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14. ABSTRACT

The objectives of this proposal are to determine whether protein kinase C epsilon (PKCε) is linked to the initiation and progression of Prostate cancer (PCa) and should be explored as a molecular target for the prevention of human PCa. PKCε, a calcium-insensitive PKC, is among the PKC isoforms expressed in both mouse and human prostate tissue. We plan to test the hypothesis that PKCε is linked to the onset, progression and metastasis PCa. Two specific aims are proposed to test this hypothesis. Specific Aim #1: To obtain the first molecular genetic evidence that PKCε is linked to the development of PCa. To accomplish this specific aim, we will employ TRAMP mice, the well established mouse model of PCa. We will deplete PKCε in TRAMP mice by crossbreeding TRAMP mice with PKCε knockout (-/-) mice. We will evaluate TRAMP-PKCε KO mice for the development and progression of PCa *in vivo*. We will determine whether the genetic loss of one (-/+) or both (-/-) PKCε alleles will attenuate the progression of PCa. Specific Aim #2: To explore the mechanisms by which PKCε may promote the progression of AI PCa. This report will review the accomplishments made over the first year of grant award with respect to these specific objectives and according to the time line proposed in the original statement of work of the project.

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FIRST ANNUAL REPORT FOR AWARD NUMBER " W81XWH-07-1-0049"

ENTITLED MOLECULAR TARGETS FOR PREVENTION OF PROSTAT CANCER.

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INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer-related deaths in men. The risk of PCa increases rapidly after age 50 in men, with two-thirds of all PCa cases found in men after age 50. PCa first manifests as an androgen-dependent (AD) disease and can be treated with androgen-deprivation therapy. Despite the initial success of androgen ablation therapy, PCa progresses from AD to androgen-independent (AI). The hormone refractory, invasive PCa is the end stage and accounts for the majority of PCa patient deaths. Defining the molecular mechanisms linked to the transition of AD PCa to a hormone refractory PCa is essential in planning strategies in the prevention and treatment of PCa.. The objectives of this proposal are to determine whether protein kinase C epsilon (PKCε) is linked to the initiation and progression of Prostate cancer (PCa) and should be explored as a molecular target for the prevention of human PCa. PKC represents a family of Phospholipid-dependent, serine/threonine protein kinases. PKCε is a calcium-insensitive PKC. Previous studies have shown, using cultured prostate cancer-derived cell lines and human PCa specimens that PKCε may play a role in the progression to AI PCa. However, the role PKCε plays in the course of PCa progression on the whole tissue level *in vivo* is unknown and that forms the focus of this proposal. We plan to test the hypothesis that PKCε is linked to the onset, progression and metastasis PCa. Two specific aims are proposed to test this hypothesis: Specific Aim #1: To obtain the first molecular genetic evidence that PKCε is linked to the development of PCa. To accomplish this specific aim, we will employ TRAMP mice. Specific Aim #2: To explore the mechanisms by which PKCε may promote the progression of AI PCa. PKCε may be a new marker for the prognosis of PCa, as well as a molecular target for the prevention and therapy of PCa. Knowledge obtained from the proposed study will help to plan strategies to manage the development of PCa. This report will review the accomplishments made over the first year of grant award with respect to these specific objectives and according to the time line proposed in the original statement of work of the project.

BODY (Key Research Accomplishments by original statement of work)

***Task 1*: Specific Aim #1: To obtain the first molecular genetic evidence that PKCε is linked to the development of PCa**. **Anticipated time to accomplish: 18-28 months**

The principle experimental approach to link PKCε to the development of PCa is to deplete PKCε in TRAMP mice. This will be accomplished by crossbreeding TRAMP mice with PKCε knockout (-/-) mice. We will evaluate TRAMP-PKCε KO mice for the development and progression of PCa *in vivo.* We will determine whether genetic loss of one (-/+) or both (-/-) PKC alleles will attenuate the progression of PCa. Our PKCε knockout (-/-) mice are on FVB background while TRAMP mice are on C57BL/6 background. Breeding of PKCε knockout (-/-) with TRAMP mice is still in progress to generate sufficient TRAMP-PKCε KO mice for the proposed experiments as illustrated below.

Experiment 1: **Effects of PKCε deletion on the development of PCa in TRAMP mice.** All mice will be randomly assigned to the indicated cohort and sacrificed at 4, 8, 12, 16, 20, 24 and 32 weeks of age. There will be 20 mice per experimental group. Since it is difficult to generate sufficient numbers of male mice for all the time points, we have divided this experiment in three separate parts.

