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Introduction

This research project strives to advance our understanding of how cell signaling pathways mediated by the protein kinase C (PKC) gene family contribute to the translational hyperactivity that accompanies the unrestrained cell cycle reentry and unlimited replicative potential of prostate cancer cells. Experiments designed to address these basic questions are intended to determine the mechanisms regulating the expression of PKC isozymes throughout the progression of prostate cancer and the metabolic consequences of enhanced expression for PKCepsilon (PKC ϵ). Molecular and biochemical approaches are being employed to specifically determine how PKC ϵ activity influences reactivation of the translational repressor protein retinoblastoma (Rb), and thereby global protein synthesis, upon entry into the G1 phase of the cell cycle.

Body

Five tasks were included in the approved Statement of Work for this project. The following progress report presents our efforts to experimentally address each of these research tasks, in the order in which they were originally presented.

Task 1. To develop a profile of changes in PKC ϵ isozyme expression during the recurrence of tumor growth in the CWR22 model.

Our data indicate that prostate cancer cells express endogenous PKC ϵ and that the recurrence of androgen-independent tumors in the CWR22 xenograft model of prostate cancer is accompanied by an increase in the levels of PKC ϵ expression (Appendix; see Wu and Terrian, 2002; Figure 1). CWR22 tumors were transplanted into nude mice containing s.c. testosterone pellets and serum testosterone levels of 4.0 ng/ml. Protein lysates for Western analysis were prepared from frozen tumors harvested from intact mice bearing androgen-stimulated tumors and castrated mice carrying recurrent (resected 150 days after castration and removal of testosterone pellets) CWR22 tumors. Western blots were prepared and probed using goat polyclonal anti- PKC ϵ (Santa Cruz, Santa Cruz, CA). Using this approach, the highest level of endogenous PKC ϵ protein expression was consistently associated with the four recurrent CWR22 tumors obtained 150 days after castration (Appendix; see Wu et al., 2002; Figure 5 and Wu and Terrian, 2002; Figure 1A). In addition to this observation, we reported several additional findings of direct relevance to the influence of PKC ϵ signaling in the growth of CWR22 prostate cancer tumors: 1) antisense PKC ϵ oligodeoxynucleotides effectively inhibit the translation of endogenous PKC ϵ in CWR-R1 cells (see below) and significantly inhibit their proliferation; 2) PKC ϵ enhances the synthesis and secretion of caveolin-1 by these prostate cancer cells by sequentially promoting the transcriptional elongation of c-myc transcripts and stimulation of androgen receptor-mediated signaling to the caveolin-1 promoter (Appendix; see Wu and Terrian, 2002; Figures 2-4).

Southern blot analyses have indicated that gains of the PKC ϵ gene, at 2p21, do not account for the increased expression of PKC ϵ in human prostate cancer cell lines (data not shown). The promoter of the human PKC ϵ gene has not been experimentally analyzed and transcriptional activation could well account for the increased levels of PKC ϵ mRNA that we have detected in 35% of the recurrent CWR22 tumor lysates tested to date. However, an analysis of the PKC ϵ promoter region was not included in the original Statement of Work and this hypothesis could not be productively tested without additional support. Therefore, a proposal to follow-up on this unexpected finding was submitted to the USAMRMC Prostate Cancer Research Program Peer Review Panel by Dr. Daqing Wu (Proposal Number PC020885). The objectives of this proposal were to identify the promoter and 5'-untranslated regions of the PKC ϵ gene and develop a fine map of the regulatory elements regulating PKC ϵ transcription and translation, respectively. A 2 kb fragment of a provisional sequence for the PKC ϵ promoter was identified on chromosome 2p21 and a computer-aided analysis predicted the presence of several potentially important binding sites for transcriptional factors such as the androgen receptor and NF κ B. The full-length 5' end sequence of the PKC ϵ gene was isolated using traditional RACE methods and was shown to be exceptionally large (~680 kb) and structurally complex (>80% G/C), two characteristics of oncogenes. Despite this significant progress, our application for a Postdoctoral Traineeship Award was reviewed 06/30/2002 and assigned an unfundable score of 2.7. For this reason, we abandoned efforts to further study the molecular regulation of the PKC ϵ promoter/5' untranslated region during the progression of prostate cancer.

Task 2. To create and analyze the growth of MDA PCa 2b variants overexpressing PKC ϵ .

Efforts to establish and maintain the growth of this prostate cancer cell line (MDA PCa 2b) were unsuccessful. Therefore, we adopted the use of an alternative model system for the study of advanced, androgen-independent, prostate cancer, the CWR-R1 cell line. Our collaborator, Dr. Christopher Gregory (University of North Carolina at Chapel Hill), first introduced this cell line in 2001 (Gregory et al., *Cancer Res.* 61:2892-2898). The CWR-R1 cell line grows with a doubling time of approximately 36 h under the conditions first established by Dr. Gregory and adopted for use in this laboratory.

Task 2a: Neomycin-resistant clones of the CWR-R1 cell line have been selected following their transfection using an empty pLXSN adenoviral retrovector (control) or the same adenoviral vector containing either the wild-type PKC ϵ gene (CWR-R1/ ϵ) or a truncated, dominant negative, construct consisting of amino acids 1-245 (CWR-R1/DN ϵ). Western blots have confirmed the expression of PKC ϵ and DN-PKC ϵ in these cell lines (Appendix; see Wu and Terrian, 2002; Figure 2A). **Task 2b:** Flow cytometric analysis of propidium iodide stained cells demonstrates that the expression of exogenous PKC ϵ significantly decreases the percentage of CWR-R1 cells within the G1 phase of cell cycle progression, a phenotypic response that recapitulates that observed previously in our work with LNCaP cells overexpressing PKC ϵ (Appendix: Wu et al., 2001, *Cancer Res.* 62:2423-2429; Figure 2A).

In addition to these cell lines, we have also establish variants of the CWR-R1 line that also stably express other important molecular components in the phosphatidylinositol 3' kinase (PI3K), phosphatidylinositol-dependent kinase (PDK1), PKC ϵ pathway. CWR-R1 and LNCaP cells expressing wild-type, constitutively active, and dominant negative mutants of the p85 and p110 subunits of PI3K, PDK1, and Akt/PKB have recently been established. Using these cell lines it will now be possible to perform a more complete and systematic analysis of the influence that this mitogenic signaling pathway has on the growth of prostate cancer cells than was originally envisioned.

Task 3. To analyze changes in the cap-dependent and -independent translational activity of prostate cancer cells overexpressing PKC ϵ .

PKC ϵ has been established as an oncogenic protein and our laboratory has recently demonstrated that this kinase affects multiple pathways involved in the growth and progression of prostate cancer. Overexpression of PKC ϵ has been shown to transform LNCaP cells into an androgen-independent variant through a rapamycin-sensitive signaling pathway, and it leads to the formation of tumors in intact nude mice as well as in castrate mice (Appendix; Wu et al, 2002). Increased levels of PKC ϵ are also associated with a decrease in time needed to transit from G1 to S phase pointing to an influence on cell cycle regulatory proteins. More specifically, PKC ϵ has been shown to associate with Raf-1, and Raf-1 activity is increased in these cells as observed by an increased level of phosphorylated extracellular signal-regulated protein kinase (ERK), another downstream target of Raf-1. Activated ERK may then be capable of inactivating retinoblastoma protein (Rb), thereby removing restrictions on cell growth and translational activity. The work conducted under Task 3 was intended to further investigate the mechanism(s) by which PKC ϵ activates the translational apparatus in prostate cancer cells. As discussed below, the results indicated that PKC ϵ might have an indirect stabilizing affect on the assembly of active polysomes during androgen withdrawal.

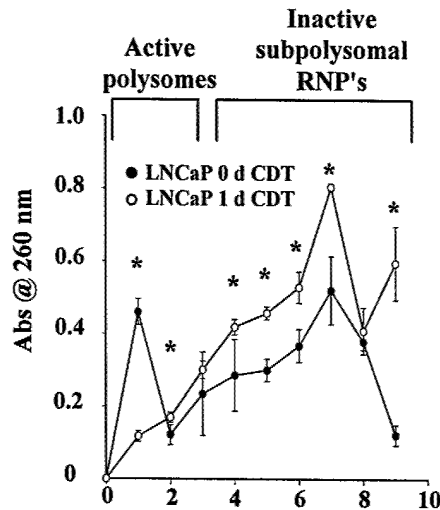
LNCaP cells overexpressing PKC ϵ demonstrate a seven-fold increase in the rate of total protein synthesis, but these cells do not exhibit a change in the rate of total RNA

synthesis (Appendix; see Wu et al., 2002; Figure 2). Therefore, the increased rate of total protein synthesis does not arise from an increased rate of transcription. Furthermore, an inhibitor of the translational elongation factor 2, *Pseudomonas* exotoxin A, was found to decrease protein synthesis in LNCaP and LNCaP cells overexpressing PKC ϵ to the same degree, indicating that PKC ϵ enhances the efficiency of protein translation at a step(s) upstream of peptide elongation. Translation initiation could, however, still account for the noted increase. This step in protein synthesis involves several factors, but there are two major points of regulation, the release of eIF-4E from its regulator 4E-BP1 and the process of ribosome assembly.

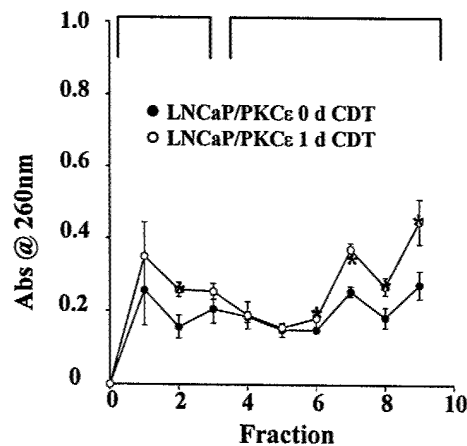
The work conducted thus far has focused on the process of ribosome assembly (Task 3a). The interaction of ribosomal subunits and other components of the translational machinery are thought to signal other undetermined events that regulate the maturation of rRNA and its incorporation into polyribosomes. First, extra sequences in immature rRNA are thought to induce the combination of ribosomal proteins and RNA molecules. Following this event, the unstable structure composed of proteins, rRNA, and mRNA is stabilized by rRNA maturation and the assembled complex is tested for functionality. If the complex has formed correctly, protein synthesis will proceed, but errors in the maturation/assembly process can lead to polyribosome disassembly. To investigate the effects of PKC ϵ on ribosome assembly, polysomal profiles were generated from a control cell line, LNCaP, and from a cell line overexpressing PKC ϵ , LNCaP/PKC ϵ (Figure 1). Cells cultured in the absence of androgens, using charcoal-dextran treated (CDT) serum, produced classic polysomal profiles in which assembled polysomes migrated the greatest distance through a linear sucrose gradient due to their higher aggregate density. A second absorbance peak closer to the top of the gradient represents the inactive subpolysomal ribonucleic proteins (RNPs) that are relatively more buoyant fragments of the mature ribosomal complexes. PKC ϵ overexpression seems to have affected ribosome disassembly during the stress of androgen withdrawal in the 1 d CDT condition. As seen in Figure 1C, androgen withdrawal caused LNCaP cells to exhibit a decrease in active polysomes relative to the control condition along with a concordant increase in inactive RNPs. However, this shift to inactivity is not seen in the LNCaP/PKC ϵ cells.

Figure 1. Polysomal profiles from LNCaP cells overexpressing PKC ϵ in complete serum and during androgen withdrawal. A. Polysomal profiles were obtained for LNCaP cells grown in RPMI + 10% FBS for 5 d (●) and for LNCaP cells grown in RPMI + 10% CDT FBS for the final 24 h (○). By convention, fraction 1 corresponds to the bottom of the gradient. Data are the means (\pm SE) of quadruplicate gradients. Asterisks (*) indicate 1 d CDT values that are significantly different according to the t-test at $p=0.05$ from corresponding values in the control condition. B. Polysomal profiles were obtained for LNCaP/PKC ϵ cells grown under the same conditions as in A. C. Comparison of changes in LNCaP and LNCaP/PKC ϵ polysomal profiles with respect to control conditions. Values for "percent change", $((1 \text{ d CDT}/0 \text{ d CDT}) \cdot 100) - 100$, were calculated for each fraction in each cell line.

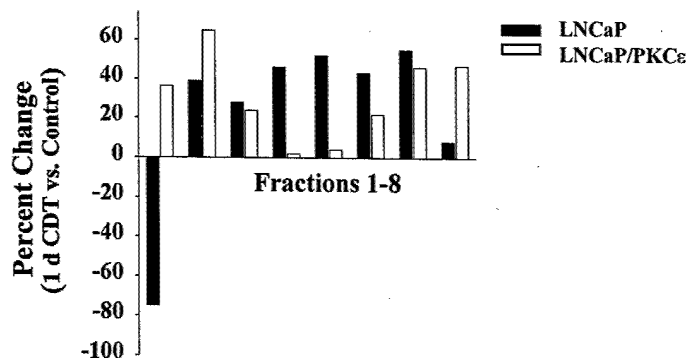
A. LNCaP



B. LNCaP/PKC ϵ



C. Percent Change



The next step in the analysis of PKC ϵ 's effect on ribosome assembly was initially intended to include an RNase protection assay (RPA). The intent was to isolate mRNA from sucrose fractions containing active polysomes and use an RPA to determine which classes of mRNA were preferentially associated with the active translational apparatus under normal conditions and during androgen withdrawal. The assay was to be designed in a way that would compare levels of representatives from four classes of mRNA: control or "housekeeping" mRNAs that are constitutively translated, cap-dependent mRNAs, cap-independent mRNAs, and internal ribosome entry site (IRES) dependent mRNAs. This comparison would have provided useful information both in determining which mechanism was responsible for the increase in total protein synthesis and in understanding which set of genes are involved in the cell's response to androgen withdrawal. For example, cap-dependent mRNAs include cyclin D1 and Rb, both of which are tied to cell cycle regulation. Cap-independent mRNAs or 5' terminal oligopyrimidine (TOP) mRNAs code for ribosomal proteins and elongation factors that might be directly involved with the observed increase in protein synthesis. Lastly, IRES dependent mRNAs include c-myc and vascular endothelial growth factor, two proteins known to play a role in tumorigenicity. Unfortunately, this endeavor was not productive. Several attempts to isolate mRNA and total RNA proved unsuccessful due to the high concentrations of sucrose in the active polysome fractions. Furthermore, the RPA required large amounts of mRNA and it became apparent that attempts to scale-up the process would lead to decreased resolution of an active polysome peak. Efforts to resolve these technical difficulties through the use of Northern blots are ongoing.

Task 4. To analyze changes in PKC ϵ biogenesis in CWR22 tumor explants and the CWR-R1 line.

As indicated in the original Statement of Work, this Task is to be addressed in Months 12-24. Accordingly, we have yet to initiate the work included under this Task in our initial year of support for this research project.

Task 5. To analyze changes in cell cycle regulatory proteins and the retinoblastoma (Rb) signaling pathway.

Although the original Statement of Work indicated that this Task would not be experimentally addressed until Months 12-36, we have initiated work on this project. While results of these studies are incomplete, the preliminary data indicate that PKC ϵ signaling promotes the growth and proliferation of human prostate cancer cells by interfering with Rb activation upon exit from M phase and in response to environmental stresses. Western blot analyses (Task 5a) suggest that this defect in Rb activation is selectively associated with increased levels of cyclin D1 expression and the activity of cyclin-dependent kinase 4 (cdk4). The work included under Task 5b and 5c are to be addressed during Months 12-24.

Our analysis of the influence of PKC ϵ signaling in tumor cell lines has also been extended to include alterations in the survival of these cells. Using a combination of primary tumor specimens, CWR22 xenograft tumors, and cultures of CWR-R1 and LNCaP cell lines, we have demonstrated that the basal level of PKC ϵ expression increases in prostatic cancer cells that survive throughout the progression of this disease by suppressing apoptotic signals propagated through the mitochondrial death signaling pathway. Gene transfer, antisense, flow cytometry, and confocal microscopy studies now provide evidence that survival signals mediated by PKC ϵ operate within the intrinsic mitochondrial pathway downstream of PI3K and upstream of cytochrome c release to block apoptosis that is triggered by phorbol esters in CWR-R1 and LNCaP cells. Proteomic profiling and coimmunoprecipitation assays indicate that PKC ϵ interacts with

the apoptotic protein Bax in recurrent CWR22 tumors *in vivo* and CWR-R1 and LNCaP cells in culture. These studies demonstrate that PKC ϵ overexpression in LNCaP cells has the potential to suppress mitochondrial targeting of Bax; thus providing a novel mechanism that PKC ϵ may employ to promote the survival of prostate cancer cells. The results of this study have recently been submitted for publication (Appendix; see McJilton et al.).

Recommended Changes and Future Work.

It is recommended that future work include a detailed analysis of the mechanism(s) by which PKC ϵ interacts with the Bax protein, to include a fine map of the interface mediating this protein-protein interaction. This recommended analysis of the antiapoptotic responses to PKC ϵ signaling was not included in the original Statement of Work. Accordingly, I am requesting that the Grants Officer provide the official approval required for incorporating this research topic into our ongoing investigations.

Key Research Outcomes

- Publication of a book edited by David M. Terrian, entitled "Cancer Cell Signaling: Methods and Protocols" In, *Methods in Molecular Biology*, Humana Press, Totowa, NJ, 2003; 218:1-333. No additional book copies currently available.
- Identification of the first oncoprotein (PKC ϵ) that is sufficient to induce the growth of LNCaP tumor xenografts in castrated nude mice.
- A detailed and comprehensive analysis of the phenotypic transformations that accompany the androgen-independent growth of LNCaP tumors in response to PKC ϵ -mediated signaling; including:
 - defects in Rb activation that are associated with increased levels of cyclin D1, constitutive activation of cdk4, and Rb hyperphosphorylation
 - hyperphosphorylation/activation of protein kinases in the ERK mitogenic signaling cascade
 - derepression of translational activity
 - increased production and expulsion of caveolin-1, and
 - increased resistance to apoptogenic stimuli.
- Molecular and biochemical evidence that the sequential activation of a PKC ϵ , MAPKs, c-Myc, and androgen receptor signaling pathway functions to drive the downstream expression of caveolin-1 in human prostate cancer cells.
- A detailed molecular explanation of the mechanism by which MAPKs link PKC ϵ signaling to the expression of c-Myc; i.e., by releasing paused RNA polymerase molecules downstream of *c-myc* promoter 2 and thereby derepressing the transcriptional elongation of the full-length gene.
- Introduction of a novel protein-protein interaction between PKC ϵ and Bax that functions to block the mitochondrial death signaling pathway in human prostate cancer cells.

Reportable Outcomes

Manuscripts

1. Wu, D, Foreman, TL, Gregory, CW, McJilton, MA, Wescott, GG, Ford, OH, Alvey, RF, Mohler, JL, and Terrian, DM: Protein kinase C-epsilon has the potential to advance the recurrence of human prostate cancer. *Cancer Res.*, 2002; 62:2423-2429.
2. Tachado, SD, Mayhew, MW, Wescott, GG, Foreman, TL, Goodman, CD, McJilton, MA, Terrian, DM: Regulation of tumor invasion and metastasis in protein kinase C epsilon-transformed NIH3T3 fibroblasts. *J. Cell. Biochem.*, 2002; 85:785-797.
3. Wu, D and Terrian, DM: Regulation of caveolin-1 expression and secretion by a protein kinase C-epsilon signaling pathway in human prostate cancer cells. *J. Biol. Chem.* 2002; 277:40449-40455.
4. Wu, D, Wescott, GG, Terrian, DM: Manipulating expression of endogenous oncogenic proteins using an antisense oligonucleotide approach in prostate cancer cells. In *Methods in Molecular Biology, Cancer Cell Signaling Methods and Protocols* (Terrian, D. M., ed.), Humana, Totowa, NJ, 2003; 218:143-153.
5. McJilton, MA, Sikes, CV, Wescott, GG, Wu, D, Foreman, TL, Gregory, CW, Weidner, DA, Mohler, JL, Ford, OH, AM Lasater, Terrian, DM: Protein kinase C-epsilon interacts with Bax and blocks the mitochondrial death signaling pathway in human prostate cancer cells. (Submitted for publication).
6. Wu, D: Inhibition of the MAP kinase pathway coordinately downregulates the metastatic potential of human prostate cancer cells. (Submitted for publication).

Books Edited

Terrian, DM: "Cancer Cell Signaling: Methods and Protocols". in, *Methods in Molecular Biology*, Humana Press, Totowa, NJ, 2003; 218:1-333.

Degrees Obtained Through Support by This Award

McJilton, Meagan A., M.S. awarded Dec. 2002; Thesis entitled, "Protein kinase C promotes the survival of human prostate cancer cells.

Sikes III, Charles Van, M.D. awarded Dec. 2002; Thesis entitled, "Characterization of a multimeric signaling complex in human prostate cancer.

Funding Applied for Based on Work Supported by This Award

USAMRMC 2002 Prostate Cancer Research Program Postdoctoral Traineeship Award, Proposal Number PC020885; entitled, "Protein kinase C-epsilon as a therapeutic target in androgen-independent prostate cancer progression." Status: unfunded.

Conclusions

Human prostate cancer cells expressing endogenous PKC ϵ are clonally selected for during the progression of this disease and, as a consequence, a majority of the cancer cells found in recurrent tumors are positive for PKC ϵ expression. Transgene and antisense experiments have illustrated that the expression of this oncoprotein has the potential to actively promote the proliferation, via Rb activation, and survival, via Bax inhibition, of recurrent prostate tumors.

"SO WHAT?" – These studies provide empirical evidence supporting the notion that PKC ϵ may provide a useful prognostic indicator and possible target for interventional therapy of organ-confined prostate cancer.

Recommended Changes on Future Work – Effort should be invested in developing a fine map of the physical determinants governing the interactions between PKC ϵ and Bax proteins. In addition, we have designed small inhibitory RNAs (siRNA) for stably suppressing the *in vivo* expression of PKC ϵ and would welcome the opportunity to test their effectiveness in inhibiting the growth of CWR22 xenograft tumors.

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- McJilton, MA, Sikes, CV, Wescott, GG, Wu, D, Foreman, TL, Gregory, CW, Weidner, DA, Mohler, JL, Ford, OH, AM Lasater, Terrian, DM. Protein kinase C-epsilon interacts with Bax and blocks the mitochondrial death signaling pathway in human prostate cancer cells. (Submitted for publication).
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- Wu, D and Terrian, DM. Regulation of caveolin-1 expression and secretion by a protein kinase C-epsilon signaling pathway in human prostate cancer cells. *J. Biol. Chem.* 2002; 277:40449-40455.

Appendices

1. Wu, D, Wescott, GG, Terrian, DM: Manipulating expression of endogenous oncogenic proteins using an antisense oligonucleotide approach in prostate cancer cells. In *Methods in Molecular Biology, Cancer Cell Signaling Methods and Protocols* (Terrian, D. M., ed.), Humana, Totowa, NJ, 2003; 218:143-153.
2. Wu, D, Foreman, TL, Gregory, CW, McJilton, MA, Wescott, GG, Ford, OH, Alvey, RF, Mohler, JL, and Terrian, DM: Protein kinase C-epsilon has the potential to advance the recurrence of human prostate cancer. Cancer Res., 2002; 62:2423-2429.
3. Wu, D and Terrian, DM: Regulation of caveolin-1 expression and secretion by a protein kinase C-epsilon signaling pathway in human prostate cancer cells. J. Biol. Chem. 2002; 277:40449-40455.
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5. McJilton, MA, Sikes, CV, Wescott, GG, Wu, D, Foreman, TL, Gregory, CW, Weidner, DA, Mohler, JL, Ford, OH, AM Lasater, Terrian, DM: Protein kinase C-epsilon interacts with Bax and blocks the mitochondrial death signaling pathway in human prostate cancer cells. (Submitted for publication).
6. Wu, D: Inhibition of the MAP kinase pathway coordinately downregulates the metastatic potential of human prostate cancer cells. (Submitted for publication).

Manipulating Expression of Endogenous Oncogenic Proteins Using an Antisense Oligonucleotide Approach in Prostate Cancer Cells

Daqing Wu, Ginger G. Wescott, and David M. Terrian

Abstract

It has been shown that antisense oligodeoxynucleotide (ODN) treatments provide an effective, specific approach to inhibiting the function of target proteins. Using this method, we have acquired additional evidence that protein kinase C-epsilon functions as an oncogenic protein in the progression of recurrent human prostate cancer. This chapter describes the use of antisense ODN to directly target cellular protein kinase C-epsilon as a potential chemotherapeutic agent for blocking the advance of prostatic adenocarcinoma to androgen-independence. Using Lipofectin® as the carrier, phosphorothioate-modified antisense ODNs were transferred into prostate cancer cells with high efficiency, effectively inhibiting the expression of endogenous protein kinase C-epsilon and the androgen-independent (AI) proliferation of several independent human prostate cancer cell lines.

Key Words: Antisense ODN; prostate cancer; PKC-epsilon.

1. Introduction

Disordered signal transduction events that are permissive to unrestricted cell-cycle progression and escape from proapoptotic signals directly contribute to the malignant transformation of cancer cells (1,2). Gene transfer studies involving the use of constitutively active/inactive mutant signaling molecules have provided a wealth of information concerning the identities of putative oncogenic

and tumor-suppressor proteins. However, stable transfections of these mutant constructs naturally lead to the induction of a complex series of direct and indirect adaptive responses in the cellular host. For this reason, alternative experimental approaches such as transient transfections, inducible expression vectors, and antisense oligodeoxynucleotides (ODN) have often been incorporated into investigations of aberrant cancer cell signaling molecules.

Various gene delivery approaches have been developed to introduce gene-specific DNA into target cells or organs using nonviral (plasmid DNA, DNA-coated gold particles, liposomes, and polymer DNA complexes) and viral (adenovirus, retrovirus, adeno-associated virus, herpes virus and pox virus) vectors/carriers. Each approach has its own strengths and weaknesses (for review, *see* **ref. 3**). Gene-specific antisense ODNs have proven to be effective in suppressing the malignant effects of aberrant cell signaling by oncogenic proteins in a variety of cancer cells. "Bystander" effects associated with these agents are minimal when compared to those induced by conventional chemotherapeutic agents because antisense constructs are capable of recognizing and downregulating the intended target without altering the expression of closely related members within the same gene family.

Protein kinase C (PKC) comprises a family of at least 11 closely related kinase isozymes (**4**). The family is conventionally subdivided into three main categories (classic, novel, and atypical PKCs) based on sequence homologies and cofactor requirements. PKC-epsilon (PKC- ϵ) was the first member of the novel PKC subfamily to be identified (**5,6**) and was subsequently shown to increase growth and cause malignant transformation when overexpressed in rodent fibroblasts by enhancing activation of Raf-1 kinase (**7,8**). PKC- ϵ is presently thought to be involved in the progression of various cancers, including cancers of the skin (**9,10**), brain (**11**), kidney (**12**), thyroid (**13**), blood (**14**), breast (**15**), colon (**16**), and prostate (**17**). Preliminary studies have established that the ectopic expression of PKC- ϵ is sufficient to transform LNCaP cells, a model of early-stage prostate cancer (CaP), into variants that are capable of establishing tumor growth in castrated mice. In clinical specimens of CaP, PKC- ϵ expression appears to be a reliable positive marker for the malignant epithelial cells that will survive androgen ablation therapy and be clonally selected during the recurrence of CaP. Thus, this oncogenic protein makes a conspicuous target for the adjunctive treatment of prostatic adenocarcinoma.

Our results to date show that downregulation of endogenous PKC- ϵ causes a block of cellular proliferation *in vitro* in androgen-independent (AI) CaP cell models (Wu, D., Terrian, D. M. [2002] *J. Biol. Chem.*, available online), providing a single gene-based therapeutic approach to CaP. Additional antisense ODNs that have been tested for their anti-tumor effects on CaP are listed in **Table 1**. In this chapter, we provide a detailed protocol using antisense ODN that is specifi-

Table 1
Some Genes Inhibited
with Antisense ODNs in Prostate Cancer Model

Target	Tumor model/cell line	References
AR	LNCaP	18
Bcl-2	Shionogi tumor	19
		20
	LNCaP	21
	PC3	22
	LNCaP	23
IGFBP-3	LNCaP	23
IGFBP-5	LNCaP	24
c-Raf	PC3	25
	DU145, PC3	26
		27
	Metalloproteinase-7	LNCaP FGC
PAR	DU145	29
PKC- α	PC3	22
TRPM-2	PC3	30

cally targeted at PKC- ϵ to study cellular signaling in AI CaP cell lines DU145, PC3, and CWR-R1. However, the protocol provided below may be easily adopted for the study of alternative gene-specific targets.

2. Materials

2.1. Cell Culture

1. Prostate cancer cell lines DU145 and PC3 were obtained from the American Type Culture Collection (Manassas, VA; ATCC HTB-81 and CRL-1435, respectively). CWR-R1 was a generous gift from Dr. C W. Gregory (The University of North Carolina at Chapel Hill).
2. a) DU145 cells were cultured in Minimum essential medium Eagle with 2 mM L-glutamine and Earle's Balanced Salts Modified (BSS) adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 0.1 mM sodium pyruvate, and supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. b) PC3 cells were cultured in F12K medium with 2 mM L-glutamine and supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. c) CWR-R1 cells were cultured in Prostate Growth Medium (PGM), comprised of Improved MEM Zinc Option (Richter's Improved MEM) supplemented with 900 μ g/L linoleic acid, 1.2 g/L nicotinamide, 100 μ g/L epidermal growth factor (EGF), 10 mg/L ITS (insulin/transferrin/selenious acid), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2% FBS (31, and personal communication). Linoleic

acid and nicotinamide were from Sigma (St. Louis, MO). EGF and ITS were from Becton Dickinson Labware (Franklin Lakes, NJ). All other cell culture reagents were purchased from Invitrogen (Rockville, MD).

3. Phosphate buffered saline (PBS) and Trypsin-EDTA (10X) were purchased from Invitrogen.
4. Cell culture dish (150 mm), Corning, Inc., (Acton, MA).
5. Tissue culture plate (24-well), Becton Dickinson Labware.

2.2. Antisense Oligonucleotide Treatment

1. TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM ethylenediamine tetraacetic acid (EDTA).
2. Phosphorothioate-modified oligonucleotides, synthesized and purified at Invitrogen (*see* Notes 1 and 2), were prepared at concentrations of 50 μ M in 10 mM TE buffer under sterile conditions, and stored at -20°C . The sequences are: antisense PKC- ϵ ODN (5'-CATGACGGCCGATGTGACCT-3') and scrambled PKC- ϵ ODN (5'-TACGCATAACGCGCTGGTGG-3') (32).
3. OPTI-MEM[®] I Reduced Serum Medium (Cat. No. 31985) and transfection reagent Lipofectin[®] (Cat. No. 18292-011) were purchased from Invitrogen.
4. Sterile tubes (15 \times 120 mm), Becton Dickinson Labware.

2.3. Lysis of Cells and Electrophoresis of Proteins (33)

1. Polyacrylamide minigel electrophoresis apparatus (Bio-Rad, Richmond, CA, Mini-Protein II system) with electrophoresis power supply.
2. Hoefer TE-22 Mighty Small Transfer Tank (Pharmacia Biotech, Piscataway, NJ) with cold water circulator.
3. IEC HN-SII Clinical centrifuge (International Equipment Company, Needham Heights, MA).
4. Sonicator.
5. Microcentrifuge.
6. Heating block.
7. Nitrocellulose paper (NCP, Bio-Rad, Cat. No. 162-015).
8. Sponges, paper towels, and plastic boxes.
9. 15 mL tubes.
10. Microcentrifuge tubes with screw-on caps.
11. Protease inhibitor stocks: store all stocks at -20°C .
 - a. Aprotinin (10 mg/mL) in deionized water.
 - b. Pepstatin A (10 mM) in dimethylsulfoxide (DMSO). Store as 20 μ L aliquots.
 - c. Leupeptin (10 mM) in distilled water.
 - d. Ovalbumin (10 mg/mL) in deionized water. Store as 200 μ L aliquots.
12. PMSF stock: 5 mg phenylmethylsulfonyl fluoride (PMSF) in 50 μ L methanol. Make fresh just before use.
13. Lysis buffer: 50 mM Tris-HCl, pH 7.5, 10 mM β -mercaptoethanol, 1% (Octylphenoxy)polyethoxyethanol (IGEPAL CA-630), 100 μ g/mL aprotinin, 2 μ M pepstatin, 10 μ M leupeptin, 100 μ g/mL ovalbumin, 100 μ g/mL PMSF.

14. Solution A: Trizma base (1.5 M, pH 8.9) and EDTA (8 mM). Store at 4°C.
15. Solution B: Trizma base (0.5 M, pH 6.8) and EDTA (8 mM). Store at 4°C.
16. Solution C: 30% acrylamide/bis solution (37.5:1) was purchased from Bio-Rad. Store at 4°C.
17. Solution D: 10% sodium dodecyl sulfate (SDS). Store at room temperature.
18. Solution E: 1.5% (w/v) ammonium persulfate. Make fresh every day.
19. 2X Western stop solution: 60 mM Trizma base, (pH 6.8), 7.5% (v/v) glycerol, 0.01% (w/v) bromphenol blue, 100 mM dithiothreitol (DTT), and 2% (w/v) SDS. Store at -20°C.
20. SDS-polyacrylamide gel electrophoresis (PAGE) molecular weight marker: Invitrogen BenchMark Prestained Protein Ladder (cat. no. 10748-010). Store at -20°C.
21. Reservoir buffer: 25 mM Trizma base, 187 mM glycine, and 0.1% (w/v) SDS. Adjust to pH 8.3. Prepare in stock solutions of 2 L and store at 4°C.
22. Transfer blot buffer: 25 mM Trizma base, 192 mM glycine, 20% (v/v) methanol. Store at 4°C.

