 cells. Cells were prepared as indicated in A, and conditioned medium collected at the time points indicated in the figure.

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3.2. PMA up-regulates the expression of EMMPRIN on the surface of MCF-7 cells.

It is well established that most of the MMP-9 secreted during cancer progression is secreted by the surrounding stroma (Reimers et al., 2004). Consequently, we looked for molecules that were capable of inducing secretion of MMPs. One such molecule is Extracellular Matrix Metalloproteinase Inducer (EMMPRIN). EMMPRIN has been shown to induce secretion of several MMPs from various cell types in the tumor microenvironment (Langzam et al., 2001). As shown in figure 2A, treatment of MCF-7 cells with PMA led to the up-regulation of EMMPRIN on the surface of MCF-7 cells. Because the cell line A431 cells were shown to over-express EMMPRIN (Toole, 2003), we used whole cell A431 cell lysate as a positive control for EMMPRIN in these experiments, figure (2A). EMMPRIN is a glycosylated protein; the degree of glycosylation is correlated with the ability to induce secretion of MMPs. Further, it was shown that deglycosylated EMMPRIN was unable to induce secretion of MMPs (Sun and Hemler, 2001). Figure 2B shows the time dependent increase in the upregulation of EMMPRIN in response to PMA. Multiple bands observed in this western blot are consistent with an increase in glycosylated species. Interestingly, the kinetics of EMMPIN expression paralleled that of MMP-9 secretion from these cells, figure 2B. These data imply that EMMPRIN is responsive to PMA, and as such may be regulated by PKC to induce MMP secretion from cancer cells and the reactive stroma.

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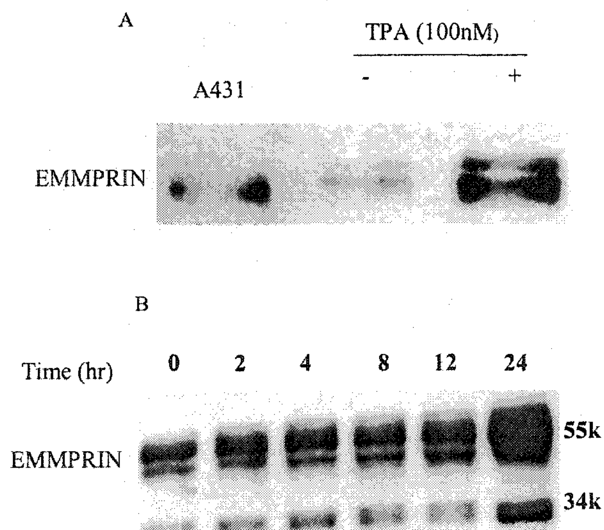

Figure 2. PMA Enhanced Expression of EMMPRIN on the surface of MCF-7 cells.

(A) Cells were seeded in growth medium and allowed to reach 90% confluence. The cells were then switched to fresh growth medium and treated with or without PMA for 24 hours. Whole cell lysates were collected and assayed by western blot, using antibodies to EMMPRIN. A431 cell lysate was used as a positive control for EMMPRIM. (B) Cells were maintained as above, after 24 hours cells were treated with PMA for the indicated times. Lysates were collected in RIPA buffer and analyzed by western blotting.

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3.3. Migratory activity of MCF-7 cells is stimulated by PMA

Cell migration has been implicated in the progression to a more metastatic phenotype. Cell migration requires the complex interplay between adhesion release and force generation, and is dependent on actin. Migration therefore necessitates dramatic morphological and cytoskeletal changes. Since both PMA and secretion of MMP-9 are implicated in promoting migration in various experimental systems, we asked whether or not treatment of MCF-7 cells with PMA affected their ability to migrate through matrigel. Shown on the left in figure 3A, is a representative field of stained nuclei of MCF-7 cells that were treated with or without PMA. Treatment MCF-7 cells with PMA induced a five fold increase in the migration cells compared to untreated cells in the modified Boyden chamber assay. These results are quantified in (B).

These results along with those obtained in the previous figures suggest a role for PKC signaling in promoting metastatic phenotypes in the poorly invasive MCF-7 breast cancer cells. Further, these seemingly distinct phenotypes may be regulated by a single pathway.

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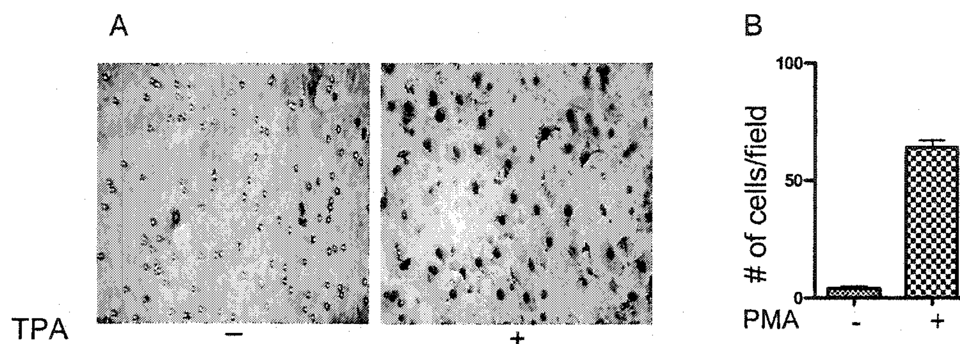

Figure 3. PMA induces migration of MCF-7 cells.
MCF-7 cells were maintained in DMEM supplemented with 10% BCS. In preparation for the experiment, cells were trypsinized and counted using a hemocytometer. Cells were then resuspended in migration medium (DMEM supplemented with .5% BSA), in the presence or absence of PMA (100nM) and 15,000 cells added to the upper well of the boyden chamber. Growth medium was added to the lower chamber as a chemo-attractant. Treated plates were then incubated at 37° C for 16 hours. Cells that had not moved to the bottom of the well were removed with a cotton swab. Those cells that had migrated through the membrane were fixed and stained. Nuclei were then counted using a light microscope; nuclei were visible as purple dots as illustrated in (A). The results obtained in (A) are quantified in (B), The data presented are representative of experiments repeated three times.

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3.4. Metastatic Phenotypes Induced by PMA are dependent upon down-regulation of PKCδ

Previous results generated in our laboratory demonstrated that down-regulation of PKCδ conspired with over-expression of c-Src to transform rat fibroblasts. Further, the macrocyclic lactone bryostatin-1, which shares many of the properties of PMA, and antagonizes those it does not possess, abrogated PMA induced transformation through the retention of PKC δ in cells (Lu et al., 1997; Szallasi et al., 1994). To begin to examine the role of PKCδ in the metastasis associated phenotypes induced by PMA in MCF-7 cells, we first established the conditions under which PMA down-regulated PKCδ in these cells. As shown in figure 4A, PMA begins to down-regulate PKCδ after 8 hours of treatment. Maximal degradation of PKCδ was evident at 24 hours post treatment. It is interesting to note that these kinetics parallel those of the appearance of MMP-9 secretion shown in figure 1. Further, prolonged treatment of MCF-7 cells with PMA failed to down regulate PKCα or PKCη, indeed, there appears to be an increase in the expression and activity of PKCα upon PMA treatment of MCF-7 cells, Figure 4A. In the presence of bryostatin-1 however, these PKC isoforms were rapidly down-regulated, figure 4B. These data suggests that retention of PKCδ in these cells by bryostatin-1 could inhibit the observed PMA induced phenotypes. The results presented here indicate that modulation of one isoform of PKC in these cells may influence the regulation of other isoforms, as in the case of PKCα and PKCδ, figure 4A.

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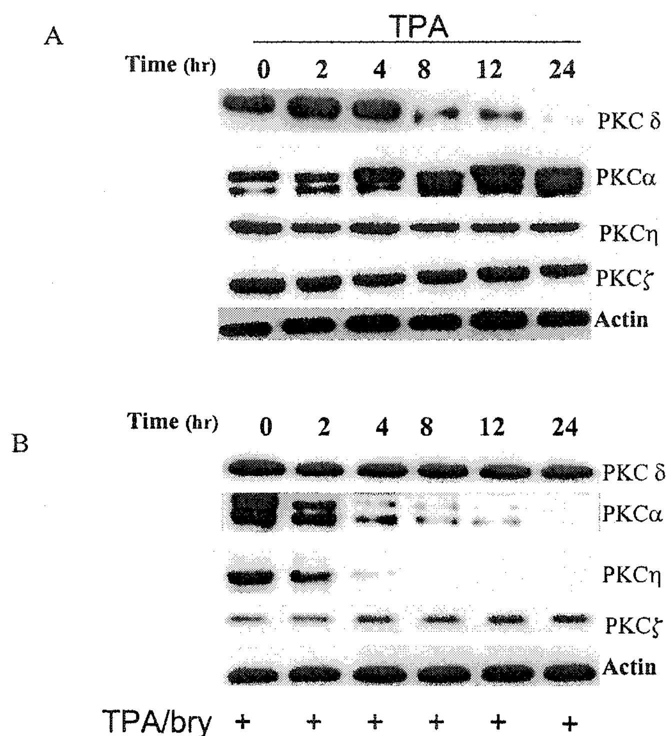

Figure 4. Prolonged treatment of MCF-7 cells with PMA down-regulates PKCδ

MCF-7 cells were treated with PMA as indicated in the figure. (A), whole cell lysates were collected at the indicated times and equal amounts of protein electrophoresed on a 10% polyacyrilamide gel. The expression levels of the major PKC isoforms expressed in MCF-7 cells were analyzed using isoform specific antibodies. (B), sub-confluent plates of cells were treated with the combination of bryostatin-1 and PMA (100nM each) whole cell lysates were collected and analyzed by western blot.