Experiment 1A: The link of PKCε to the progression to AI PCa. In this experiment, there will be 40 male mice in each genotype (TRAMP+, PKCε +/+); (TRAMP+, PKCε -/+); and (TRAMP+, PKCε -/-). Both TRAMP and PKCε KO will be on C57BL/6 background. At 12 weeks of age, 20 mice of each genotype will be castrated. For castration, mice will be anesthetized with sodium pentobarbital (65 mg/kg, administered intraperineally) and an incision will be made across the lower abdomen to allow access to the testes. The ductus deferens will then be cauterized and the testes removed. The incision will be closed by staples, which will then be removed two weeks post operation. At 32 weeks of age, all mice will be sacrificed.

Cohorts	Sacrificed at: 32 weeks	
	Intact	Castrated
Experiment with (C57BL/6 TRAMP x C57BL/6 PKCε KO)		
TRAMP/PKCε (+/+)	20	20
TRAMP/PKCε (+/-)	20	20
TRAMP/PKCε (-/-)	20	20

Experiment 1B: The link of PKCε to the initiation of PCa. In this experiment, 20 male mice of each genotype will be sacrificed at 4, 8 and 12 weeks of age to determine whether PKCε deletion prevents the development of early lesion (PIN) in TRAMP mice.

Cohorts	All intact, sacrificed at:		
	4 weeks	8weeks	12 weeks
Experiment with (C57BL/6 TRAMP x C57BL/6 PKCε KO)			
TRAMP/PKCε (+/+)	20	20	20
TRAMP/PKCε (+/-)	20	20	20
TRAMP/PKCε (-/-)	20	20	20

Experiment 1C: The link of PKCε to the development of PCa at the post-initiation phase of prostate carcinogenesis. In this experiment, mice of each genotype will be sacrificed at 16, 20, and 24 weeks of age.

Cohorts	All intact, sacrificed at:		
	16 weeks	20weeks	24 weeks
Experiment with (C57BL/6 TRAMP x C57BL/6 PKCε KO)			
TRAMP/PKCε (+/+)	20	20	20
TRAMP/PKCε (+/-)	20	20	20
TRAMP/PKCε (-/-)	20	20	20

Experiment 2: To determine the effects of PKCε deletion on the progression of PCa in TRAMP mice on (C57BL/6 TRAMP x FVB/N F1) background. In this experiment, we will have 40 male mice in each genotype (TRAMP+, PKCε +/+); (TRAMP+, PKCε -/+); and (TRAMP+, PKCε -/-). At 12 weeks of age, 20 mice of each genotype will be castrated. At 28 weeks of age, all mice will be sacrificed.

Cohorts	Sacrificed at: 28 weeks	
	Intact	Castrated
Experiment with (C57BL/6 TRAMP x FVB PKCε KO)		
TRAMP/PKCε (+/+)	20	20
TRAMP/PKCε (+/-)	20	20
TRAMP/PKCε (-/-)	20	20

Task 2: **Specific Aim #2: To explore the mechanisms by which PKCε may promote the progression of AI PCa. Anticipated time to accomplish: 24-36 months**

During first year of the DOD grant award period we characterized changes in the level of expression of PKCε and PKCε associated signaling component Stat3 in PCa tissue derived from both human and TRAMP mice. These results are published in CANCER RESEARCH (Aziz, Moammir H., Manoharan, Herbert T., Church, Dawn R., Dreckschmidt ,Nancy E., Zhong, Weixiong, Oberley, Terry D., Wilding, George, and Verma, Ajit K. Protein kinase Cε interacts with Stat3, phosphorylates Stat3Ser727 and regulates its constitutive activation in prostate cancer. **Cancer Res. 67: 8828-8838, 2007) (23).** Our major findings are summarized below:
Both PKCε and constitutively activated Stat3 are overexpressed in PCa and their expression levels correlate with PCa aggressiveness. PKCε overexpression in PCa accompanied: 1) increased in the levels of IL-6 and phosphorylated Jak-1 and Jak-2, 2) increased phosphorylation of PI3K and AKT, 3) decreased expression of cyclin-dependent protein kinase inhibitors (p21 and p27) and 4) upregulation of anti-apoptotic (Bcl-2, Bcl-xL, survivin) and proliferative (COX-2) markers. PKCε interacts with Stat3 and phosphorylates Stat3Ser727. PKCε-mediated Stat3Ser727 phosphorylation correlated with Stat3 DNA-binding and transcriptional activities as well as PCa progression. These results indicate that PKCε activation is essential for constitutive activation of Stat3 and PCa progression **(see Cancer Research paper in the appendix).**