2.4. Immunoblot Detection of PKC-ε

1. Stir plate.
2. X-ray film (Kodak BioMax ML, cat. no. 178-8207) and film cassette.
3. Saran wrap®.
4. Trizma buffered saline (TBS): Dissolve 40 mL 1 M Trizma HCl, pH 7.4, and 3.6 g NaCl in 4 L deionized water. Store at room temperature. Chill prior to use for washing blots.
5. TBS-Tween: Add 500 µL polyoxyethylenesorbitan monolaurate (Tween-20) to 1 L TBS. Store at 4°C.
6. 5% TBS blotto: Dissolve 5 g nonfat milk in 100 mL TBS. Store at 4°C. Make fresh on the day of use.
7. Enhanced chemiluminescence (ECL) detection solution: Mix 40 mL of 0.1 M Trizma HCl, pH 8.5, 4 mL of 0.68 mM p-Coumeric acid, 80 µL of 100 mg/mL 5-amino-2, 3-dihydro-1,4-phthalazinedione (luminol) in a glass flask and cover with foil because of the light sensitivity of luminol. Add 400 µL 3% hydrogen peroxide once in the darkroom. Must be made fresh on day of use.
8. Primary antibody: Polyclonal rabbit antihuman PKC-ε antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).
9. Secondary antibody: Antirabbit horseradish-peroxidase-conjugated IgG was purchased from Amersham (Arlington Heights, IL). Add 12.5 µL of the appropriate secondary antibody to 50 mL TBS blotto. Store at -20°C.

2.5. Cell Viability Assay

1. 24-well tissue culture plates.
2. Hemacytometer.
3. Trypsin-EDTA (1X).
4. Trypan blue solution (0.4%) was from Sigma.

3. Methods

3.1. Antisense Oligonucleotide Treatment

The following protocol is to transfect the cells in one well of a 24-well tissue culture plate. For other applications (such as Western blotting), use plate(s) of different size, and adjust the reagents accordingly.

1. Culture cells (37°C, 5% CO₂) on 150 mm tissue culture plate in corresponding medium to 60–70% confluence. Serum-starve cells overnight.
2. Aspirate medium; wash the cells once with PBS. Add 5 mL Trypsin-EDTA onto the cells. Incubate at 37°C for 10 min.
3. Count the cell numbers using Trypan blue and a hemacytometer. Suspend cells at 10⁵ cells/mL medium, and plate in 24-well plates at 1 mL per well. Culture for 24 h before oligonucleotide treatment (*see Note 3*).
4. Prepare transfection solution A (for each transfection): dilute 4–20 µL ODN stock (50 µM) in 50 µL OPTI-MEM I reduced serum medium at room temperature.
5. Prepare transfection solution B (for each transfection): incubate 2–10 µL Lipofectin in 50 µL OPTI-MEM I medium at room temperature for 30 min.
6. Combine solution A and solution B, mix gently, and incubate at room temperature for 15 min (*see Note 4*).
7. Set two controls: OPTI-MEM I medium without Lipofectin and ODN, OPTI-MEM I medium with Lipofectin only.
8. Add an additional 900 µL of OPTI-MEM I medium to the solution from **steps 6** and **7**, giving final ODN concentrations of 200 nM–1 µM. Mix gently.
9. Wash the cells twice with 1 mL OPTI-MEM I medium.
10. Overlay the ODN/Lipofectin mixture onto cells, and incubate at 37°C for 6 h.
11. Aspirate ODN/Lipofectin complex, wash the cells twice with 1 mL OPTI-MEM I medium, then add fresh medium containing 10% FBS and appropriate concentrations of ODNs, but no Lipofectin. Incubate the cells at 37°C for 18 h (*see Note 5*).
12. Aspirate medium, add fresh medium containing 10% FBS and appropriate concentrations of ODNs. Incubate the cells at 37°C for a further 48 h.

3.2. Cell Viability Assay

Cellular proliferation can be assessed using either the MTT (thiazolyl blue) assay, or the number of viable cells may be counted using a hemacytometer and trypan blue staining, as follows. Cell count should be performed in triplicate in 24-well tissue culture plates.

1. Aspirate medium; wash the cells once with PBS. Add 1 mL Trypsin-EDTA onto the cells. Incubate at 37°C for 10 min.
2. Take 50 µL Trypsin-treated cells and mix with 50 µL Trypan blue. Add to a hemacytometer. View under the microscope and count viable cells.

3.3. Lysis of Cells and Electroblothing (33)

The following protocol is for a 150 mm plate. For different plate sizes, adjust the reagents accordingly.

1. Aspirate medium; wash the cells once with PBS. Add 5 mL Trypsin-EDTA onto the cells (150 mm plate). Incubate at 37°C for 10 min.
2. Transfer to a 15-mL tube and add 5 mL of medium containing FBS.
3. Sediment at 1000g for 5 min on IEC HN-SII clinical centrifuge.
4. Pipet off supernatant and discard.
5. Resuspend cell pellet in 1 mL PBS, and transfer to microcentrifuge tubes.
6. Sediment at full speed (12,500g) for 30 s on microcentrifuge. Discard the supernatant.
7. Resuspend cell pellet in 200–500 μ L lysis buffer depending on pellet size.
8. Sonicate resuspended cell pellet for 10–20 s and incubate on ice for 30 min.
9. Centrifuge at 12,500g on microcentrifuge for 10 min at 4°C.
10. Transfer supernatants to new microcentrifuge tubes. Remove 5–50 μ L aliquot for protein concentration determination. Add one-half volume of 2X Western stop solution to remaining samples. Boil for 3–5 min. Run SDS-PAGE or freeze samples at –20°C.
11. For Western blotting of PKC- ϵ , prepare 10% acrylamide separating gel (enough for four minigels): Add 3.75 mL solution A, 10.0 mL solution C, 14.45 mL deionized water, 0.2 mL solution D, 1.0 mL solution E, 25 mL *N, N, N', N'*-tetramethylethylenediamine (TEMED). Swirl gently to mix. Do not add solution E and TEMED until ready to pour gels.
12. Prepare 5% acrylamide stacking gel (enough for four minigels): Add 2.5 mL solution B, 5.0 mL solution C, 11.3 mL deionized water, 0.25 mL solution D, 2.5 mL solution E, 25 μ L TEMED. Swirl gently to mix. Do not add solution E and TEMED until ready to pour gels.
13. Pour the gel(s) and polymerize.
14. Load equal amounts of sample protein into wells. Load prestained molecular weight markers on the same gel.
15. Run the gel(s) at 200 V until lowest molecular weight marker reaches the bottom of the separating gel (approx 1 h).
16. Turn off power supply when SDS-PAGE is complete. Remove the gel from the glass plate and assemble the transfer sandwich consisting of the following layers: black side of gel cassette (negative); 6 mm foam sponge; blotter paper; gel; NCP; blotter paper; two 3-mm foam sponges; and gray side of gel cassette (positive). Run at 400 mA for 90 min.

3.4. Enhanced Chemiluminescence (ECL) Detection

1. Block the membrane with 50 mL TBS blotto for 1 h at room temperature.
2. Prepare the primary antibody for incubation with membrane. Mix appropriate primary antibody with TBS blotto at the recommended dilution. Incubate the blot in primary antibody diluted in TBS blotto overnight at 4°C.

3. Wash the blot as follows: TBS for 10 min; TBS-Tween for 10 min; TBS-Tween for 10 min; TBS for 10 min; and TBS for 5 min.
4. Prepare secondary antibody conjugate for incubation with the membrane. Mix appropriate secondary antibody with TBS blotto at the recommended dilution. Incubate the blot in secondary antibody diluted in TBS blotto for 1 h at room temperature.
5. Wash the blot as in **step 3**.
6. Mix ECL detection solution. Add 3% H₂O₂ just before using in the dark room.
7. Drain excess buffer from the washed blot, and place it in a clean plastic box.
8. Add ECL detection solution. Incubate for precisely 1 min at room temperature.
9. Remove blot from detection reagent, drain excess reagent. Lay blot on Saran Wrap®, avoiding air pockets. Place in film cassette, keeping the smooth side up.
10. Notch film (Kodak BioMax ML) in same place as blot. Place piece of film on top of blot, matching notches and close the film cassette. Expose the film for 1 min and develop it immediately. Estimate exposure time of subsequent film(s) on the basis of signal strength. We found that 3 d of treatment with 1 μ M antisense ODN significantly reduced the endogenous expression of PKC- ϵ in AI CaP cell lines DU145, PC3, and CWR-R1 (data not shown).

4. Notes

1. Though it is suggested (as the authors will do) that the oligonucleotide used in antisense treatment should be highly purified, such as, by reverse phase high-performance liquid chromatography (HPLC) or PAGE, to assure the specificity of antisense ODN as well as to minimize the cellular toxicity, we found that the quality of cartridge-purified ODN was adequate for routine treatment of cells on 150 mm plates. Be sure to include those controls (sense, mismatch, or scrambled ODN) no matter which kind of purification method is selected.
2. Instead of using the “fully-phosphorothioated” oligonucleotide, we found that those ODNs only with modification to the four bases in the 5' most position and the three bases next to the last one in the 3' most position are stable and effective for most applications.
3. For successful transfection with ODN, cell density must be optimized, which may vary among cell types. For the three CaP cell lines (DU145, PC3, CWR-R1) used in this protocol, 40–60% confluency was optimal. Cell confluency should be maintained constant in experiments to get reproducible results.
4. ODN/Lipofectin complex may appear cloudy after mixture, but that will not affect transfection (34). If too much visible precipitate appears, try to incubate the mixture for a shorter time (5–10 min).
5. Generally, it is not suggested to treat the cells with phosphorothiotide ODN alone, because phosphorothiotides are very nonspecifically active at the cell membrane (35). However, we found that treatments with 1 μ M “naked” ODN for a further 66 h following a 6 h incubation in ODN/Lipofectin complex could more effectively downregulate the protein level of PKC- ϵ in cells, without significant difference in cellular morphology when compared to the procedure using only ODN/Lipofectin complex for 6 h.

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Protein Kinase C ϵ Has the Potential to Advance the Recurrence of Human Prostate Cancer¹

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ABSTRACT

Prostatic epithelial cells that are capable of surviving in the absence of androgenic steroids were found to express protein kinase C ϵ (PKC ϵ), an oncogenic protein capable of promoting autocrine cell-signaling events. Gene transfer experiments demonstrated that PKC ϵ overexpression was sufficient to transform androgen-dependent LNCaP cells into an androgen-independent variant that rapidly initiated tumor growth *in vivo* in both intact and castrated male nude mice. This transformation was associated with an accelerated rate of androgen-independent LNCaP cell proliferation, resistance to apoptosis, hyperphosphorylation of the mitogen-activated protein kinase extracellular signal-regulated kinase and transcriptional repressor protein retinoblastoma, and increased expression of E2F-1 and other 5'-cap-dependent mRNAs, including the G₁ cyclins, c-myc, and caveolin-1. Coimmunoprecipitation experiments indicated that PKC ϵ was associated with members of the extracellular signal-regulated kinase signaling cascade and the scaffolding protein caveolin-1. Caveolin-1, produced by LNCaP cells overexpressing PKC ϵ , was released into the medium, possibly through a Golgi-independent route, and significant growth inhibition was observed when these cells were cultured in the presence of an anti-caveolin-1 antiserum. Finally, antisense experiments established that endogenous PKC ϵ plays an important role in regulating the growth and survival of androgen-independent prostate cancer cells. This study provides several independent lines of evidence supporting the hypothesis that PKC ϵ expression may be sufficient to maintain prostate cancer growth and survival after androgen ablation.

INTRODUCTION

Prostatic epithelia normally depend on a functional androgen receptor signaling pathway for survival and undergo programmed cell death in response to androgen ablation therapy (1). This accounts for the clinical regression such treatments initially produce among CaP³ patients treated by orchiectomy. However, a relapse of tumor growth is common, and these recurrent tumors grow in an AI manner, are highly metastatic, and respond poorly to chemotherapy (2). Thus, oncogenic proteins that actively maintain the growth and survival of CaP cells after androgen ablation make conspicuous targets for the treatment of advanced CaP. Many laboratories have attempted to identify these proteins through comprehensive analyses of differential gene expression between androgen-dependent and AI CaP cell lines and prostatectomy specimens (3, 4). However, there is currently no

direct evidence to support the concept of a dominant oncogene in recurrent CaP (defined as any protein that is alone sufficient to initiate the growth of AI tumors *in vivo*). Here we report the identification of such an oncogene.

PKC ϵ is a member of the AGC family of Ser/Thr protein kinases that is known to have oncogenic potential (5) and to be associated with the progression of many cancers (6-9). Although there is recent evidence that PKC ϵ expression is elevated in tissue biopsies collected from patients with organ-confined CaP (10), the role of this isozyme in the progression to androgen independence has not been investigated. The activation of PKC α and PKC δ induces apoptosis in LNCaP cells, an intensively studied androgen-sensitive CaP cell line, but not AI (DU145 and PC3) CaP cell lines (11-13). This finding indicates that at least some members of this gene family are capable of differentially regulating the growth and survival of CaP cells. Given the reciprocal functions of PKC isozymes in various cell types (5) and the oncogenic activity of PKC ϵ , we hypothesized that this isozyme may oppose the proapoptotic influence of PKC α and PKC δ in CaP.

There is evidence that caveolae might represent an important locus for PKC action (14, 15), and a positive correlation between caveolin-1 expression and the progression of human CaP has been described recently (16, 17). Caveolin-1 and -2 form homo- and hetero-oligomers on the inner membrane surface of caveolae and may serve as a scaffold for the assembly of multimeric signaling complexes that often include multiple components of the ERK cascade and certain members of the PKC family (14, 18). Exactly how these caveolin-associated signaling complexes function is unknown, although the overexpression of caveolin-1 alone is not sufficient to stimulate the AI proliferation of LNCaP cells (19). We also do not understand the mechanisms that control the intracellular trafficking of caveolin-1 or how this membrane-type V protein gets rerouted into the secretory pathway of human CaP cells. However, aberrant transport is necessary for caveolin-1 to function as an autocrine/paracrine factor, now known to contribute to CaP metastasis and cell survival after androgen ablation (17).

In the present study, investigation of human CaP cell lines indicated a relationship between PKC ϵ expression and androgen independence. To better understand whether the expression of PKC ϵ could be of functional importance in CaP progression, we stably transfected LNCaP cells with a retroviral vector containing PKC ϵ cDNA. This analysis revealed that PKC ϵ overexpression was sufficient to transform LNCaP cells into an AI variant that rapidly initiated tumor growth, in the absence of Matrigel, in both intact and castrated male nude mice. This transformation of LNCaP growth was accompanied by changes in the expression of key cell cycle regulatory proteins, hyperphosphorylation of protein kinases in the ERK mitogenic signaling cascade, derepression of biosynthetic processes, increased production and expulsion of caveolin-1, and resistance to apoptosis. Finally, when antisense PKC ϵ ODNs were used to specifically block the expression of endogenous PKC ϵ in DU145 and PC3 AI CaP cells, we observed a significant inhibition of Raf-1 and ERK phosphorylation, caveolin-1 expression, and their AI proliferation. This study

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³ The abbreviations used are: CaP, prostate cancer; AI, androgen independent; ERK, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; CDT, charcoal dextran-treated serum; RT-PCR, reverse transcription-PCR; DHT, 5 α -dihydrotestosterone; ODN, oligodeoxynucleotide; PKC, protein kinase C; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; RB, retinoblastoma protein.

provides data from gene transfer and antisense experiments demonstrating that PKC ϵ expression may contribute to recurrent tumor growth in the absence of testicular androgens.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. LNCaP, DU145, and PC3 CaP cell lines were obtained from American Type Culture Collection (Manassas, VA; ATCC CRL-1740, HTB-81, and CRL-1435, respectively). All cell culture reagents were purchased from Invitrogen (Rockville, MD). LNCaP cells were maintained in culture in RPMI 1640 containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, and 1.5 g/l sodium bicarbonate and supplemented with 10% FBS and 100 units/ml penicillin and 100 mg/ml streptomycin. DU145 cells were cultured in MEM Eagle with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM nonessential amino acids, 0.1 mM sodium pyruvate, and supplemented with 10% FBS and 100 units/ml penicillin and 100 mg/ml streptomycin. PC3 cells were cultured in F12K medium with 2 mM L-glutamine and supplemented with 10% FBS and 100 units/ml penicillin and 100 mg/ml streptomycin. Where indicated, cells were cultured in serum-free medium or in medium in which CDT FBS (Hyclone, Logan, UT) was substituted for untreated FBS. Cellular proliferation was assessed using either the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue) assay, according to the manufacturer's specifications (Sigma Chemical Co., St. Louis, MO), or the number of viable cells were counted, in triplicate, using a hemacytometer and trypan blue staining. Each assay was performed in triplicate in at least three independent experiments.

Expression Plasmid and Transfection into LNCaP Cells. LNCaP cells were infected with pLXSN recombinant retrovirus (LNCaP/v) or pLXSN harboring the gene for p3/PKC ϵ (LNCaP/ ϵ) as described previously (20). Stably expressing cells were selected and subcloned by limiting dilution in 500 μ g/ml G418, and resultant subclones were then screened for PKC ϵ protein expression and *in vitro* kinase activity as described previously (20).

Assessment of *in Vivo* Tumor Growth. Intact and surgically castrated nude male mice (NU/NU-*nu*BR) were purchased from Charles River Laboratories (Wilmington, MA) and inoculated s.c., into the dorsal flanks left and right of the midline, with 1×10^6 cells suspended in 250 μ l of PBS/site and routinely inspected for tumor growth and morbidity for up to 10 weeks. Cell cultures used in these studies were free of *Mycoplasma* contamination. Solid tumor volumes were calculated by the formula: length \times width \times depth \times 0.5236.

Immunoblot Analyses. Immunoblot analyses were performed as described previously (20). Antibodies purchased from Santa Cruz Biotechnology, Inc. were raised against caveolin-1 (clone N-20), cyclin D1 (C-20), cyclin E (M-20), ERK1 (K-23), c-Myc (C-19), PKC α (C-15), PKC ϵ (C-15), c-Raf-1 (C-12), and upstream binding factor-1 (H-300). Antibodies purchased from BD Transduction Laboratories (Lexington, KY) were raised against cyclin D3 (C28620), phospho-ERK1/2 (12D4), and the retinoblastoma protein (RB; clone 2). Anti-phospho-Raf-1 (Ser259; 9421) and anti-phospho-RB (Ser807/Ser811; 9308S) antisera were from Cell Signaling Technology (Beverly, MA). Anti-E2F-1 antibody (KH129) was purchased from Geneka Biotechnology (Montreal, Quebec) and anti- β -actin antibody (JLA20) was purchased from Oncogene Research Products (Boston, MA).

RT-PCR Analyses. The SuperScript One-Step RT-PCR System (Invitrogen) was used to analyze LNCaP variants for changes in the steady-state concentrations of PKC ϵ and β -actin mRNA. The PKC ϵ sense and antisense primers were 5'-AGC CGG CTT CTG GAA ACT CCC-3' and 5'-AGC TGC CTT TGC CTA ACA CCT TGA T-3', respectively. The human β -actin primers were purchased from Invitrogen. The following cycling conditions were used in a GeneAmp Thermal Cycler 2400 (Perkin-Elmer): cDNA synthesis and predenaturation in 1 cycle of 50°C for 30 min and 94°C for 2 min followed by 40 cycles of amplification at 94, 58, and 72°C for 1 min each. The final extension was performed in a single cycle at 72°C for 10 min, and RT-PCR products were analyzed using 5% PAGE. The only products visible on these gels are shown in Fig. 5A and corresponded to either a 353-bp (β -actin) or 380-bp (PKC ϵ) fragment.

Assays of Cell Cycle Progression. Subconfluent cultures of cells were collected by mild trypsinization and gentle centrifugation, washed twice in

PBS, fixed in 70% ice-cold methanol, incubated with RNase (20 units), and stained with PI (50 μ g/ml). The DNA content of 1×10^4 PI-stained cells was analyzed by flow cytometry (FACSscan) at 488 nm excitation gated to exclude debris. Scattered light was excluded from the PI signal using the FL2 band-pass filter. The percentage of cells in each phase of the cell cycle was determined using a ModFit 5.02 computer program.

Apoptosis Detection. Subconfluent LNCaP cells and their derivatives (3.5×10^4 cells/well in 24-well plates) were seeded and cultured in complete medium for 24 h at 37°C. Adherent cells were then incubated for an additional 24 h in fresh medium, with or without PMA (100 nM). Medium containing anoikis was transferred to microcentrifuge tubes and sedimented, whereas adherent cells were removed from tissue culture plates by trypsinization and transferred to tubes containing the corresponding anoikis. After centrifugation, cell pellets were washed with PBS, resedimented, and suspended in 10 μ l of PBS containing a 2- μ l aliquot of a dye solution containing 100 μ g/ml acridine orange (Sigma Chemical Co.) and 100 μ g/ml ethidium bromide (Sigma Chemical Co.) in PBS. Cells were examined by epifluorescence microscopy (Nikon Microphot-FX; excitation, 450–490 nm; barrier, 520 nm). The nuclei of apoptotic cells contained uniformly stained condensed or fragmented chromatin. One hundred cells were scored in triplicate for each cell line and treatment condition. Three independent experiments were conducted, and data are expressed as the percentage of apoptotic cells. Caspase-3 proteolytic activity was measured using the PharMingen (San Diego, CA) assay kit, according to the manufacturer's specifications. Data are expressed as the percentage change in proteolytic activity measured in PMA-treated *versus* untreated cell cultures after a 6-h exposure to PMA.

Assays of Protein Synthesis. The kinetics of total protein synthesis were analyzed by measuring the rate of L-[4,5- 3 H]leucine (153 Ci/mmol; Amersham) incorporation into the specified LNCaP subline after growing for 3 days in serum-free medium. Briefly, subconfluent cultures were washed with PBS and cultured for 12 h in leucine-free RPMI 1640 (Invitrogen) before the addition of radiolabel (4 μ Ci/ml) to the medium. Where indicated, test compounds were added to the leucine-free medium prior to the radiolabel. Cells were then incubated at 37°C for the specified time, washed with excess PBS, and lysed in 100 μ l of MPER protein extraction reagent (Pierce Corp., Rockford, IL). Results are expressed as cpm/mg protein. Under these conditions, >95% of the radioactivity incorporated by LNCaP cells was inhibited by 10 μ g/ml cycloheximide (data not shown).

Coimmunoprecipitation Assays. Cells were grown in CDT for 3 days before lysis in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol (w/v), 1% Triton X-100 (w/v), 1 mM EGTA, 1.5 mM MgCl $_2$, 0.4 mM phenylmethylsulfonyl fluoride, 2 μ M pepstatin, 0.1 mg/ml aprotinin, and 1 mg/ml leupeptin. Cellular debris was sedimented by centrifugation (10 min at 12,500 \times g), and the resulting supernatants were precleared by adding 60- μ l aliquots of protein agarose A (Invitrogen) to each sample, mixing for 30 min at 4°C, and sedimentation. Precleared lysates were transferred to sterile microcentrifuge tubes containing either a mouse IgG (1.5 μ g, control), anti-PKC ϵ , or a GST-anti-Raf-1 agarose conjugate (Upstate Biotechnology, Lake Placid, NY) and mixed overnight by rotation at 4°C. Protein agarose A (75 μ l) was added and incubated for 1 h at 4°C before centrifugation (5 min at 12,500 \times g). Supernatants were discarded, and immunoprecipitates were washed three times using excess lysis buffer before solubilizing the final pellets in 60 μ l of a standard SDS-PAGE sample buffer. Masking of the ERK 1/2 bands (M_r 42,000–44,000) by the monomeric IgG heavy-chains (M_r ~50,000) was avoided by solubilizing the immune complexes at room temperature in SDS-PAGE buffer, rather than boiling, and allowing the IgG heavy-chains to dimerize (M_r ~100,000) under this condition.

Antisense PKC ϵ ODN. Phosphorothioate ODNs were obtained from Invitrogen. Sequences for the antisense PKC ϵ ODN and corresponding scrambled control ODN were exactly as specified (21). Subconfluent (70–80%) DU145 and PC3 cultures were washed with Opti-MEM 1 (Invitrogen) before introducing a mixture of ODN (1 μ M) and lipofectin (2 μ g/ml; Invitrogen). After 6 h at 37°C, the cells were washed twice with serum-free medium and incubated 18 h in lipofectin-free medium containing ODN and fresh CDT. Medium was replenished, and the CaP cells were incubated for an additional 3 days in CDT before harvesting using trypsinization.

Data Analysis. Values shown are representative of three or more experiments, unless otherwise specified, and treatment effects were evaluated using

a two-sided Student's *t* test. Errors are SEs of averaged results, and values of $P < 0.05$ were taken as a significant difference between means.

RESULTS AND DISCUSSION

PKC ϵ Causes Androgen-independent Growth and Tumorigenicity. In a recent study of PKC isozyme patterns in specimens of early prostatic adenocarcinoma, there appeared to be a significant increase in PKC ϵ expression compared with control benign tissues (10). To determine whether signals transduced through PKC ϵ had the potential to contribute to the AI progression of CaP, LNCaP cells overexpressing PKC ϵ were established using the pLXSN retroviral vector. The pooled population of PKC ϵ overexpressing LNCaP cells (LNCaP/ ϵ) were selected for by their collective resistance to G418, and a representative subclone (LNCaP/ ϵ 3) was isolated from this pool of transfectants by limiting dilution and maintained in culture. The pLXSN vector control line was called LNCaP/v. LNCaP/ ϵ and LNCaP/ ϵ 3 cells expressed equivalent levels of PKC ϵ (Fig. 2C) and, compared with parental and vector controls, the catalytic activity of PKC ϵ was increased, in the absence of an exogenous PKC activator, by 4.3- and 5.8-fold in adherent LNCaP/ ϵ and LNCaP/ ϵ 3 sublines, respectively (not shown). LNCaP cells are PTEN^{-/-} (22) and, because PTEN plays a dominant role in suppressing PKC ϵ activity (23), this may account for the increased basal activity of PKC ϵ in the LNCaP/ ϵ and LNCaP/ ϵ 3 sublines.

When cultured in complete medium, LNCaP cells maintained a functional androgen receptor signaling pathway (Fig. 1A) and gradually became arrested in G₁ upon androgen removal (Ref. 24 and Fig. 1B). FACS analysis of PI-stained LNCaP cells indicated that >90% of these parental cells were in G₁ after a 96-h incubation in CDT (data

not shown). The forced expression of PKC ϵ dramatically altered this phenotypic response to androgen withdrawal and enabled LNCaP cells to proliferate in the absence of androgens (Fig. 1B) or in serum-free medium (not shown). LNCaP cells overexpressing PKC ϵ (LNCaP/ ϵ) remained responsive to the growth-promoting effects of DHT (Fig. 1A), but their expression of androgen receptor and prostate-specific antigen proteins was substantially reduced and could only be detected after prolonged film exposures (not shown). The conservation of androgen responsiveness in the presence of a declining steady-state concentration of androgen receptor protein suggested that the overexpression of PKC ϵ in LNCaP cells could be associated with androgen hypersensitivity. These results prompted investigation into the effects of PKC ϵ overexpression on the tumorigenicity of LNCaP cells in the absence and presence of testicular androgens.

Mycoplasma-free LNCaP/ ϵ 3 cells (1×10^6 /site) were injected s.c. into intact and castrated male nude mice. Matrigel was not used as an adjunct in any of these experiments, and all cells were injected alone as a suspension in PBS. Within 3–4 weeks, tumors appeared with a take rate of 100%, and the onset of tumor growth was more rapid in castrated animals (Fig. 1C). A similar result was obtained using the heterogeneous LNCaP/ ϵ subline in castrated mice (Fig. 1C). No tumors formed during a 10-week observation period when an equal number of LNCaP or LNCaP/v cells were injected without Matrigel into intact male nude mice. The finding that LNCaP cells overexpressing PKC ϵ grew tumors *in vivo* more rapidly in castrated than intact male mice is similar to that of LNCaP variants that have been clonally selected in CDT medium for AI growth, e.g., LNCaP 104-S (25) and CL-1 (26). Forced overexpression of the HER-2 receptor tyrosine kinase also enables LNCaP cells to form xenograft tumors in castrated nude mice but only if mixed with Matrigel (27), a potent inducer of LNCaP cell growth. These studies indicate that PKC ϵ has the potential to advance the progression of CaP and may be sufficient to initiate recurrent tumor growth in the absence of testicular androgens.

PKC ϵ Accelerates Transit through the G₁ Restriction Point. FACS analysis of exponentially multiplying LNCaP/ ϵ 3 cells revealed that PKC ϵ overexpression decreased the number of cells in the diploid compartment and increased the number of cells in S-phase, relative to their parental counterparts (Fig. 2A). In complete medium, LNCaP and LNCaP/v cells doubled in 55 h and had an average G₁ phase duration of 42 h (76% in G₁ \times 55 h = 41.8 h). In contrast, the LNCaP/ ϵ and LNCaP/ ϵ 3 lines had an average doubling time of 19 h and traversed G₁ in ~8 h (43% in G₁ \times 19 h = 18.2 h). Thus, increased expression of PKC ϵ accelerated G₁ to S transit of LNCaP cells.

PKC ϵ and ERK Signaling to RB. The G₁ arrest associated with androgen ablation in LNCaP cells is dependent on the growth-restraining activity of the RB family of transcriptional repressor proteins (28); androgen independence may be achieved through signals that disrupt the function of pocket proteins such as RB, p130, and p107. Collectively, the data in Fig. 2 provide strong support for this hypothesis. Raf-1 is an imminent, if not direct, target of PKC ϵ (29) that is capable of inactivating RB (30). Coimmunoprecipitation experiments showed that PKC ϵ remained constitutively associated with phospho-Raf-1 and ERK 1/2 in both quiescent LNCaP cells and the AI-LNCaP/ ϵ cells that continued to proliferate after 3 days in CDT (Fig. 2B, upper panel). The association of PKC ϵ with Raf-1 was confirmed by using a GST-anti-Raf-1 agarose conjugate to perform a reciprocal pull-down of endogenous Raf-1 from precleared LNCaP and LNCaP/ ϵ lysates. The lower panel in Fig. 2B shows that Raf-1 immunoprecipitates from these two cell lines contained equivalent amounts of PKC ϵ protein. In contrast to LNCaP cells, however, PKC ϵ immunoprecipitates from LNCaP/ ϵ cells pulled down caveolin-1 (Fig.

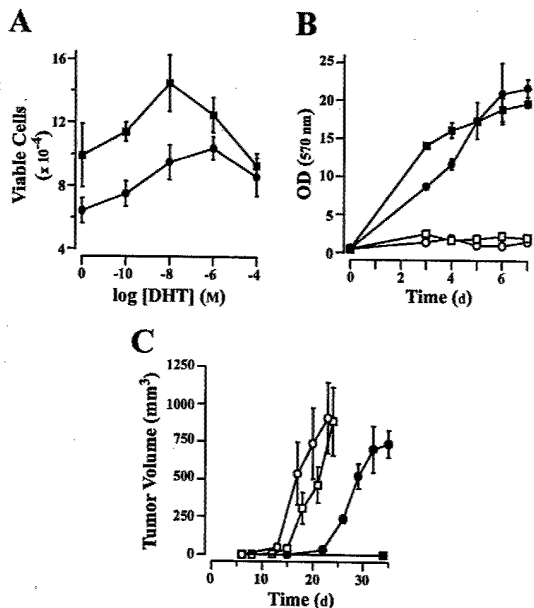


Fig. 1. Growth characteristics of LNCaP cells overexpressing PKC ϵ . A, growth-promoting effects of increasing concentrations of DHT on LNCaP (●) and LNCaP/ ϵ (■) cells incubated at 37°C for 3 days in CDT. Cells were serum starved overnight before the introduction of DHT, and cell proliferation was measured by counting the total number of viable cells/plate by trypan blue exclusion. Data are the means of triplicate determinations in three independent experiments; bars, SE. B, growth of LNCaP (○), LNCaP/v (□), LNCaP/ ϵ (●), and LNCaP/ ϵ 3 (■) cells in CDT medium. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are the means of triplicate determinations in three independent experiments; bars, SE. C, intact (closed symbols) and castrated (open symbols) male nude mice were injected s.c. with 1×10^6 LNCaP/LNCaP/v (■), LNCaP/ ϵ (□), or LNCaP/ ϵ 3 (● and ○) cells suspended in 0.25 ml of PBS. Data are the mean tumor volumes for each group of eight mice; bars, SE.