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3.5. Bryostatin-1Abrogates the PMA induced metastatic phenotypes

To further dissect the role of PKCδ in the PMA induced metastatic phenotypes in MCF-7 cells we treated cells with bryostatin-1, either alone or in combination with PMA. As expected, bryostatin-1 was able to block the PMA induced secretion of MMP-9 figure (5A), and up-regulation of EMMPRIN (5B). Treatment of MCF-7 cells with the combination of PMA and bryostatin-1 also abrogated PMA induced migration (5C). Figure (5D) shows retention of PKCδ by bryostatin-1. The suggestion here is that the PKC isoform responsible for the observed effects is PKCδ, since this was the only isoform afforded protection from down-regulation by bryostatin-1. These data further supports the hypothesis that down-regulation of PKCδ enhanced phenotypes associated with progression to a more metastatic state. Alternatively, expression of PKCδ was necessary to inhibit the observed phenotypes.

Because drugs can have multiple effects on cells, we further tested the ability of PKCδ to mediate the observed phenotypes by using a dominant negative mutant of PKCδ this mutant contains substitutions in the kinase domain that results in lack of activity. MCF-7 cells were transfected with this dominant negative mutant in duplicate using lipofectamine 2000 according to the manufacturer's instructions. Cells were then selected in G418 for 14 days after which colonies were pooled and used for experiment. In figure (6A), we present western blot analysis of MCF-7 cells transfected with or without a dominant negative mutant of PKCδ. As evident in (6A), the dominant negative mutant enhanced the expression of EMMPRIN in MCF-7 cells. Interestingly, there

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appears to be dosage effects of the dominant negative mutant and expression of EMMPRIN, figure 6A. To further assay the role of PKCδ in phenotypes induced by PMA, conditioned medium was collected from cells growing in serum free medium and analyzed by zymography. We observed secretion of MMP-9 upon transfection of MCF-7 cells with the dominant negative mutant of PKCδ, figure (6B). These data along with the results presented in figure 5, strongly implicate down-regulation of PKCδ in promoting tumorigenesis.

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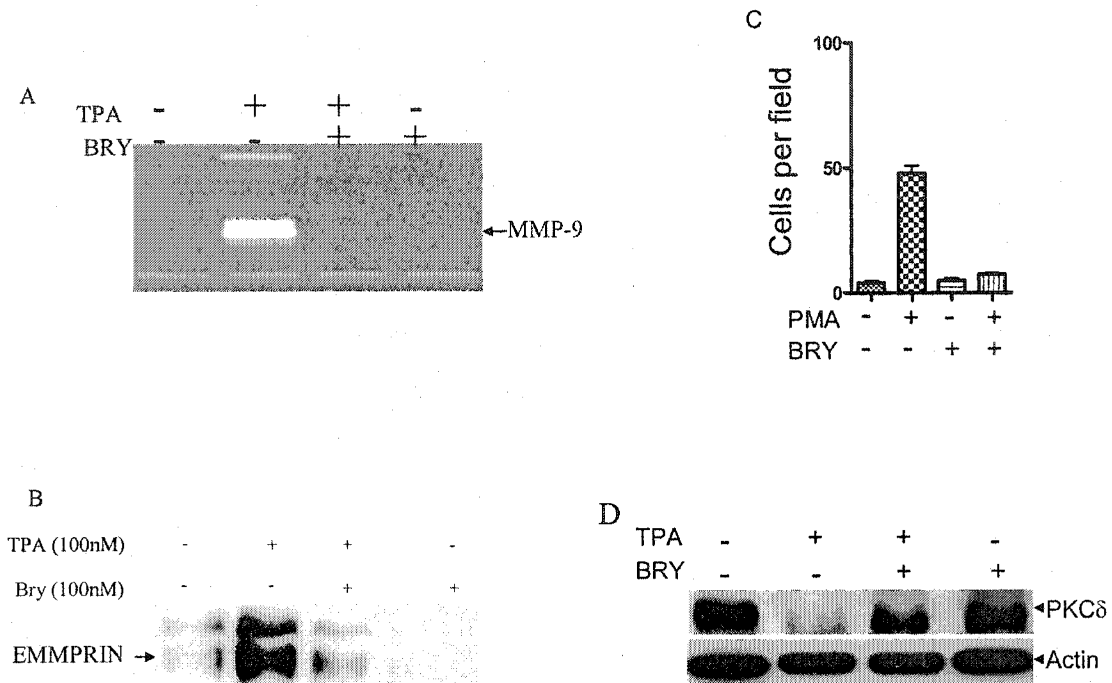

Figure 5. Bryostatin-1 Abrogates PMA-induced Metastatic Phenotypes

MCF-7 cells were treated with PMA and bryostatin-1, either alone or in combination. A, Conditioned medium was collected after a 24 hr incubation period. A volume of conditioned medium normalized to cell number was analyzed by zymography for MMP-9 secretion. B, Cells were treated as outlined in A. Whole cell lysates were collected and analyzed by western blot using antibodies raised against EMMPRIN. C, Motility of MCF-7 cells in the presence of bryostatin-1 was determined by transwell migration assays shown in figure 1. D,

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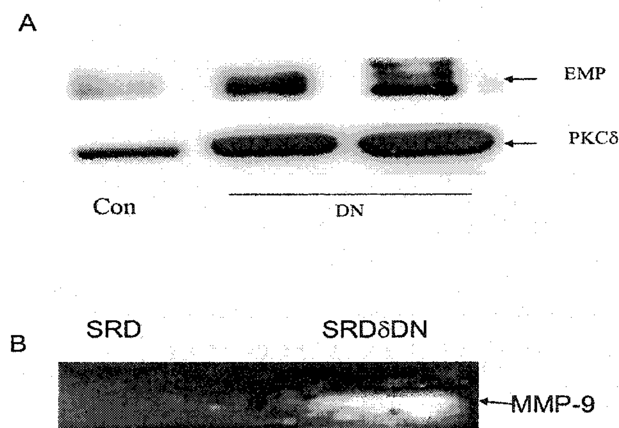

Figure 6. Dominant negative mutant of PKCδ induces metastasis associated phenotypes in MCF-7 cells.

MCF-7 cells were transfected with plasmids containing dominant negative mutants of PKCδ (pPKCδ), or an empty vector. A, 36 hr post transfection, whole cell lysates were prepared and assayed by western blot using antibodies raised against EMMPRIN. B, Cells were transfected as in A, 24 hr after transfection, plates were shifted to serum free medium supplemented with .5% BSA for an additional 24 hours. Conditional medium was then collected and assayed by zymography.

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3.6. PKCδ Expression Correlates with Reduced Cell Motility in MCF-7 & BT-549 Breast Cancer Cells

BT-549 breast cancer cells have been reported to have little or no expression of PKCδ (Nieves-Neira and Pommier, 1999), whereas MCF-7 cells have been shown to have high levels of PKCδ expression (Abbas et al., 2004). In Fig. 6A, the level of PKCδ in these two cell lines was compared directly, and as reported previously, there were high levels of PKCδ in MCF-7 cells, whereas PKCδ expression was undetectable in the BT-549 cells. Having established the differential level of PKCδ expression, the ability of the BT-549 cells to migrate in a modified Boyden chamber assay was examined. As shown in Fig. 6B, the BT-549 cells were highly mobile relative to the MCF-7 cells, which have previously been shown to be weakly mobile (Johnson et al., 1999; Liu et al., 2002). There was about a ten-fold difference in the ability of BT-549 cells to cross the Boyden chamber membrane relative to the MCF-7 cells. These data reveal a correlation between PKCδ expression and reduced cell motility.

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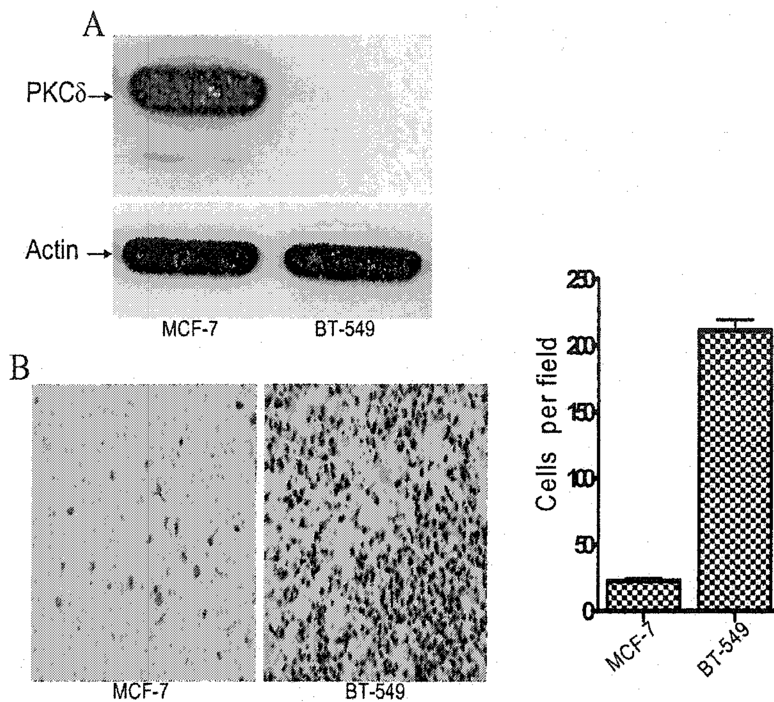

Figure 7. Expression level of PKCδ in MCF-7 and BT-549 human breast cancer cells.

(A) Whole cell lysates from MCF-7 and BT-549 cells were prepared, normalized to total protein, and subjected to western blot analysis using antibodies raised against PKCδ and actin. (B) Cells were placed in the modified Boyden chambers and transwell migration assays performed. The left panel shows typical fields of stained cells, quantification is shown on the right. Error bars represent the standard deviation of 3 different fields that were counted. The data presented are representative of experiments repeated 2 times.