KEY RESEARCH ACCOMPLISHMENT

PKCε and constitutively activated Stat3 play roles in the transition of AD PCa to AI PCa. Novel findings are our observation that:

1. PKCε associates with Stat3 and regulates Stat3 activation.
2. Stat3 is activated by phosphorylation at both tyrosine705 and serine727 residues. PKCε activation transduces multiple signals involving inhibition of apoptotic pathways and promotion of cell survival pathways. PKCε-mediated cell survival pathway involves constitutive activation of Stat3.
3. PKCε is an initial signal that regulates activation of Stat3.
4. PKCε and Stat3, the proteins with oncogenic traits, should be explored as potential targets for prevention of AI PCa.

PLANS: We will continue our experiments proposed under specific aims in the grant proposal. We anticipate no change in our original plans.

REPORTABLE OUTCOMES

A publication in CANCER RESEARCH (Aziz, Moammir H., Manoharan, Herbert T., Church, Dawn R., Dreckschmidt ,Nancy E., Zhong, Weixiong, Oberley, Terry D., Wilding, George, and Verma, Ajit K. Protein kinase Cε interacts with Stat3, phosphorylates Stat3Ser727 and regulates its constitutive activation in prostate cancer. (**Cancer Res. 67: 8828-8838, 2007).**

Patents and licenses – NONE

Degrees obtained – NONE

Development of cell lines, tissue or serum repositories – NONE

Informatics – NONE

Funding applied for based on work supported by this award : NONE

Employment or research opportunities applied for – NONE

CONCLUSIONS

Prostate cancer is the most common type of cancer in American men and ranks second to lung cancer in cancer-related deaths. While 1 in 6 men will get prostate cancer during his lifetime, 1 in 34 will die of this disease. The American Cancer Society estimates that there will be about 232,090 new cases of PCa in the US in 2006. About 30,350 men will die of this disease. Prostate epithelial cells are dependent on the male hormone androgen for survival and enter programmed cell death following hormone oblation resulting in involution of the prostate gland. Early PCa is typically diagnosed as androgen-dependent and is treated with anti-androgen drugs or using a procedure termed castration, which involves removal of the androgen producing testes. Despite androgen therapy, some of the cancer cells still survive and grow to form PCa. The PCa that grows after hormone therapy is called androgen independent (AI) PCa. This invasive PCa is the end stage and accounts for the majority of PCa patient deaths. The management of locally advanced prostate cancer is difficult and complex because the cancer often becomes hormone-insensitive and unresponsive to current chemotherapeutic agents. Knowledge about the regulatory molecules involved in the transformation to AI prostate cancer is essential for the rational design of agents to prevent and treat prostate cancer. Recently we found a protein termed protein kinase C epsilon (PKCε), which may play a role in the formation of advanced prostate cancer. The level of this protein is increased in prostate cancer tissue as compared to the normal prostate. The proposed study is aimed at validating the role of this protein in the progression of prostate cancer. Knowledge obtained from the proposed study will help to plan strategies to manage the development of PCa. This PKCε protein may be a new marker for the prognosis of PCa, as well as a molecular target for the prevention and therapy of PCa.

REFERENCES: None

APPENDICES:

1. Cancer Research paper

Protein Kinase Cε Interacts with Signal Transducers and Activators of Transcription 3 (Stat3), Phosphorylates Stat3Ser727, and Regulates Its Constitutive Activation in Prostate Cancer