2B, upper panel). It has been reported that PKCε remains physically associated with Raf-1 in a biochemically inactive signaling complex when fibroblasts are forced into quiescence by serum starvation (29) and that the scaffolding domain of caveolin-1 inhibits the *in vitro* catalytic activity of PKCα and PKCζ (15). These observations suggested that the caveolin-based PKCε:Raf-1:ERK 1/2 complex may not be actively signaling in LNCaP/ε cells. However, immunoblot analyses of whole cell lysates indicated that the general levels of Raf-1 and ERK 1/2 phosphorylation remained elevated in both LNCaP/ε and LNCaP/ε3 cells after 3 days in CDT, in comparison to either the parental or vector controls (Fig. 2C). These experiments implied that PKCε was able to colocalize with caveolin-1 and Raf-1 while indirectly promoting ERK phosphorylation (activation). The fact that PKCε remains biochemically active when linked to caveolin-1 has been attributed to the unique location of the caveolin-binding motif in this isozyme (*i.e.*, subdomain IV of PKCε *versus* subdomain IX of the PKCα catalytic domain, see Ref. 15). On the basis of these and other studies, we hypothesized that PKCε has the potential to activate members of the ERK cascade that are capable of deregulating the cell cycle progression of LNCaP cells through hyperphosphorylation of RB (30).

In the absence of androgen and/or serum, LNCaP cells undergo a

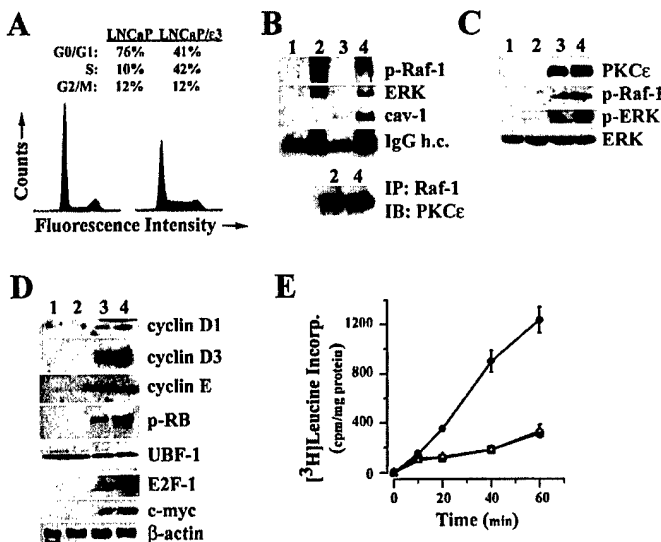


Fig. 2. Effects of PKCε on ERK signaling to RB, and translational activity. A, PI staining and flow cytometric analysis of asynchronous and subconfluent cultures of LNCaP (left) and LNCaP/ε3 (right) cells. Data represent the means of triplicate determinations. B, coimmunoprecipitation of PKCε-associated proteins from LNCaP and LNCaP/ε cells. Upper panel, LNCaP and LNCaP/ε cells were grown for 3 days in CDT before isolating PKCε immunoprecipitates (Lanes 2 and 4) from precleared lysates containing 1% Triton X-100. Controls (Lanes 1 and 3) were not exposed to the anti-PKCε antiserum before the addition of secondary IgG. Immunoprecipitates were solubilized in equal volumes of SDS sample buffer, and equal sample volumes were resolved by SDS-PAGE and immunoblotted using anti-phospho-Raf-1, anti-ERK 1/2, or anti-caveolin-1 antibodies. The relative amounts of IgG heavy chains (h.c.) loaded for each sample are shown as a control. Lower panel, endogenous Raf-1 was immunoprecipitated (IP: Raf-1) from LNCaP and LNCaP/ε cell lysates (Lanes 2 and 4, respectively), using an anti-Raf-1 agarose conjugate, and immunoblotted using an anti-PKCε antiserum (IB: PKCε). Results are representative of two independent experiments. C, immunoblot analysis of whole cell lysates isolated from LNCaP, LNCaP/ε, LNCaP/ε3 cells after a 3-day incubation in CDT (Lanes 1–4, respectively). Lysates were probed using antibodies raised against PKCε, phosphorylated Raf-1, phosphorylated ERK 1/2, and total ERK 1/2 protein. Results are representative of four independent experiments. D, LNCaP, LNCaP/ε, LNCaP/ε3 cells (Lanes 1–4, respectively) were grown in serum-free medium for 3 days. Cells were harvested, whole cell lysates were prepared, and equal protein was resolved by SDS-PAGE. The endogenous proteins specified were detected by immunoblotting with their respective antibodies. Results are representative of two independent experiments. E, time course of [³H]leucine incorporation by LNCaP (○), LNCaP/ε (□), and LNCaP/ε3 (●) cells after growing for 3 days in serum-free medium. Data are the means of triplicate determinations in three independent experiments; bars, SE.

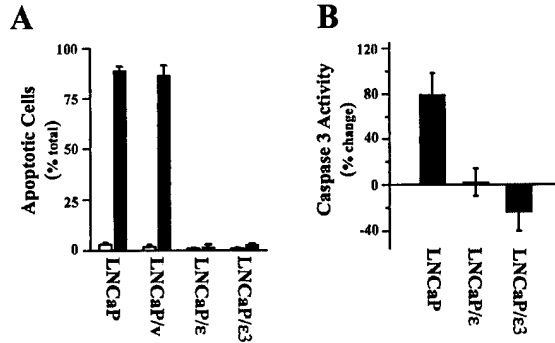


Fig. 3. PMA responsiveness of LNCaP cells overexpressing PKCε. A, apoptotic response of LNCaP, LNCaP/ε, LNCaP/ε3 cells during a 24-h exposure to either 10% FBS (□) or 10% FBS containing 100 nM PMA (●). Apoptotic cells were identified and counted using the acridine orange staining method; a total of 100 stained cells were analyzed per plate. Data represent the mean percentage of stained cells that were apoptotic, counted in triplicate in three independent experiments; bars, SE. B, caspase 3 proteolytic activity measured in LNCaP, LNCaP/ε, and LNCaP/ε3 cells after 6 h incubation in the absence or presence of 100 nM PMA. Data are expressed as the percentage change in proteolytic activity resulting from exposure to PMA and are the means of triplicate determinations in three independent experiments; bars, SE.

programmed response that is characterized by reduced levels of G₁ cyclin-dependent kinase and RB activities (24). The data in Fig. 2D show that the overexpression of PKCε was sufficient to disrupt this inherent cellular response. Compared with the parental and vector controls, both LNCaP/ε and LNCaP/ε3 cells maintained elevated levels of inactive/phosphorylated RB and continued to express elevated levels of cyclins D1, D3, and E and the E2F-1 transcription factor after 3 days in serum-free medium. Other cell cycle regulators and transcription factors were either unaltered in their expression (p21^{waf1/cip1} and upstream binding factor-1) or showed no consistent changes (p27^{kip1} and TATA binding protein-1).⁴ PKCε overexpression also increased cellular levels of c-myc (Fig. 2D), which is an oncogene with functional E2F binding sites in the promoter DNA sequence that is up-regulated in expression during the progression to androgen independence in some CaPs (3). Taken together, the data point to PKCε as an active regulator of the ERK to RB signaling pathway in LNCaP cells.

PKCε Stimulates Protein Synthesis and Cellular Proliferation. Abnormal stimulation of the translation apparatus may constitute a major step toward tumor development (31), and our studies demonstrate that under the stress of serum starvation, the autocrine growth of LNCaP/ε and LNCaP/ε3 cells was associated with a 7-fold increase in their rate of general protein synthesis, measured as the incorporation of [³H]leucine over time (Fig. 2E). Although the cellular content of DNA was increased 4-fold in LNCaP/ε3 cells (Fig. 2A), their rate of total RNA synthesis ([³H]uridine incorporation) did not change relative to the parental or vector controls (data not shown). PKCε appears to stimulate translation upstream of the polypeptide elongation stage. *Pseudomonas* exotoxin A, an inhibitor of elongation factor-2, decreased protein synthesis with equal potency in all cell lines with a maximum inhibitory effect of 96% at 10 μg/ml (data not shown).

PKCε Renders LNCaP Cells Resistant to the Apoptotic Effects of PMA. PMA induces a massive apoptogenic response in LNCaP cells (13). Greater than 80% of parental or vector control cells cultured in the presence of PMA (100 nM) for 24 h tested positive for apoptosis using acridine orange staining of the cells (Fig. 3A). In contrast, apoptosis was rarely observed when LNCaP/ε or LNCaP/ε3 cells were treated with equimolar concentrations of PMA. In LNCaP cells, the apoptosis induced by PMA was preceded by the stimulation

⁴ D. Wu and D. M. Terrian, unpublished data.

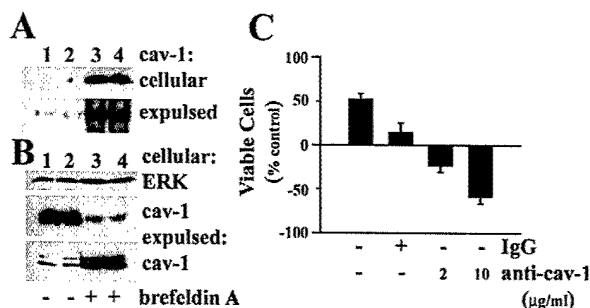


Fig. 4. Brefeldin A-induced expulsion of caveolin-1 and survival of LNCaP cells. *A*, immunoblot analysis of caveolin-1 in cell lysates (*cellular*) and conditioned media (*expulsed*) from LNCaP, LNCaP/v, LNCaP/ε, and LNCaP/ε3 sublines (*Lanes 1–4*, respectively) cultured in serum-free medium for 3 days. *B*, LNCaP/ε (*Lanes 1 and 3*) and LNCaP/ε3 (*Lanes 2 and 4*) cells were cultured for 24 h in serum-free medium in the absence (*Lanes 1 and 2*) or presence (*Lanes 3 and 4*) of 10 μM brefeldin A. Equal amounts of cellular and expelled protein was loaded per lane (confirmed by staining with Coomassie Brilliant blue) and resolved by SDS-PAGE. Cellular and released caveolin-1 protein was detected by immunoblotting, and ERK 1/2 protein was detected in cell lysates, as a control for equal loading. No ERK 1/2 protein was detected in the conditioned medium. *C*, cell viability measured by trypan blue exclusion. LNCaP cells were incubated in serum-free medium as a control or in conditioned medium from LNCaP/ε3 cells for 3 days before counting viable cells. The conditioned medium contained either no additives, IgG (10 μg/ml), or anti-caveolin-1 antibody (2 or 10 μg/ml). The percentage of viable cells was expressed relative to the number in serum-free medium alone. Data represent the means of triplicate determinations; bars, SE.

of caspase 3 proteolytic activity, whereas no change in the activity of caspase 3 was detected in LNCaP/ε or LNCaP/ε3 cells after a 6-h exposure to 100 nM PMA (Fig. 3B). It has been proposed recently that both dephosphorylation (activation) of RB and the repression of c-myc transcription are prerequisite events for PKC-mediated apoptosis in LNCaP cells (31). The present studies indicate that PKCε overexpression renders LNCaP cells resistant to the apoptogenic effects of PMA (Fig. 3) while also preventing the activation of RB or c-myc repression during androgen ablation (Fig. 2D). Additional studies of the response of LNCaP/ε cells to PMA should reveal whether PKCε signaling uncouples the apoptotic response of LNCaP cells to PMA by either preventing RB activation or c-myc repression.

PKCε Enhances the Synthesis and Expulsion of Caveolin-1.

Caveolin-1 is an unusually versatile membrane protein that not only regulates cholesterol trafficking and the assembly of signaling complexes within the cell but is also able to function as an autocrine/paracrine factor when secreted by CaP cells (17). PKCε overexpression significantly increased both the steady-state levels of cellular caveolin-1 and the “secretion” of this protein by LNCaP cells (Fig. 4A). Control (LNCaP and LNCaP/v) and PKCε overexpressing LNCaP cells were grown in serum-free medium for 3 days in the absence of any exogenous secretagogues before cell lysates and medium were analyzed for caveolin-1 by immunoblotting. In a separate experiment, LNCaP/ε3 cells were cultured in serum-free medium for 1 day in the absence or presence of brefeldin A (10 μM) to determine whether caveolin-1 must enter the default secretory pathway via the lumen of the ER/Golgi apparatus to exit the cell. Exposure to brefeldin A significantly depleted the intracellular pool of caveolin-1 and increased the amount of this protein in conditioned medium (Fig. 4B). This finding supports the notion that caveolin-1 may accumulate into preexisting lipid droplets in the presence of brefeldin A (32) and be released as a lipoprotein that remains associated with a fibrous protein(s) after dissolution of the lipid droplet by Triton X-100 (33). Our studies also confirm an earlier report that caveolin-1 has the potential to stimulate the survival of CaP cells (17). Medium preconditioned by LNCaP/ε3 cells (3 days) had a positive effect on the growth/survival of LNCaP cells held in serum-free medium (Fig. 4C). Treatment of the LNCaP/ε3 conditioned medium with anti-caveolin-1 antiserum completely blocked this growth-promoting response and

significantly reduced the viability of LNCaP cells ($P < 0.001$) compared with the IgG-treated medium (Fig. 4C). These studies suggest that PKCε had the potential to ensure the survival of CaP cells by promoting the expression and expulsion of caveolin-1.

With the capacity to coordinately deregulate the proliferation and survival of CaP cells, PKCε could play a dominant role in the clonal expansion of prostatic epithelial cells that naturally overexpress this oncoprotein in recurrent CaP. At the same time, however, we remained circumspect and questioned whether the proliferative autonomy of LNCaP/ε cells could have arisen as an artifact because of the forced overexpression of this oncoprotein rather than the activation of a normal program for oncogenesis. In this regard, it is important to note that essentially the same phenotypic changes were observed in two independently transfected and heterogeneous pools of LNCaP/ε cells (selected in G418 but not subcloned). Nevertheless, it was evident that alternative experimental strategies would be required to critically examine the importance of PKCε in the proliferation and survival of CaP cells. For this reason, we extended our analysis of PKCε expression to include additional models of the progression to androgen independence in CaP and used complimentary antisense strategies to determine whether AI CaP cells are dependent for their proliferation or survival on the expression of endogenous PKCε.

Increased Steady-State Concentrations of PKCε in AI CaP Cell Lines. Immunoblot analysis revealed that the levels of endogenous PKCε were increased in AI DU145 and AI PC3 cell lines in comparison with the androgen-dependent LNCaP cell line (Fig. 5A). PKCε protein was not detected in immunoblots of whole cell lysates prepared from subconfluent cultures of LNCaP cells, although the protein could be detected in PKCε immunoprecipitates (not shown). Compared with the LNCaP cell line, AI DU145 and AI PC3 cells ex-

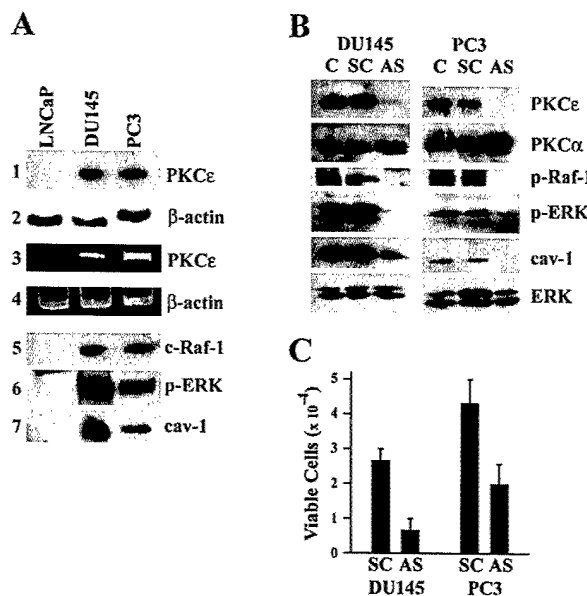


Fig. 5. PKCε expression in CaP cell lines and the antiproliferative effects of antisense PKCε in AI-CaP cells. *A*, immunoblot analysis of protein lysates (*panels 1, 2, and 5–7*) and RT-PCR of total RNA (*panels 3 and 4*) from subconfluent cultures of CaP cell lines. The endogenous PKCε, c-Raf-1, caveolin-1, phospho-ERK 1/2, and β-actin (loading control) proteins were detected by immunoblot analysis with their respective antibodies. Equal amounts of RNA (1 μg) were subjected to RT-PCR, and products of the predicted size encoding PKCε and β-actin were detected (*panels 3 and 4*). The results shown are representative of two experiments. *B*, subconfluent cultures of DU145 and PC3 cells were exposed to lipofectin alone (*C*), lipofectin plus 1 μM scrambled control (*SC*) PKCε ODN, or 1 μM antisense (*AS*) PKCε ODN for 3 days in CDT. Protein lysates were analyzed for endogenous proteins by immunoblotting using the respective antibodies. The results shown are representative of two independent experiments. *C*, after ODN treatment, as in *B*, viable cells were counted using the trypan blue exclusion assay. Data represent the means of triplicate determinations in three independent experiments; bars, SE.

pressed elevated levels of PKC ϵ , Raf-1, phospho-ERK 1/2, and caveolin-1 (Fig. 5A). In DU145 and PC3 cells, the increased level of PKC ϵ protein was associated with an increase in the amount of a RT-PCR product encoding PKC ϵ (Fig. 5A), implying that the PKC ϵ gene may be transcriptionally up-regulated in some AI CaP cells. Alternatively, the AI DU145 and AI PC3 cell lines may have been derived from metastases that had grown to be relatively homogeneous for PKC ϵ -positive cells *in vivo*, possibly via a selection process favoring the survival of these cells.

Antiproliferative Effects of Antisense PKC ϵ ODNs in AI CaP Cells. To investigate the role of endogenous PKC ϵ in the growth of AI CaP cells, an antisense PKC ϵ ODN was used for selective down-regulation of expression of this isoenzyme in DU145 (PTEN^{+/+}) and PC3 (PTEN^{-/-}) CaP cells. DU145 and PC3 cells were preincubated with either the scrambled or antisense PKC ϵ ODN (1 μ M) for 3 days, and lysates were analyzed by immunoblotting for changes in the steady-state concentration of PKC α , PKC ϵ , phospho-Raf-1, phospho-ERK 1/2, caveolin-1, and ERK 1/2. Regulation of PKC ϵ mRNA was selectively and effectively down-regulated by the antisense PKC ϵ ODN, whereas the steady-state concentrations of PKC α and ERK 1/2 remained unaltered (Fig. 5B). Under identical conditions, neither lipofectin alone nor lipofectin plus the scrambled PKC ϵ ODN inhibited PKC α , PKC ϵ , caveolin-1, or ERK1/2 synthesis in these two cell lines (Fig. 5B). The decreased expression of PKC ϵ induced by the antisense PKC ϵ ODN was associated with sequence-specific reductions in the phosphorylation of Raf-1 and ERK 1/2 and the expression of caveolin-1 in both the DU145 and PC3 cell lines (Fig. 5B). In addition, after a 3-day incubation, 1 μ M antisense PKC ϵ ODN inhibited the growth of DU145 and PC3 cells in complete medium by ~45%, relative to the scrambled PKC ϵ ODN control. The growth-inhibitory effects of this treatment were augmented when these same cells were grown in CDT. When DU145 and PC3 cells were cultured for 3 days in CDT, 1 μ M antisense PKC ϵ ODN inhibited the AI growth of DU145 and PC3 cells by 75 and 54%, respectively (Fig. 5C). These results are consistent with the suggestion that PKC ϵ may function upstream of Raf-1 in the ERK signaling cascade, where it plays an important role in sustaining the expression of caveolin-1 and the AI growth and proliferation in both PTEN^{+/+} (DU145) and PTEN^{-/-} (PC3) CaP cells.

The major finding of the present study was that PKC ϵ is an oncogenic protein with the potential to induce AI growth of LNCaP tumors in castrated animals. Now that there is direct evidence that PKC ϵ is capable of functioning as a complete oncogene in the LNCaP tumor model, the signaling mechanism(s) that confer this potential should be rigorously investigated. Our gene transfer experiments demonstrate that PKC ϵ overexpression transforms LNCaP cells into AI tumor cells that recapitulate many hallmark features of recurrent CaP. The overexpression of PKC ϵ leads to an uncontrolled and accelerated proliferation of LNCaP cells associated with the constitutive activation of the ERK signaling cascade, hyperphosphorylation of the transcriptional repressor RB, and the increased expression of E2F-1 and other m⁷ GTP cap-dependent mRNAs, including the G₁ cyclins, c-myc, and caveolin-1. Although there is no question that Raf-1 is a downstream target of PKC ϵ , Ras/Raf induction alone is insufficient to promote the AI proliferation of LNCaP cells (34, 35). Therefore, PKC ϵ must signal to additional downstream targets, possibly within the caveolin-1 signaling complex, to overcome the growth-regulatory signals that normally control the cell cycle progression of LNCaP cells. Caveolin-1 differentially influences the biochemical activity of its binding partners, up-regulating ligand-dependent androgen receptor signaling (36) while inhibiting the activity of proapoptotic (PKC α and PKC δ) but not oncogenic (PKC ϵ) isozymes of PKC (14, 15). It is of note that multiple oncoproteins, and PKC

isozymes, have been overexpressed in LNCaP cells without producing the phenotype of LNCaP/ ϵ cells. Although many important details need to be further investigated, this study demonstrates that PKC ϵ may play an important role in the progression to androgen independence in some human prostate cancers.

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Regulation of Caveolin-1 Expression and Secretion by a Protein Kinase C ϵ Signaling Pathway in Human Prostate Cancer Cells*

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Caveolin-1, androgen receptor, c-Myc, and protein kinase C ϵ (PKC ϵ) proteins are overrepresented in most advanced prostate cancer tumors. Previously, we demonstrated that PKC ϵ has the capacity to enhance the expression of both caveolin-1 and c-Myc in cultured prostate cancer cells and is sufficient to induce the growth of androgen-independent tumors. In this study, we have uncovered further evidence of a functional interplay among these proteins in the CWR22 model of human prostate cancer. The results demonstrated that PKC ϵ expression was naturally up-regulated in recurrent CWR22 tumors and that this oncoprotein was required to sustain the androgen-independent proliferation of CWR-R1 cells in culture. Gene transfer experiments demonstrated that PKC ϵ had the potential to augment the expression and secretion of a biologically active caveolin-1 protein that supports the growth of the CWR-R1 cell line. Antisense and pharmacological experiments provided additional evidence that the sequential activation of PKC ϵ , mitogen-activated protein kinases, c-Myc, and androgen receptor signaling drove the downstream expression of caveolin-1 in CWR-R1 cells. Finally, we demonstrate that mitogen-activated protein kinases were required downstream of PKC ϵ to derepress the transcriptional elongation of the *c-myc* gene. Our findings support the hypothesis that PKC ϵ may advance the recurrence of human prostate cancer by promoting the expression of several important downstream effectors of disease progression.

Caveolin-1 is an unusually versatile membrane protein that not only regulates cholesterol trafficking and the assembly of multimeric signaling complexes within the cell but is also able to function as an autocrine/paracrine factor when secreted by human prostate cancer cells (1). For an integral membrane protein that is normally transported to, and retained by, the plasma membrane (2), the latter finding was unexpected. However, there is now good evidence that biologically active caveolin-1 escapes into the medium of cultured human and mouse prostate cancer cells, where this protein functions to suppress apoptosis and regulate their androgen responsiveness (1, 3). Moreover, the finding that caveolin-1 antibodies suppress the growth and metastasis of prostate cancer tumors in mice (1)

clearly indicates that the aberrant sorting and secretion of caveolin-1 has the potential to promote the progression of this disease *in vivo*. This is significant because metastatic prostate cancer cells are generally more resistant to apoptosis and express increased levels of caveolin-1, a prostate cancer progression marker that positively correlates with the Gleason score of biopsies from patients after undergoing radical prostatectomy (4, 5).

We do not know how caveolin-1 becomes misrouted in prostate cancer cells, but this can be experimentally induced by enhancing caveolin-1 expression or forcing cells to accumulate excess levels of this protein in the endoplasmic reticulum (ER)¹ (2, 3). Recent studies suggest that, under these conditions, caveolins may be redistributed to lipid droplets that are in continuity with the ER membrane (2). Although these droplets can bud through the plasma membrane, there is no direct evidence that lipid droplets transport caveolins from the ER to plasma membrane. Therefore, identifying and characterizing the signaling pathways that are capable of enhancing caveolin-1 expression and secretion, while promoting the survival and recurrent growth of prostate cancer tumors in the absence of testicular androgens, will be imperative to understanding the progression of this disease. We have recently shown that a PKC ϵ -mediated signaling pathway meets each of these four criteria and may be sufficient to advance the recurrence of some prostate cancers (3).

PKC ϵ is an oncoprotein that glandular epithelial cells express in malignant but not benign prostatic tissues (6). PKC ϵ contributes to prostatic oncogenesis by accelerating the rate of androgen-independent cell proliferation and tumor growth, blocking the mitochondrial death (apoptotic) signaling pathway, stimulating mitogen-activated protein kinases (MAPK), and derepressing the translation of 5'-cap-dependent mRNAs, including the G₁ cyclins, caveolin-1, and c-Myc (3). In this study, we have questioned whether a functional interplay between specific molecular components within this repertoire of responses to PKC ϵ signaling are responsible for driving the expression of endogenous caveolin-1. The PKC ϵ -induced up-regulation of c-Myc expression is likely to deliver a powerful gain-of-function step in the progression of prostate cancer. This is because an important suppressor of c-Myc oncogenic activity, Bin1 (7), is commonly deleted in metastatic prostate tumors (8). Because compensatory alterations in Max expression do not occur in any human cancer, c-Myc should be fully active in prostate cancer cells expressing elevated levels of PKC ϵ and c-Myc. However, it is less certain how the constitutive activa-

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¹ The abbreviations used are: ER, endoplasmic reticulum; AR, androgen receptor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; ODN, oligodeoxynucleotide; PKC ϵ , protein kinase C ϵ ; RT, reverse transcription.

tion of c-Myc might influence the expression of caveolin-1 in prostate cancer cells. In rodent prostate cancer cells, c-Myc has been reported to repress caveolin-1 expression at the transcriptional level (9). At the same time, there is evidence that c-Myc-Max heterodimers interact with a novel exonic region of the androgen receptor (AR) gene to enhance AR autoregulation in the human PC3 prostate cancer cell line (10). The latter finding may be important because testosterone stimulates caveolin-1 transcription in the androgen-sensitive LNCaP prostate cancer cell line (11). Thus it is possible that LNCaP cells expressing increased levels of PKC ϵ either overcome the repression of caveolin-1 transcription by c-Myc or harness the oncogenic activity of this oncoprotein to indirectly enhance caveolin-1 expression via the AR signaling pathway.

In this study, we demonstrate that the expression of PKC ϵ is naturally up-regulated in recurrent CWR22 xenograft tumors and that endogenous PKC ϵ is required to sustain the proliferation/survival of CWR-R1 cells, a cell line selected for androgen-independent growth from recurrent CWR22 tumors (12). Most of the phenotypic responses to PKC ϵ overexpression that we have previously observed in LNCaP cells (3) were recapitulated in CWR-R1 cells overexpressing this oncoprotein, including the enhanced expression and secretion of biologically active caveolin-1. Antisense experiments demonstrate that the *c-myc* gene is a downstream target of PKC ϵ that is required for the constitutive expression of caveolin-1 in CWR-R1 cells. Our data are in agreement with the hypothesis that PKC ϵ -mediated signals enhance *c-myc* expression by reducing transcriptional attenuation (pause) at the 3' end of exon 1 and, thereby, reinitiate transcriptional elongation (13). Studies using the MAPK kinase (MEK) inhibitor PD98059 indicate that PKC ϵ stimulates the elongation of *c-myc* transcripts through a MEK-dependent signaling pathway(s). Finally, we report that AR activity is required downstream of c-Myc to sustain the translation of caveolin-1 in CWR-R1 cells. The cumulative evidence supports the notion that PKC ϵ transduces important mitogenic and survival signals that are propagated to the caveolin-1 gene through the sequential activation of MEK, ERK, c-Myc, and AR in androgen-independent CWR-R1 cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The CWR-R1 cell line was clonally selected for androgen-independent growth from recurrent CWR22 xenograft tumors (12). These cells were cultured in Richter's improved minimal essential medium (Invitrogen) supplemented with 100 ng/ml epidermal growth factor (Becton Dickinson Labware, Franklin Lakes, NJ), 10 μ g/ml insulin/transferrin/selenium (BD Biosciences; Bedford, MA), 10 mM nicotinamide, 900 ng/ml linoleic acid (Sigma), 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2% fetal bovine serum. Where specified, CWR-R1 cells were cultured in serum-free medium in the absence or presence of either brefeldin A (10 μ M, 24 h; Calbiochem, San Diego, CA) or the MEK inhibitor PD98059 (50 μ M, 48 h; Calbiochem). Subconfluent cultures of CWR-R1 cells were also exposed to the anti-androgen flutamide (1 μ M, Sigma) for 48 h in complete medium.

Expression Plasmid and Transfection into CWR-R1 Cells—CWR-R1 cells were infected with either the empty pLXSN recombinant retrovirus (CWR-R1/v) or pLXSN harboring the gene for p3/PKC ϵ (CWR-R1/ ϵ) as described previously (14). Stably expressing cells were selected and subcloned by limiting dilution in 500 μ g/ml G418, as described previously (3).

Immunoblot Analyses—Immunoblot analyses were performed as described previously (15). Antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA) were raised against AR (N-20), caveolin-1 (N-20), ERK1 (K-23), c-Myc (C-19), and PKC ϵ (C-15). Anti- β -actin antibody (JLA20) was purchased from Oncogene Research Products (Boston, MA).

Antisense Oligodeoxynucleotide Treatment—Phosphorothioate oligodeoxynucleotides (ODN) were obtained from Invitrogen. Sequences

for the antisense PKC ϵ and c-Myc ODNs, and their corresponding scrambled or sense controls, were exactly as specified (3, 16). CWR-R1 cells were treated with ODNs as described previously (3). Briefly, subconfluent (70–80%) cultures were washed with Opti-MEM 1 (Invitrogen) before introducing a mixture of ODN and Lipofectin (2 μ g/ml; Invitrogen). After 6 h at 37 °C, the cells were washed twice with Opti-MEM 1 and incubated 18 h in Lipofectin-free medium containing ODN and 2% fetal bovine serum. Medium was replenished, and the cells were incubated for an additional 3 days in complete medium containing ODN before harvesting using trypsinization.

RT-PCR Analyses—CWR-R1 cells (1×10^6) were collected by trypsinization and centrifugation at $2,000 \times g$ for 5 min. Total RNA was prepared using an RNeasy Mini kit (Qiagen, Valencia, CA). The Superscript one-step RT-PCR system (Invitrogen) was used to analyze the relative abundance of *c-myc* exon 1 and/or exon 2 transcripts. RT-PCR primers for human *c-myc* were derived from the published sequences. The sequences of the primers and their positions in the gene sequence are as follows: c-Myc, 5'-primer, 5'-CCTGCCTCGAGAAGGGCAGGGCTTCT-3' (48–73); 3'-primer within exon 1, 5'-TCAGAGAAGCGGGTCCTGGCAGCGGGGAAGT-3' (453–486); 3'-primer within exon 2, 5'-GTTGGTGAAGCTAACGTTGAGGGGCATCGTCGCGGGA-3' (549–585). A RT-PCR primer and control set (Invitrogen), containing RT-PCR primers for β -actin, was used to confirm equal loading of all samples. RT-PCR reactions were performed in a PerkinElmer Life Sciences DNA thermal cycler GeneAmp PCR system 2400. The thermal cycling conditions comprised a cycle of cDNA synthesis and pre-denaturation at 45 °C for 15 min and 94 °C for 2 min, followed by 35 cycles of amplification at 94 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. RT-PCR products were analyzed using 5% PAGE. The size of the products for human *c-myc* exon 1, exon 2, and β -actin were 439, 538, and 353 bp, respectively.

Preparation of Conditioned Medium—CWR-R1 cells were serum-starved overnight and cultured in fresh serum-free medium for 3 days before collecting the conditioned medium. Possible contamination of membranous caveolin-1 was minimized by a sequential centrifugation of the conditioned medium; $1,000 \times g$ for 5 min followed by $20,000 \times g$ for 30 min. Trichloroacetic acid and deoxycholic acid were added to the supernatants to final concentrations of 10 and 0.015%, respectively. After 1 h on ice, the mixtures were centrifuged at $10,000 \times g$ for 10 min, and the resultant pellets were dissolved in 3 M Tris buffer. For *in vitro* viability assays, caveolin-1 antibody (Santa Cruz Biotechnology) was added to conditioned, or complete, medium and incubated for 16 h at 4 °C. Normal rabbit IgG (R & D Systems Inc., Minneapolis, MN) was used as a control.

Data Analysis—Values shown are representative of three or more experiments, unless otherwise specified, and treatment effects were evaluated using a two-sided Student's *t* test. Errors are S.E. values of averaged results, and values of $p < 0.05$ were taken as a significant difference between means.