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3.7. PKCδ suppresses the ability of BT-549 breast cancer cells to migrate.

To determine whether the lack of PKCδ was important for the migration of the BT-549 cells, these cells were transiently transfected with either a PKCδ expression vector (pSRD-PKCδ) or an empty vector control. The level of PKCδ was verified by western blot (Figure 8A). As shown in Fig. 8B&C, the PKCδ expression vector suppressed the ability of the BT549 cells to migrate by more than 50%. Thus, the lack of PKCδ in the BT-549 cells is apparently critical for their ability to migrate.

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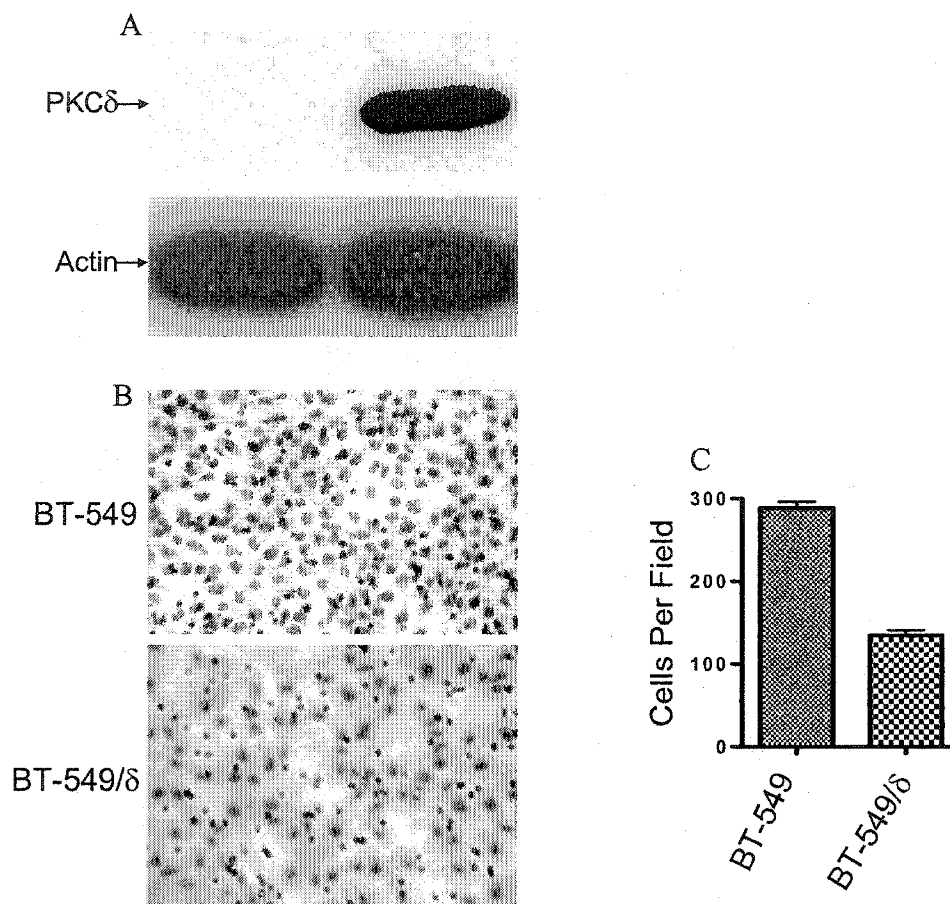

Figure 8. PKCδ suppresses the ability of BT-549 cells to migrate.

(A) Western blot depicting PKCδ expression in cells transfected with the pSRD-PKCδ or the empty vector. BT-549 cells were transiently transfected with either pSRD-PKCδ or an empty vector control as described in Materials and Methods. 36 hr post transfection, cells were subjected to the Boyden chamber assay (B). (C) Quantitaation of the results obtained in (B).

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3.8. Mouse embryo fibroblasts (MEFs) from PKCδ knockout mice have increased cell motility.

We next examined the mobility of MEFs from wild type and PKCδ knockout mice (Miyamoto et al., 2002). MEFs were generated from wild type and PKCδ knockout mouse embryos. The MEFs were added to modified Boyden chambers and cell motility was examined. In figure (9A) it is shown that wild type MEFs have very little mobility, whereas MEFs from PKCδ knockout mice were very mobile with more than a five fold number cells passing through the Boyden chamber membranes see quantitation at right. We verified the lack of PKCδ in the MEFs by western blot, as shown in figure (9B) Thus, MEFs lacking PKCδ have acquired the ability to migrate. These data further support the hypothesis that PKCδ suppresses the ability migrate and that the loss of PKCδ increases cell motility. The data also show that the effect of PKCδ on cell motility can be observed in normal rodent fibroblasts as well as in human breast cancer cells.

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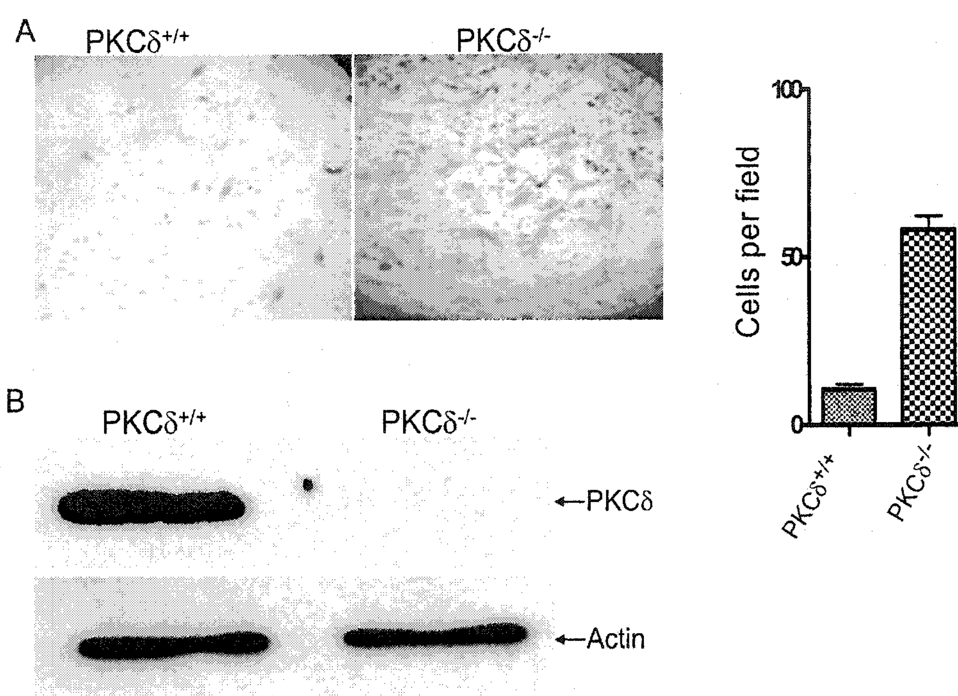

Figure 9. MEFs from PKCδ knockout mice have increased cell motility.

(A) Wild type and PKCδ knockout MEFs were examined for motility as described in figure 1. (B) PKCδ levels in wild type and PKCδ MEFs were verified by western blot analysis.

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3.9. ***PMA Activates C-src in MCF-7 Cells***

The role of the non-receptor kinase c-Src in tumor progression is controversial, since Src is not found mutated in human cancers, and over-expression studies showed c-Src to be weakly transforming at best. However, previous work from our laboratory showed that c-Src over-expression coupled with down-regulation of PKCδ was able to transform rodent fibroblasts (Lu et al., 1998). Further, c-Src can mediate transformation by enabling signal transduction from the epidermal growth factor receptor tyrosine kinase family (Biscardi et al., 1997). Consequently, we stimulated MCF-7 cells with phorbol ester over a 24 hour period and assayed for c-Src activity using an antibody that recognizes tyrosine 416 of c-Src only when phosporylated. As demonstrated in figure 10, c-Src appears to be activated in response to PMA in MCF-7 cells. Src is rapidly phosphorylated on tyrosine 416 with in 5 min of addition of phorbol ester. PMA has been shown to induce tyrosine posphorylation of proteins in various cell lines. These proteins include FAK and Paxillin (Zachary et al, 1993, Emkey & Kahn, Bell et al, 1999). Tyrosine phosphorylation was dependent on PKC, since down-regulation of PKC using prolonged treatment with phorbol ester completely abolished the phosphorylation response (Liu and Heckman, 1998). To further confirm the activation of c-Src, we assayed for the c-Src substrate FAK by western blot using phospho-specific antibodies to tyrosine 397 of FAK. We found that PMA treatment led to the phosphorylation of FAK

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on 397 (figure 10). Paxillin is a 68kDa docking protein that is observed in focal adhesions, where it associates with other members of the complex including FAK (Turner et al., 1990). Phosphorylation of paxillin enables its association with other proteins such as Csk (Birge et al., 1993). As shown in figure 10, treatment of MCF-7 cells with PMA induced a time dependent increase in phosphorylation of paxillin. Taken together, these data suggests that PMA is able to influence rearrangement of the cytoskeleton by a mechanism involving c-Src in MCF-7 cells.

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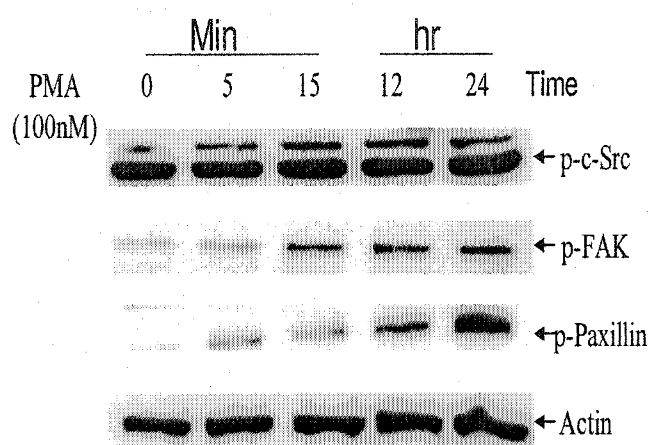

Figure 10. PMA activates Src in MCF-7 cells.