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Abstract

Prostate cancer is the most common type of cancer in men and ranks second only to lung cancer in cancer-related deaths. The management of locally advanced prostate cancer is difficult because the cancer often becomes hormone insensitive and unresponsive to current chemotherapeutic agents. Knowledge about the regulatory molecules involved in the transformation to androgen-independent prostate cancer is essential for the rational design of agents to prevent and treat prostate cancer. Protein kinase Cε (PKCε), a member of the novel PKC subfamily, is linked to the development of androgen-independent prostate cancer. PKCε expression levels, as determined by immunohistochemistry of human prostate cancer tissue microarrays, correlated with the aggressiveness of prostate cancer. The mechanism by which PKCε mediates progression to prostate cancer remains elusive. We present here for the first time that signal transducers and activators of transcription 3 (Stat3), which is constitutively activated in a wide variety of human cancers, including prostate cancer, interacts with PKCε. The interaction of PKCε with Stat3 was observed in human prostate cancer, human prostate cancer cell lines (LNCaP, DU145, PC3, and CW22rv1), and prostate cancer that developed in transgenic adenocarcinoma of mouse prostate mice. In reciprocal immunoprecipitation/blotting experiments, prostatic Stat3 coimmunoprecipitated with PKCε. Localization of PKCε with Stat3 was confirmed by double immunofluorescence staining. The interaction of PKCε with Stat3 was PKCε isoform specific. Inhibition of PKCε protein expression in DU145 cells using specific PKCε small interfering RNA (*a*) inhibited Stat3Ser727 phosphorylation, (*b*) decreased both Stat3 DNA-binding and transcriptional activity, and (*c*) decreased DU145 cell invasion. These results indicate that PKCε activation is essential for constitutive activation of Stat3 and prostate cancer progression. [Cancer Res 2007;67(18):8828–38]

Introduction

Prostate cancer is the second leading cause of cancer-related deaths in men (1). The risk of prostate cancer increases rapidly after age 50, with two thirds of all prostate cancer cases found in men after age 50. Prostate cancer first manifests as an androgen-dependent disease and can be treated with androgen deprivation therapy. Despite the initial success of androgen ablation therapy, prostate cancer progresses from androgen dependent to androgen independent. The hormone-refractory invasive prostate cancer is the end stage and accounts for the majority of prostate cancer patient deaths (2–4). Defining the molecular mechanisms linked to the transition of androgen-dependent prostate cancer to an androgen-independent prostate cancer is essential for planning strategies in the prevention and treatment of prostate cancer. Both protein kinase Cε (PKCε) and signal transducers and activators of transcription 3 (Stat3) have been shown to play roles in the development of androgen-independent prostate cancer (5, 6).

Stats comprise a family of six [Stat1 (α and β isoforms), Stat2 and Stat3 (α and β isoforms), Stat4, Stat5 (α and β isoforms), and Stat6] latent transcription factors, which reside in the cytoplasm and are encoded by seven distinct genes (7). Stat activation is linked to cell proliferation, differentiation, apoptosis, embryogenesis, and immune responses (8–11). Stats exhibit functional divergence in their roles in oncogenesis. Stat3 and Stat5 promote cell survival, whereas Stat1 has been associated with growth-inhibitory effects (12, 13). Constitutively activated Stats, particularly Stat3, are found in several human cancers [e.g., head and neck squamous cell carcinoma (SCC), breast, ovary, prostate, and lung; refs. 14–18]. Because naturally occurring mutations of Stat3 have not been observed, constitutive activation of Stat3 seems to be mediated by aberrant growth factor signaling (7, 8). Tyrosine phosphorylation of Stat3 (Tyr^{705}) is mediated by a wide variety of polypeptides and is essential for Stat3 dimerization and nuclear translocation. Stat3 also has a conserved Ser^{727} residue, which is a target for phosphorylation (19). Evidence indicates that cooperation of both tyrosine and serine phosphorylations is necessary for full activation of Stat3 (20). The identity of the protein kinase responsible for Stat3Ser727 phosphorylation in prostate cancer is unknown.

PKC is a major intracellular receptor for the mouse skin tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA; ref. 21). PKC represents a large family of phosphatidylserine (PS)-dependent serine/threonine kinases (22–25). Based on structural similarities and cofactor dependency, 11 PKC isoforms have been classified into three subfamilies: classic (cPKC), novel (nPKC), and atypical (aPKC) isoforms. The cPKCs (α, βI, βII, and γ) are dependent on PS, diacylglycerol (DAG), and Ca^{2+}. The nPKCs (δ, ε, η, and *u*) retain responsiveness to DAG and PS but do not require Ca^{2+} for full activation. The aPKCs (λ and ς) only require PS for their activation (22). PKC isoforms exhibit functional specificity in their signals to oncogenesis (22). PKCε participates in the regulation of diverse cellular functions, including gene expression, neoplastic transformation, cell adhesion, mitogenicity, and cellular motility.