RESULTS

PKC ϵ Expression in Human Prostate Cancer CWR22 Tumors—Androgen-independent human prostate cancer cell lines (DU145 and PC3) express elevated levels of endogenous PKC ϵ , relative to the androgen-sensitive LNCaP cell line (3). In this study, we have extended this analysis by performing an immunoblot analysis of PKC ϵ expression during the *in vivo* progression of prostate cancer using the CWR22 xenograft model. CWR22 tumors grow subcutaneously in intact male mice and undergo complete regression after castration but recur as androgen-independent tumors after several months (17). This reliable pattern of tumor regression and recurrence made it possible to compare the steady-state levels of endogenous PKC ϵ in androgen-dependent and recurrent CWR22 tumor lysates. An analysis of seven pairs of such tumors indicated that castration induced a reproducible and significant increase in the levels of PKC ϵ protein in CWR22 tumor cells (Fig. 1A). This increase may have resulted from the selective outgrowth of preexisting androgen-independent clones or an up-regulation of PKC ϵ transcription/translation following androgen ablation. Results of a quantitative immunohistochemical analysis of PKC ϵ expression in individual cancer cells within benign and recurrent primary tumor specimens indicate that the overall increase in PKC ϵ protein

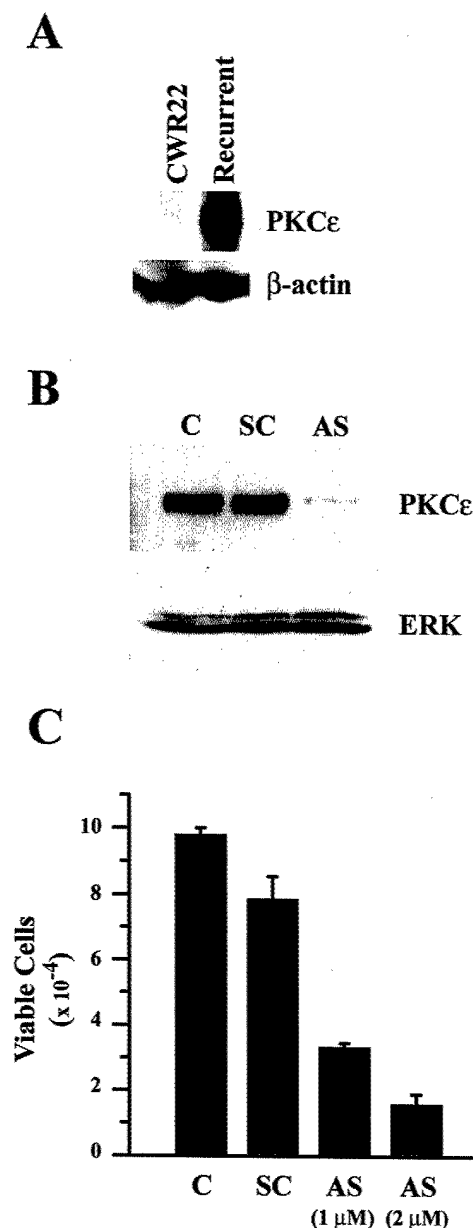


FIG. 1. PKC ϵ protein levels in CWR22 tumors and the anti-proliferative effects of antisense PKC ϵ in CWR-R1 cells. *A*, immunoblot analysis of endogenous PKC ϵ and β -actin (loading control) in lysates of CWR22 and recurrent tumors. Immunoblots are representative of two experiments using a total of 14 tumor lysates. *B*, subconfluent cultures of CWR-R1 cells were exposed to Lipofectin alone (*C*), Lipofectin plus 2 μ M scrambled control (*SC*) PKC ϵ ODN, or 2 μ M antisense (*AS*) PKC ϵ ODN for 3 days. PKC ϵ and ERK1/2 protein levels in CWR-R1 whole cell lysates were analyzed by immunoblotting. The results shown are representative of two independent experiments. *C*, subconfluent cultures of CWR-R1 cells were treated, as in *B*, with Lipofectin alone (*C*), scrambled control (*SC*) ODN, and either 1 or 2 μ M antisense (*AS*) PKC ϵ ODN, as indicated. After 3 days, viable cells were counted using the trypan blue exclusion assay. Data represent the means of triplicate determinations in three independent experiments. Bars, S.E.

shown in Fig. 1A most likely arose from the clonal selection of PKC ϵ -positive glandular epithelial cells.²

Antiproliferative Effects of Antisense PKC ϵ ODNs in CWR-R1 Cells—The CWR-R1 cell line is an androgen-independent derivative of recurrent CWR22 tumors that, unlike DU145 and

PC3 cells, expresses a functional mutated AR (H874Y) with diminished ligand specificity (12). This finding suggests that the CWR-R1 model may more accurately recapitulate the genetic composition of recurrent tumors than the immortalized DU145 or PC3 cell lines. We have recently shown that antisense PKC ϵ ODN significantly inhibits the androgen-independent proliferation of DU145 and PC3 cells (3). In this study, we have extended this analysis to include the androgen-independent CWR-R1 cell line. The results shown in Fig. 1C indicated that by specifically interfering with normal PKC ϵ function, even in the presence of many other PKC isozymes, it was possible to significantly compromise the ability of CWR-R1 cells to sustain their normal rate of proliferation. Translation of PKC ϵ mRNA was selectively and effectively down-regulated by exposing CWR-R1 cells to antisense PKC ϵ ODN without altering the steady-state concentration of ERK1/2 (Fig. 1B). Under identical conditions, neither Lipofectin alone nor Lipofectin plus the scrambled PKC ϵ ODN inhibited PKC ϵ or ERK expression in CWR-R1 cells. The decreased expression of PKC ϵ induced by the antisense PKC ϵ ODN was associated with a sequence-specific and dose-dependent inhibition of CWR-R1 growth/survival (Fig. 1C). After a 3-day exposure to 2 μ M PKC ϵ AS ODN, there was ~75% reduction in the number of viable CWR-R1 cells, relative to the number of CWR-R1 cells growing in the presence of an equimolar concentration of the scrambled ODN. These and other data suggest that PKC ϵ may be an important factor in the pathways governing growth and proliferation within genetically diverse androgen-independent prostate cancer cells (3).

PKC ϵ Enhances Caveolin-1 Synthesis and Secretion by CWR-R1 Cells—In the LNCaP cell line, PKC ϵ overexpression stimulates the expression and secretion of biologically active caveolin-1 (3). CWR-R1 cells express moderate levels of both endogenous PKC ϵ and caveolin-1 (Fig. 3) but did not accumulate sufficient amounts of caveolin-1 in their culture medium to be detected by immunoblotting. However, if the signaling networks linking PKC ϵ to caveolin-1 have been retained during acquisition of an androgen-independent growth capacity in the CWR-R1 cell line, PKC ϵ overexpression would be expected to both increase caveolin-1 expression and secretion. To test these predictions, we established CWR-R1 variants harboring either an empty pLXSN retroviral vector (CWR-R1/v) or the PKC ϵ cDNA (CWR-R1/ ϵ). An immunoblot analysis of whole cell lysates confirmed that CWR-R1/ ϵ , but not CWR-R1/v, cells maintained high levels of PKC ϵ in subconfluent cultures while also augmenting the expression of caveolin-1 (Fig. 2A). At the same time, PKC ϵ overexpression stimulated the secretion (expulsion) of caveolin-1 from CWR-R1 cells (Fig. 2B). When treated with brefeldin A, to collapse the Golgi apparatus into the ER (18), it was possible to detect small amounts of caveolin-1 in CWR-R1 conditioned medium (Fig. 2C, top left panel) and in the CWR-R1/ ϵ cell line it was evident that this protein escaped via a Golgi-independent route of vesicle/lipid droplet-mediated transport. Following a 24-h exposure to brefeldin A (10 μ M), the amounts of caveolin-1 detected in CWR-R1/ ϵ conditioned medium and cell lysates coordinately increased and decreased, respectively (Fig. 2C). In contrast, brefeldin A did not alter the steady-state concentrations of the cytosolic protein ERK1/2 in CWR-R1/ ϵ cells. These results indicated that any genetic aberrations that may be peculiar to the LNCaP cell line do not account for the powerful influence of PKC ϵ -mediated signaling on the synthesis and post-translational sorting of caveolin-1 in human prostate cancer cells.

PKC ϵ -induced Caveolin Secretion Promotes the Proliferation/ Survival of CWR-R1 Cells—The proliferation of CWR-R1 cells was inhibited by 25% when the complete growth medium was

² M. A. McJilton, C. V. Sikes, G. G. Wescott, D. Wu, T. L. Foreman, C. W. Gregory, D. A. Weidner, J. L. Mohler, O. H. Ford, A. M. Lasater, and D. M. Terrian, submitted for publication.

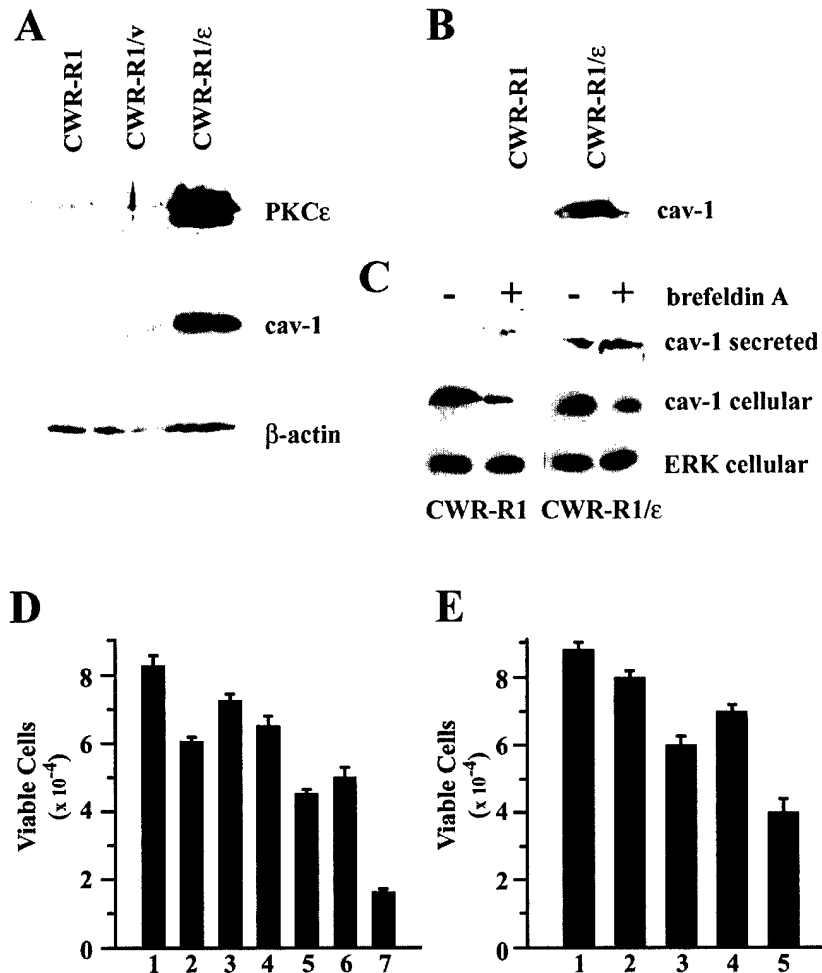


FIG. 2. PKC ϵ regulation of caveolin-1 expression and secretion by the CWR-R1 cell line. *A*, immunoblot analysis of PKC ϵ , caveolin-1 (*cav-1*), and β -actin protein levels in subconfluent cultures of CWR-R1, CWR-R1/v, and CWR-R1/ε cell sublines. *B*, immunoblot analysis of caveolin-1 levels in protein precipitates of serum-free medium conditioned by subconfluent cultures of CWR-R1 or CWR-R1/ε cells for 24 h. *C*, CWR-R1 and CWR-R1/ε cells were incubated in serum-free medium for 24 h in the absence or presence of 10 μ M brefeldin A, as indicated. Cell lysates and the conditioned medium from the same cultures were analyzed by immunoblotting for caveolin-1 and ERK1/2 proteins, as indicated. The ERK1/2 proteins were not detected in the conditioned medium (data not shown). Equal loading of secreted proteins was confirmed by staining with Coomassie Brilliant Blue. Results shown are representative of three independent experiments. *D*, proliferation of CWR-R1 cells was measured using the trypan blue exclusion assay. Subconfluent cultures of CWR-R1 cells were grown for 3 days in complete medium (lane 1) or in medium containing only 2% charcoal-dextran-treated serum (lane 2) or the same medium as in lane 2, supplemented with serum-free medium that had been preconditioned by CWR-R1/ε cell cultures (lanes 3–7). The CWR-R1/ε conditioned medium either contained no additives (lane 3), 2 μ g/ml IgG (lane 4), or anticaveolin antibody (lane 5), 10 μ g/ml IgG (lane 6), or anticaveolin-1 antibody (lane 7). Data represent the means of triplicate determinations. Bars, S.E. *E*, same as in *D*, except that all experiments were performed using complete medium. CWR-R1 cells were cultured for 3 days in complete medium alone (lane 1) or medium containing either IgG (lanes 2 and 4 correspond to 2 or 10 μ g/ml, respectively) or anticaveolin antibody (lanes 3 and 5 correspond to 2 or 10 μ g/ml, respectively). Data represent the means of triplicate determinations. Bars, S.E.

replaced with one containing no exogenous growth factors and charcoal-dextran treated fetal bovine serum (Fig. 2*D*, bars 1 versus 2). Under these conditions, a cell-free medium that had been preconditioned by CWR-R1/ε cells partially reversed this suppression of CWR-R1 autocrine growth and an antibody raised against the N terminus of caveolin-1 not only reversed this modest growth promoting effect of CWR-R1/ε conditioned medium but titrated down the proliferation rate of CWR-R1 cells to as low as 25% of the untreated controls (Fig. 2*D*). These results confirm recent reports that highly malignant mouse and human prostate cancer cells generally depend on caveolin-1 as an autocrine/paracrine growth factor to sustain their proliferation/survival (1, 3). Equivalent concentrations of IgG were significantly less effective in suppressing the autocrine growth and proliferation of CWR-R1 cells. These results indicate that caveolin-1 secreted by prostate cancer cells may either activate mitogenic signaling pathways and/or neutralize the growth suppressive effects of unknown factors that accu-

multate in the medium of CWR-R1 cells under autocrine growth conditions. Anti-caveolin-1 antibody also significantly inhibited the proliferation/survival of CWR-R1 cells grown in complete medium (Fig. 2*E*), suggesting that secreted caveolin-1 makes an important contribution to the optimal growth of these cells in the absence and presence of androgens.

c-Myc Expression and AR Signaling Are Both Required for PKC ϵ to Enhance Caveolin-1 Expression in CWR-R1 Cells—The positive influence that PKC ϵ exerts on the expression of *c-Myc* and caveolin-1 in androgen-sensitive (LNCaP) and androgen-independent (CWR-R1) prostate cancer cells may be of clinical relevance, as increased levels of both *c-Myc* and caveolin-1 proteins are common in advanced prostate cancer and appear to be independent markers of progressive disease (19). However, these two molecular signatures of advanced prostate cancer had not previously been linked at a functional level. To the contrary, when *c-Myc* has been overexpressed in various ways and in both rodent and human prostate cancer cells, investiga-

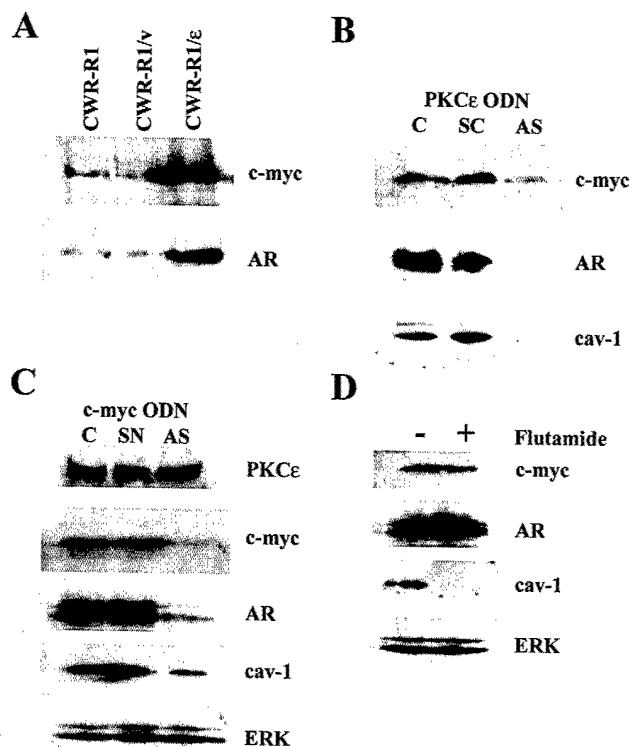


FIG. 3. Both c-Myc expression and AR signaling are required for PKC ϵ to enhance caveolin-1 expression in CWR-R1 cells. A, immunoblot analysis of c-Myc and AR proteins in cell lysates (50 μ g/lane) prepared from subconfluent CWR-R1, CWR-R1/v, and CWR-R1/ ϵ cell cultures. Equivalent loading was confirmed using anti- β -actin antibodies, in Fig. 2A. Immunoblots shown are representative of three independent comparisons. B, subconfluent cultures of CWR-R1 cells were exposed to Lipofectin alone (C), Lipofectin plus 2 μ M scramble control (SC) PKC ϵ ODN, or 2 μ M antisense (AS) PKC ϵ ODN for 3 days. Cell lysates (60 μ g/lane) were analyzed for endogenous c-Myc, AR, and caveolin-1 (cav-1) proteins by immunoblotting using appropriate antibodies. Immunoblots shown are representative of two independent experiments. C, subconfluent cultures of CWR-R1 cells were exposed to Lipofectin alone (C), Lipofectin plus 1 μ M sense (SN) c-Myc ODN, or 1 μ M antisense (AS) c-Myc ODN for 3 days. Cell lysates (60 μ g/lane) were analyzed for endogenous PKC ϵ , c-Myc, AR, caveolin-1, and total ERK1/2 (loading control) by immunoblotting using appropriate antibodies. Immunoblots are representative of two independent experiments. D, subconfluent cultures of CWR-R1 cells were incubated in the absence or presence of 1 μ M flutamide, as indicated, for 48 h. Cell lysates were analyzed for endogenous c-Myc, AR, caveolin-1, and ERK1/2 (loading control) by immunoblotting. Immunoblots shown are representative of two independent experiments.

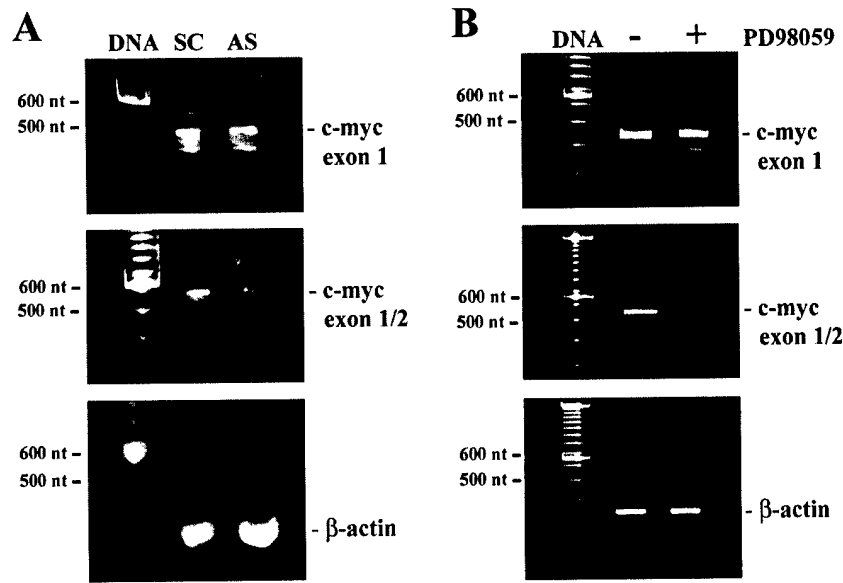
tors have observed a repression of caveolin-1 transcription (9). This implied that at least some of the important downstream responses induced by the enforced expression of c-Myc may not accurately recapitulate the natural progression of those to be found in prostate cancer. We therefore questioned whether PKC ϵ might be capable of transducing signals that either bypassed the transcriptional repression of the caveolin-1 gene by c-Myc or was actually reliant on the physiological influence of this oncoprotein for the up-regulation of caveolin-1 expression. As discussed below, the latter explanation would be consistent with independent reports that AR is a short-lived protein that is degraded by a ubiquitin-mediated proteolytic pathway that may be inhibited by c-Myc-regulated genes (20, 21).

To determine whether PKC ϵ overexpression was capable of simultaneously augmenting the expression of both endogenous c-Myc and AR proteins, we initially performed an immunoblot analysis of whole cell lysates prepared from subconfluent cultures of CWR-R1, CWR-R1/v, and CWR-R1/ ϵ cells. Immunoblots shown in Fig. 3A demonstrated that such an expression

profile could be induced by PKC ϵ overexpression in the CWR-R1 cell line. Next, we performed a series of antisense experiments to determine whether the expression of endogenous PKC ϵ was an important factor in the regulation of c-Myc, AR, and/or caveolin-1 expression in the CWR-R1 cell line. Artificial down-regulation of PKC ϵ with antisense ODN effectively inhibited the translation of either ERK1/2 or β -actin in CWR-R1 cells (Figs. 1B and 3B). Importantly, the levels of these proteins were not different in CWR-R1 cells treated with Lipofectin alone or Lipofectin combined with the scrambled PKC ϵ ODN. As an additional test, we treated subconfluent cultures of CWR-R1 cells with antisense c-Myc ODN. These studies demonstrated that c-Myc translation is required to maintain normal levels of AR and caveolin-1 proteins but not endogenous PKC ϵ or ERK1/2 (Fig. 3C). Controls treated with the sense c-Myc ODN maintained a basal level of AR and caveolin-1 proteins, indicating that the expression profiles observed in these experiments were specifically targeted toward at least two of the putative downstream effectors of endogenous c-Myc in CWR-R1 cells (Fig. 3C). Finally, we used the anti-androgen flutamide to determine whether AR-dependent signaling selectively influences caveolin-1 expression. When subconfluent cultures of CWR-R1 cells were incubated in the presence of complete growth medium plus flutamide (1 μ M for 48 h), the levels of caveolin-1 protein were decreased without altering the levels of endogenous c-Myc, AR, or ERK1/2 (Fig. 3D). The data from these studies support the notion that elevated levels of caveolin-1 expression may be maintained in CWR-R1 cells through the activation of a cascade that relies on the basal expression of endogenous PKC ϵ and c-Myc and the ligand-dependent activation of a downstream effector for the AR signaling pathway.

PKC ϵ Relies on a MAPK Signaling Pathway to Promote the Transcriptional Elongation of c-myc Transcripts in CWR-R1 Cells—Exon 1 of the c-myc gene is a relatively large, untranslated, leader sequence that contains two alternative initiation sites. Despite its lack of coding potential, this exon exerts an important influence on expression of the c-myc gene. Following recruitment and activation of a pre-initiation complex, the presence of a paused RNA polymerase restricts the transcriptional elongation of c-myc beyond the 3' end of exon 1 (22). In erythroid progenitor cells, erythropoietin is able to override this repression of c-myc elongation by activating a PKC ϵ - and MEK-dependent pathway (13, 23). To determine whether downstream effectors of PKC ϵ regulate the transcriptional activity of the c-myc gene through a similar mechanism in CWR-R1 cells, we performed a series of RT-PCR assays to compare the relative steady-state concentrations of c-myc exon 1 versus exons 1 and 2. For c-myc, factors capable of diminishing the ratio of exon 1 (transcriptional initiation) to exon 2 (transcriptional elongation) increase the levels of c-myc transcripts. Using primers targeted for nucleotides 48–73 of exon 1 or nucleotides 453–486 within exon 2 of the human c-myc gene (see "Experimental Procedures"), we consistently detected relatively higher basal levels of the exon 1 transcript in control CWR-R1 cells treated with the scrambled PKC ϵ ODN (Fig. 4, A and B). When the expression of endogenous PKC ϵ was artificially down-regulated using antisense ODN, there was no change in the levels of exon 1 transcripts, whereas transcriptional elongation of c-myc exon 2 was significantly inhibited (Fig. 4A). At the same time, antisense PKC ϵ ODN treatments did not alter transcription of the β -actin gene (Fig. 4A). Finally, an inhibitor of MEK1/2 activity (PD98059) was used to determine whether this mitogenic pathway propagates PKC ϵ signals to the c-myc gene in CWR-R1 cells. The results shown in Fig.

FIG. 4. PKC ϵ promotes the transcriptional elongation of *c-myc* transcripts via a MAPK signaling pathway. A, RT-PCR analysis of total RNA from subconfluent cultures of CWR-R1 cells incubated with scrambled (SC) PKC ϵ ODN or antisense (AS) PKC ϵ ODN for 3 days. Equal amounts of total RNA (1 μ g) were subjected to RT-PCR and products of the predicted size encoding exon 1 (top panel) and exon 1/2 (middle panel) of the human *c-myc* gene and β -actin (bottom panel) were detected. The results shown are representative of two independent experiments. B, RT-PCR analysis of total RNA from subconfluent cultures of CWR-R1 cells incubated in the absence or presence of PD98059 (50 μ M), as indicated, for 48 h. Equal amounts of total RNA (1 μ g) were subjected to RT-PCR and products of the predicted sizes, as in A, were detected. The results shown are representative of two experiments.



4B demonstrate that the signal by PKC ϵ to *c-myc* requires constitutive MEK activity to override the transcriptional repression of *c-myc* in CWR-R1 cells. In contrast, PD98059 treatments did not alter the initiation of *c-myc* transcription or the levels of β -actin transcripts in CWR-R1 cells (Fig. 4B). This finding is consistent with our observation that the MEK/ERK pathway is an imminent, if not direct, target of PKC ϵ in LNCaP cells (3) and provides convincing evidence that this signaling cascade has the potential to release paused RNA polymerase molecules downstream of *c-myc* promoter 2 and promote transcription of its two coding exons in CWR-R1 cells.

DISCUSSION

This study has unraveled the hierarchical organization of at least one signaling pathway that PKC ϵ engages to maintain the basal expression of c-Myc, AR, and caveolin-1 in human CWR-R1 prostate cancer cells. Several independent lines of evidence from gene transfer, antisense, and pharmacological experiments are presented in this paper that support the notion that PKC ϵ is capable of sequentially activating MEK, ERK, *c-myc*, and AR to promote caveolin-1 expression and the proliferation of CWR-R1 cells in culture. Detailed descriptions of the factors governing the activity of the molecular components featured within this PKC ϵ signaling cascade have been proposed and additional intermediary events should be anticipated (9, 10, 13). There is no clear and direct evidence that explains how caveolin-1 is diverted from the classic exocytotic pathway to reach, and cross, the plasma membrane and then function as an autocrine/paracrine factor in prostate cancer progression. Determining the mechanisms controlling caveolin-1 biogenesis and bioactivity in prostate cancer cells will be a significant prerequisite for understanding the malignant progression of this disease. Although growth of CWR-R1 cells is androgen-independent (12), we have demonstrated that PKC ϵ has the potential to promote the proliferation of prostate cancer cells in the presence and absence of testicular androgens *in vivo* (3). Therefore, it should be anticipated that androgen status might not be a critical determinant of how PKC ϵ influences the growth of these cells. However, we have now presented evidence of a functional link between PKC ϵ -mediated signal transduction and the androgen-independent proliferation of a variety of prostate cancer cells (Ref. 3 and this paper). Identification of *c-myc*, AR, and caveolin-1 as important factors in the propagation of growth/survival signals downstream of PKC ϵ

provides crucial support for the hypothesis that these statistically independent predictors of disease progression may actually be interdependent components of a significant regulatory pathway that permits some prostate cancer cells to escape the apoptotic penalty that is generally imposed by androgen withdrawal.

Multiple factors contribute to the progression of prostate cancer. Although there is no question that Raf-1 is a downstream target of PKC ϵ , Ras/Raf induction alone is insufficient to promote the androgen-independent proliferation of prostate cancer cells (24). PKC ϵ must therefore signal to additional downstream targets to promote caveolin-1 expression and the androgen-independent growth of LNCaP and CWR-R1 cells (3). This would be consistent with the inability of the MEK inhibitor PD98059 to completely inhibit *c-myc* transcriptional elongation in CWR-R1 cells (Fig. 4B) and the androgen-independent proliferation of DU145 and PC3 cells.³ Nuclear factor κ B is an attractive alternative candidate because this transcription factor is a known downstream target of PKC ϵ (25) that transcriptionally activates *c-myc* in a variety of cell types (26). In the case of the MEK/ERK-dependent arm of the PKC ϵ signaling cascade, we have now demonstrated that the PKC ϵ -dependent activation of MEK/ERK permits RNA polymerases to resume elongation of *c-myc* transcripts beyond exon 2.

Even minor fluctuations in the transcriptional activity of c-Myc can have profound consequences. Decreasing c-Myc levels by one-half prolongs the cell cycle (27), and unscheduled c-Myc activity is tumorigenic in many cell types (28). The essential and strict control of c-Myc activity is provided by a variety of mechanisms, including chromatin remodeling, long distance interactions between regulatory sequences, proximal *cis*-sequences within the promoter, alternative sites of transcriptional initiation, and repressors of transcriptional elongation (29, 30). *c-myc* has a three-exon/two-intron structure and contains two major promoters (P1 and P2) and two minor promoters (P0 and P3). Transcriptional initiation at the *c-myc* P2 promoter is repressed by a ME1a1 site at position -40 bp relative to the P2 start site (30). After initiation at the upstream P1 promoter, RNA polymerase II seems to pause at the ME1a1 site and requires further activation signals for processive transcription and elongation. It has been suggested that

³ D. Wu and D. M. Terrian, unpublished observations.

the P1 promoter may repress P2 activity and that ME1a1 binding factors are required for opening up the DNA strand of the *c-myc* P2 promoter region (30). However, the identity of functional ME1a1 binding factors and the signals regulating their interaction with the *c-myc* promoter have not yet been well established. The present study provides the first evidence that PKC ϵ signaling through MEK/ERK may be capable of reinitiating *c-myc* transcription in CWR-R1 cells. A potential mechanism for this step in the PKC ϵ to caveolin-1 signaling cascade may involve the inhibition of pocket protein (pRb, p107, and p130) recruitment to the E2F site located at position -64 bp relative to the P2 promoter (31). This suggestion is in line with the observation that PKC ϵ overexpression leads to the hyperphosphorylation (inactivation) of Rb in LNCaP cells (3).

A consistent decrease in the steady-state concentration of AR protein but not mRNA (Fig. 3 and data not shown) accompanied antisense neutralization of c-Myc in the CWR-R1 cell line. Previous studies demonstrated that levels of AR transcripts do not differ between CWR22 and recurrent CWR22 tumors, whereas recurrent CWR22 tumors and CWR-R1 cells maintain significantly elevated levels of AR protein and decreased AR degradation rates (12). Androgen binding prolongs the half-life of AR by forestalling ubiquitin-mediated proteosomal degradation (20, 32) and, in the absence of hormone, Hsp90 stabilizes the unactivated form of the receptor (33). c-Myc regulates functionally diverse sets of genes, and recent studies have implicated c-Myc in the regulation of ubiquitin-dependent proteolysis. Burkitt's lymphoma cells express a constitutively active c-Myc and increased levels of deubiquitinating enzymes (21). These responses to c-Myc activation inhibit ubiquitin-mediated proteolysis in lymphoma cells and could potentially contribute to AR stabilization in recurrent CWR22 tumors. The AR promoter does not contain a consensus E box for direct c-Myc transactivation (10), and there is no evidence of a change in AR mRNA in CWR-R1 cells. These observations reinforce the idea that there may be a regulatory link between c-Myc activation, changes in proteolysis, and AR stability in human prostate cancer cells.

Previous studies demonstrated that caveolin-1 expression is regulated at the transcriptional level by testosterone in prostate cancer cells (11). However, the mechanisms responsible for the transactivation/derepression of the caveolin-1 gene have not been defined. The gene encoding caveolin-1 is localized to the q31.1 region of human chromosome 7 and contains a TATA-less promoter with adjacent E2F/DP-1 and Sp1 consensus sequences and sterol regulatory elements (34). No androgen response elements have been identified in this gene promoter. Although demethylation of CpG islands embedded within the first and second exons of caveolin genes could potentially augment the rate of transcriptional initiation, the data to support such a model remain inconclusive. Given the compelling evidence linking AR and caveolin-1 expression to the recurrence of prostate cancer (5, 12), investigations into the mechanisms controlling AR signaling to the caveolin-1 gene will certainly be of interest to future prostate cancer researchers.

Although many important details need to be established, our findings imply an important role for PKC ϵ , MEK, ERK, c-Myc, and AR in the regulation of caveolin-1 expression by human prostate cancer cells. The potential for PKC ϵ to directly contribute to the recurrence of prostate cancer has been estab-

lished (3), and the signaling cascade we have identified in this study may explain at least one of the ways by which this oncoprotein functions to advance disease progression.

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Regulation of Tumor Invasion and Metastasis in Protein Kinase C Epsilon-Transformed NIH3T3 Fibroblasts

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Abstract Protein kinase C epsilon is an oncogenic, actin nucleating protein that coordinately regulates changes in cell growth and shape. Cells constitutively expressing PKCε spontaneously acquire a polarized morphology and extend long cellular membrane protrusions. Here we report that the regulatory C1 domain of PKCε contains an actin binding site that is essential for the formation of elongate invadopodial-like structures, increased pericellular metalloproteinase activity, *in vitro* invasion of a Matrigel barrier, and the invasion and metastasis of tumors grown *in vivo* by PKCε-transformed NIH3T3 fibroblasts in nude mice. While removing this small actin binding motif caused a dramatic reversion of tumor invasion, the deletion mutant of PKCε remained oncogenic and tumorigenic in this experimental system. We propose that PKCε directly interacts with actin to stimulate polymerization and the extension of membrane protrusions that transformed NIH3T3 cells use *in vivo* to penetrate and degrade surrounding tissue boundaries. *J. Cell. Biochem.* 85: 785–797, 2002. © 2002 Wiley-Liss, Inc.