MCF-7 cells were treated with PMA (100nM), for the indicated times. Whole cell lysates were prepared in RIPA buffer and analyzed by western blot using antibodies against proteins as indicated in the figure.

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3.10. Inhibition of c-Src blocks PMA-induced phenotypes in MCF-7 cells

To further ascertain the role of c-Src in the metastasis-associated phenotypes induced by PMA, we pre- treated MCF-7 cells with the Src family kinase inhibitor PP2, and then added PMA for 24 hours. In parallel experiments cells were subjected to the transwell migration assay and zymographic assays. As shown in figure 11, inhibition of src family kinase's abrogated both the secretion of MMP-9 and the migration of MCF-7 cells through the boyden chamber, (A & B) respectively. Further, the inactive analog PP3 was without effect; figure 11 (C & D). Taken together, these data suggest that PMA induced c-Src activity, coupled with down-regulation of PKCδ could enhance the metastatic potential of MCF-7 cells.

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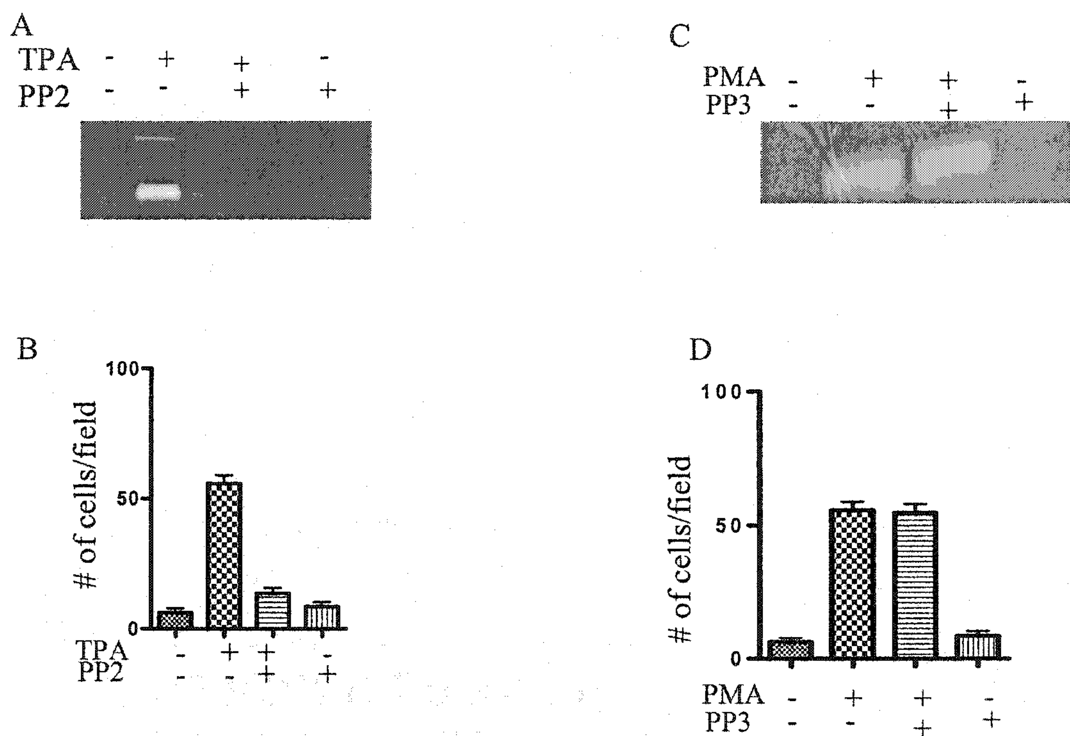

Figure 11. Inhibition of Src family kinases block PMA induced phenotypes

MCF-7 cells were pretreated for 1hr with PP2 (10μM) or the inactive analog PP3 (10μM) followed by treatment with 100nM PMA. As demonstrated in figure 11(A), inhibition of Src family kinases inhibited the PMA induced secretion of MMP-9, and (B) migration of MCF-7 cells (B). PP3 was without effect inhibiting either the secretion of MMP-9 or migration induced by PMA, C & D respectively.

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3.11. Treatment of MCF-7 cells with PMA induces expression of the EGFR

Previous work demonstrated that one consequence of long term treatment of cells with PMA is the up-regulation of the Epidermal Growth Factor Receptor (EGFR). The EGFR is frequently over-expressed in breast cancer, and has been shown to cooperate with activated c-Src to promote metastasis in breast and prostate cancer. Our lab has recently shown that inhibition of PKCδ either through use of the specific inhibitor rottlerin, or use of a dominant negative mutant, was able to transform cells over-expressing the EGFR (Hornia et al., 1999). Consequently, we treated MCF-7 cells with PMA and assayed for changes in the level of the EGFR by western blot. As evidenced in figure (12A), prolonged treatment of these cells with PMA occasioned a dramatic increase in the level of the EGFR in MCF-7 cells. Up-regulation was observed at 12hr after treatment and was maximal at 24hr. (B), we assayed the ability of bryostatin-1 to inhibit the PMA induced expression of the EGFR in MCF-7 cells by western bolt of whole cell lysates of cells

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treated with or without bryostatin-1 and PMA. As demonstrated in figure (12B), bryostatin-1 was able to block the up-regulation of the EGFR in PMA treated cells, indicating a role for PKCδ in this process. To further establish the influence of PKCδ in regulation of the EGFR in MCF-7 cells, we transfected MCF-7 cells with a dominant negative mutant of PKCδ. 36hr post transfection, whole cell lysates were collected and analyzed by western blot for EGFR expression. The results are presented in figure (12C). As demonstrated in the figure, modulation of PKCδ by dominant negative mutant resulted in a three fold increase in expression of the EGFR over background. These results offer strong evidence for a role for PKCδ in regulation of the EGFR.

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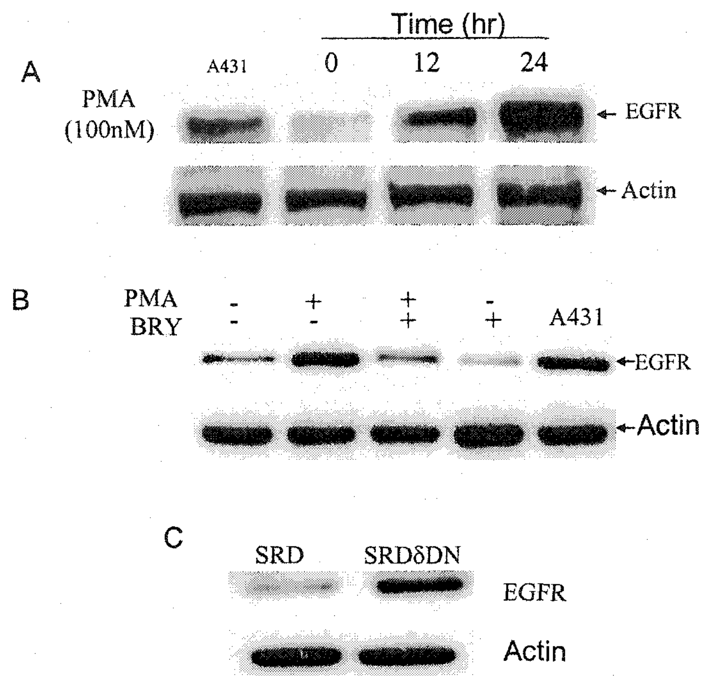

Figure 12. Prolonged treatment of MCF-7 cells with PMA up-regulates the EGFR.

A, MCF-7 cells were treated with PMA (100nM) for the indicated times, whole cell lysates were then collected and analyzed by western blotting using antibodies raised against the EGFR and actin. B, Cells were treated with PMA and bryostatin-1 either alone or in combination (100nM each). After 16 hr incubation, whole cell lysates were prepared and analyzed by western blot. C, MCF-7 cells were transiently transfected with plasmids containing a dominant negative PKCδ (pSRD-PKCδ) or an empty vector.

36 hr after transfection, cells were harvested in RIPA buffer and lysates analyzed by western blotting for EGFR expression.

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3.12. Discussion

The evidence presented here supports the hypothesis that PKCδ is able to suppress PMA induced phenotypes associated with migration and metastasis in culture. This effect was observed in two breast cancer cell lines and in mouse embryo fibroblasts. Differential expression of PKC isoforms were seen in normal vs. maglignant urothelium, with normal tissue and low grade tumors showing high expression of PKCδ which decreased with increasing stage and grade of transitional cell carcinoma (Cerda et al., 2001). In addition, increased expression of PKC δ inhibited both anchorage-dependent and independent growth and reduced survival in a human colon cancer cell line (Jackson and Foster, 2004). Numerous studies have implicated PKCδ in tumor suppression, due to its ability to modulate the cell cycle. (for review see (Jackson and Foster, 2004). Further support for a tumor suppressing effect of PKCδ is provided by studies showing a pro-apoptotic role for this PKC isoform, reviewed in (Brodie and Blumberg, 2003).

