Protein Kinase Cε Has the Potential to Advance the Recurrence of Human Prostate Cancer

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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 Cε (PKCe), an oncogenic protein capable of promoting autocrine cell-signaling events. Gene transfer experiments demonstrated that PKCe 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 S-cap-dependent mRNAs, including the G1 cyclins, c-myc, and caveolin-1. Immunoprecipitation experiments indicated that PKCe 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 PKCe, 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 antisera. Finally, antisense experiments established that endogenous PKCe 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 PKCe 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 with orchietomy. 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.

PKCe 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 PKCe 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 PKCα and PKCδ 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 isoforms in various cell types (5) and the oncogenic activity of PKCe, we hypothesized that this isozyme may oppose the proapoptotic influence of PKCα and PKCδ 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 PKCe expression and androgen independence. To better understand whether the expression of PKCe could be of functional importance in CaP progression, we stably transfected LNCaP cells with a retroviral vector containing PKCe cDNA. This analysis revealed that PKCe 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 PKCe ODNs were used to specifically block the expression of endogenous PKCe 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
provides data from gene transfer and antisense experiments demonstrating that PKCe 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. 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 (LNCaPφ) or pLXSN harboring the gene for p3/PKCα (LNCaPα) as described previously (20). Stably expressing cells were selected and subcloned by limiting dilution in 500 μg/ml G418, and resultant subclones were then screened for PKCe 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 × 10⁶ cells suspended in 250 μl 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 × width × depth × 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 N20), cyclin D1 (C-20), cyclin E (M-20), ERK1 (K-23), c-Myc (C-19), PKCα (C-15), PKCε (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 (122D4), 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 PKCe and β-actin mRNAs. The PKCe sense and antisense primers were 5’-AGC CGG CTT CTT GAA ACT CCC-3’ and 5’-AGC TGC TCT GTA 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 preamplification in 1 cycle of 50°C for 30 min and 94°C for 2 min followed by 40 cycles of amplification at 94°C, 58°C, 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 (PKCe) 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 μg/ml). The DNA content of 1 × 10⁶ PI-stained cells was analyzed by flow cytometry (FACScan) at 488 nm excitation gated to exclude debris. Scattered light was excluded from the PI signal using the FL2 bandpass 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 × 10⁶ 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 anokis 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 anokis. After centrifugation, cell pellets were washed with PBS, resedimented, and suspended in 10 μl of PBS containing a 2-μl aliquot of a dye solution containing 100 μg/ml acridine orange (Sigma Chemical Co.) and 100 μg/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 [3H]Leucine (153 Ci/mmol; Amer sham) 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 μCi/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 μl 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 μg/ml cycloheximide (data not shown).

Comun precipitation 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 EDTA, 1.5 mm MgCl₂, 0.4 mm phenylmethylsulfonyl fluoride, 2 μM peptatin, 0.1 mg/ml aprotinin, and 1 mg/ml leupeptin. Cellular debris was sedimented by centrifugation (10 min at 12,500 × g), and the resulting supernatants were precleared by adding 60-μl aliquots of protein argarose 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 μg, control), anti-PKCa, or a GST-anti-Raf-1 agarose conjugate (Upstate Biotechnology, Lake Placid, NY) and mixed overnight by rotation at 4°C. Protein argarose A (75 μl) was added and incubated for 1 h at 4°C before centrifugation (5 min at 12,500 × g). Supernatants were discarded, and immunoprecipitates were washed three times using excess lysis buffer before solubilizing the final pellets in 60 μl of a standard SDS-PAGE sample buffer. Masking of the ERK 1/2 bands (M₁, 42,000–44,000) by the monomeric IgG heavy-chains (M₂, ~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 (M₃, ~100,000) under this condition.

Antisense PKCe ODN. Phosphorothioate ODNs were obtained from Invitrogen. Sequences for the antisense PKCe 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 μM) and lipofectin (2 μg/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 test. Errors are SEs of averaged results, and values of \( P < 0.05 \) were taken as a significant difference between means.

**RESULTS AND DISCUSSION**

**PKCe 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 PKCe expression compared with control benign tissues (10). To determine whether signals transduced through PKCe had the potential to contribute to the AI progression of CaP, LNCaP cells overexpressing PKCe were established using the pLXSN retroviral vector. The pooled population of PKCe overexpressing LNCaP cells (LNCaP/e3) were selected for by their collective resistance to G418, and a representative subclone (LNCaP/e3) was isolated from this pool of transfectants by limiting dilution and maintained in culture. The pLXSN vector control line was called LNCaP/v, LNCaP/e1 and LNCaP/e3 cells expressed equivalent levels of PKCe (Fig. 2C) and, compared with parental and vector controls, the catalytic activity of PKCe was increased, in the absence of an exogenous PKC activator, by 4.3- and 5.8-fold in adherent LNCaP/e and LNCaP/e3 sublines, respectively (not shown). LNCaP cells are PTEN+/− (22) and, because PTEN plays a dominant role in suppressing PKCe activity (23), this may account for the increased basal activity of PKCe in the LNCaP/e and LNCaP/e3 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 PKCe 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 PKCe (LNCaP/e3) 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 PKCe in LNCaP cells could be associated with androgen hypersensitivity. These results prompted investigation into the effects of PKCe overexpression on the tumorigenicity of LNCaP cells in the absence and presence of testicular androgens.

**Myxoma-free LNCaP/e3 cells** (1 \( \times 10^6 \)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/e 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 PKCe 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 PKCe has the potential to advance the progression of CaP and may be sufficient to initiate recurrent tumor growth in the absence of testicular androgens.