Key words: protein kinase C; actin; membrane protrusion; invadopodia; metalloprotease; mRNA stability; mitogenicity

As transformed cells progressively become more proliferative and highly invasive, a complex molecular machinery is assembled that enables the tumor to actively invade dense structural barriers and spread to healthy tissues [Aguirre Ghiso et al., 1999]. During the acquisition of this invasive and metastatic phenotype, distinct, and spatially segregated, multimeric signaling networks are engaged to constitutively activate mitogenic and proteolytic cascades [Aguirre Ghiso et al., 1999]. Among others, the protein kinase C (PKC) family of

proteins has been shown to be positively associated with the metastatic spread and invasiveness of human prostate cancer cells [O'Brian, 1998], astroglial brain tumors [Sharif and Sharif, 1999], urinary bladder carcinomas [Busso et al., 1994], gastric cancer cells [Perletti et al., 1998], thyroid carcinomas [Knauf et al., 1999], mammary adenocarcinoma cells [Lavie et al., 1998; Kiley et al., 1999], and murine melanoma cells [LaPorta and Comolli, 1997]. While members of the PKC family play heterogeneous roles in regulating gene expression, cell growth, proliferation, and apoptosis, PKCε remains the only isoenzyme with established oncogenic activity. Indeed, PKCε has been shown to act as a dominant oncogene in rodent fibroblasts [Cacace et al., 1993; Mishak et al., 1993; Cai et al., 1997; Ueffing et al., 1997; Cacace et al., 1998; Wang et al., 1998], colonic epithelial cells [Perletti et al., 1998], and hepatocytes [Perletti et al., 1996]. PKCε was the first member of the novel (classified AGC-IIB) subfamily of lipid-regulated protein serine/threonine kinases to be characterized, and it is presently understood that PKCε may participate in the regulation of diverse cellular

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functions, including the modulation of gene expression [Soh et al., 1999], neoplastic transformation [Cacace et al., 1993; Mishak et al., 1993], mitogenicity [Cai et al., 1997; Ueffing et al., 1997], cell adhesion [Miranti et al., 1999], and motility [Winder et al., 1998].

To date, gene transfer studies have most carefully detailed the cellular consequences of PKC ϵ hyperactivity in preneoplastic rodent fibroblasts. This substantial body of work demonstrates that PKC ϵ overproduction causes malignant transformation and all characteristic changes in structural morphology, serum- and anchorage-dependence of cell cycle progression, and the ability to form tumors in experimental animals [Cacace et al., 1993; Mishak et al., 1993]. While the molecular basis for this disordered growth control remains controversial; there is now evidence that PKC ϵ may be capable of directly activating the classic mitogenic signaling pathway involving Ras and Raf-1 [Cai et al., 1997; Ueffing et al., 1997; Cacace et al., 1998]. More recently, transforming growth factors β (TGF- β) have been proposed as alternative candidates for the downstream effector of PKC ϵ [Cai et al., 1997; Ueffing et al., 1997]. PKC ϵ transformed fibroblasts secrete increased amounts of biologically active TGF- β 2, TGF- β 3, and an uncharacterized epidermal growth factor-like mitogen, implying that growth autocrine loops may account for the oncogenic activity of PKC ϵ .

Beyond stimulating autocrine and anchorage-independent growth, excessive or inappropriate PKC ϵ activity may contribute to tumor progression by regulating morphogenic processes that are relevant for the invasive and/or metastatic behavior of malignant tumor cells. In previous studies, we investigated the potential involvement of PKC ϵ in cytoskeletal remodeling and reported that this isoenzyme of PKC contains an actin binding motif that positions it within a cytoskeletal matrix where many PKC substrates are localized [Prekeris et al., 1996; Prekeris et al., 1998; Hernandez et al., 2001]. While phorbol esters facilitated the *in vitro* interactions between recombinant PKC ϵ and purified α -actin [Prekeris et al., 1998], these PKC agonists are not required to observe the morphogenic effects of PKC ϵ overexpression in living cells [Hernandez et al., 2001]. In the present investigation, we have confirmed that PKC ϵ overexpression induces the transformation of preneoplastic NIH3T3 fibroblasts

and have extended this work by identifying a specific protein-protein interaction that is required for these transformed cells to penetrate and degrade tissue boundaries *in vivo*. Of central importance is the observation that the transformation induced by PKC ϵ was contingent on productive interactions between this kinase and the actin-based cytoskeletal network. The biological significance of this protein-protein interaction was confirmed by the demonstration that an internal deletion of the PKC ϵ actin-binding motif completely abrogated the metastatic spread of cancer cells in athymic (*nu/nu*) nude mice, without significantly altering the growth of primary tumors. Our results demonstrate that PKC ϵ relies on this signal peptide to regulate pleiotropic and interdependent cellular processes that are each capable of contributing to the metastatic potential of cancer cells.

MATERIALS AND METHODS

All reagents used in this study were of the highest grade available and purchased from the Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. All media, streptomycin, G418, bovine calf serum, and fetal calf serum were purchased from Invitrogen (Rockville, MD). Athymic (*nu/nu*) male mice were from Charles River Laboratories, Inc. (Wilmington, MA). 3,3'-Diocetadecyloxycarbocyanine perchlorate (Di O) was from Molecular Probes (Eugene, OR). Antisera raised against PKC ϵ (clone C-15) and matrix metalloproteinase-9 (clone M-17) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [6- 3 H]Thymidine (25 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NJ). NIH3T3 fibroblasts transformed with a constitutively active Harvey-*ras* oncogene was a gift from Dr. C. Kent (University of Michigan; Ann Arbor, MI) and NIH3T3 cells transformed with the *v-src* oncogene were donated by Dr. J. McCubrey (Brody School of Medicine at East Carolina University). The pLXSN retroviral vector was originally obtained from Dr. A.D. Miller (University of Washington; Seattle, WA).

Single-Cell Cloning

NIH3T3 mouse embryo fibroblasts (ATCC No. CRL-1658) were infected with pLXSN recombinant retrovirus (L) or pLXSN harboring genes for either wild-type PKC ϵ (L ϵ) or a PKC ϵ mutant

containing an internal deletion from codons 1013 to 1039 of the mouse PKC ϵ gene (corresponding to an actin binding motif; accession number AF028009) that has been designated L $\Delta\epsilon$. The pLXSN vector contains an SV40 driven *neo* gene, ampicillin resistance marker, and 5' viral LTR promoter controlling stable overexpression of PKC ϵ . Polyclonal populations of infected cells were selected using the aminoglycoside antibiotic G418 (500 μ g/ml) and subcloned by limiting dilution [Prekeris et al., 1998]. The NIH3T3 fibroblasts and all clonal derivatives were routinely grown in Dulbecco's modified Eagle medium (DMEM) with glucose (4.5 g/L), sodium bicarbonate (3.7 g/L), the antibiotics penicillin (100 U/ml) and streptomycin (100 μ g/ml), and 10% bovine calf serum (CS).

Assays of Growth in Culture

Cells were seeded at a density of 2×10^4 cells per 35 mm plate in 3 ml of DMEM plus 10% CS and maintained with media changes every 2 days. The average doubling time was determined by counting the total number of cells per plate, in triplicate, at 24 h intervals. Viable cells were identified by trypan blue exclusion and counted using a hemacytometer. The appearance of transformed foci was scored using an inverted phase-contrast microscope at $4 \times$ magnification. Estimates of cell growth rates were based on measurements collected after an initial lag period, and during the log phase, of cell proliferation. Saturation density was measured after 4 days of postconfluent growth with daily medium changes. To assay serum-independent cell proliferation, NIH3T3 cells were preconditioned for 2 days in DMEM containing 0.5% CS to ensure correct depletion of serum; the cultures were then replated, in triplicate, on day 0 in the same medium at 2×10^4 cells per plate. Assays of anchorage-independent growth were performed by suspending 2×10^4 cells in 2 ml 0.33% Bacto-agar in DMEM containing 10% fetal calf serum (FCS) and overlaid onto a 7 ml layer of 0.5% agar on 60 mm culture dishes. These dishes were placed into a humidified 100 mm plate and the agar was supplemented with 0.5 ml DMEM plus 10% FCS every 4 days. After 14 days, colonies were stained and counted.

Tumorigenicity Assays

Nude male mice were inoculated subcutaneously, into the dorsal flanks left and right of

the midline, with 1×10^6 cells suspended in 200 μ l phosphate-buffered saline (PBS) per site and routinely inspected for tumor growth and morbidity for up to 90 days. The cell cultures used in these studies were free of *Mycoplasma* contamination. When evident, solid tumors were measured with a caliper on the days indicated. The animals were sacrificed when tumors had grown to a considerable size, but before the tumors caused visible distress to their hosts. Histological examinations were performed, in a blinded manner, by a professional histopathologist (John F. Bradfield, DVM, East Carolina University, Greenville, NC) using standard methods. Four-micron thick sections of formalin-fixed tissue were cut and stained with hematoxylin and eosin for examination by light microscopy.

Establishment of Explant Cell Lines

Tumor-bearing animals were sacrificed, localized primary and metastatic tumors were excised using sterile technique, finely minced to form explants, and plated onto 100 mm culture dishes in DMEM containing 10% CS. After a 4 h incubation at 37°C, the explanted tissue was removed by aspiration and adherent cells were washed twice with PBS and propagated in fresh growth media for in vitro passage and characterization. Metastases collected for this purpose were found to be growing as localized hepatic tumors that were well separated from the primary mass.

Mitogenic Activity

Mitogenicity was assayed according to the method of Cacace et al. [1998]. Conditioned medium (CM) was prepared by growing cells to a confluency of $\sim 70\%$ in 6-well plates, starving cells for 24 h in DMEM plus 0.5% CS, and then conditioning 1 ml of the same media for 36 h. Aliquots (100 μ l) of the CM from donor cells were then transferred to 96-well plates containing confluent cultures of untransformed NIH3T3 cells that had been starved for 24 h, in DMEM plus 0.5% CS, and washed two times with PBS. After 18 h of stimulation with CM, cells were incubated with [3 H]thymidine (2.5 μ Ci/ml) for 3 h, washed, treated with 10% ice-cold trichloroacetic acid (TCA), and DNA precipitates were solubilized with 50 μ l of formic acid [Cacace et al., 1998]. [3 H]Thymidine incorporation was measured using scintillation counting.

Protrusive Activity of Cells Grown in a Matrigel Matrix

Cells were stained with 5 μM Di O in a suspension of 1×10^5 cells/ml DMEM for 30 min at 37°C. After washing with PBS, equal volumes of the stained cells and Matrigel were gently mixed and 1 ml aliquots of this cell suspension were seeded into pre-cooled 2 well chamber slides (LAB-TEK, Naperville, IL). Cells embedded in this Matrigel matrix were incubated overnight at 37°C and visualized using a Nikon Microphot-FXA fluorescence microscope at 40 \times magnification.

Cell Invasion Assays

Assays of in vitro invasion were performed using 24-well Matrigel-coated polycarbonate chambers (Transwell™ 8 μm pore size filters). Cell suspensions (5×10^4 cells in 200 μl) were seeded into the upper chambers of individual Transwell inserts and a chemoattractant (DMEM plus 10% CS) was added to the lower chamber. Cells were allowed to invade for 22 h at 37°C before fixing and staining with Diff-Quick (Baxter, McGraw Park, IL). Cells that had migrated to the under surface of the filter were counted under a 100 \times microscope objective.

Zymography Assays

The collagenolytic activities of secreted MMPs were assayed by acrylamide gel zymography. Subconfluent flasks of the specified cell line were washed with PBS and then incubated at 37°C in DMEM plus 0.5% CS for 30 h. The CM was collected and centrifuged at 13,500g for 10 min to sediment cell debris and soluble proteins were then precipitated from the supernatant with an equal volume of 100% EtOH at -20°C for 3 h. Precipitates were solubilized in equal volumes of nonreducing sample buffer (2% SDS, 10% glycerol in 62.5 mM Tris, pH 6.8), and samples were loaded onto a 10% SDS-PAGE gel that had been impregnated with 1 mg/ml gelatin and run at 100 V for 2 h. Caseinolytic activity was assayed by casein zymography according to the method of Heussen and Dowdle [1980]. The SDS was removed, using two 30 min incubations in 2.5% Triton X-100, and incubated for 24-48 h at 37°C in developing buffer (5 mM CaCl_2 , 1 mM ZnCl_2 , 50 mM Tris, pH 7.5). Gels were then stained with Coomassie brilliant blue, washed to

remove excess dye, dried, and scanned using the Adobe Photoshop™ 5.5 computer program (San Jose, CA).

Assays of mRNA Stability

Stability of the mRNAs for MMP-2 and MMP-9 was measured using the transcriptional block and chase method of Ziegler et al. [1990]. Briefly, subconfluent cultures were exposed to actinomycin D (20 $\mu\text{g/ml}$) at time 0, to block RNA synthesis, and poly(A) RNA was isolated at the times specified using the Oligotex™ protocol (Qiagen Inc., Valencia, CA). Oligonucleotide probes used for hybridization to the MMP-2 and MMP-9 mRNAs were identical to those employed previously by Greene et al. [1997] and were labeled using [γ - ^{32}P]ATP and hybridized according to the KinaseMax™ and Multi-NPA™ protocols, respectively (Ambion, Inc., Austin, TX). Densitometric analysis was performed using the BioRad Gel Doc 2000 Gel Documentation system and Quantity One 4.1.1 software (Hercules, CA).

Immunoblotting

Western blot analyses were performed as described [Prekeris et al., 1998].

Data Analysis

All of the assays performed in this study were conducted at least three times and found to be reproducible. Only representative data are presented and numeric data represent the mean and standard deviation of duplicate or triplicate determinations from at least three independent experiments, as described in the figure legends. Treatment effects were evaluated using a two-sided Student's *t*-test and values of $P < 0.05$ were taken as a significant difference between means.

RESULTS

PKC ϵ Interacts With Actin to Promote Tumor Progression to a Metastatic Stage

NIH3T3 fibroblasts were transformed by constitutively overexpressing the full length PKC ϵ cDNA. The oncogenic potential of PKC ϵ was abundantly clear in our in vitro assays of cellular transformation. Cells that overexpressed PKC ϵ (L ϵ) were refractile, extended elongate cellular protrusions, formed small dense foci in confluent cultures, and displayed a significantly increased saturation density and

TABLE I. Growth Properties of NIH3T3 Cell Lines Overproducing PKC ϵ or a Deletion Mutant Lacking an Actin-Binding Motif (APKC ϵ)

Cell line	Foci formation	Maximum cell density (cells/cm ² × 10 ⁻⁴)	Growth in agar	Doubling time (h)	Tumor formation in nude mice	Metastases in nude mice
3T3	-	7.1 ± 1.2	-	16.6 ± 0.5	ND	ND
L	-	9.5 ± 0.9	-	16.3 ± 1.0	0/4	0/4
L ϵ -3	+	ND	+	ND	8/8	8/8
L ϵ -5	+	9.8 ± 0.3	ND	16.0 ± 0.3	4/4	2/4
L ϵ -7	+	15.9 ± 0.3 ^a	+	13.6 ± 0.5 ^a	14/16	13/16
L $\Delta\epsilon$ -2	+	ND	ND	ND	4/4	0/4
L $\Delta\epsilon$ -5	+	12.5 ± 0.2 ^a	++	23.7 ± 1.9 ^a	ND	ND
L $\Delta\epsilon$ -9	+	19.9 ± 0.7 ^a	++	21.3 ± 2.5 ^a	16/16	0/16

ND, not determined.

^aP < 0.05 versus 3T3 control cell line.

anchorage-independent growth in soft agar (Table I). These observations were made in the absence of phorbol esters and were consistent with that previously reported [Mishak et al., 1993; Hernandez et al., 2001]. A pLXSN vector control cell line (L) was indistinguishable from the parental cell line in each of these assays (Table I). To identify determinants of PKC ϵ induced transformation, we have examined the role of specific signal peptides that are unique to this member of the PKC family. In the present study, we have targeted an actin binding motif that is located within the C1 domain of PKC ϵ [Prekeris et al., 1996; Prekeris et al., 1998] using a PCR-based deletion mutagenesis approach: NIH3T3 cell lines overexpressing this deletion mutant (called L $\Delta\epsilon$) resembled the L ϵ cell lines in certain respects; these cells were also refractile, formed foci, had an increased saturation density, and grew in soft agar (Table I). In fact, the L $\Delta\epsilon$ cell lines consistently formed larger colonies in soft agar than those formed by the L ϵ cells (Table I).

The *in vivo* tumorigenicity that PKC ϵ imparted to NIH3T3 cells did not prove to be dependent on the localization of PKC ϵ within the cytoskeletal matrix, as L $\Delta\epsilon$ variants remained > 90% tumorigenic in nude mice (Table I and Fig. 1A). Remarkably, however, this mutation of PKC ϵ conferred an attenuating phenotype that only became evident during advanced stages of tumor progression. Nude mice inoculated with variants of the L ϵ cell lines (L ϵ clones 3 and 7) formed tumors within 5 weeks (Fig. 1A), and histopathologic evaluation of these tumor-bearing mice provided clear evidence of active invasion by the tumor cells into the surrounding tissue (Fig. 2A). Although occasional central areas of necrosis were found in some of the L ϵ tumors, there were abundant vascular channels

scattered throughout all sections (Fig. 2C). Evidence of direct hematogenous metastasis to the liver was also apparent, as nests of tumor cells were found in the centrilobular and periportal areas (Fig. 2E). Indeed, metastases were found in several locations of the mesentery and, within 6 weeks, were present in more than 80% of the mice injected with L ϵ cells (Table I). In contrast, L $\Delta\epsilon$ cells formed paraneoplastic (primary) tumors that grew more slowly than L ϵ tumors (Fig. 1A) and never progressed to an invasive or metastatic stage (Table I). Histological analyses have failed to detect any evidence of L $\Delta\epsilon$ cell dissemination in the heart, lungs, brain, liver, spleen, kidneys, or gastrointestinal tract. The primary L $\Delta\epsilon$ tumors were character-

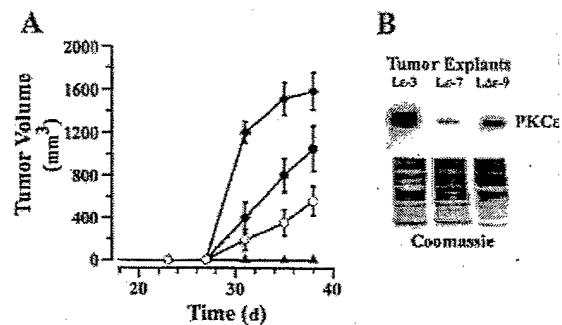


Fig. 1. A: Tumorigenic effects of PKC ϵ overexpression in murine NIH3T3 fibroblasts. Each cell line was injected s.c. into male nude mice and the volume of paraneoplastic tumors was calculated by the formula: length × width × depth × 0.5236. Each data point represents the mean tumor volume in each experimental group containing at least four mice (see Table I). Sublines tested included the NIH3T3 and vector controls (both ▲), L ϵ -3 (◆), L ϵ -7 (●), and L $\Delta\epsilon$ -9 (○) fibroblasts. **B:** Immunoblot analysis of PKC ϵ expression in L ϵ -3, L ϵ -7, and L $\Delta\epsilon$ -9 explants of primary tumors passaged in culture five times prior to the preparation of whole cell lysates, 10 μ g total protein loaded per lane. Coomassie-stained gel shown below was a control to confirm equal loading of all lanes.

ized by distinct tumor margins and extensive central necrosis and hemorrhage (Fig. 2B, D). Although the volume of L $\Delta\epsilon$ tumors was initially less than the L ϵ tumors, this did not account for the apparent absence of L $\Delta\epsilon$ cell metastasis. Respective final volumes of the L ϵ and L $\Delta\epsilon$ primary tumors ultimately reached $1590 \pm 160 \text{ mm}^3$ and $1440 \pm 123 \text{ mm}^3$ at the time of sacrifice. Finally, immunoblot analyses of primary tumor explants growing in culture for five passages demonstrated that the level of PKC ϵ and Δ PKC ϵ expression in the host animal did not correlate with the metastatic potential of the transformed NIH3T3 cell lines (Fig. 1B). In these cultured tumor explants, the nonmetastatic L $\Delta\epsilon$ -9 cells expressed an intermediate level of PKC ϵ in comparison to the two metastatic lines examined (L ϵ -3 and L ϵ -7). These studies implied that disrupting the interactions

between PKC ϵ and the cytoskeleton prevented metastasis but not tumor growth.

The L ϵ -7 and L $\Delta\epsilon$ -9 cell lines were selected as representatives in the comparative analyses that follow, unless otherwise indicated. Although their levels of PKC ϵ and Δ PKC ϵ protein expression differed in tumor explants (Fig. 1B), direct comparisons of 16 different L ϵ and L $\Delta\epsilon$ clones indicated that these two sublines (L ϵ -7 and L $\Delta\epsilon$ -9) provided the closest approximation in terms of their levels of PKC ϵ protein expression in culture [Hernandez et al., 2001].

PKC ϵ Interacts With Actin to Promote Mitogenicity But Not Autocrine Proliferation

It is presently understood that in rodent fibroblasts, the transforming potential of PKC ϵ is strictly dependent on the production of autocrine growth factors [Cai et al., 1997; Ueffing et al., 1997; Cacace et al., 1998]. The important finding that mitogens secreted by fibroblasts overexpressing PKC ϵ have a potent stimulatory effect on DNA synthesis supports this model. We have confirmed that CM collected from NIH3T3 cells overexpressing PKC ϵ has a mitogenic effect on the incorporation of [^3H]thymidine into parental NIH3T3 cells (Fig. 3A). The PKC ϵ -induced production of autocrine growth factors would also be expected to diminish the requirement of L ϵ cells for exogenous growth factors or serum [Cacace et al., 1993]. However, growth curves indicated that neither L ϵ -7 nor L ϵ -3 cells actively proliferated in the absence of serum, although both were more capable of surviving under these conditions than the L $\Delta\epsilon$ -9, vector control, or parental cell lines (Fig. 3B data not shown). In contrast, the number of viable Ha-*ras* transformed NIH3T3 cells doubled in the absence of serum (Fig. 3B). This was an important result because it indicated that PKC ϵ may be capable of inducing both oncogenic transformation and tumorigenesis without rendering NIH3T3 cells autocrine in growth. Analysis of the L $\Delta\epsilon$ -9 mutants provided additional support for this conclusion. CM collected from L $\Delta\epsilon$ -9 cells had a minor but insignificant effect on the synthesis of DNA by their parental counterparts (Fig. 3A), and the survival of L $\Delta\epsilon$ -9 cells was serum-dependent (Fig. 3B). Therefore, the PKC ϵ -induced transformation of NIH3T3 cells appears to be associated with an increased production of mitogenic growth factors but does not engage the full program required for

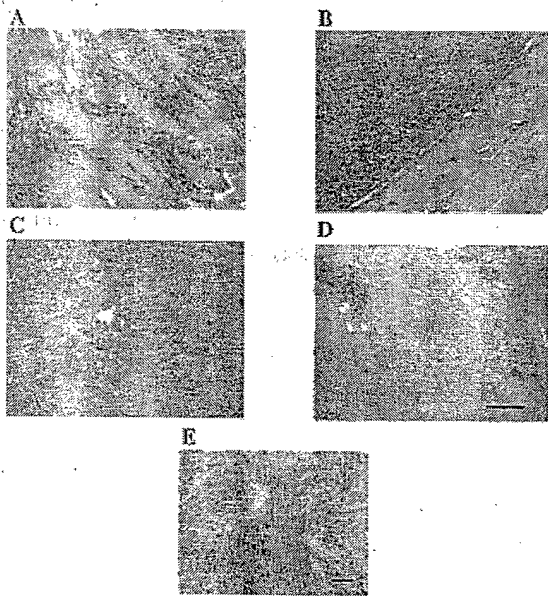


Fig. 2. Histological visualization of tumor cells after microsectioning, staining with hematoxylin and eosin and visualization under $400\times$ magnification. Tumors illustrated were representative of those examined. A: Primary tumor established by L ϵ -7 fibroblasts showing indistinct, expansile borders and extensive tumor infiltration. B: Intact boundary between a primary tumor of transformed L $\Delta\epsilon$ -9 fibroblasts (upper left) and abdominal tissue of the host (lower right). C: Core region of a L ϵ -7 tumor with vascular channels and occasional evidence of hemorrhage. D: Central necrosis of a L $\Delta\epsilon$ -9 tumor and accumulations of extracellular fibrillar hyalin material interpreted to be collagen. Calibration bar is $20 \mu\text{m}$. E: Metastatic L ϵ -7 tumor cells found in a periportal area of the liver 38 d postinoculation. Calibration bar is $20 \mu\text{m}$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

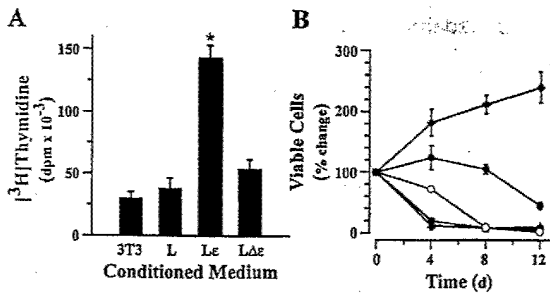


Fig. 3. A: [³H]Thymidine incorporation by NIH3T3 cells following an 18 h exposure to medium that had been preconditioned by parental (3T3) or vector (L) controls and the Le-7 (Le) or LΔε-9 (LΔε) cell lines. Data are the mean with SD of triplicate determinations from three independent experiments. B: Proliferation of NIH3T3 (■), vector controls (▲), and NIH3T3 cells overexpressing Le-7 (●), LΔε-9 (○), or Ha-Ras (◆) in the presence of 0.5% serum for the time indicated. Cell proliferation was measured by counting the total number of viable cells per plate by trypan blue exclusion. Data are the mean with SD of triplicate determinations from three separate experiments.

genuine autocrine growth. Host-derived growth factors may be responsible for supporting the *in vivo* growth of Le and LΔε primary tumors.

PKC ϵ Interacts With Actin to Extend Invadopodia and Augment the Invasiveness of NIH3T3 Fibroblasts

Although NIH3T3 and neuroblastoma cells that overexpress PKC ϵ rapidly form elongate cellular processes on a tissue culture dish [Fagerstrom et al., 1996; Prekeris et al., 1996; Zeidman et al., 1999], it remained unclear whether these cells would also form the invadopodial-like extensions that are characteristic of invasive tumor cells [Monsky et al., 1994]. For this reason, we examined the morphology of NIH3T3, L, Le, and LΔε cells that had been stained with a fluorescent membrane dye, Di O, and cultured overnight in a complex and three-dimensional biological matrix of the Engelbreth-Holm sarcoma-derived Matrigel [Albini et al., 1987]. As illustrated in Figure 4A, Le-7 cells formed long thin protrusions into the Matrigel that closely resembled invadopodia [Monsky et al., 1994], while the LΔε-9 clone had maintained a completely rounded morphology within this three-dimensional matrix (Fig. 4B). Under these conditions, parental and vector controls also remained rounded and did not form membrane extensions. Digital images of the invasive Le-7 cells captured several instances in which membrane extensions at the leading edge of the cell were actually seen to

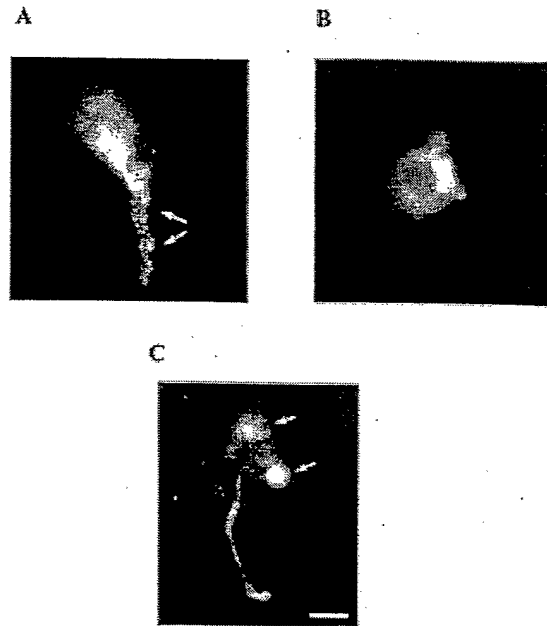


Fig. 4. A: Fluorescence images of an invadopodial-like extension (arrows) from the Di O-stained plasma membrane of a Le-7 fibroblast after suspension for 24 h within a three-dimensional Matrigel matrix. B: Di O-stained LΔε-9 cells grown under identical conditions to those used in A. C: Digital image of a Di O-stained Le-7 cell penetrating an 8- μ m pore of a Matrigel coated membrane, as viewed from the undersurface. Two presumptive pores (arrows) mark sites of potential cellular invasion and entry into the lower chamber of the Transwell insert. Calibration bar is 10 μ m and images are representative of either six (A and B) or two (C) independent experiments.

be penetrating through the 8 μ m pores of a Matrigel membrane while traversing into the lower chamber of a Transwell apparatus. Figure 4C shows an example of this invasive behavior, where an elongate invadopodial extension can be seen protruding through a pore of the Matrigel membrane. This visual evidence provided convincing proof that PKC ϵ -transformed NIH3T3 cells could employ their invadopodial-like processes to traverse an artificial extracellular matrix (ECM).

A variety of cytoskeleton-dependent processes become involved in regulating the attachment, migration, and invasion of tumor cells during the progressive stages of metastasis. However, our results indicated that changes in cell adhesion and motility could not account for the invasive phenotype of PKC ϵ -transformed NIH3T3 cells. Le cells were more adherent on a fibronectin matrix than their parental cells, apparently due to an upregulation of integrin β 1 avidity, and less motile in chemotaxis, chemokinesis, and cell scattering assays [Mayhew

et al., unpublished communication]. Accordingly, we proceeded to investigate the effect of PKC ϵ overexpression on the production of activated proteases. A variety of ECM-degrading proteases have been associated with invadopodia [Monksy et al., 1994; Chen, 1996]. To obtain a quantitative assessment of the in vitro invasiveness of PKC ϵ -transformed NIH3T3 cells, we performed invasion assays with Matrigel as the matrix barrier. Using this approach, it was demonstrated that the invasiveness of L ϵ -7 cells was four-fold greater than the corresponding vector controls (Fig. 5A). Moreover, this striking increase in invasive activity was completely absent in the L $\Delta\epsilon$ -9 deletion mutant (Fig. 5A).

The oncogenes *v-src* and *Ha-ras* coordinately upregulate the expression of the urokinase plasminogen activator (uPA) and its receptor (uPAR) in NIH3T3 cells while downregulating the production of caveolin-1, an integral membrane protein that interacts with uPAR to assemble kinase-rich domains within focal adhesions [Koleske et al., 1995; Aguirre Ghiso et al., 1999; Wei et al., 1999]. An immunoblot analysis of cell lysates prepared from NIH3T3 cells overexpressing *v-src*, *Ha-ras*, PKC ϵ or Δ PKC ϵ revealed that caveolin-1 expression was only decreased in the invasive *v-src*, *Ha-ras*, and L ϵ sublines (Fig. 5B). To investigate the role of uPA or the MMPs in ECM degradation, we established whether the vector controls and PKC ϵ -transformed NIH3T3 cells differentially secreted these zymogens. Samples of CM from each cell line were assayed for caseinolytic and collagenolytic activity. Figure 5C illustrates that uPA activity did not accumulate in the medium of NIH3T3 cells overexpressing wild-type PKC ϵ , whereas caseinolytic activity was consistently detected in the medium conditioned by vector controls and L $\Delta\epsilon$ -9 cells. Therefore, the invasive behavior of PKC ϵ -transformed NIH3T3 cells did not appear to depend on the upregulation of caseinolytic activity. We next examined the effect of this oncogene on the production of the type IV collagenases/gelatinases MMP-2 and MMP-9. The mouse homologue of proMMP-9 (gelatinase B) is an inactive zymogen of 105 kDa [Tanaka et al., 1993]. Using an antibody that only recognizes this latent form of MMP-9, it was determined that the ectopic overexpression of wild-type PKC ϵ , but not Δ PKC ϵ , increased the secretion of proMMP-9 without altering

the cellular levels of this protein (Fig. 5D). This result reflects the fact that fibroblasts constitutively secrete proMMP-9. At the same time, however, the 105 kDa form of this collagenase was not the major species detected in zymograms of the CM from either the control or PKC ϵ -transformed NIH3T3 cell lines. Rather, we consistently detected the presence of a secreted 96–98 kDa fragment of murine MMP-9 activity that was not upregulated in PKC ϵ overexpressing clones relative to the control cell lines under basal growth conditions (Fig. 5E). It is also important to note that MMP-9 activity in CM from L $\Delta\epsilon$ -9 cell cultures was significantly decreased and was not consistently detected under these conditions (Fig. 5E, lane 4). Immunoblot analyses were also performed using antibodies raised against MMP-2; however, we were unable to detect immunospecific bands for this proteolytic enzyme. Gelatin zymographies, on the other hand, clearly showed that CM from L ϵ -7 and L $\Delta\epsilon$ -9 clones contained increased levels of collagenolytic activity corresponding to 70–76 kDa proteins, possibly including MMP-2 (Fig. 5E). The collagenolytic activities of both the 70–76 kDa and 96–98 kDa proteins were inhibited by the addition of either EDTA or the synthetic MMP inhibitor phenanthroline to the CM (data not shown). Moreover, ultracentrifugation of the CM did not lower the levels of soluble MMP-2 (70–76 kDa) or MMP-9 (96–98 kDa) activity (data not shown); implying that neither MMP was primarily associated with membrane vesicles [Ginestra et al., 1998]. These data indicate that the production of endogenous MMPs but not uPA was upregulated in PKC ϵ -transformed NIH3T3 fibroblasts. In comparison to the nonmetastatic L $\Delta\epsilon$ -9 cells, L ϵ -7 cells produced increased levels of both MMP-2 and MMP-9 activity when cultured under identical conditions (Fig. 5E).