While PKCδ was able to suppress metastasis associated phenotypes in the cell lines in the forgoing study, over-expression of PKCδ was reported to correlate with increased metastatic potential in rat mammary tumor cell lines (Kiley et al., 1999a; Kiley et al., 1999b) Further, it has also been reported that PKCδ is required for sustained migration in cells over-expressing the EGFR (Kruger and Reddy, 2003). Also while PKCδ over-expression was able to inhibit the proliferation of BL6 murine melanoma cells, it enhanced the in vivo metastatic pontential (La Porta et al., 2000). This study serves to illustrate the highly complex nature of PKCδ singaling in cancer. These apparently conflicting data attest to the notion that PKCδ can influence downstream

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signaling either negatively or positively. We recently posited the hypothesis that PKCδ may function as a stress response kinase that provides backup support during both proliferative and non-prolifferative crisis (Jackson and Foster, 2004). Viewed in this light, it is not surprising that PKCδ can function to both suppress and enhance metastasis associated phenotypes in different cancer cells with different genetic alterations.

It was previously shown that down-regulation of PKCδ was implicated in the transformation of rat fibroblasts that over-expressed a tyrosine kinase (Lu et al., 1997). Over-expression of a tyrosine kinase is a common genetic alteration in human cancer (Biscardi et al., 2000). Further, depletion of PKCδ prevented apoptosis in response to serum withdrawal in cells having enforced expression of the tyrosine kinase Src (Zhong et al., 2002). Survival signaling has been linked to increased cell motility and invasion (Assert et al., 1996; Romanova et al., 1998). Data provided in this study further link survival signaling to increased motility and invasive behavior in culture. It is possible that the ability to prevent apoptosis may be linked to mechanisms that stimulate migration away from stressful conditions. This would provide a strong logic for linking the two activities. It may also provide evidence that the genetic changes that lead to metastasis involve the same genes that ensure suppression of default apoptotic programs early in tumorigenesis.

To begin to construct a mechanism by which PKCδ down regulation might influence progression to metastasis, we looked at PKC expression in MCF-7 cells treated with PMA. As shown in figure 4A, PMA treated cells failed to down regulate PKCα in MCF-7 cells. Indeed there appears to be an increase in the level of PKCα in response to PMA treatment. Interestingly, an inverse relationship apparently exists between these

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isoforms of PKC (Ways et al., 1995). In addition, MCF-7 cells over-expressing PKCα show down-regulation of PKCδ (Murakami et al., 2002; Oka et al., 2003). There are also reports in the literature in which PKCδ appears to inhibit PKCα (Joseloff et al., 2002). These studies together with data presented in figure 4 suggest the possibility that PMA induction of metastasis associated phenotypes in MCF-7 cells depends upon differential modulation of the alpha and delta isoforms of PKC.

Numerous studies have shown interactions between Src family kinases and PKCδ. The functional significance of PKCδ-Src interaction is unclear. Src family kinases have been reported to both activate and inhibit PKCδ. In mouse keratinocytes, inhibition of Src blocked tyrosine phosphorylation of PKCδ and increased PKC activity (Blake et al., 1999). Src was also shown to promote degradation of PKCδ by a mechanism involving phosphorylation of PKCδ on tyrosine 311 (Shanmugam et al., 1998) . In addition, PMA was shown to promote an association between Src and PKCδ in MCF-7 cells (Chen et al., 2001). This association between active Src and PKCδ was limited to the cytosolic fraction, and was inhibitory toward PKCδ. Evidence presented in this thesis is consistent with the above mentioned studies. As showed in figure 10, PMA occasioned a rapid increase in active Src in MCF-7cells. Treatment of MCF-7 cells with PMA also resulted in phosphorylation of FAK and paxillin, both of which are involved in the formation of focal adhesions and migration. It is therefore possible that PMA mediated increases in active Src might facilitate the inhibition or down-regulation of PKCδ and consequently promote metastasis.

Because PMA treatment of MCF-7 cells is known to induce multiple effects, this suggested to us that PMA might be activating a major pathway. PMA has been shown to

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up-regulate the EGFR upon long term treatment. Activation of the EGFR by PMA was shown to be a necessary step in PMA mediated signal transduction and is therefore involved in PMA induced tumor promotion (Welsh et al., 1991). Further, activation of the EGFR can induce multiple cellular and biological responses in vivo and in vitro, including mitogenesis and cell motility. The EGFR is phosphorylated on threonine 645 by PKC (Jimenez de Asua and Goin, 1992). In this study, down-regulation of PKC blocked induction of lamellipodia retraction. PKC phosphorylation of the EGFR, results in reduction of binding affinity of the receptor for its ligands.

In figure 12A, we show that treatment of MCF-7 cells with PMA induced increases in the level of the EGFR upon long term treatment. Further, MCF-7 cells transfected to express a dominant negative mutant of PKCδ showed increased EGFR expression figure 12C. These data suggests the possibility that PKCδ can modulate the expression and or activity of the EGFR, and hence mitogenic and motility signaling mediated by the EGFR.

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Chapter IV Survival Signaling by TGF-β1 in breast cancer cells

Introduction

Apoptosis or programmed cell death is a genetically controlled process which functions in normal development of the organism. Cells undergoing apoptosis display distinct morphological changes including nuclear condensation, membrane blebbing, and fragmentation of genomic DNA (Kerr et al., 1972). PKCδ, a member of the novel PKCs has been implicated in various processes within the cell. These processes include cell cycle regulation, differentiation, migration and apoptosis. The role of PKCδ in apoptosis is complex. For the most part, PKCδ has been implicated as a pro-apoptotic molecule (Blass et al., 2002; Cross et al., 2000; Emoto et al., 1995). For example, the PKCδ selective inhibitor rottlerin blocked the activation of caspases, the generation of the 40Kd catalytically active fragment of PKCδ and apoptosis induced by DNA damage (Basu, 2003), Further, we have previously shown that down-regulation of PKCδ provided a survival signal in cells deprived of growth factors (Zhong et al., 2002). However, PKCδ has also been implicated as an anti-apoptotic molecule, particularly in breast cancer (Kilpatrick et al., 2002; McCracken et al., 2003; Ni et al., 2003). These studies implicated PKCδ in protecting human breast cancer cells against DNA damage induced

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cell death, however the mechanism by which this protection occurs remains largely unexplored.

Rapamycin, is an immunosuppressant that has shown exquisite sensitivity for the inhibition of mammalian Target of Rapamycin (mTOR). Rapamycin has been shown to induce apoptosis in cells lacking functional p53 or p21 (Hosoi et al., 1999; Huang et al., 2003). Apoptosis induced by rapamycin is dependent on a mechanism involving the Stress Activated Protein Kinase/Jun N-Terminal Kinase (SAPK/JNK) pathway and 4E-Bp1. In cells containing functional p53 or p21, rapamycin induces growth arrest in the G1 phase of the cell cycle. Rapamycin has been shown to exert its growth suppressing effects via inhibition of the evolutionarily conserved regulator mTOR (Brown et al., 1994). Interestingly, mTOR has been implicated in the serum dependent activation of PKCδ (Parekh et al., 1999). A role for PKCδ downstream of mTOR has also been demonstrated (Kumar et al., 2000). This group showed that PKCδ could interact with mTOR, and that this association was required for the phosphorylation and inactivation of 4E-BP1 thereby stimulating cap dependent initiation of protein translation. The immunosuppressant rapamycin has also been shown to directly inhibit PKC activity in some cells (Rokaw et al., 1996). The breast cancer cell line MDA-MB- 231 has been shown to be resistant to rapamycin (Chen et al., 2004; Chen et al., 2003). These experiments showed that in the presence of serum, rapamycin failed to affect cell viability. Rapamycin was also shown to inhibit the serum induced phosphorylation of PKCδ in some cell lines; we therefore sought to determine the nature of the involvement of PKCδ in anti-apoptotic signaling in human breast cancer cells.

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Results

4.1. Rapamycin Induces apoptosis in the absence of serum in MDA-MB-231 cells.

Previous work in our lab showed that MDA-MB-231 breast cancer cells were resistant to rapamycin, requiring significantly higher concentrations of the drug in order to induce growth arrest and of apoptosis compared to MCF-7 cells. Further, we noticed that the stress of serum whitdrawal did not increase cell death above background in 231 cells. However, treatment with rapamycin in the absence of serum resulted in the induction of cell death in MDA-MB-231 cells, (figure 13A & C). Adding serum to the medium inhibited rapamycin induced cell death (A). In these experiments, MDA-MB-231 cells were serum deprived for 24hr then treated with or without 20μM rapamycin for a further 24hr. Both attached and floating cells were then collected and analyzed for viability by trypan blue exclusion assay. The ability of rapamycin to induce cell death in MDA-MB-231 cells was verified by FACs analysis, (figure 13B & C). A clear increase in the sub G1 population was observed after 24hr of treatment with rapamycin, (C). These experiments confirmed the ability of rapamycin to activate pro-apoptotic pathways under stressful conditions in MDA-MB- 231 cells.