**PKCe Accelerates Transit through the G1 Restriction Point.** FACS analysis of exponentially multiplying LNCaP/e3 cells revealed that PKCe 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/e and LNCaP/e3 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 PKCe accelerated G1 to S transit of LNCaP cells.

**PKCe 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 proteins (28); androgen independence may be achieved through signals that disrupt the function of pocket proteins such as RB, p130, and RB. Collectively, the data in Fig. 2 provide strong support for this hypothesis. Raf-1 is an imminent, if not direct, target of PKCe (29) that is capable of inactivating RB (30). Immunoprecipitation experiments showed that PKCe remained constitutively associated with phospho-Raf-1 and ERK 1/2 in both quiescent LNCaP cells and the AI-LNCaP/e3 cells that continued to proliferate after 3 days in CDT (Fig. 2B, upper panel). The association of PKCe 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/e lysates. The lower panel in Fig. 2B shows that Raf-1 immunoprecipitates from these two cell lines contained equivalent amounts of PKCe protein. In contrast to LNCaP cells, however, PKCe immunoprecipitates from LNCaP/e cells pulled down caveolin-1 (Fig. 2A, lower panel).


It has been reported that PKCe 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 PKCe and PKCζ (15). These observations suggested that the caveolin-based PKCe:Raf-1:ERK 1/2 complex may not be actively signaling in LNCaP/e 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/e and LNCaP/e3 cells after 3 days in CDT, in comparison to either the parental or vector controls (Fig. 2C). These experiments implied that PKCe was able to colocalize with caveolin-1 and Raf-1 while indirectly promoting ERK phosphorylation (activation). The fact that PKCe 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 PKCe versus subdomain IX of the PKCα catalytic domain, see Ref. 15). On the basis of these and other studies, we hypothesized that PKCe 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 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 PKCe was sufficient to disrupt this inherent cellular response. Compared with the parental and vector controls, both LNCaP/e and LNCaP/e3 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 (p21wafl/cip1 and upstream binding factor-1) or showed no consistent changes (p27kip1 and TATA binding protein-1).3 PKCe 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 PKCe as an active regulator of the ERK to RB signaling pathway in LNCaP cells.

**PKCe 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/e and LNCaP/e3 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/e3 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). PKCe 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).

**PKCe Renders LNCaP Cells Resistant to the Apoptotic Effects of PMA.** PMA induces a massive apoptotic 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/e or LNCaP/e3 cells were treated with equimolar concentrations of PMA. In LNCaP cells, the apoptosis induced by PMA was preceded by the stimulation

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3 W. Wu and D. M. Terrian, unpublished data.
of caspase 3 proteolytic activity, whereas no change in the activity of caspase 3 was detected in LNCaP 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 expressed 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 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 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.

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\(\alpha\), Raf-1, phospho-ERK 1/2, and caveolin-1 (Fig. 5A). In DU145 and PC3 cells, the increased level of PKC\(\alpha\) protein was associated with an increase in the amount of a RT-PCR product encoding PKC\(\alpha\) (Fig. 5A), implying that the PKC\(\alpha\) 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\(\alpha\)-positive cells in vivo, possibly via a selection process favoring the survival of these cells.

**Antiproliferative Effects of Antisense PKC\(\alpha\) ODNs in AI CaP Cells.** To investigate the role of endogenous PKC\(\alpha\) in the growth of AI CaP cells, an antisense PKC\(\alpha\) ODN was used for selective down-regulation of expression of this isozyme in DU145 (PTEN\(^{–/-}\)) and PC3 (PTEN\(^{–/-}\)) CaP cells. DU145 and PC3 cells were preincubated with either the scrambled or antisense PKC\(\alpha\) ODN (1 \(\mu\)M) for 3 days, and lysates were analyzed by immunoblotting for changes in the steady-state concentration of PKC\(\alpha\), PKC\(\epsilon\), phospho-Raf-1, phospho-ERK 1/2, caveolin-1, and ERK 1/2. Translation of PKC\(\epsilon\) mRNA was selectively and effectively down-regulated by the antisense PKC\(\alpha\) ODN, whereas the steady-state concentrations of PKC\(\alpha\) and ERK 1/2 remained unaltered (Fig. 5B). Under identical conditions, neither lipofectin alone nor lipofectin plus the scrambled PKC\(\alpha\) ODN inhibited PKC\(\alpha\), PKC\(\epsilon\), caveolin-1, or ERK 1/2 synthesis in these two cell lines (Fig. 5B). The decreased expression of PKC\(\alpha\) induced by the antisense PKC\(\alpha\) 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 \(\mu\)M antisense PKC\(\alpha\) ODN inhibited the growth of DU145 and PC3 cells in complete medium by \(\sim\)45%, relative to the scrambled PKC\(\alpha\) 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 \(\mu\)M antisense PKC\(\alpha\) 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\(\alpha\) 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\(\alpha\) 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\(\alpha\) 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\(\alpha\) overexpression transforms LNCaP cells into AI tumor cells that recapitulate many hallmark features of recurrent CaP. The overexpression of PKC\(\alpha\) 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 \(m^2\) 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\(\alpha\), Ras/Raf induction alone is insufficient to promote the AI proliferation of LNCaP cells (34, 35). Therefore, PKC\(\alpha\) 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\(\alpha\) and PKC\(\beta\)) but not oncogenic (PKC\(\alpha\)) isozymes of PKC (14, 15). It is of note that multiple oncoproteins, and PKC

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