Because tumor cell interactions with the surrounding stroma play such a central role in regulating the in vivo expression of type IV collagenases, CM from tumor explants were also assayed for collagenolytic activity. Explants from primary (paralumbur; L ϵ -7 and L $\Delta\epsilon$ -9) and secondary (hepatic metastasis; L ϵ -7) tumors were cultured in serum-free medium for 30 h and samples of their CM were analyzed using gelatin zymography. In comparison to the CM from a secondary L ϵ -7 tumor explant, medium conditioned by the primary tumor explant had accumulated higher levels of collagenolytic

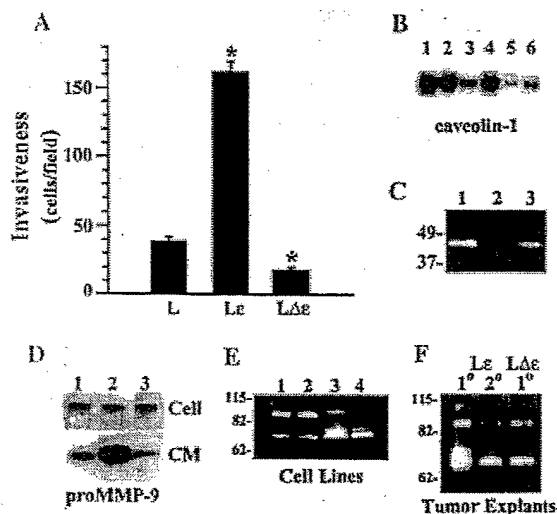


Fig. 5. A: In vitro invasion of PKC ϵ -transformed NIH3T3 fibroblasts. Cell suspensions (5×10^4 cells in 200 μ l) of vector controls (L) or cells overexpressing either PKC ϵ (L ϵ) or an actin binding null mutant (L $\Delta\epsilon$) were placed into the upper wells of individual Transwell inserts containing 8- μ m pore size polycarbonate membranes precoated with Matrigel (0.78 mg/ml). Cells were allowed to invade for 22 h at 37°C followed by the fixation and staining of cells. Cells that passed through the membrane were counted at 100 \times magnification. Data are the mean with SD of triplicate determinations in four separate experiments. B: Western blot analysis of caveolin-1 expression in parental and vector control cells (lanes 1 and 2, respectively) and in NIH3T3 cells that have been experimentally transformed by a known oncogene; i.e., PKC ϵ , Δ PKC ϵ , Ha-ras, or v-src (lanes 3–6, respectively). Samples containing an equal amount of protein (50 μ g) were loaded into each lane. C: Zymographic analysis of caseinolytic activity in CM collected from NIH3T3 cells overexpressing an empty pLXSN vector, PKC ϵ , or Δ PKC ϵ (lanes 1–3, respectively). Results are representative of six individual experiments. D: Production and secretion of proMMP-9 in NIH3T3 clones, loaded in the order specified in B. Samples of cell lysate (Cell, 25 μ g protein) and conditioned medium (CM, 15 μ g protein) were loaded into each lane and results are representative of at least two independent experiments. E: Gelatin zymography of collagenolytic activity in CM collected from NIH3T3, vector control, L ϵ -7, and L $\Delta\epsilon$ -9 cells (lanes 1–4, respectively). Results are representative of ten experiments. F: Gelatin zymography of CM collected from the explants of primary (1 $^\circ$) tumors grown in nude mice inoculated with either L ϵ -7 cells (lane 1) or L $\Delta\epsilon$ -9 cells (lane 3) and a metastatic (2 $^\circ$) tumor established in the former animal (lane 2). Explants were prepared from four individual animals and this is one representative experiment.

activity (Fig. 5F, lane 1 vs. 2). Both of these tumors were originally taken from the same animal. The nonmetastatic L $\Delta\epsilon$ -9 tumor explant appeared to produce as much MMP-2 and MMP-9 as the secondary L ϵ -7 tumor (Fig. 5F). These results indicated that the level of collagenolytic activity produced by PKC ϵ -transformed

NIH3T3 cells may be influenced by local factors within the surrounding environment.

PKC ϵ Overproduction Selectively Augments MMP-9 mRNA Stability

Several members of the type IV collagenase/gelatinase family (MMP-1, -3, -9, -10, and -11) have been shown to be responsive to PKC activation [Johnson et al., 1999; Sehgal and Thompson, 1999]. In the case of MMP-9, increases in PKC activity have been related to increased levels of total MMP-9 RNA [Crowe and Brown, 1999] that may result from direct effects on gene transcription or the regulation of nontranscriptional pathways [Johnson et al., 1999; Sehgal and Thompson, 1999]. Our interest in the effects of PKC ϵ overexpression on MMP-9 mRNA stability was less than intuitive and only emerged following an extensive analysis of alternative possibilities that are most appropriately summarized at this time. First, we examined the possibility that PKC ϵ might indirectly upregulate the activity of MMPs by stimulating the plasminogen activator systems within the proteolytic cascade [Aguirre Ghiso et al., 1999]. However, we found no evidence of uPA activity in media conditioned by L ϵ cultures (Fig. 5C). Second, we considered the possibility that PKC ϵ could upregulate the expression of MMP-2 and/or MMP-9 transcripts. In NIH3T3 cells, PKC ϵ signaling transactivates multiple pathways that converge on the serum response element (SRE) in the *c-fos* promoter [Soh et al., 1999], a member of the AP-1 family that has been shown to regulate MMP-9 promoter activity [Crowe and Brown, 1999]. Therefore, we performed a series of transient-transfection assays with NIH3T3, L ϵ , and L $\Delta\epsilon$ cells using SRE, AP-1, and NF- κ B driven luciferase reporter plasmids under various growth conditions. Identical results were obtained using these cell lines (data not shown). Third, we found no evidence of uPA or MMP activity associated with membrane vesicles isolated from the CM of L ϵ cultures, suggesting that vesicle shedding [Ginestra et al., 1998] did not play a major role in the in vitro invasiveness of these cells. Fourth, biochemical assays revealed that the level of MMP-2, but not MMP-9, pericellular activity was substantially increased in the CM of both invasive L ϵ -7 and noninvasive L $\Delta\epsilon$ -9 cultures. More importantly, MMP-2 activity appeared to remain elevated in the primary tumors, where MMP-9 activity

could also be detected, and in either case the level of collagenolytic activity was always highest in explants prepared from invasive L ϵ -7 tumors (Fig. 5F). Most recently, we have tested the hypothesis that excessive PKC ϵ activity might differentially influence the half-life of MMP-2 and MMP-9 transcripts in the invasive L ϵ -7 cell line. Cells were exposed to actinomycin D (a transcriptional inhibitor) for different times and the levels of the MMP-2 and MMP-9 poly(A)⁺ mRNA were determined.

As illustrated in Figure 6, Northern blots revealed that the MMP-2 mRNA was significantly more stable than the MMP-9 mRNA in NIH3T3 fibroblasts, with half-lives corresponding to about 24 h and 5 h, respectively (Fig. 6B). Interestingly, while MMP-2 mRNA stability was not altered by the constitutive expression of PKC ϵ in cultured NIH3T3 fibroblasts, the half-life of MMP-9 mRNA was markedly increased in L ϵ -7 cells (Fig. 6B). Although the stability of MMP-9 mRNA was not assayed in L $\Delta\epsilon$ cells, it is important to note that these fibroblasts did not accumulate detectable levels of MMP-9 activity in culture (Fig. 5E). Therefore, we have shown that the overexpression of full length PKC ϵ in NIH3T3 fibroblasts selectively stabilizes MMP-9 mRNA and augments the secretion of a latent form of this zymogen (Figs. 5D and 6). To investigate whether the prolonged half-life of MMP-9 mRNA can sustain the production of an active protease in the absence of ongoing transcriptional activity, we examined the effects of actinomycin D on the secretion of MMP-9 by L ϵ -7 cells. L ϵ -7 cells were incubated for 20 h in the absence or presence of 20 μ g/ml actinomycin D and then CM samples were assayed for collagenolytic activity. Under these conditions, MMP-9 secretion was unimpaired (Fig. 6C). In contrast, collagenolytic activity was not detected in CM collected from L ϵ -7 cells exposed to either 100 μ M cycloheximide (an inhibitor of protein synthesis) or 10 μ M brefeldin A (an inhibitor of the Golgi secretory pathway) for 20 h (Fig. 6C). These data indicate that the translational control of MMP-9 expression may have an important influence on the invasiveness of PKC ϵ -transformed NIH3T3 cells.

DISCUSSION

Elementary features relevant to the program employed by PKC ϵ to constitutively activate cell

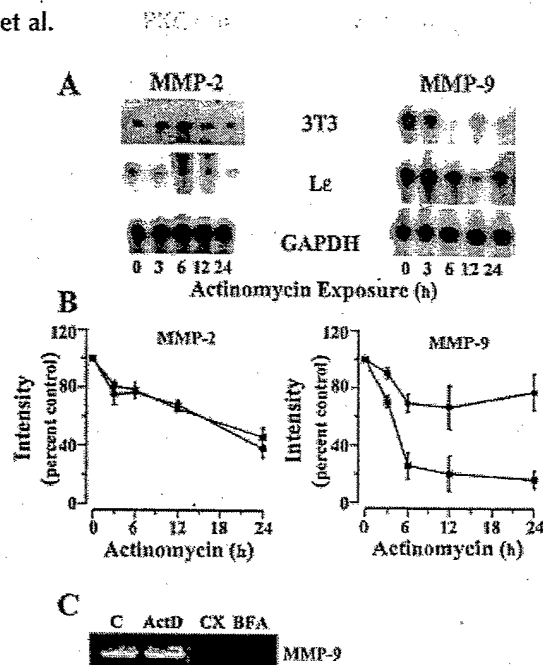


Fig. 6. Northern blot analysis for type IV matrix metalloproteinase transcripts in NIH3T3 cells (■) and transformants overexpressing PKC ϵ (L ϵ -7; ●). A: Polyadenylated mRNA (2 μ g/lane) was isolated from cultures exposed to actinomycin D (ActD, 20 μ g/ml) for the specified time and used to detect the specific mRNA transcripts. A GAPDH probe was used as an internal control. B: The BioRad Gel Documentation system was used for densitometric quantitation and the band intensity of untreated cells was arbitrarily defined as 100% control. The data are the mean with SD of three independent experiments. C: Gelatin zymography of collagenolytic activity in the CM collected from L ϵ -7 cells following a 20 h exposure to serum-free medium (C) or the same medium supplemented with 20 μ g/ml ActD, 100 μ M cycloheximide (CX), or 10 μ M brefeldin A (BFA). Results shown are representative of two independent experiments.

cycle progression and cellular transformation remain unsolved; however, it now seems clear that this isoenzyme must productively interact with an actin-based cytoskeleton to influence the metastatic progression of transformed NIH3T3 fibroblasts. Previous studies have stressed the importance of autocrine signaling in the *in vitro* transformation and *in vivo* tumorigenesis that is induced by excessive PKC ϵ activity [Mishak et al., 1993; Cai et al., 1997; Ueffing et al., 1997]. Yet, in the present study it has been demonstrated that Δ PKC ϵ -transformed NIH3T3 cells remained tumorigenic *in vivo* despite a pronounced reversion of their mitogenicity and autocrine growth capacity. This is a significant result because it shows that the contribution of autocrine growth loops may be dispensable in the programming of PKC ϵ induced transformation and tumorigenesis.

Moreover, this underlines the fact that there can be important differences between *in vitro* evidence of transformation and *in vivo* evidence of tumorigenic and metastatic activity. It is speculated that L $\Delta\epsilon$ tumor cells may derive the factor(s) required for tumor growth, but not intravasation, from the host stroma in the model we have employed. Other findings presented in this, and a second [Hernandez et al., 2001], report advance the notion that PKC ϵ may interact with actin to directly promote polymer assembly and the extension of invadopodial-like processes, while indirectly stimulating the production of MMP-2 and MMP-9 zymogens. As a consequence, PKC ϵ may coordinately regulate the metastatic potential of transformed NIH3T3 cells.

Metastatic cascades are characterized by a multistep process in which cytoskeletal rearrangements play a prominent role. The transition to an invasive phenotype occurs relatively late in tumor progression and is associated with a loosening of cell attachment to the primary mass, aberrant motility, membrane protrusion, and penetration of the surrounding stroma or ECM [Monsky et al., 1994; Chen, 1996; Aguirre Ghiso et al., 1999]. Clear histopathologic evidence of active invasion by the L ϵ tumor cells into host tissue has now been presented, while tissue margins bordering L $\Delta\epsilon$ tumor cells remained intact. These results show that the internal deletion of a small actin-binding motif was sufficient to dramatically attenuate the highly invasive phenotype of PKC ϵ -transformed NIH3T3 fibroblasts. This report features a fascinating aspect of tumor invasion by demonstrating that the regulatory C1 domain of PKC ϵ participates in the generation of protrusive cell shape changes and activation of a proteolytic cascade. Our results support the hypothesis that PKC ϵ activation and actin polymerization are both critical events driving the expression of MMP-9 by invasive tumor cells [Chintala et al., 1998; Hernandez et al., 2001].

Mechanical pressure must be generated to establish the membrane protrusions observed in fibroblasts [Hernandez et al., 2001 and this report] and neuroblastoma cells [Fagerstrom et al., 1996] overexpressing PKC ϵ . It is now abundantly clear that the prerequisite force is created by the N-terminal regulatory domain of PKC ϵ and that this morphogenetic activity is independent of kinase activity and not diminished by truncation of the entire catalytic

domain [Zeidman et al., 1999]. Other mechanistic details of the operational machinery are also becoming apparent. Studies with a cell-free system reveal that binary interactions between PKC ϵ and actin promote the *de novo* nucleation and elongation of new filaments, either by decreasing the critical concentration of actin and/or the off-rate at pointed ends of pre-existing filaments [Hernandez et al., 2001]. Deletion analysis further indicates that the actin binding motif of the PKC ϵ C1 domain designates an essential interface in these protein-protein interactions. Based on these and other observations [Prekeris et al., 1996, 1998], we have proposed that PKC ϵ may generate protrusive forces in a living cell by nucleating *de novo* polymerization and stabilizing polymerization intermediates [Hernandez et al., 2001], in a manner that would be reminiscent of the mechanism employed by the Arp 2/3 complex for lamellipod extension [Bailly et al., 1999; Borisy and Svitkina, 2000].

Microscopic images have now been captured in which it was evident that L ϵ , but not L $\Delta\epsilon$, cells make use of membrane protrusions at their leading edge to penetrate and degrade Matrigel during the *in vitro* invasion of a microporous membrane. These polarized structures have been called invadopodial extensions, actin-containing protrusions associated with high local concentrations of ECM-degrading proteases [Monsky et al., 1994; Chen, 1996]. Independent biochemical evidence that the processes formed by L ϵ cells actually contain localized hot spots of protease activity remains to be established. However, we have previously shown that PKC ϵ co-localizes with actin within these membrane extensions [Prekeris et al., 1998], and the present data indicate that MMP-2 and MMP-9 were functional components of the invasive program induced by constitutive expression of PKC ϵ in NIH3T3 fibroblasts. Intracellular signaling pathways associated with transformation and the biogenesis of tumor-associated proteases have recently been reviewed [Aguirre Ghiso et al., 1999]. Here, we propose that PKC ϵ interacts with actin protomers to coordinately penetrate and degrade the natural tissue boundaries imposed by tumor-bearing athymic mice. The later could be accomplished by regulating any number of molecular interactions within the proteolytic cascade [Aguirre Ghiso et al., 1999]. However, these and other studies provide certain clues as to how the positioning of

PKC ϵ within an actin-based cytoskeletal matrix might promote tumor cell invasion. By upregulating the production of TGF- β , prostatic carcinomas manage to increase the half-life of the mature MMP-2 protein and stability of proMMP-9 mRNA [Johnson et al., 1999]. While the importance of TGF- β in the transformation and tumorigenesis of PKC ϵ -transformed NIH3T3 fibroblasts may be debated, several independent laboratories have now reported that TGF- β is secreted by these transformants and contributes to mitogenicity in this experimental system [Cai et al., 1997; Ueffing et al., 1997; Cacace et al., 1998]. We have presented evidence suggesting that PKC ϵ may not be capable of engaging the full program required for genuine autocrine growth of NIH3T3 fibroblasts. As an alternative hypothesis, we propose that the enhanced production of PKC ϵ by NIH3T3 cells might play an important role in regulating the program required for tumor cell invasion. According to this model, PKC ϵ would prompt actin polymerization and cell shape changes through direct protein-protein interactions [Hernandez et al., 2001] and upregulate the expression of TGF- β and other growth factors by phosphorylating cytoskeletal-associated substrates that are capable of transactivating their cognate promoters in a Raf-1 independent manner [Ueffing et al., 1997; Cacace et al., 1998]. Once activated, these growth factors could advance the invasiveness of PKC ϵ -transformed fibroblasts through the post-transcriptional upregulation of MMP-2 and MMP-9 activity.

The major finding of the present study is that the deletion of a small actin binding motif within the C1 domain of PKC ϵ resulted in a clear-cut reversion of the invasive phenotype established by PKC ϵ -transformed NIH3T3 fibroblasts growing in culture or as tumors in vivo. This result strongly suggests that the subcellular localization of PKC ϵ is an important determinant in the progression of tumor invasion and metastasis in this experimental system. Many important details concerning the complex oncogenic signaling cascade that operates downstream of PKC ϵ remain to be established. It is our working hypothesis that PKC ϵ directly interacts with actin to establish membrane protrusions that PKC ϵ -transformed NIH3T3 cells are capable of using to penetrate and degrade tissue boundaries in a primary tumor.

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**Protein Kinase C ϵ Interacts with Bax and Blocks the
Mitochondrial Death Signaling Pathway in Human Prostate Cancer Cells**

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Running Title: PKC ϵ complexes with Bax and promotes CaP cell survival

Key Words: Apoptosis; LNCaP; CWR22; recurrent prostate cancer; phorbol ester; PI3-K

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Abstract

Prostatic glandular epithelial cells express protein kinase C ϵ (PKC ϵ), an oncoprotein with the potential to promote recurrence of human prostate cancer. Using a combination of primary tumor specimens, CWR22 xenograft tumors, and cultures of CWR-R1 and LNCaP cell lines, we demonstrate that the basal level of PKC ϵ expression increases in prostatic cancer cells that survive throughout the progression of this disease by suppressing apoptotic signals propagated through the mitochondrial death signaling pathway. Gene transfer, antisense, flow cytometry, and confocal microscopy studies provide evidence that survival signals mediated by PKC ϵ operate within the intrinsic mitochondrial pathway downstream of phosphatidylinositol-3'-kinase and upstream of cytochrome c release to block apoptosis that is triggered by phorbol esters in CWR-R1 and LNCaP cells. Proteomic profiling and coimmunoprecipitation assays indicate that PKC ϵ interacts with the apoptotic protein Bax in recurrent CWR22 tumors *in vivo* and CWR-R1 and LNCaP cells in culture. PKC ϵ overexpression in LNCaP cells has the potential to suppress mitochondrial targeting of Bax that may represent a mechanism that PKC ϵ employs to promote the survival of prostate cancer cells.

Introduction

Prostatic epithelial cells that survive androgen deprivation therapy engage multiple molecular pathways to escape their normal apoptotic fate, to restore the expression of androgen-responsive genes, and to disrupt the growth-restraining activity of the retinoblastoma (Rb) family of transcriptional repressor proteins (Gregory *et al.*, 1998; Fribourg *et al.*, 2000; Mousses *et al.*, 2001). The present study has focused on the first of these hallmarks of advanced prostate cancer (CaP) because defects in the apoptotic machinery are likely to be an important contributing factor in the resistance of some CaP cells to chemotherapy or irradiation and to the recurrence of CaP after androgen deprivation. One well characterized example of the progression to an apoptosis-resistant stage is provided by the differential responses of androgen-dependent and -independent (AI) CaP cell lines to agents such as phorbol 12-myristate 13-acetate (PMA) (Powell *et al.*, 1996; Zhao *et al.*, 1997; Henttu and Vihko, 1998; Fujii *et al.*, 2000; Gschwend *et al.*, 2000). As documented in these and other studies, PMA is a protein kinase C (PKC) agonist that selectively activates a mitochondrial (intrinsic) death signaling pathway in androgen sensitive (LNCaP) but not AI (DU145 and PC3) CaP cell lines. Additional examples of agents that preferentially induce cell death in the LNCaP model of early CaP include bryostatin 1, thapsigargin, N-(4-hydroxyphenyl)retinamide, and inhibitors of phosphatidylinositol-3'-kinase (PI3-K) (Chen *et al.*, 1999; Gschwend *et al.*, 2000; Murillo *et al.*, 2001; Engedal *et al.*, 2002). Thus, AI-CaP cells are extremely resistant to the apoptogenic signals triggered by diverse agents.

Understanding the survival strategies employed by certain AI-CaP cells could be instructive in targeting the proteins that permit these cells to overcome the stress of androgen deprivation and chemotherapy. Because intracellular signaling pathways responsible for triggering the programmed death of normal CaP cells must be understood fully, the apoptotic response of LNCaP cells to PMA has interested CaP researchers for many years. A number of reports have established that both PKC α and

PKC δ directly participate in the PMA-induced apoptosis of LNCaP cells (Powell *et al.*, 1996; Fujii *et al.*, 2000; Gschwend *et al.*, 2000; Garcia-Bermejo *et al.*, 2002). It has been proposed that ceramide biosynthesis may be prerequisite for the apoptotic events induced by PKC δ (Garzotto *et al.*, 1998; Sumitomo *et al.*, 2002), but the relationship between these signaling molecules is highly complex and remains controversial (Grant and Spiegel, 2002). Alternative explanations for the PKC-mediated apoptosis of LNCaP cells have emphasized the potential involvement of the *c-myc* oncogene (Zhao and Day, 2001) and c-Jun N-terminal Kinase (JNK; Engedal *et al.*, 2002). There is good evidence that PMA-induced apoptosis of LNCaP cells is preceded by the activation of Rb and transcriptional repression of *c-myc*. Neither of these molecular responses to PMA treatment are observed in DU145 cells (Zhao and Day, 2001), implying that disruption of this key regulatory pathway may be sufficient to abrogate apoptosis in CaP cells. More recently, it was reported that PMA enhances JNK activity in LNCaP, but not DU145 nor PC3, cells and that introduction of the JNK Inhibitory Protein (JIP) significantly inhibits PMA-induced apoptosis of LNCaP cells (Engedal *et al.*, 2002). These data suggest that JNK may play a pivotal role downstream of PKC α /PKC δ in the mitochondria-dependent apoptotic pathway of LNCaP cells. Moreover, protein kinase D (PKD), a downstream effector and substrate of PKC, has the potential to bypass this requirement for JNK activity by directly phosphorylating the N-terminus of c-Jun (Hurd *et al.*, 2002).

Evidence of an antagonism among the PKC family members that LNCaP cells express has recently been presented (Wu *et al.*, 2002). In this paper, we reported that PKC ϵ overexpression significantly inhibits the activation of caspase 3 and apoptogenic effects of PMA in LNCaP cells. These findings suggested that PKC ϵ was capable of playing a predominant role in determining the fate of CaP cells following androgen deprivation. PKC ϵ overexpression was sufficient to transform LNCaP cells into an AI variant that grew rapidly in both intact and castrate male nude

mice. In the present study, we sought to define the mechanism by which PKC ϵ regulates the apoptotic machinery that prostate cancer cells employ during their transition to recurrent CaP.

Primary specimens of benign prostatic hyperplasia (BPH) and AI-CaP, CWR22 xenograft tumors, and the CWR-R1 and LNCaP cell lines were examined. PKC ϵ immunostaining was detected in the cytoplasm of epithelial cells in BPH specimens and the intensity of this staining increased during the progression to AI-CaP. Characteristic of AI-CaP cell lines, CWR-R1 cells were resistant to the apoptogenic effects of PMA and antisense experiments demonstrated that endogenous PKC ϵ was a required molecular component of this survival pathway. Flow cytometry and confocal scanning laser microscopy provided evidence that PKC ϵ overexpression was capable of blocking the liberation of mitochondrial cytochrome c and loss of transmembrane potential ($\Delta\psi_m$) that are typical of the response pattern of LNCaP cells to PMA exposure. Using a proteomic approach, we found that PKC ϵ preferentially interacted with a Bax-like protein in recurrent CWR22 xenograft tumors and then confirmed that such an interaction occurred naturally in living cells using reciprocal coimmunoprecipitation assays. Finally, we report that the apoptotic protein Bax was required for LNCaP cells to commit to PMA-triggered apoptosis and that, under these conditions, Bax redistribution to mitochondria was suppressed by the overexpression of PKC ϵ . Such a mechanism may represent one of the means whereby PKC ϵ enables CaP cells to escape destruction by chemotherapeutic intervention.

Results

Enhanced expression of endogenous PKC ϵ during the progression of CaP

Immunoperoxidase staining of PKC ϵ was weak and predominantly confined to the basal and columnar epithelial cells of BPH (Fig. 1B). In CaP, the intensity of this immunostaining for PKC ϵ was higher than in BPH and could be easily detected in the cytoplasm of recurrent CaP

cells (Fig. 1C). Weak immunostaining could also be detected in the stroma of some prostate tissues, but this was not consistently observed. The results suggest that expression of PKC ϵ by human prostate epithelial cells is enhanced, and may favor their survival and autonomous growth, as prostate tissue evolves from benign to malignant and recurrent CaP. This interpretation is supported by previous data (Wu *et al.*, 2002) and would be consistent with the pattern of immunostaining observed in the present study (Fig. 1). Thus, PKC ϵ may play a consequential role in establishing a phenotype that could be prerequisite for the development and progression of CaP.

PKC ϵ protects LNCaP and CWR-R1 cells from the apoptotic effects of PMA

Previously, we reported that PKC ϵ overexpression prevented the apoptotic effects of PMA on LNCaP cells (Wu *et al.*, 2002). In this report, equimolar concentrations (100 nM) of PMA and thymeleatoxin, a selective agonist of conventional PKC isoforms, produced similar apoptotic responses in LNCaP cells (Figs. 2A and 2B). Even under conditions favoring the activation of PKC α (thymeleatoxin), an apoptotic protein in LNCaP cells (Powell *et al.*, 1996), the protective effects of PKC ϵ overexpression remained dominant and apoptosis was rarely observed (Fig. 2B). The results were similar in a heterogeneous population of LNCaP cells overexpressing PKC ϵ (LNCaP/ ϵ) and a subclone selected from this population (LNCaP/ ϵ 3). The AI CWR-R1 cell line was studied to determine whether PKC ϵ might regulate apoptosis in recurrent CaP. CWR-R1 was derived from a CWR22 xenograft that recurred several months after castration (Gregory *et al.*, 2001a). CWR-R1 expresses moderate levels of PKC ϵ in culture and are resistant to the apoptotic effects of PMA (Fig. 2C). These characteristics made it possible to conduct an antisense experiment to determine whether endogenous PKC ϵ influenced the susceptibility of CaP cells to PMA. The antisense PKC ϵ oligodeoxynucleotide (ODN; Wu *et al.*, 2002)

selectively and effectively down-regulates translation of PKC ϵ mRNA in the CWR-R1 cell line (Wu *et al.*, manuscript submitted). PKC ϵ expression was decreased using the antisense PKC ϵ ODN. A five-fold increase in the percentage of apoptotic CWR-R1 cells occurred after a 20 h exposure to 100 nM PMA (Fig. 2C). CWR-R1 cells appear to rely on endogenous PKC ϵ to survive the apoptotic signals triggered by PMA.

PKC ϵ selectively blocks the mitochondrial apoptotic pathway

Two major signaling pathways control the activity of initiator caspases and apoptotic cell death: an intrinsic mitochondrial pathway and an extrinsic death receptor pathway. In LNCaP cells, PMA triggers apoptosis via the intrinsic path at a site upstream of cytochrome c release (Majumder *et al.*, 2000). However, PKC ϵ has been reported to suppress the activity of the extrinsic death receptor pathway in a variety of cells (Shinohara *et al.*, 2001; Chang and Tepperman, 2001). Thus, PKC ϵ may also disrupt the activity of this second arm of the apoptotic machinery. The protocol of Kulik *et al.* (2001) was used to drive LNCaP cells into apoptosis using cycloheximide and the death receptor ligand tumor necrosis factor α (TNF- α) (Fig. 3). Under these conditions, the overexpression of PKC ϵ afforded no protection from the apoptotic signals triggered by TNF- α (Fig. 3). This would indicate that the death receptor pathway feeds into the apoptotic machinery downstream, or independent, of PKC ϵ -mediated survival signals in LNCaP cells.

PKC ϵ operates at a site upstream of cytochrome c release

The effects of PMA treatment on the $\Delta\psi_m$ and cytochrome c distribution within LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells were compared to more precisely locate the site of PKC ϵ action. Within 6 h, PMA significantly reduced the $\Delta\psi_m$ of LNCaP cells (Fig. 4A) and, within 12 h, the subcellular distribution of cytochrome c immunostaining shifted from a normal filamentous

shape characteristic of mitochondrial staining (Gao et al., 2001) to a punctuate and weaker intensity of staining (Fig. 4B). Neither of these mitochondrial responses to PMA were observed in either of the LNCaP variants overexpressing PKC ϵ (Fig. 4). These results supported the conclusion that the pro-survival signals produced by PKC ϵ in LNCaP cells operate at a site upstream of cytochrome c release.

Evidence that PKC ϵ activates an unconventional cell survival pathway in LNCaP cells

Constitutive signaling through the PI3-K pathway, upstream of cytochrome c release, appears to be required to suppress the spontaneous activation of the intrinsic mitochondrial pathway in LNCaP cells (Lin et al., 1999). Downstream of PI3-K, Akt acts to directly phosphorylate the apoptotic protein Bad at Ser¹³⁶ (Datta et al., 1997), which provides a binding site for the chaperone protein 14-3-3 (Zha et al., 1996). The finding that PKC ϵ overexpression was associated with the hyperphosphorylation of Akt at Thr³⁰⁸ in healthy LNCaP cells (Fig. 5A) was consistent with a mechanism involving the cytosolic sequestration of Bad by 14-3-3 proteins. However, the maturation of Akt into a catalytically competent kinase requires the phosphorylation of multiple sites; the phosphorylation of Akt at Thr³⁰⁸ is required, but not sufficient, to activate Akt (Nicholson and Anderson, 2002). Therefore, LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells were treated with the PI3-K inhibitor LY294002 to inhibit both PI3-K and downstream Akt activity. A significant number of LNCaP cells become apoptotic when exposed to LY294002 in the presence of cycloheximide (Fig. 5B). However, LY294002 had no effect on the survival of LNCaP cells overexpressing PKC ϵ (Fig. 5B). Thus, PKC ϵ appeared to generate pro-survival signals that made the normal requirement for PI3-K activity dispensable.

p90^{RSK} is a downstream effector of PKC ϵ that directly phosphorylates Bad at Ser¹¹² (Tan et al., 1999; Bertolotto et al., 2000), an alternative site to Ser¹³⁶ for inducing the sequestration of

Bad with 14-3-3 proteins. LNCaP, LNCaP/ε, and LNCaP/ε3 cells were treated with PMA for 24 h before immunoprecipitating Bad from whole cell lysates and probing for 14-3-3ζ to test whether the overexpression of PKCε had enhanced this protein-protein interaction. The results of this analysis indicated that PKCε did not augment or stabilize interactions between Bad and 14-3-3ζ in the PMA-resistant LNCaP/ε or LNCaP/ε3 cell lines (Fig. 5C). Thus, PKCε appears to operate by a Bad-independent mechanism downstream of PI3-K and upstream of cytochrome c release.

The temporal relationship between Rb activation, c-myc repression, and PMA-induced apoptosis of LNCaP cells is convincing (Zhao and Day, 2001). Moreover, such a mechanism is consistent with the observation that each of these cellular responses to PMA can be attenuated by forcing LNCaP cells to overexpress PKCε (Wu *et al.*, 2002). However, LNCaP/ε and LNCaP/ε3 cells that express increased levels of c-myc under basal conditions (Fig. 5D) remained viable after a 20 h exposure to apoptogenic concentrations of PMA, despite the loss of c-myc expression (Figs. 2 and 5E, LNCaP/ε cells). Therefore, PKCε was able to overcome the PMA-triggered mortality of LNCaP cells without preventing the repression of c-myc expression.