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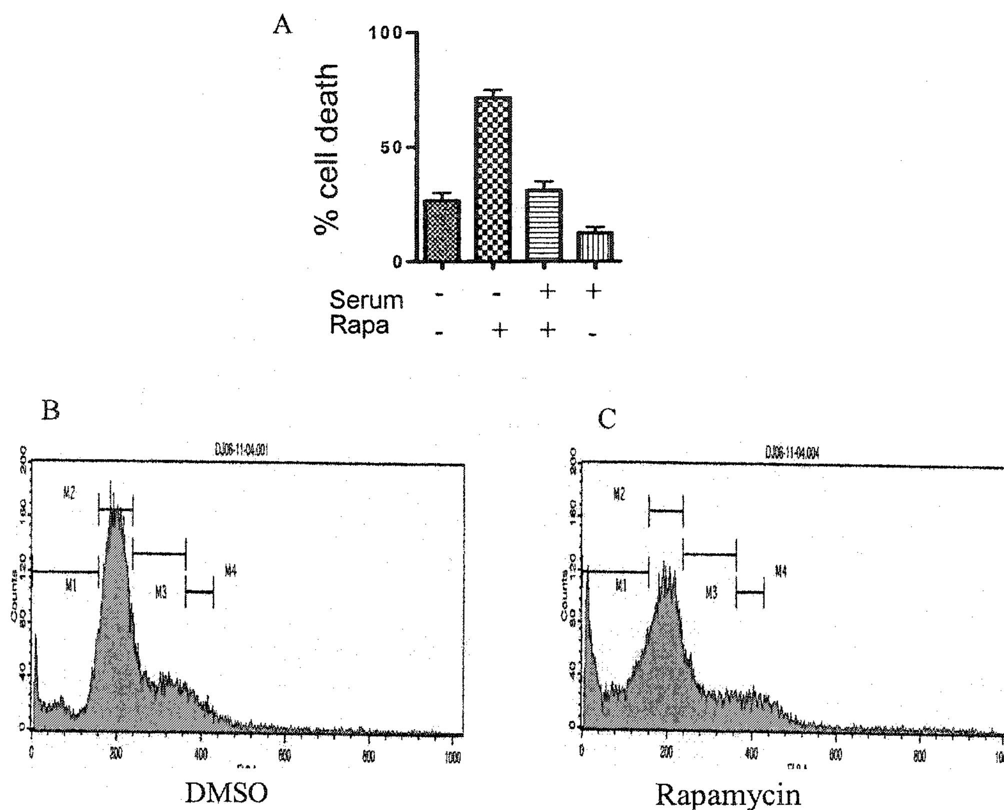

Figure 13 Induction of apoptosis by rapamycin in MDA-MB-231 cells.

MDA-MB-231 cells were maintained in DMEM supplemented with 10% BCS. Cells were then placed in serum free medium with or without rapamycin (20μM) for 24hr. A, after incubation, both adherent and floating cells were collected and cell viability determined by trypan blue exclusion. B, 231 cells were prepared as in A, fixed in 100% ethanol, and nuclei were stained using propidium iodide. FACS was performed using FACS vantage.

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4.2. Transforming Growth Factor-β1 inhibits rapamycin induced cell death in MDA-MB-231 cells.

The inability of rapamycin to induce cell death in the presence of serum suggested to us that some component of serum specifically inhibited rapamycin induced cell death. Serum growth factors are known to provide survival signals to cells in culture. Normal breast epithelial tissue is exquisitely sensitive to growth inhibition by Transforming Growth Factor-β1, and because TGF-β1has been implicated in the suppression of apoptosis induced by growth factor withdrawal (Lee et al., 1999), we reasoned that the inhibitory component in serum may be TGF-β1. Consequently, MDA-MB-231 cells were subjected to serum deprivation for 24hrs, followed by treatment with TGF-β1 in the presence or absence of rapamycin. After the 24hr incubatory period, attached and floating cells were collected and analyzed by trypan blue exclusion for viability. We found that TGF-β1 was as effective as serum in blocking rapamycin induced cell death in 231 cells (14A). This effect was not observed when MDA-MB-231 cells were treated with IGF-1 figure (14B), suggesting that the component of serum responsible for the inhibition of rapamycin induced apoptosis was TGF-β1. To further establish the effect of TGF-β1 in rapamycin induced cell death, we treated MDA-MB-231 cells growing in 10% serum with a neutralizing antibody to TGF-β1. As shown in figure (15), neutralizing TGF-β1 in serum rescued the ability of rapamycin to induce apoptosis in MDA-MB-231 cells. These data provide evidence supporting the hypothesis that TGF-β1 is sufficient to abrogate rapamycin induced cell death in MDA-MB-231 cells.

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4.3. Rapamycin and TGFβ-1 combine to induce a more complete growth arrest than either agent alone

Cells sensitive to rapamycin are growth arrested in G1 phase of the cell cycle upon administration of nanomolar quantities of the drug (Noh et al., 2004). However we recently showed that these cells require significantly high concentrations of rapamycin in order to ensure growth arrest (Chen et al., 2003). One possible explanation for the observed effects of TGF-β1 on rapamycin induced cell death is that these two agents may compliment each others ability to induce growth arrest. We tested this hypothesis in MDA-MB-231, growing in asynchronous culture. 5×10^3 cells per well were added to a 96 well plate and allowed to attach overnight. The next day the cells were treated with rapamycin (R), rapamycin and TGF-β1 (R&T) or TGF-β1 alone for 48hr. Cells were then harvested and assayed for thymidine incorporation into DNA. As shown in (17A), the combination rapamycin and TGF-β1 induced a more complete growth arrest than either agent alone. We did however observe an approximately 20% reduction in cell growth in the presence of rapamycin, and about a 30% reduction in the presence of TGF-β1 compared to control, when these agents were applied alone, figure 16(A). The results obtained in (A) were corroborated in sequential counting experiments. 2×10^5 cells were seeded in 100mM tissue culture plates and allowed to attach overnight. The following day, plates were treated as indicated in figure 16B. Cell counts were then made on sequential days using a hemocytometer. Counting experiments further show that where as rapamycin and TGF-β1 displayed minimal effects on growth inhibition in MDA-MB-231 cells when applied as a single agent, the two drugs combined to effectively inhibit the growth of 231 cells by greater than 75% of control. These results suggest the

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possibility that MDA-MB-231 cells are protected form rapamycin induced death by a mechanism involving inhibition of the cell cycle.

4.4. Growth arrest induced by rapamycin and TGFβ-1 is dependent on p21 and dephosphorylation of pRb.

To begin to dissect the mechanism of growth arrest induced by rapamycin and TGF-β1 in MDA-MB-231 cells, we treated cells with rapamycin and TGF-β1 either alone or in combination. We then assayed by immunoblot for known cell cycle proteins. As shown in figure (17A), TGF-β1 induced p21 expression in 231 cells; induction of p21 was relatively rapid with maximal induction occurring at 2hr after treatment. PMA treated lysates was used as a positive control for p21 expression. We observed no induction of p21 expression in the absence of TGF-β1, or in the presence of rapamycin (B). TGF-β has been shown to inhibit the phosphorylation of pRb at multiple serine/threonine sites, including ser 807/811 (Hu and Zuckerman, 2001). Consequently, we analyzed lysates from cells treated with the combination of rapamycin and TGF-β1 by western blot. We found that the combination of rapamycin and TGF-β1 induced dephosphorylation of pRb to a greater extent than that seen with either agent alone (C). These results indicated the possibility that rapamycin can along with TGF-β1, induce growth arrest in MDA-MB-231 cells. Growth arrest under the conditions tested in these experiments is correlated with the induction of p21 and dephosphorylation of pRb. These results further suggest that rapamycin and TGF-β1, by inducing p21, can protect cells from apoptosis.

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4.5. The ability of TGF-β1 to induce expression of p21 is dependent upon PKCδ

Numerous studies have implicated PKCδ in the regulation of p21(Frey et al., 1997; Fukumoto et al., 1997; Shanmugam et al., 2001). Further, there appears to be a correlation between TGF-β1 signaling and PKCδ, that is in a variety of cells, TGF-β1 signaling requires active PKCδ (Olivier et al., 1996; Runyan et al., 2003). Consequently, we sought to determine whether or not TGF-β1 signaling in MDA-MB-231 cells was mediated by PKCδ under the conditions tested. To answer this question, we transfected MDA-MB-231 cells with siRNA against PKCδ, 60 hr post transfection, cells were treated with rapamycin and or TGF-β1 for a further 12hr. In figure (18A), we demonstrate effective knock down of PKCδ by western blot. MCF-7 lysates were used as a positive control for PKCδ expression. The ability of TGF-β1 to induce p21 expression was tested in MDA-MB-231 cells transfected with siRNA against PKCδ. In figure (18B), we show that knock down of PKCδ was sufficient to abrogate induction of p21 by TGF-β1.

4.6. Knock down of PKCδ abrogated the ability of TGF-β1 to inhibit rapamycin induced apoptosis.

To further establish a role for PKCδ in TGF-β1 mediated signaling in MDA-MB-231 cells, we transfected cells with siRNA against PKCδ, cells were then treated with rapamycin and or TGF-β1 and analyzed by western blot using antibodies against the death substrate Poly (ADP-ribose) polymerase (PARP). During apoptosis, PARP is cleaved to generate a stable 85 kDa fragment; generation of this fragment has been used as an indication of apoptotic cell death. As shown in figure 19, knock down of PKCδ

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resulted in significant PARP cleavage, suggesting involvement of PKCδ in mediating the TGF-β1 blockade of rapamycin induced apoptosis.

4.7. Smad 2/3 proteins are required for inhibition of growth and apoptosis in MDA-MB-231 cells.

TGF-β1 receptor signaling occurs most often via Smad proteins(Chen et al., 1998), however, TGF-β1 can also signal by modulation of the activity of other multipurpose pathways. These include the phosphatidylinositol-3 kinase (PI3K) pathway, the Ras/Raf, JNK and P38 pathways; as well as the cytoskeletal regulators, Rho A and Rac (Massague, 1998; Massague and Wotton, 2000). Further, PKC has been shown to regulate Smad signaling(Runyan et al., 2003; Yakymovych et al., 2001). We therefore probed MDA-MB-231 lysates from cells transfected with siRNA against PKCδ and untransfected controls with antibodies that recognized phosphorylated Smad 2/3. As demonstrated in figure 20, down-regulation of PKCδ severely reduced the phosphorylation of Smad 2/3 in MDA-MB-231 cells. These results along with the data presented in figures 19 and 20, argue the possibility that the effects of TGF-β1 observed in this cell line is mediated by modulation of Smad 2/3 by PKCδ.