If JNK activation is required for PMA-induced apoptosis of LNCaP cells (Engedal *et al.*, 2002), PKCε overexpression may promote survival of these cells by interfering with this apoptotic signal. To test this, the effects of PMA on the phosphorylation (activation) of JNK at Thr¹⁸³/Tyr¹⁸⁵ was evaluated in parental and LNCaP/ε cells. This analysis confirmed that PMA enhances the phosphorylation of JNK in LNCaP cells (Fig. 5F), as previously reported (Engedal *et al.*, 2002), and demonstrated that this post-translational modification was not sufficient to induce the apoptotic death of LNCaP cells. JNK1 was hyperphosphorylated in both the LNCaP and LNCaP/ε cell lines after a 24 h exposure to PMA, despite their marked differences in

sensitivity to this agent (Fig. 5F). These data imply that PKC ϵ may be able to bypass the apoptotic signals triggered by JNK1 activity in LNCaP Cells.

Identification of a novel interaction between PKC ϵ and Bax

PKC ϵ interacts with a diverse array of structural, stress-related, and signaling proteins in cardiomyocytes, where PKC ϵ signaling is required to survive an ischemic insult (Ping *et al.*, 2001). However, the cell type-specific mechanisms that define the assembly of PKC ϵ signaling modules have not been resolved. For this reason, PKC ϵ immunocomplexes prepared from CWR22 and recurrent CWR22 tumor lysates were analyzed proteomically. Silver-stained 2D gels indicated PKC ϵ was associated with at least 18 different proteins, including β -actin, in CWR22 tumor lysates (Fig. 6A, β -actin). Fewer proteins coimmunoprecipitated with PKC ϵ in recurrent CWR22 tumor lysates (Fig. 6B). Superimposition of these proteomic profiles, from three independent experiments, indicated that several interactions with PKC ϵ were unique to either the CWR22 or recurrent CWR22 tumors while other interactions were common to both (manuscript in preparation). Among the subset of PKC ϵ interacting proteins that were unique to recurrent CWR22 tumors, a protein spot with coordinates (pI \sim 5.1 and $M_r \sim$ 23 kDa) approximates those of the apoptotic protein Bax (Fig. 6B, spot 2). Coimmunoprecipitation studies confirmed that Bax was present in PKC ϵ immunoprecipitates of CaP cells in culture (Figs. 6C and 6D). In contrast to tumor lysates, however, PKC ϵ /Bax complexes were detected in both androgen sensitive (LNCaP) and AI (CWR-R1 and LNCaP/ ϵ) CaP cell lines. Although PKC ϵ immunoprecipitates prepared from LNCaP/ ϵ cultures appeared to contain more Bax protein than their parental counterpart (Fig. 6C), this was most likely a result of ectopic PKC ϵ overexpression. PKC ϵ was immunoprecipitated from CWR-R1 lysates and immunoblotted with

Bax antibody to determine whether PKC ϵ /Bax complexes were also endogenous to the CWR-R1 model of recurrent CaP (Fig. 6D). PKC ϵ associated with Bax in this model of AI-CaP and, using the reciprocal approach, anti-Bax antibodies coimmunoprecipitated endogenous PKC ϵ from CWR-R1 cell lysates (Fig. 6D). Neither of these proteins was detected in controls that had been treated with IgG alone (Fig. 6D) and the concentration of detergent used in these studies (0.1% IGEPAL) does not induce the spontaneous oligomerization of Bax (Suzuki *et al.*, 2002). Thus, an initial proteomic analysis led to the identification of a novel *in vivo* interaction between PKC ϵ and Bax in CaP cells.

Bax mediates the commitment of LNCaP cells to PMA-induced apoptosis

Although there is convincing evidence that Bax activation and insertion into the outer mitochondrial membrane is sufficient to permit the efflux of cytochrome c (Pavlov *et al.*, 2001), the importance of this protein in the PMA-induced apoptosis of LNCaP cells has not been investigated. LNCaP cells were treated with a combination of antisense Bax ODN that has been shown to suppress Bax expression in several cell types (Dibbert *et al.*, 1999) to determine whether Bax participated in the commitment of LNCaP cells to the apoptosis induced by PMA. We have been unable to detect Bax protein in total cell lysates of untreated LNCaP cells so the extent to which antisense Bax ODN downregulated Bax expression in this cell line could not be determined. Even so, antisense Bax ODN significantly inhibited the apoptotic effects of PMA in LNCaP cells and this effect of the ODN was sequence-specific (Fig. 7). This result reinforced the notion that PMA triggers apoptosis through a Bax-dependent mechanism in LNCaP cells.

PKC ϵ remains associated with Bax and disrupts Bax redistribution in LNCaP/ ϵ cells

The balance between apoptosis and survival often depends on the manner in which apoptotic and anti-apoptotic members of the Bcl-2 protein family interact with one another (Antonsson *et al.*,

2001), although some reports disagree with this view (Knudson and Korsmeyer, 1997; Metcalfe *et al.*, 1999). Taken together, the available evidence suggests that Bax conformation is an important determinant of its subcellular localization (Suzuki *et al.*, 2002). It was therefore important to determine whether PKC ϵ remained associated with Bax in a manner that influenced its subcellular redistribution in LNCaP cells that had been rendered resistant to the apoptotic effects of PMA. To address this question, PKC ϵ was immunoprecipitated from LNCaP and LNCaP/ ϵ cell lysates, after a 24 h exposure to PMA, and immunoblotted using anti-Bax antibody. This analysis indicated that PMA may have weakened the interaction between PKC ϵ and Bax in apoptotic LNCaP cells, whereas the Bax interaction with PKC ϵ was not altered in LNCaP/ ϵ cells (Fig. 8). Next, the capability of PKC ϵ to influence the mitochondrial targeting of Bax in LNCaP cells was investigated. LNCaP and LNCaP/ ϵ cells were incubated in the absence or presence of PMA for 12 h prior to staining mitochondrial membranes, using Mitotracker Green FM, and endogenous Bax, using anti-Bax primary antibodies followed by TXRD-conjugated secondary antibodies. Immunofluorescence-based confocal microscopy confirmed the mitochondrial localization of Bax in PMA-treated LNCaP cells and indicated that the overexpression of PKC ϵ was capable of diverting Bax from its intended intracellular target (Fig. 9). Superimposed images of mitochondrial and Bax staining patterns of apoptotic, PMA-treated, LNCaP cells merged extensively while LNCaP cells overexpressing PKC ϵ displayed a more localized merging of these intracellular markers (Fig. 9). These and the data discussed above have provided the first evidence to support the hypothesis that Bax is required for PMA-induced apoptosis in CaP cells and that a novel direct, or indirect, interaction between PKC ϵ and Bax has the potential to influence the commitment of CaP cells to apoptosis.

Discussion

PKC ϵ expression favors the survival and autonomous growth of human prostate epithelial cells during the development and progression of CaP (Wu *et al.*, 2002). Until now, the components linking this oncoprotein to the apoptotic machinery have not been identified. The enhanced expression of PKC ϵ , and expansion of PKC ϵ -positive cells, from BPH to recurrent CaP suggests an important role for PKC ϵ in the development and progression of CaP. Recent attempts to characterize genes linked to CaP suggest that gains of the PKC ϵ gene, at 2p21, are unlikely to account for the increased expression of this oncoprotein in recurrent CaP. The promoter of the human PKC ϵ gene has not been experimentally analyzed and transcriptional activation could account for the increased levels of PKC ϵ mRNA and protein that are evident in AI DU145 and PC3 cells, relative to their LNCaP counterparts (Wu *et al.*, 2002). While this possibility we be examined in future experiments, the data presented here suggest that PKC ϵ may enhance resistance to apoptosis, conferring a survival advantage to CaP cells. We have demonstrated that PKC ϵ is capable of supplanting the requirement for constitutive PI3-K activity and neutralizing mitochondrial death signals generated by other members of the PKC family, c-myc, and JNK. Our results also suggest that Bax is required for LNCaP cells to commit to apoptosis after receipt of PMA-generated death signals and have presented evidence of an association between PKC ϵ and Bax in CaP tumors and cultured cells. Of course, our data do not address whether this interaction is direct or indirect, although we would suspect the latter. Identification of PKC ϵ as a Bax-interacting protein, and the overriding influence of this protein on the mitochondrial death signaling pathway of CaP cells, provides crucial support for the hypothesis that PKC ϵ may be an important and early determinant for CaP cell survival.

In this paper, we report several substantive findings that should contribute to continuing advances in our understanding of the molecular mechanisms that PKC ϵ employs in CaP cells to overcome programmed cell death. Data presented in this report show that PKC ϵ is capable of engaging 'unconventional' mechanisms to selectively block the mitochondrial apoptotic pathway in CaP cells at a site downstream of PI3-K and upstream of cytochrome c release. The pro-survival signal(s) produced by PKC ϵ are unconventional because this oncoprotein permitted LNCaP cells to escape the apoptotic effects of PMA and an inhibitor of PI3-K, without blocking several consequential and well established apoptogenic effects of these agents. LNCaP cells overexpressing PKC ϵ remained viable, in the presence of apoptotic concentrations of PMA, without enhancing the cytosolic sequestration of Bad by 14-3-3 chaperones and despite the downregulation of c-myc expression and JNK hyperphosphorylation. These and other observations imply that PI3-K, JNK, and c-myc operate upstream of PKC ϵ in the mitochondrial apoptotic pathway and that PKC ϵ -mediated survival signals are Bad-independent.

Directly linking PI3-K to PKC ϵ is phosphoinositide-dependent kinase 1 (PDK-1), the enzyme required to surmount the first and rate-limiting step in the biogenesis of a mature PKC ϵ enzyme. While there are conflicting reports (Parekh *et al.*, 1999; Cenni *et al.*, 2002), the cumulative data suggest that PDK-1 phosphorylates PKC ϵ at the activation-loop Thr⁵⁶⁶ to initiate the autophosphorylation of a conserved turn motif (Thr⁷¹⁰) and hydrophobic motif (Ser⁷²⁹) by PKC ϵ itself. In HEK293 and NIH 3T3 cells, the phosphorylation of PKC ϵ at Thr⁵⁶⁶ may depend on the production of PI3-K lipid products. PKC ϵ sustained the survival of LNCaP cells during a 12 h exposure to cytotoxic concentrations of the PI3-K inhibitor LY294002. One explanation for this result is that a kinase-independent mechanism mediated the survival of LNCaP cells overexpressing PKC ϵ . Alternatively, it is possible that PDK-1 is constitutively active in PTEN^{-/-}

LNCaP cells and that this activity sustained the maturation and downstream signaling by PKC ϵ in the presence of LY294002. As a key regulator of polyphosphatidylinositol dephosphorylation, PTEN normally plays an important role in limiting the activity of PDK-1 and we have found that the proliferation of LNCaP/ ϵ cells is dependent on constitutive PDK-1 activity (unpublished data). However, PKC ϵ -mediated survival signals are not dependent on the forced expression of this protein in PTEN^{-/-} CaP cells, as we have shown that the expression of endogenous PKC ϵ is also required for PTEN^{+/+} CWR-R1 cells to resist the apoptosis that is triggered by PMA (this study and our unpublished data).

Many details regarding the mechanisms controlling Bax oligomerization and bioactivity have not been resolved. It is widely accepted that the C-terminal tail of Bax contains a conserved hydrophobic motif that appears to be critical for the mitochondrial targeting of Bax and its apoptotic activity (Antonsson, 2001). While mutational studies have also implicated the Bax BH3 domain in Bax-mediated oligomerization and apoptosis, the role of this domain is less certain. Antibodies raised against a Bax BH3 epitope indicate that a conformational rearrangement exposing this domain precedes the mitochondrial translocation of this protein in response to apoptotic stimuli (Gilmore *et al.*, 2000). However, a structural analysis of Bax shows that its C-terminal α 9 helix, which contains the BH3 domain, establishes an extremely stable interface with the hydrophobic BH3-binding pocket of this protein (Suzuki *et al.*, 2002). Thus, the stability of this intramolecular interaction may prevent dimer formation at this site in the cytosol of living cells. Alternative mechanisms for the formation of Bax homo- and hetero-oligomers include helical packing/bundling at the interface between Bax and its binding partners (Suzuki *et al.*, 2002). The N-terminus of PKC ϵ contains a C2-like domain that may be important for its intracellular targeting and interactions with other proteins (Schechtman and Mochly-

Rosen, 2001). However, crystal structures of the PKC ϵ -C2 domain (residues Met¹ to Gly¹³⁸) consist of an eight-stranded, antiparallel, β -sandwich (Ochoa *et al.*, 2001) that would not be suited for helical packing/bundling with a protein such as Bax. PKC ϵ does not contain a BH3-like domain and, until the complete crystal structure of PKC ϵ has been resolved, it is not possible to predict whether the surface of this protein presents a suitable interface for interacting directly with Bax protein. Given the numerous proteins that are present in PKC ϵ modules within CWR22 tumors *in vivo*, the association between PKC ϵ and Bax is likely to be indirect and may be mediated by one or more intermediary proteins. In any case, PKC ϵ overexpression is capable of suppressing Bax translocation from the cytosol to mitochondria in LNCaP cells exposed to cytotoxic concentrations of PMA. The studies described emphasize the importance of addressing the basic question of how PKC ϵ influences the bioactivity of Bax and developing a detailed molecular description of this novel protein interaction.

In summary, we have identified one of the possible mechanisms that PKC ϵ employs to propagate survival signals in CaP cells. PKC ϵ -mediated survival signals can supplant the requirement for constitutive signaling through the PI3-K pathway in LNCaP cells and endogenous PKC ϵ is a required component of the CWR-R1 survival pathway that operates to protect these cells from PMA-induced apoptosis. By altering the topological response of Bax within the mitochondrial death signaling pathway, PKC ϵ may subvert the programmed death of selected CaP cells and promote CaP progression. Certain ligands of the death receptor pathway should bypass the cell survival signals mediated by PKC ϵ and may provide a novel and effective chemotherapeutic approach.

Materials and methods

Immunohistochemical analysis of PKC ϵ

Prostate specimens were acquired in compliance with the guidelines of the University of North Carolina at Chapel Hill Clinical Cancer Protocol Review Committee and Institutional Review Board (Chapel Hill, NC). Histological diagnoses were verified by examination of frozen and corresponding formalin-fixed, paraffin-embedded tissues (Gregory *et al.*, 1999). Samples of BPH from ten men were obtained from the transition zone of radical prostatectomy specimens. Samples of AI-CaP were obtained by transurethral resection from 25 men who exhibited increasing serum prostate-specific antigen values and developed urinary retention from locally recurrent CaP more than one year after surgical castration. Formalin-fixed, paraffin-embedded AI-CaP samples were used to construct tissue microarrays. Sections of BPH and tissue arrays of CaP were processed for immunostaining by incubating the slides in a pressure cooker at 100°C (Biocare Medical, Walnut Creek, CA) for 1 min 45 s followed by preincubation in normal goat serum for 15 min at room temperature and a phosphate-buffered saline (PBS) wash. Slides were incubated with antihuman PKC ϵ IgG at a 1:500 dilution for 1 h followed by incubation with goat anti-rabbit secondary antibody for 15 min. Immunoperoxidase reaction products were detected using diaminobenzidine. Specificity of immunostaining was determined by omitting the primary antibody or peptide competition of the primary antibody using PKC ϵ (C-15P) blocking peptide (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell lines and culture conditions

LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA; ATCC CRL-1740) and maintained in culture as previously described (Wu *et al.*, 2002). The CWR-R1 cell line was clonally selected for AI growth from recurrent CWR22 xenograft tumors as described (Gregory *et al.*,

2001a). LNCaP cell culture reagents were purchased from Invitrogen (Rockville, MD). CWR-R1 cells were cultured in Richter's Improved MEM (Invitrogen, Santa Ana, CA) supplemented with 100 ng/ml epidermal growth factor and 10 µg/ml insulin/transferrin/selenium (BD Biosciences; Bedford, MA), 10 mM nicotinamide, 900 ng/ml linoleic acid, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2% fetal bovine serum (FBS; Invitrogen).

Expression plasmid and transfection into LNCaP cells

LNCaP cells with pLXSN recombinant retrovirus harboring the gene for p3/PKCε (LNCaP/ε), and a subclone derived by limiting dilution (LNCaP/ε3), were prepared as described (Prekeris *et al.*, 1998; Wu *et al.*, 2002).

Apoptosis detection

Subconfluent LNCaP, LNCaP/ε, and LNCaP/ε3 cells (3.5×10^4 cells per well in 24-well plates) were cultured in complete medium for at least 24 h at 37 °C. Adherent cells were then incubated for an additional 20 h in fresh media, with or without PMA (100 nM) or thymeleatoxin (100 nM). In separate experiments, cells were treated with cycloheximide (3 µg/ml) with or without LY294002 (25 µM) or tumor necrosis factor α (TNF-α, 10 ng/ml) for 12 h. Apoptosis assays performed using the CWR-R1 cell line and an antisense PKCε ODN are described below.

Apoptotic cells were identified using acridine orange and ethidium bromide staining, as before (Wu *et al.*, 2002). One hundred cells were scored in triplicate for each cell line and treatment condition. Three independent experiments were conducted and data were expressed as the percentage of apoptotic cells.

Optical recording of $\Delta\psi_m$

LNCaP, LNCaPε, and LNCaP/ε3 cells were plated on 10-cm plates and cultured in complete medium for at least 24 h at 37 °C. Adherent cells were then incubated for an additional 6 h in

fresh media, with or without PMA (100 nM). Any cells having undergone anoikis were collected and combined with adherent cells in sterile microfuge tubes placed on ice. Aliquots of each cell suspension, containing 5×10^5 cells, were transferred to fresh microfuge tubes and incubated in the dark with PBS containing 1.0 μ M JC-1 monomer (Molecular Probes, Eugene, OR) for 30 min at room temperature. After washing twice with cold PBS, cell fluorescence was recorded using a FACScan flow cytometer (Becton Dickinson, Lincoln Park, NJ). A total of 1×10^4 cells were analyzed per sample and the data are expressed as the percentage of cells displaying a PMA-dependent fluorescence shift from green (FL1) to red (FL2), indicative of a fall in the $\Delta\psi_m$ within a given cell.

Immunoblot analyses

Immunoblot analyses were performed as described previously (Prekeris *et al.*, 1996). Antibodies purchased from Santa Cruz Biotechnology were raised against Bad (H-168), Bax (P-19), JNK1 (clone FL), phospho-JNK (Thr183/Tyr185; G-7), c-myc (C-19), PKC ϵ (C-15), and 14-3-3 ζ (C-16). Other antibodies included: anti-Akt1 (559028) from BD PharMingen (Franklin Lakes, NJ) and anti-phospho-Akt (Thr308) from New England BioLabs (Beverly, MA).

Coimmunoprecipitation

Whole cell lysates were prepared from cultures of LNCaP, LNCaP/ ϵ , and CWR-R1 cells and from frozen CWR22 and recurrent CWR22 tumors as described previously (Gregory *et al.*, 2001b; Wu *et al.*, 2002). Tumor lysates were precleared with 20 μ l of protein A agarose (Invitrogen) at 4°C for 30 min before sedimenting the agarose beads at 13,000 x g for 5 min. Pre-cleared supernatants were incubated with polyclonal anti-PKC ϵ antiserum on a rotator overnight at 4°C. Immunocomplexes were precipitated by adding 50 μ l Protein A agarose to each lysate, incubating on a rotator for 1 h at 4°C, and centrifuging at 13,000 x g for 5 min at

4°C. After four washings with immunoprecipitation buffer, immunoprecipitates were solubilized in 40 µl boiling SDS sample buffer. Immunoprecipitates were also prepared from subconfluent cell cultures grown for 24 h in either the absence or presence of 100 nM PMA as described previously (Wu *et al.*, 2002).

Two-dimensional electrophoresis

PKCε immunoprecipitates, prepared from CWR22 and recurrent CWR22 tumor lysates, were separated by 2-D electrophoresis. The protein A-anti-PKCε complex was solubilized with 250 µl 2-D buffer (9.5 mM deionized urea, 4% CHAPS, 18 mM DTT, 0.01% bromophenol blue, 2% 3-10 Immobilized pH Gradient [IPG] buffer) and resolved on IPG strips (Amersham Pharmacia, Piscataway, NJ) by isoelectric focusing (IPGPhor, Amersham Pharmacia). IPG strips were then re-equilibrated and the second dimension electrophoresis was performed for between 60K and 100K total V/hours. Gels were then stained, dried, digitized, and analyzed using DECODON Delta2D (DECODON, Greifswald, Germany) software for the differential display of CWR22 and recurrent CWR22 2-D gels and a search engine designed to predict the identity of proteins isolated on 2-D gels (www.ca.expasy.org/tools/tagident.html). Data presented are representative of three independent experiments.

Antisense ODN treatments

Phosphorothioate ODNs were obtained from Invitrogen. Sequences for the antisense PKCε and Bax ODNs, and corresponding scrambled control ODNs, were exactly as specified (Dibbert *et al.*, 1999; Wu *et al.*, 2002). Subconfluent (70-80%) LNCaP and CWR-R1 cultures were washed with Opti-MEM 1 (Invitrogen) before introducing a mixture of ODN and lipofectin (2 µg/ml; Invitrogen). Antisense and scrambled PKCε ODNs were used at a final concentration of 1 µM with the CWR-R1 cell line. A mixture of two antisense Bax ODNs were used at a final

concentration of 5 μ M and the pair of control scramble Bax ODNs were used at an equimolar concentration. After 6 h at 37 °C, the cells were washed twice with serum-free medium and incubated 56 h in lipofectin-free media containing fresh ODN.

Immunohistochemical analysis of Bax and cytochrome c

LNCaP (2×10^4) and LNCaP/ ϵ (5×10^3) cells were plated on 25-mm German glass coverslips (Electron Microscopy Sciences, Ft. Washington, PA) in 10-cm dishes. Cells were incubated 48 h in RPMI, containing 10% FBS, before introducing either PMA (100 nM) or an equal volume of 0.1% dimethylsulfoxide (vehicle control). Following a 12 h exposure, the media was removed and coverslips were washed with 1 ml PBS, fixed with 3.7% paraformaldehyde (15 min), incubated with 0.2% Triton X-100 (5 min), blocked in 1% bovine serum albumin (5 min), and incubated with either 2.5 μ g/ml anti-cytochrome c (556432; BD PharMingen) or 1.6 μ g/ml anti-Bax (P-19; Santa Cruz Biotechnology) antibodies for 1 h; all steps were performed at room temperature. After a PBS wash, the slides were incubated in the dark with either 2 μ g/ml goat anti-rabbit IgG TXRD conjugate (Southern Biotechnology Associates, Birmingham, AL) or 3 μ g/ml goat anti-mouse alexa conjugate (Molecular Probes, Eugene, OR) for 45 min at room temperature. After another PBS wash, slides immunostained for Bax were incubated 15 min in PBS containing 100 nM Mitotracker Green FM (Molecular Probes). All coverslips were rinsed a final time with PBS and affixed to glass microscope slides using Prolong anti-fade fixative (Molecular Probes). Cells were examined for immunofluorescence using a LSM 510 confocal microscope (Zeiss, Jena, Germany) and LSM 5 Image Examiner software. Control slides were incubated with equimolar concentrations of goat anti-rabbit IgG TXRD or goat anti-mouse alexa conjugates alone and showed no immunoreactivity.

Data analysis

Values shown are representative of three or more experiments, unless otherwise specified, and treatment effects were evaluated using a two-sided Student's *t* test. Errors are standard errors of the mean (SE) of averaged results and values of $P < 0.05$ were considered to represent a significant difference between means.

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Figure Legends

Figure 1. PKC ϵ immunoperoxidase staining in human prostate tissue. (A) BPH negative controls stained in the absence of primary antibody. (B) BPH tissue. Note the weak immunostaining of PKC ϵ within the characteristic small, closely spaced glands of BPH. (C) A representative example of the PKC ϵ immunostaining typically observed in recurrent CaP. $\times 400$.

Figure 2. PKC ϵ can protect CaP cells from apoptosis induced by PKC agonists. (A and B) Comparison of the apoptotic responses of LNCaP, LNCaP/ ϵ , and LNCaP/ $\epsilon 3$ cells during 20 h exposure to 10% FBS containing either 100 nM PMA (A, closed bars) or thymeleatoxin (B, closed bars). Controls (open bars) were cultured in 10% FBS alone. Apoptotic cells were identified and counted using the acridine orange and ethidium bromide staining method; a total of 100 stained cells were analyzed per plate. Data represent the mean percentage of stained cells that were apoptotic (\pm SE), counted in triplicate in three independent experiments. (C) Subconfluent cultures of CWR-R1 cells were exposed to lipofectin alone or 1 μ M antisense (AS) PKC ϵ ODN for 2 days, as indicated, prior to culturing cells in the absence (open bars) or presence (closed bars) of 100 nM PMA for 20 h. Apoptotic cells were identified and counted as in (A) above. Data represent the mean percentage of stained cells that were apoptotic (\pm SE), counted in triplicate in two independent experiments.

Figure 3. PKC ϵ does not protect LNCaP cells from apoptosis induced by TNF- α . Subconfluent cultures of LNCaP, LNCaP/ ϵ , and LNCaP/ $\epsilon 3$ cells were serum starved for 24 h, incubated with cycloheximide (3 μ g/ml, 30 min), and either treated with TNF- α (100 ng/ml, closed bars) or vehicle alone (open bars) for 12h prior to staining and counting apoptotic cells. Data represent the mean percentage of stained cells that were apoptotic (\pm SE), counted in triplicate in three independent experiments.

Figure 4. PKC ϵ blocks the PMA-triggered loss of $\Delta\psi_m$ and release of cytochrome c in LNCaP cells. (A) Subconfluent cultures LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells were incubated in the absence or presence of 100 nM PMA for 6 h, stained in suspension with 1.0 μ M JC-1, washed twice, and analyzed using by flow cytometry. Data are expressed as the percentage of cells displaying a PMA-dependent fluorescence shift indicative of a fall in the $\Delta\psi_m$ within a given cell and represent the mean (\pm SE) of triplicate determinations in three independent experiments. (B) Comparison of cytochrome c distribution in LNCaP and LNCaP/ ϵ cells stained with anti-cytochrome c following a 12 h incubation in the absence (control) or presence of 100 nM PMA. When cytochrome c has accumulated within mitochondria, staining is fibrous and becomes more punctate and weaker in intensity when cytochrome c is released from the mitochondria. Confocal images shown are representative of those observed in three independent experiments. Scale bar is 10 μ m.

Figure 5. Evidence that PKC ϵ operates downstream of PI3-K, JNK, and c-myc to suppress the apoptotic effects of PMA on LNCaP cells. (A) Immunoblot analysis of total Akt and the phosphorylation of Akt at Thr³⁰⁸ (p-Akt) in subconfluent cultures of LNCaP, LNCaP/v, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells (lanes 1 to 4, respectively) growing in complete media. (B) Subconfluent cultures of LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells were serum starved for 24 h, incubated with cycloheximide (3 μ g/ml, 30 min), and treated with LY294002 (25 μ M, closed bars) or vehicle alone (open bars) for 12h prior to staining and counting apoptotic cells. Data represent the mean percentage of stained cells that were apoptotic (\pm SE) and counted in triplicate. (C) LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells (lanes 1 to 3, respectively) were treated with 100 nM PMA for 24 h before immunoprecipitating Bad from whole cell lysates and immunoblotting with anti-14-3-3 ζ antibody. Results are representative of two independent

assays. (D) Immunoblot analysis of c-myc protein in subconfluent cultures of LNCaP, LNCaP/v, LNCaP/ε, and LNCaP/ε3 cells (lanes 1 to 4, respectively) growing in complete media. Results are representative of three independent assays. (E) Immunoblot analysis of c-myc protein in LNCaP/ε cells cultured in the presence of 100 nM PMA for 0, 12, 24, or 48 h. Results are representative of two independent assays at each time point. (F) Immunoblot analysis of total JNK1 and JNK2 and the phosphorylation of JNK1 at Thr¹⁸³/Tyr¹⁸⁵ (p-JNK1) in subconfluent cultures of LNCaP and LNCaP/ε cells incubated for 24 h in the absence or presence of 100 nM PMA. Samples were loaded for equal amounts of total JNK1 protein and results are representative of three independent assays.

Figure 6. Identification of a novel interaction between PKCε and Bax. A. Representative 2D gels illustrating proteomic profiling of PKCε immunoprecipitates from CWR22 (A) and recurrent CWR22 (B) tumor lysates. Comparative 2D electrophoresis analysis revealed significant changes in the identity and number of protein spots in PKCε complexes isolated from CWR22 and recurrent CWR22 tumors. Protein 1 is β-actin (panels A and B) and protein 2 is the apoptotic protein Bax (see panel B). (C) Equal amounts of precleared LNCaP and LNCaP/ε whole cell lysates were subjected to immunoprecipitation with anti-PKCε antibodies and immunoblotted using anti-Bax antibody. (D) Reciprocal coimmunoprecipitation of PKCε/Bax complexes from equal amounts of precleared CWR-R1 whole cell lysates. Lysates were subjected to immunoprecipitation (IP) with the secondary IgG alone (left lane) or with anti-PKCε or anti-Bax antibodies and immunoblotted (IB) using the reciprocal antibody. Results are representative of three independent assays.

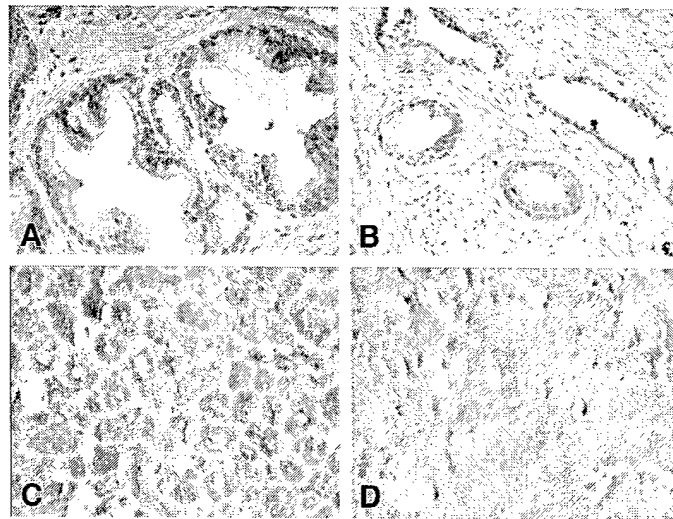
Figure 7. Antisense Bax ODN inhibit PMA-triggered apoptosis in LNCaP cells. Subconfluent cultures of LNCaP cells were treated with lipofectin alone (C) or lipofectin plus 5 μM antisense

(AS) or scrambled sense (SC) Bax ODNs for 6 h and maintained for 56 h in lipofectin-free media. Cells were then incubated in fresh media alone (open bars) or media containing 100nM PMA (closed bars) for 24 h before staining and counting apoptotic cells. Data are expressed as the percent of total cells exhibiting staining indicative of apoptosis and are the means (\pm SE) of triplicate determinations in three independent experiments.

Figure 8. Coimmunoprecipitation of PKC ϵ /Bax complexes from apoptotic LNCaP and healthy LNCaP/ ϵ cell lysates. LNCaP and LNCaP/ ϵ cells were incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 100 nM PMA for 24 h prior to cell lysis. Equal amounts of precleared whole cell lysates were then subjected to immunoprecipitation with the secondary IgG alone (lanes 1 and 3) or with anti-PKC ϵ primary antibodies (lanes 2 and 4) and immunoblotted using anti-Bax antibody. Results shown are representative of three independent experiments.

h.c.: IgG heavy chains.

Figure 9. PKC ϵ alters the topological response of Bax to PMA in LNCaP cells. Simultaneous confocal measurements of Mitotracker distribution (left) and Bax distribution (middle) in LNCaP and LNCaP/ ϵ cells cultured for 12 h in the absence (control) or presence (PMA) of 100 nM PMA. The overlap of green (Mitotraker) and red (Bax) fluorescence gives the yellow color shown in the right panel. All fields have been magnified similarly. Bax colocalization with the staining pattern of Mitotracker was extensive in LNCaP cells treated with PMA (see arrows). In contrast, focal sites of yellow staining were also evident in PMA-treated LNCaP/ ϵ cells but there was a consistent decrease in the extent to which these staining patterns overlapped. Results are representative of six independent experiments. Scale bar is 10 μ m.



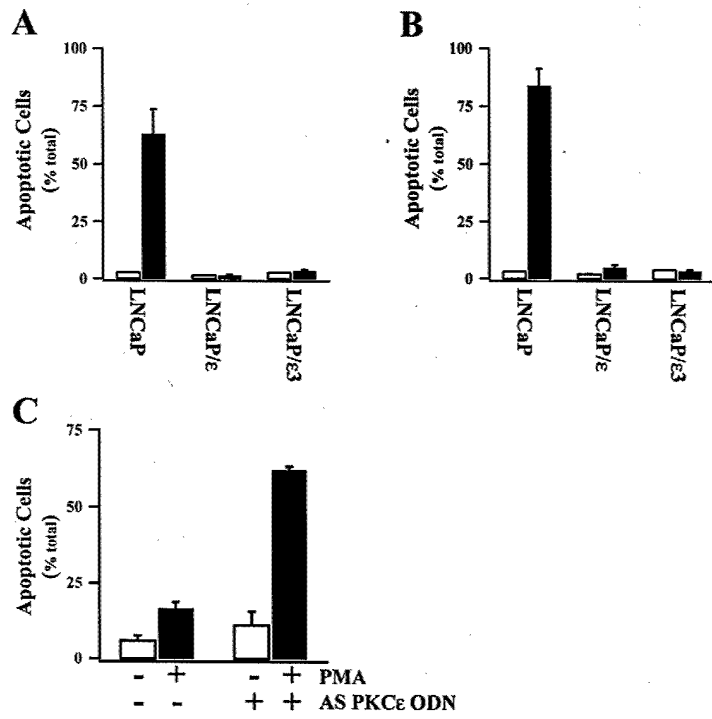
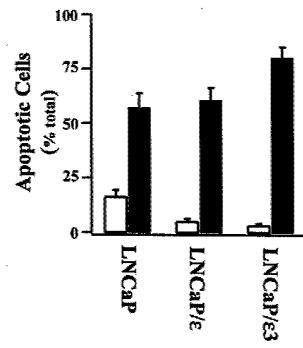
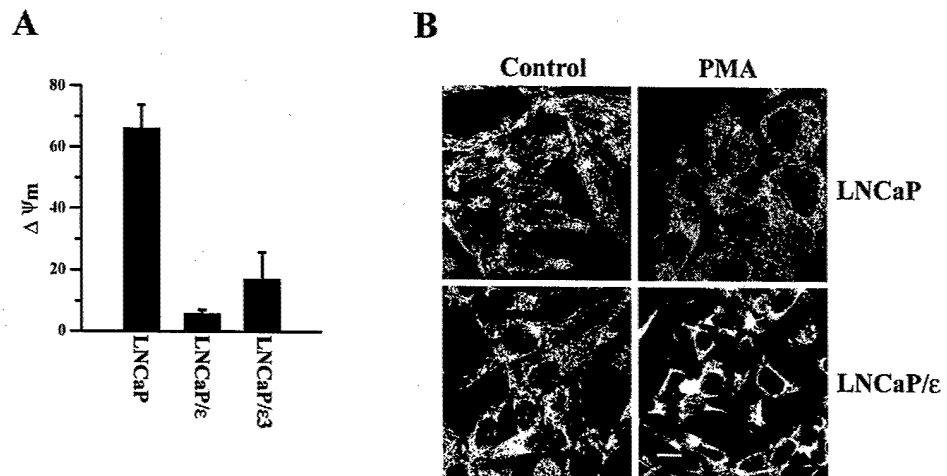
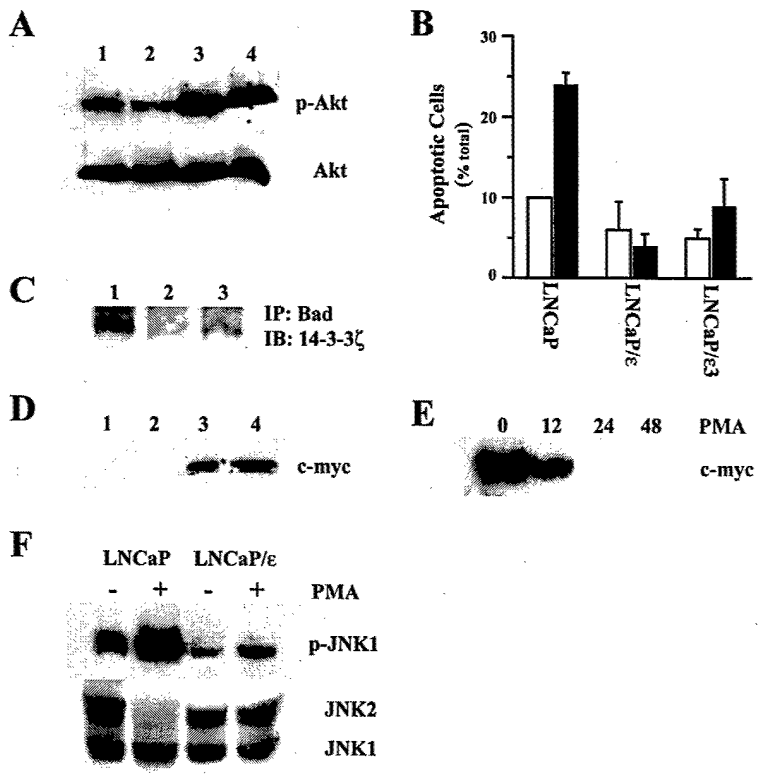
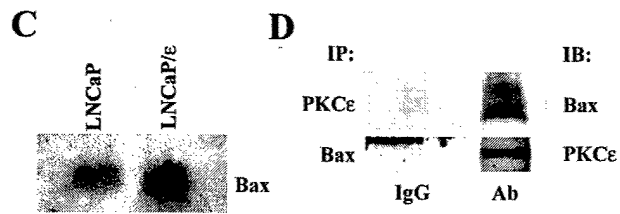
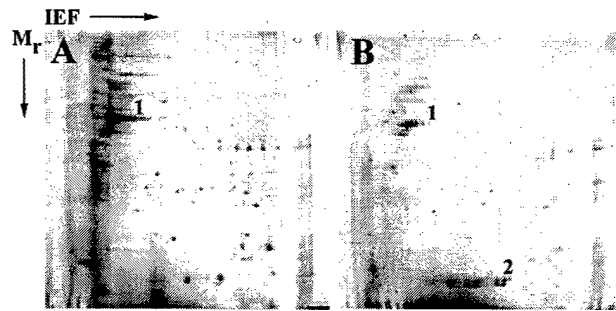


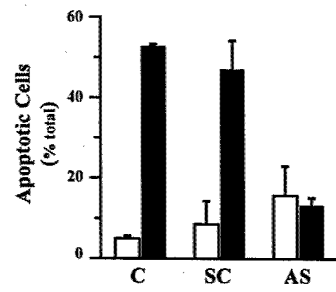
Figure 3

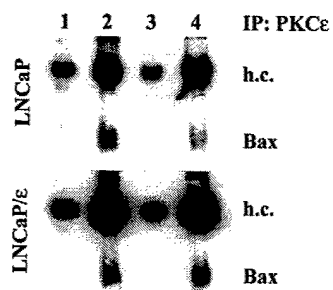


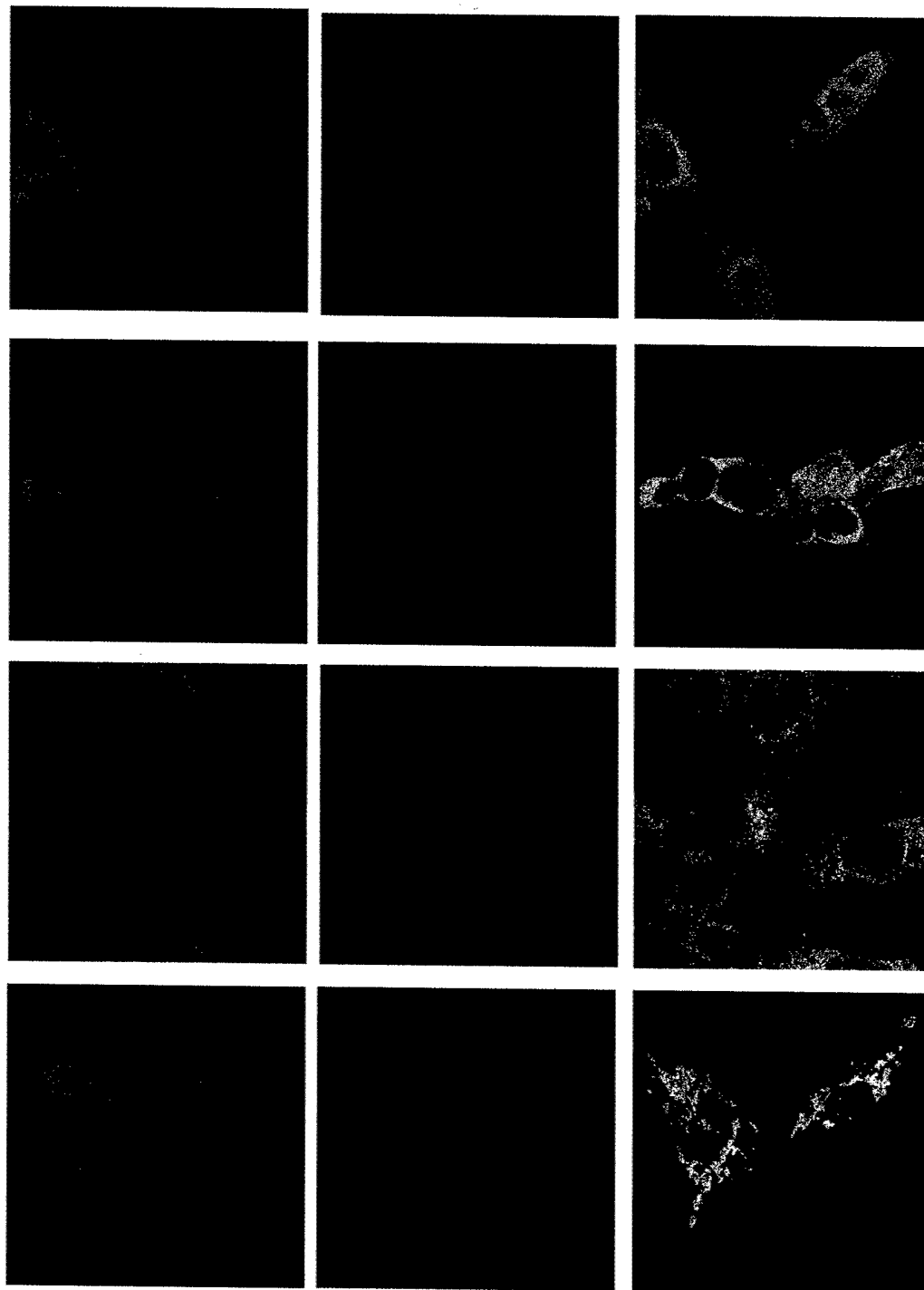












**Inhibition of the MAP Kinase Pathway Coordinately Downregulates the Metastatic
Potential of Human Prostate Cancer Cells¹**

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Running Title: Raf/ERK Signaling and Prostate Cancer

Key Words: Five

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Abstract

Components of the mitogen-activated protein kinase (MAPK) signal transduction pathway have been prominently implicated in the malignant progression of human prostate carcinoma. Activated Raf, MAPK kinase (MEK), and extracellular signal-regulated kinase-1 and -2 (ERK1/2) appear to promote cell survival, proliferation, and invasiveness in the more advanced stages of androgen-independent (AI) prostate cancer (CaP). For this reason I have examined the effects of inhibiting Raf and MEK on the growth of the highly invasive and AI CaP cell lines DU145 and PC3. An anti-Raf oligodeoxynucleotide (ODN) effectively downregulated Raf expression in both cell lines, while potently suppressing cell growth and inducing apoptosis. The MEK inhibitor PD98059 had similar but less potent effects on cell growth and survival. In the presence of bombesin, DU145 cells secreted the matrix metalloprotease MMP-9 and actively invaded Matrigel-coated transwell membranes. Either the anti-Raf ODN or PD98059 alone significantly inhibited these *in vitro* markers of a metastatic phenotype. The data presented here further implicate the Raf/MEK/ERK signaling cascade in the regulation of cellular apoptosis, MMP-9 production, and modulation of CaP metastasis.

Key Words: Raf/ERK, prostate cancer, growth, apoptosis, metastasis.

Introduction

Prostatic carcinoma often emerge from endocrine therapy as highly invasive and hormone-independent cancers that are refractory to conventional therapies and a common cause of death due to cancer among Western men. Although a number of genetic and epigenetic changes have been associated with prostate cancer (CaP), the cellular mechanisms that directly contribute to the malignant progression of localized CaP to metastases remain unknown [1]. Advanced stages of CaP are often associated with elevated levels, and the autocrine production, of multiple polypeptide growth factors that could potentially support the growth and proliferation of tumors in the absence of circulating androgens. For example, increased expression of epidermal growth factor (EGF), transforming growth factor (TGF)- β 1, heparin-binding EGF (HB-EGF), insulin-like growth factor (IGF)-1, interleukin-6, keratinocyte growth factor (KGF) and others have been associated with advanced stages of CaP [2-4]. Neuroendocrine cells may also contribute to the growth of advanced CaP by secreting elevated levels of neuropeptides that are often accompanied by aggressive malignancies and a poor prognosis [5].

Polypeptide growth factors and neuropeptides induce mitogenesis by activating well-characterized signal transduction pathways, including the mitogen-activated protein kinases (MAPKs) [6-9]. The classic MAPK cascade, which involves the kinase Raf, MAPK kinase (MEK), and ERK, has been implicated in the coordinate regulation of cell growth, differentiation, survival, death and migration [10]. Constitutively active mutants of Raf-1 and MEK1 are transforming in many cell systems and MEK hyperactivity has been observed in numerous types of cancer [11-15]. More to the point, elevated levels of activated MAP kinases are evident in high-grade and advanced stage prostate tumors,

where these signaling molecules have been linked to cell proliferation and the acquisition of androgen-independent (AI) CaP [14, 16-18].

Tumor invasion is a multistage process in which cellular motility becomes associated with the controlled proteolysis and compositional alteration of the extracellular matrix (ECM), basement membrane and interstitial stroma by tumor cells. Elevated levels of the type IV collagenases/gelatinases matrix metalloproteinase (MMP-2 and MMP-9) and plasmin-dependent ECM degradation are strongly associated with tumor invasion and the metastasis of malignant tumors [19]. MMP-9 degrades type IV collagen and MMP-9 mRNA levels are highly expressed in a variety of CaP cell lines, where there is a significant correlation between the levels of MMP-9 expression and tumor invasiveness [20-22].

A role for MAP kinase signaling cascades in the regulation of MMP-9 gene expression has recently become apparent. Indeed, these pathways connect several cell surface receptor tyrosine kinases to the nuclear MMP-9 transcriptional machinery by upregulating the levels of c-Fos and Jun-D, transcription factors required for optimal MMP-9 promoter activity [23]. Ligand-dependent regulation of MMP-9 expression is clearly related to cellular invasiveness and the capacity of multiple receptor tyrosine kinases to sustain MAP kinase activity [15, 24-26]. Bombesin-like neuropeptides are mitogens [8, 9] that highly invasive CaP cells frequently express in abundance [27, 28]. Bombesin has been reported to enhance *in vitro* CaP cell motility through tyrosine phosphorylation of focal adhesion kinase and integrin-associated proteins [29]. Moreover, this growth factor selectively increases the production of MMP-9 in invasive (PC3 and DU145) CaP cell lines [30].

In a study it was shown that, in comparison to the LNCaP cell line, the key molecular components of the Raf/MAP kinase signal transduction pathway are naturally upregulated in PC3 and DU145 cells that have progressed to the AI and invasive stages of advanced CaP [18, 31]. Coimmunoprecipitation experiments indicated that certain members of the MAPK signaling pathway, *i.e.* c-Raf-1 and ERKs, are associated with the scaffolding protein caveolin-1 and the oncoprotein protein kinase C epsilon (PKC ϵ). Moreover, I found that the Raf/MAP kinase signaling cascade could be activated by the overexpression of PKC ϵ in LNCaP cells, which in turn may lead to the upregulation of c-Myc expression, and increased expression and expulsion of caveolin-1 in CaP the LNCaP and CWR-R1 cell lines [18, 31]. In the present study, I have confirmed that constitutive signaling through the Raf/MAPK cascade is required for sustained growth, and to prevent cell death, in DU145 and PC3 CaP cells. Evidence that the Raf/MAPK pathway regulates the expression of MMP-9 in these invasive CaP cell lines will also be presented. These data support a critical role for Raf/MAPK activity in prostate tumor progression.

Materials and methods

Cell Culture. DU145 and PC3 CaP cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in MEM or F12 medium, supplemented with 10% fetal bovine serum (FBS), as instructed by the supplier. Phosphorothioate-modified oligodeoxynucleotides (ODN) were synthesized and purified by Invitrogen (Carlsbad, CA). Sequences for the anti-c-Raf (CGP69846A/ISIS5132) and mismatch control ODNs were exactly as specified [32, 33]. Each ODN was stored at -20°C in 10 mM Tris (pH8.0) containing 1 mM EDTA and brought to room temperature in 500 μ l of Opti-MEM 1 reduced serum medium containing 20 μ g/ml lipofectin

(Invitrogen, Carlsbad, CA) immediately prior to use. Subconfluent (70-80%) DU145 and PC3 CaP cells were washed with Opti-MEM 1 before introducing the ODN and lipofectin mixture at final concentrations of 200 nM and 2 µg/ml, respectively. After an incubation of 6 h at 37°C, the cells were washed twice with serum-free medium. DU145 and PC3 cells were then incubated for 18 h in lipofectin-free media containing 500 nM ODN and fresh 10% FBS. This media was then replenished and the CaP cells were incubated for an additional 48 h prior to harvesting by trypsinization.

Cell Growth Assays. DU145 or PC3 cells (5×10^4) were seeded onto tissue culture plastic in 6-well plates. After 24 h, the cell cultures were washed with serum-free medium and treated with either the specified ODN (see above) or the MEK inhibitor PD98059 (20 or 50 µM; Calbiochem, San Diego, CA). Following an incubation of 48 h, the number of viable cells was counted, in triplicate, using a hemocytometer. Cell viability was evaluated by trypan blue staining.

Preparation of conditioned medium. After reaching a confluency of 80-90%, DU145 cells were cultured in serum-free medium for 48 h. Supernatants from conditioned media (CM) were prepared by centrifugation (13,500 x g for 10 min) to remove cellular debris and soluble proteins were then precipitated with an equal volume of 100% ethanol at -20°C for 3h. For treatment with bombesin (Sigma, St. Louis, MO), cell cultures were exposed to 100 ng/ml bombesin, in the absence or presence of PD98059 (20 or 50 µM), throughout the 48 h of conditioning. Ethanol precipitates of CM were dissolved in sample buffer and analyzed by Western blotting.

Western blot analysis. Western blots were prepared as described previously [18] and probed using mouse monoclonal anti-c-Raf-1 (Transduction Laboratories, San Diego,

CA) and anti-MMP-9 (Calbiochem, San Diego, CA) and a rabbit polyclonal raised against ERK1/2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Matrigel invasion. CaP cell invasion was assayed using modified Boyden chambers containing Matrigel-coated filters (Becton-Dickinson Labware, Bedford, MA). DU145 cells were pretreated with antisense or mismatch ODNs to c-Raf-1 or PD98059, as described above. Equal numbers of viable and adherent cells (5×10^4) were then plated onto the upper chamber in 500 μ l of serum-free medium containing 0.1% bovine serum albumin (BSA). Lower chambers contained 750 μ l of a medium containing 10% fetal bovine serum (FBS) as the chemoattractant. Following an incubation of 20 h at 37°C, the filter separating the two chambers was fixed and stained with DiffQuik (Baxter, McGaw Park, IL). Cells that had migrated through the 8 μ m pores to reach the undersurface of the filter were counted under a 100 x microscope objective. Five random fields were counted to calculate the mean number of cells per field.

DNA fragmentation. Detached and adherent CaP cells were pooled and sedimented by centrifugation (3,000 x g, 5 min). Cell pellets were washed twice using ice-cold PBS and resuspended in lysis buffer (TE buffer, pH 7.5, containing 0.2% NP-40 and 5 mg/ml proteinase K). After an incubation of 20 min at 37°C, RNaseA was added to a final concentration of 5 mg/ml and incubated for an additional 30 min at 37°C. Equal amounts of DNA were electrophoresed in 1.8% agarose gels impregnated with 0.1 μ g/ml ethidium bromide for 2 h at 60 V. DNA fragmentation was visualized by ultraviolet transillumination.

Statistical analysis. Only representative data are presented and numeric data represent the mean and standard deviation of triplicate determinations from at least three

independent experiments. Where specified, treatment effects were evaluated for statistical significance ($p < 0.05$; two-sided Student's *t* test).

Results and Discussion

DU145, PC3 and LNCaP cells are model systems with distinct growth characteristics and phenotypes that may reflect the heterogeneity of CaP. DU145 and PC3 cells are AI and metastatic, whereas LNCaP cells are androgen-responsive and non-metastatic. In previous work, I have sought to understand the molecular alterations that promote CaP progression by using the LNCaP model system to carry out gain-of-function studies to identify genes that are capable of accelerating tumor growth in an androgen-deprived environment. PKC ϵ overexpression was found to transform LNCaP cells into a phenotype that was highly reminiscent of AI CaP cells and this transformation was associated with Raf and ERK hyperphosphorylation, which may be important in regulating the growth and survival of AI CaP cells [18]. In the present study, I have employed a loss-of-function strategy in which the objective was to identify signaling molecules, specifically within the Raf/ERK/MAPK pathway, that highly invasive and AI CaP cells require in order to avoid death or spontaneous reversion into more benign cancer cells.

Silencing c-Raf-1 expression in AI CaP cells. DU145 and PC3 cells express substantially increased basal levels of c-Raf-1 and sustained phosphorylation of ERK1/2 (The202/Tyr204) when compared with LNCaP cells [18], which is consistent with the suggestion that the Raf//MEK/ERK pathway may be constitutively active in AI CaP cells [35]. In the present study, an antisense ODN against c-Raf-1 (BGP 69846A/ISIS5132) was used to down-regulate the expression of c-Raf-1 in DU145 and PC3 cells. As shown

in Fig.1A, the CGP 69846A ODN inhibited the expression of endogenous c-Raf-1 effectively. The corresponding mismatch ODN had no significant effect on c-Raf-1 expression. This result demonstrated that the CGP 69846A ODN could be used to effectively down-regulate the expression of endogenous c-Raf-1 in CaP cells.

Growth inhibitory effects of anti-Raf-1 ODN and PD 98059 in AI CaP cells.

Current models of CaP progression hold that members of the classic MAPK gene family plays a dominant role in enhancing tumor growth, while the principal survival signaling pathway is mediated by phosphatidylinositol 3'-kinase (PI3K)-Akt/protein kinase B [36, 37]. However, these growth factor responsive signaling pathways may perform redundant/overlapping functions in CaP cells [38-42]. For example, c-Raf-1 expression appears to be required to suppress apoptotic signals and to maintain a positive growth advantage in most tumor cells [43-45]. However, it has been reported that the activation of an exogenous, estradiol-inducible, c-Raf-1 construct produces a paradoxical inhibition of LNCaP cell proliferation [35]. In contrast, the growth of DU145 or PC3 cells was unaffected by this exogenous c-Raf-1 construct. In the present study, we have taken advantage of the potent effects that the CGP 69846A ODN exerts on the expression of endogenous c-Raf-1 to determine whether the constitutive activation of the Raf/MAPK pathway in DU145 and PC3 cells confers a growth advantage. At a concentration of 200 nM, anti-Raf ODN significantly inhibited the growth/proliferation of these AI CaP cells (Fig.1B). After a 72 h exposure to the CGP 69846A ODN, the number of viable DU145 and PC3 cells had decreased by 30-40% of the mismatch ODN and untreated control values (Fig.1B).

ERK1/2 mediates c-Raf-1-dependent signal transduction and the expression of ERK1/2 appears to be increased in primary CaP specimens, relative to normal human prostate tissue [14]. Because ERK1/2 is a phosphoprotein substrate for the kinase MEK and was likely to mediate the growth stimulatory effects of endogenous c-Raf-1 in DU145 and PC3 cells, we analyzed the growth of these cells in the presence of a pharmacological inhibitor of MEK activation, PD 98059. Figure 1C illustrates the results of these experiments. When allowed to proliferation for 72 h in the presence of this MEK inhibitor, PD 98059 produced a dose-dependent inhibition of DU145 and PC3 cell proliferation. Under these conditions, the number of viable DU145 and PC3 cells was decreased by about 30% in the presence of 50 μ M PD 98059 (Fig.1C). These results provided additional and independent support for the notion that AI CaP cells may employ the MAPK signaling pathway to gain, and sustain, a positive growth advantage. Interestingly, while the growth inhibitory effects of PD 98059 implicate the downstream MEK/ERK in this Raf-dependent survival signaling pathway, this pharmacological inhibitor of MEK activation was consistently less efficacious than the antisense approach employed for suppressing the growth of DU145 and PC3 cells. This observation reinforces the suggestion that activated c-Raf-1 may be capable of regulating multiple downstream signaling cascades, independent of MEK/ERK.

Silencing c-Raf-1 expression induces apoptosis in AI CaP cells. DU145 and PC3 cells treated with anti-c-Raf-1 ODN often displayed a shrunken morphology and subsequent detachment from the cell culture plastic, both features characteristic of apoptosis. Lipofectin in the absence of ODN or presence of the mismatch ODN did not produce these noticeable changes in cell morphology and attachment. These observations

promoted an investigation into the effects of abrogating c-Raf-1 expression on a signature event in the late stages of apoptosis, *i.e.* DNA degradation. The results show that an antisense ODN known to decrease the expression of c-Raf-1 in AI CaP cells (Fig.1A) was also a strong inducer of cell apoptosis (Fig.2). While some DNA fragmentation could be detected in PC3 cells that had been treated with lipofectin in the absence of ODN or presence of the mismatch ODN (Fig.2, lanes 6 and 7, respectively), this was not seen in DU145 cells exposed to the same conditions (Fig.2, lanes 2 and 3). These data suggest that the basal expression of endogenous c-Raf-1 may be required to suppress spontaneous activation of apoptotic signals in cultured AI CaP cells.

Raf/ERK-mediated production of proMMP-9 and Matrigel invasion. Each step in the malignant progression of a tumor cell is decisive and if a tumor cell fails to accomplish any step in the sequence, it remains nonmetastatic. Multiple MMPs contribute to, and are required for, tumor cell invasion and metastasis. However, the type IV collagenase/gelatinases (MMP-2 and MMP-9) have been most directly implicated in the progression of CaP [46-48]. Xenografts of DU145 and PC3 CaP cells rapidly form tumors and metastases *in vivo* [19], and it has been shown that MMPs are produced that directly contribute to the degradation of the ECM and tumor invasion. Because the level of MMP-9 expression strongly correlates with the metastatic potential of AI CaP cells [49] and MMP-9 promoter activity is dependent on Raf/MAP kinase signaling cascades [15, 23, 50], it was reasoned that Raf/MAPK inhibition might suppress the invasiveness of AI CaP cells. Accordingly, experiments were conducted to test this hypothesis by measuring the effects of c-Raf-1 antisense ODN and PD 98059 on the production of proMMP-9 by DU145 cells in culture. To detect proMMP-9 in the media conditioned by

these cells, it proved necessary to supplement the media with the neuropeptide bombesin (Fig. 3A). Under these conditions, the pharmacological inhibition of MEK activity (PD 98059) diminished proMMP-9 production/secretion by DU145 cells and silencing the expression of c-Raf-1 (CGP 69846A ODN) produced substantial decreases in the level of proMMP-9 detected by Western blotting (Fig.3A). These results implied that bombesin receptors may employ a Raf/ERK-dependent pathway to augment the expression of proMMP-9 in AI CaP cells, but did not verify that elevated levels of metalloprotease activity were indeed present in the CM. To address the later issue, *in vitro* assays of Matrigel invasion were performed using the DU145 cell line and modified Boyden chambers. Unlike zymography assays of collagenase/gelatinase enzymatic activity, for cells to be scored positive in a Matrigel invasion assay both enzymatic activity and chemotaxis are required. The results illustrated in Figure 3.B show that DU145 CaP cells met both of these criteria and that treatments designed to specifically inhibit the Raf/ERK pathway (CGP 69846A ODN and PD 98059) in these cells significantly decreased the invasiveness of these AI CaP cells. Of central importance here is the finding that c-Raf-1 represents a key signaling molecule that is required to complete the assembly of a metastatic CaP cell; *i.e.* loss of growth regulation, antiapoptotic signaling, and invasion of the ECM. This analysis and the work of others suggest that the Raf/MAP kinase pathway may regulate the invasiveness of AI CaP cells by increasing the levels of total MMP-9 RNA, either through direct effects on gene transcription [23] or the regulation of nontranscriptional pathways [49].

The major finding of the present study is that AI CaP cell lines like DU145 and PC3 will undergo a spontaneous reversion to a growth inhibited, apoptotic and less

invasive phenotype when the expression of endogenous c-Raf-1 has been abrogated. Identification of c-Raf-1 as a molecular component that may be required to sustain the malignant growth and dissemination of these tumor cells provides additional support for the hypothesis that this protein kinase may be a legitimate therapeutic target in advanced stages of AI prostate cancer [43].

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Figure legends

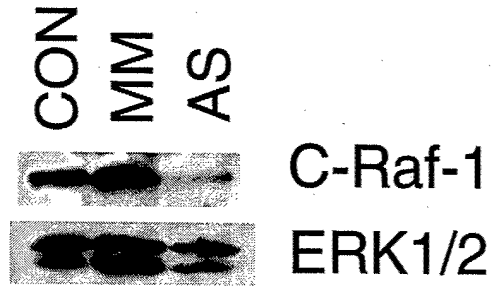
Figure 1. Effects of inhibition of Raf/ERKs signaling pathway on the growth and proliferation of CaP cells. (A) Western blot of the inhibitory effects of anti-Raf ODN on the expression of endogenous c-Raf-1. DU145 cells were treated with 200 nM antisense (AS) ODN against c-Raf-1 for 72 h. 100 μ g proteins from cell lysis were run on 10% SDS-PAGE. Cell treated with lipofectin only (CON), and mismatch (MM) ODN were used as controls. (B) Inhibitory effects of AS ODN against c-Raf-1 on the growth of DU145 and PC3 cells. Treatment with ODN was carried out as in (A). Cells were trypsinized and counted. (C) DU145 and PC3 cells were treated with MEK inhibitor PD 98059 (20 or 50 μ M, respectively) for 48 h. DMSO was used as control. Cells were trypsinized and counted.

Figure 2. DNA fragmentation analysis of apoptosis in CaP cells. DU145 and PC3 cells were treated with AS ODN against c-Raf-1 in comparison to lipofectin and MM ODN treated cells. Both detached and attached cells were harvested, their DNA were isolated and analyzed by 1.8% agarose gel electrophoresis. Treatment with ODN was carried out as in Figure 1 (A). Lanes 1 and 5, 1 kb DNA ladder (Invitrogen); Lane 2, lipofectin-treated DU145; Lane 3, MM-ODN-treated DU145; Lane 4, AS-ODN-treated DU145; Lane 6, lipofectin-treated PC3; Lane 7, MM-ODN-treated PC3; Lane 8, AS-ODN-treated PC3.

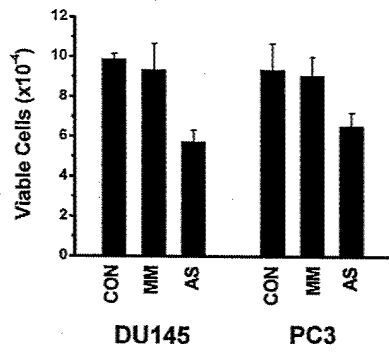
Figure 3. Effects of inhibition of Raf/ERKs signaling pathway on the bombesin-induced MMP-9 secretion and invasiveness of DU145 cells. (A) DU145 cells were treated with ODN and PD 98059 as in Figure 1 (A) and (C). Conditioned media were collected and run on 10% SDS-PAGE. Antibody to MMP-9 was employed in Western

blotting. The complete transfer of proteins was confirmed by staining the SDS-PAGE gel with the Coomassie blue. (B) DU145 cells were treated with ODN and PD98059 as in Figure 1 (A) and (C). Cells were trypsinized and counted; 5×10^4 cells were seeded into the upper chamber of inserts containing Matrigel-coated filters. Cells were allowed to migrate for 20 h, and invasive cells were stained and counted under microscope at a 100 x magnification. Five random fields were recorded.

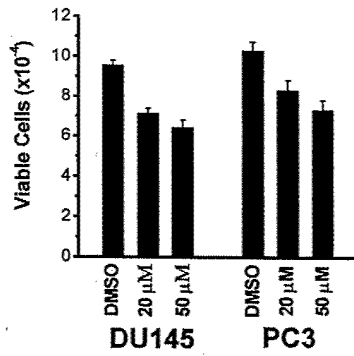
A



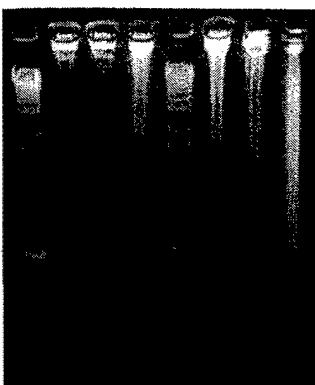
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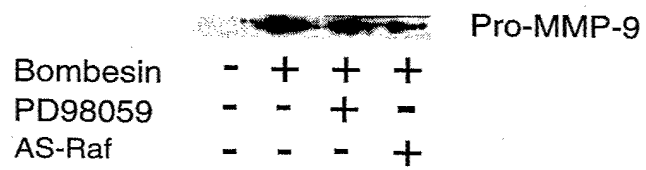
C



12345678



A



B