Discussion

TGF-β1 is a multifunctional cytokine that has been implicated in cell growth, differention and apoptosis. Early studies implicated TGF-β1as a tumor suppressor that can induce apoptosis in some cell lines (Massague, 1998). However, TGF-β1 has also been implicated in the inhibition of apoptosis (Mazars et al., 2000). Loss of TGF-β sensitivity is frequently observed in tumors derived from cells that are normally sensitive

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to TGF-β. The effects of TGFβ on growth arrest usually occurs via the Rb pathway, since inactivation of this pathway by expression of the human papillomavirus HPV-16 E7 protein confirs resistance to TGF-β (Blain and Massague, 2000). Evidence presented here in addressed the mechanism by which serum inhibits rapamycin induced apoptosis in MDA-MB-231 cells. We found that whereas treatment of MDA-MB-231 cells with rapamycin induced apoptosis in serum free medium, this effect was lost in the presence of serum. These experiments suggested to us that some growth factor present in serum provided survival signals to counteract the apoptotic influences of rapamycin. To test this hypothesis, we treated MDA-MB-231 cells with rapamycin and TGF-β1, or rapamycin and or IGF-1. We observed inhibition of rapamycin induced cell death in the presence of TGF-β1 but not IGF-1 in cells grown in serum free medium. These experiments are consistent with the observations of other groups in which the ability of TGF-β1 was able to block apoptosis in these cells under conditions of growth factor withdrawal was reported (Huang et al., 2000).

Herein, we present evidence that the inhibition of apoptosis by TGF-β1 is dependent on the PKCδ mediated induction of p21. Treatment of MDA-MB-231 cells with TGF-β1 rapidly induced p21 protein expression in these cells. Induction of p21 was evident at one hour post treatment and was maximal at 2hr, figure (17A). Interestingly, although rapamycin did not induce p21 when applied alone, in the presence of TGF-β1, we observed a synergistic response to rapamycin and TGF-β1, figure (17B). We show that induction of p21 by PKCδ in these cells is Smad dependent. PKC has been shown to phosphorylate Smad3 on serine 70; this modification was shown to inhibit TGF-β1 induced cell death (Yakymovych et al., 2001). Phosphorylation of Smad2/3 inhibits its

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DNA binding ability, but does not affect the transactivation function of Smad2/3. Thus it is possible that application of TGF-β1 under the conditions tested in the forgoing experiments led to transactivation of the p21 promoter by PKCδ. These data suggest a model in which rapamycin induces apoptosis in MDA-MB-231 cells subjected to growth factor withdrawal by inhibiting mTOR and PKCδ. Inhibition of these two proteins in this manner could then lead to apoptosis mediated by 4E-BP1, see figure (21A). In the presence of serum or TGF-β1, rapamycin may still be able to inhibit signaling from mTOR, however, TGF-β1 mediated activation of PKCδ results in the up-regulation of p21. p21 can then mediate growth arrest and survival of MDA-MB-231 cells under the conditions tested (B). TGF-β1 has been shown to directly activate PKCδ, although the mechanism by which such activation might occur is not clear (Runyan et al., 2003). Further, PKC has been shown to phosphorylate Smad 2/3 in the Smad MH1 domain, suggesting a regulatory role for PKC in TGFβ signaling (Yakymovych et al., 2001). Thus PKCδ may function as part of the TGF-β1 signaling pathway to negatively regulate cell growth.

PKCδ has been shown to influence apoptosis in both negative and positive ways. However, the mechanism whereby PKCδ might inhibit apoptosis is known. One method by which PKCδ might inhibit apoptosis is by induction of expression of p21. (Huang et al., 2001), showed that under serum free conditions, over-expression of p21 or p53 protected cells from rapamycin induced apoptosis. These results are consistent with our observations that PKCδ mediated induction of p21 inhibited apoptosis in MDA-MB-231 cells. This hypothesis also helps explain the results of other investigators who reported

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an inhibitory role for PKCδ in breast cancer cells (Kilpatrick et al., 2002; McCracken et al., 2003).

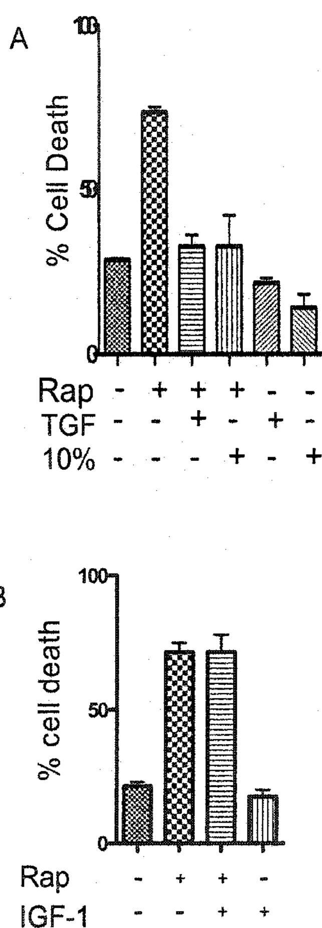

Figure 14. TGF-β1 inhibition of rapamycin induced apoptosis.
MDA-MB-231 cells were seeded in growth medium for 24hr. The cells were then stressed by serum withdrawal for 24hr, after which they were rinsed and treated with rapamycin (15μM) and or TGF-β1 (10nM) in the presence or absence of 10% serum as indicated. Cells were then harvested and viability assessed by trypan blue exclusion, A. B, cells were prepared as in A, then treated with rapamycin (15μM) and or IGF-1. Cell viability was assayed as in A.

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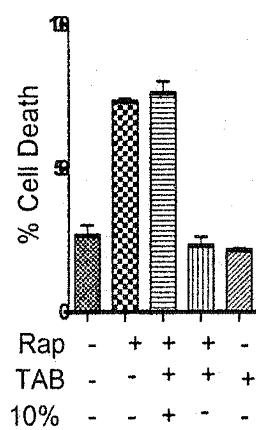

Figure 15. Neutralizing antibody to TGF-β1 potentiates rapamycin induced cell death in 10% serum.

MDA-MB-231 cells were treated with rapamycin (15μM) and or TGF-β1 (10ng/ml) in the presence or absence of 10% serum for 20hr. The cells were then harvested and assayed by trypan blue exclusion.

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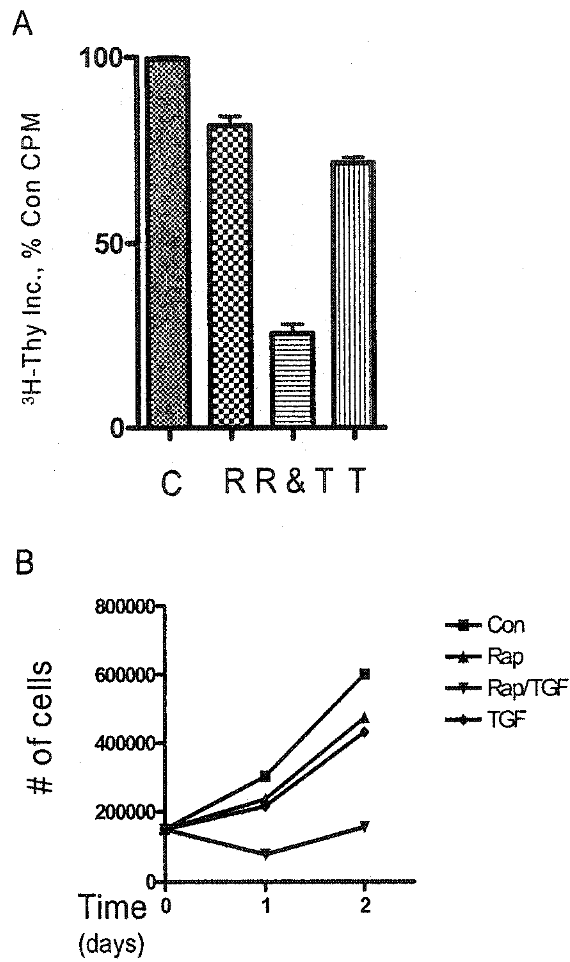

Figure 16. Rapamycin and TGF-β1 combine to inhibit the growth of MDA-MB-231 cells.

Asynchronously growing MDA-MB-231 cells were treated with rapamycin and TGF-β1 as indicated. (A) cells were incubated in drug for 48hr, after which growth was analyzed by thymidine incorporation into DNA. (B) Sequential cell counts, MDA-MB-231 cells were treated as indicated in the figure; duplicate plates were harvested and counted using a hemocytometer.

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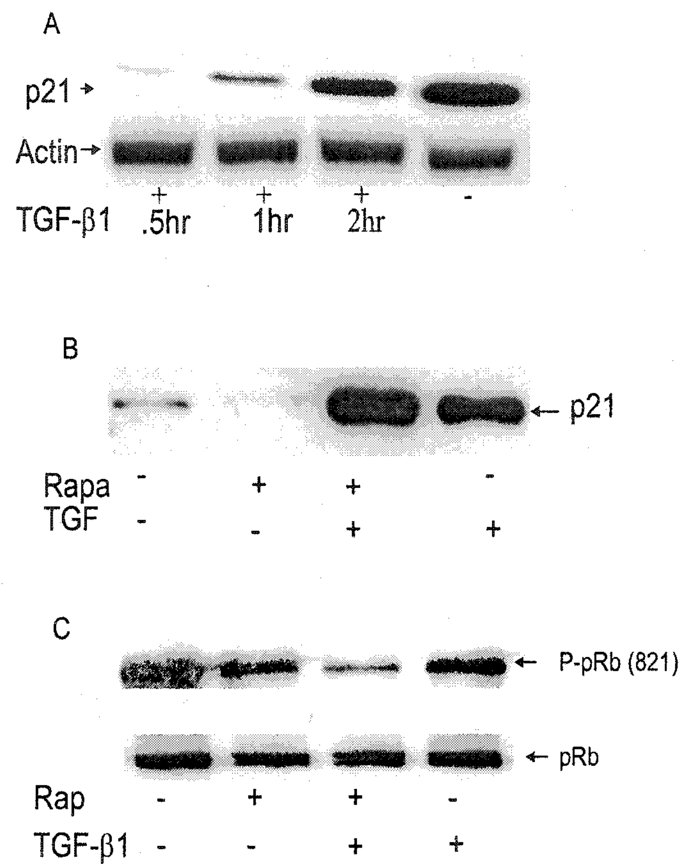

Figure 17. TGF-β1 treatment induced p21 expression and dephosphorelation of pRb.

Cells were treated with TGF-β1(10ng/ml), for the indicated times, whole cell lysates were then collected and analyzed by western blot using antibodies to p21. PMA treated MCF-7 cell lysates were used as a positive control A. B, MDA-MB-231 cells were treated with rapamycin and or TGF-β1 for 6hr, whole cell lysates were collected in modified RIPA buffer and analyzed by western blot. C, whole cell lysates were prepared from cells treated as in B, and analyzed by western blot using phospho-specific antibodies to pRb.

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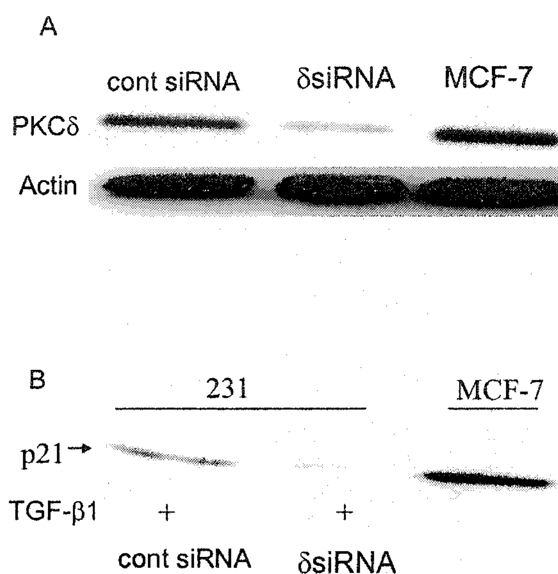

Figure 18. TGF-β1 induction of p21 in MDA-MB-231 cells is mediated by PKCδ.

MDA-MB-231 cells were transfected with 2μg of siRNA against PKCδ or a control siRNA. (A) 72hr post transfection, cells were harvested in RIPA buffer, and whole cell lysates analyzed by western blot using antibodies raised against PKCδ. (B), Cells were transfected as in A, 72 hr after transfection, plates were shifted to serum free medium containing 10ng/ml TGF-β1 for 2hr. Whole cell lysates were then collected and assayed by western blot.

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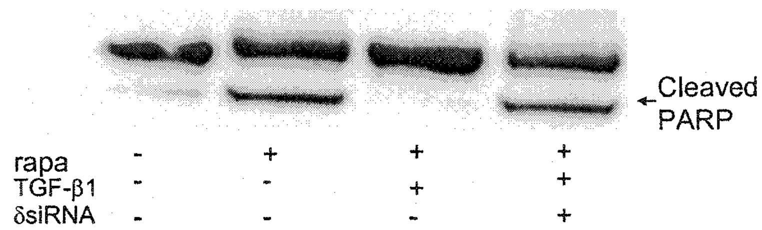

Figure 19. PKCδ mediates TGF-β1 blockade of rapamycin induced apoptosis.

MDA-MB-231 cells were transfected with siRNA against PKCδ. 48hr after transfection, cells were rinsed in serum free medium then subjected to serum withdrawal for a further 24hr. Both floating and attached cells were collected and whole cell lysate prepared. Whole cell lysates were analyzed by western blot using antibodies against PARP.

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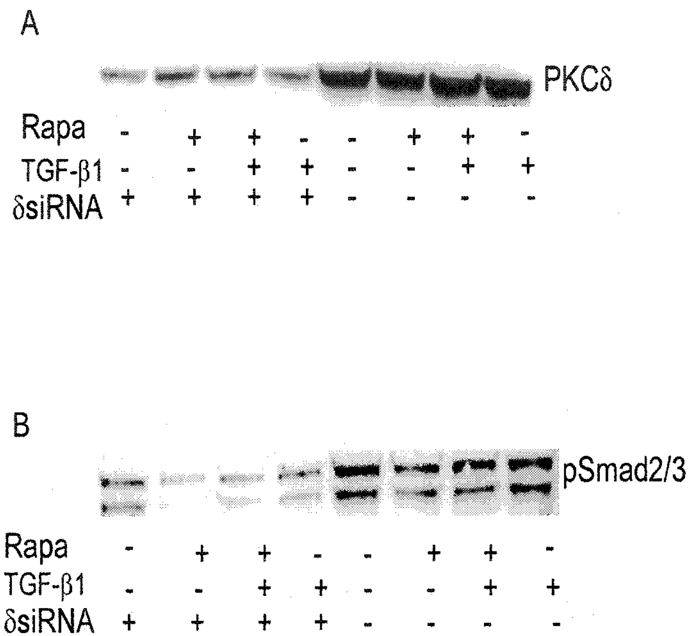

Figure 20. Down-regulation of PKCδ in MDA-MB-231 cells inhibits phosphorylation of Smad 2/3.

A, Cells were transfected with 2μg of siRNA against PKCδ, 48hr post transfection,transfected cells as well as control plates were treated with rapamycin and or TGF-β1 for 6hr as indicated in the figure. Cells were harvested by scraping into RIPA buffer, and whole cell lysates prepared and subjected to western analysis using antibodies to PKCδ. B, Membranes used in A were stripped and reprobed with a phospho-specific antibody to Smad2/3.

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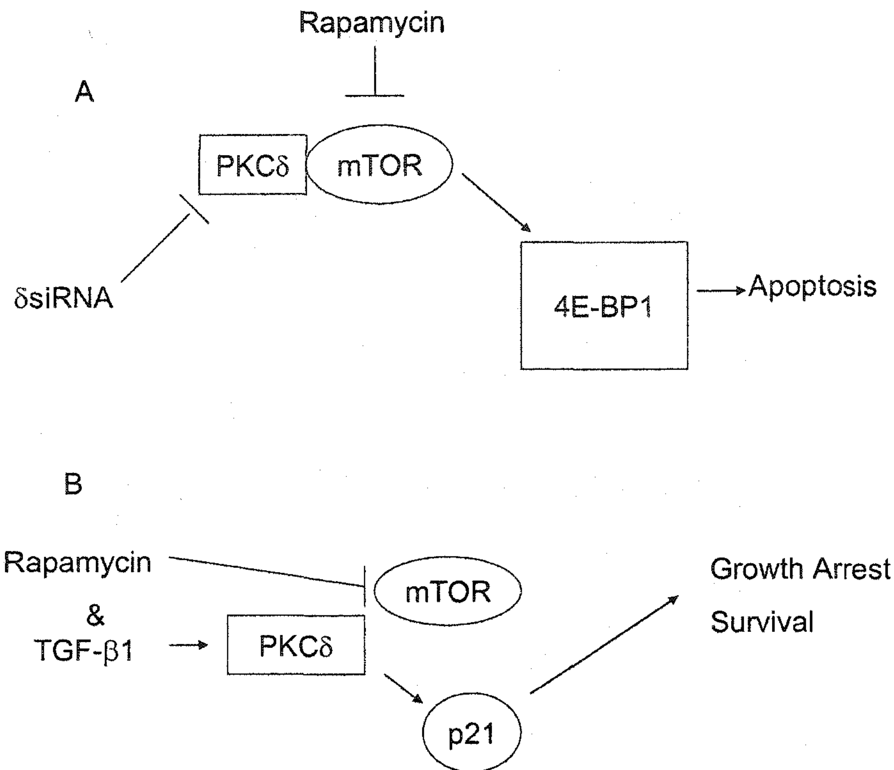

Figure 21. Proposed Mechanism for the effects of Rapamycin and TGF-β1 on the cell cycle

Inhibition of mTOR by rapamycin and or inhibition of PKCδ enable 4E-BP1 mediated induction of apoptosis (A). However, in the presence of serum or TGF-β1, the ability of rapamycin to inhibit both mTOR and PKCδ might be relieved there by allowing PKCδ to up-regulate p21 expression in MDA-MB-231cells. Induction of p21 can then inhibit apoptosis, there by ensuring survival of 231 cells.

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Chapter V

Conclusion

A role for PKCδ in human cancer has not been established. To the best of our knowledge, no loss of function mutations has been isolated from patient material thus far. However, it is intresting to note that PKCδ was localized to chromosome 3p in a region in which there is considerable loss of heterozygosity (LOH) in a wide range of tumors (Huppi et al., 1994; Zabarovsky et al., 2002). LOH on chromosome 3p has led to speculation that perhaps several tumor suppressor genes are clustered at the loci between 3p12 and 3p21(Braga et al., 2002). Sincee PKCδ is localized to 3p14, is plausible that PKCδ might be lost during tumor progression. Results presented herein and by other investigators suggest the possibility that PKCδ expression may indeed be down-regulated in human cancer. One means of down-regulating endogenous PKCδ might be through methylation of the PKCδ promoter. It will be interesting to explore this possibility in those cancer cell lines reported to show low expression of PKCδ. In this thesis, we provide preliminary evidence for regulation of the EGFR expression by PKCδ. We previously showed that PKCδ can modulate basal transcription of the tumor suppressor p53 in human cancer cell lines having wild type p53 (Abbas et al., 2004). Thus it is plausible that PKCδ can also regulating the transcription of the EGFR. Experiments to test this hypothesis are currently ongoing in our laboratory.

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Chapter VI

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