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Overexpression of PKCε in rodent fibroblasts led to increase in growth rates, anchorage independence, and tumor formation in nude mice. Additionally, PKCε overexpression transformed nontumorigenic rat colonic epithelial cells (reviewed in ref. 22). PKCε is a transforming oncogene (26) and a predictive biomarker of various human cancers (27), including prostate cancer (5). We found PKCε is linked to the development of SCC elicited either by the 7,12-dimethylbenz(a)anthracene-TPA protocol (28–30) or by repeated UV radiation (UVR) exposures (31, 32). PKCε is also overexpressed in human prostate cancer (33). Overexpression of PKCε transformed androgen-dependent LNCaP tumor cells to androgen-independent cells (5). The transformation of androgen-dependent LNCaP cells to an androgen-independent variant was associated with increased cell proliferation and resistance to apoptosis (34).

We and others have shown, using both *in vivo* experimental animal models and human cancer–derived cell lines, that PKCε-mediated oncogenic activity is linked to its ability to promote cell survival (16, 26, 34). However, the mechanisms by which PKCε signals cell survival remain elusive. We present here for the first time in prostate cancer that Stat3, which is constitutively activated in a wide variety of human cancers, is a protein partner of PKCε. PKCε interacts with Stat3, phosphorylates Stat3Ser727, and increases both DNA-binding and transcriptional activity of Stat3. PKCε-mediated Stat3Ser727 phosphorylation seems to be essential for constitutive activation of Stat3 and prostate cancer cell invasion.

Materials and Methods

Chemicals, antibodies, and assay kits. The antibodies and sources of the antibodies used in this study were as follows: PKCε, Stat3, phosphorylated Stat3Tyr705 (pStat3Tyr705), phosphatidylinositol 3-kinase (PI3K; p85), PI3K (p110), p21, p27, Bcl-2, Bcl-xL, survivin, cyclooxygenase-2 (COX-2), β-actin, donkey anti-goat immunoglobulin (IgG)-FITC for PKCε, and donkey anti-rabbit IgG-rhodamine for Stat3 (Santa Cruz Biotechnology); phosphorylated AKT (pAKT; Ser^{473}), pAKT (Thr^{308}), AKT, phosphorylated Janus-activated kinase (pJAK) 1 ($Tyr^{1022/1023}$), and pJAK2 ($Tyr^{1007/1008}$; Cell Signaling Technology); and phosphorylated Stat3Ser727 (pStat3Ser727; BD Biosciences). Blocking peptides for PKCε and Stat3 were obtained from Santa Cruz Biotechnology. Immunocomplex PKC assay kit was from Biotrak assay system (Amersham Biosciences). Double-stranded Stat3 consensus DNA-binding motif 5′-GATCCTTCTGGGAATTCCTAGATC was obtained from Santa Cruz Biotechnology. PKCε small interfering RNA (siRNA) and siRNA transfection reagents were purchased from Dharmacon, Inc. Dual-Glo luciferase reporter assay kit was purchased from Promega Corp. Cell Invasion Assay kit was purchased from Chemicon International. Plasmid pLucTKS3 was obtained from Dr. James Turkson (University of Central Florida, Orlando, FL).

Human specimens. Prostate cancer specimens were obtained at radical prostatectomy. The University of Wisconsin Human Subjects Committee approved the use of these human prostate specimens. Tissue microarrays were obtained from Folio Biosciences.[4] Tissue microarrays were produced by relocating tissue from conventional histologic paraffin blocks (Folio Biosciences). This was done using a needle to biopsy a standard histology section and placing the core into an array on a recipient paraffin block. Optimal sectioning of arrays was obtained with ~5-μm sections. Using this technology, each tissue is treated in an identical manner to avoid slide-to-slide variations in the conventional sections. The tissue microarrays allowed the entire cohort to be analyzed in one batch on a single slide. Specificities of the tissue microarrays were as follows: fixative, formalin; core size, 1.5 mm; section thickness, 5 μm; control quality, pathology of every 10th slide confirmed by board-certified pathologist associated with the company; >95% tissue core retention; and validation, validated for immunohistochemistry.

Animals. Transgenic adenocarcinoma of mouse prostate (TRAMP) mice were obtained both from Dr. Norman Greenberg (Fred Hutchinson Cancer Research Center, Seattle, WA; ref. 35) and from The Jackson Laboratory and screened for the presence of the SV40 large T antigen gene by PCR as detailed on The Jackson Laboratory Web site.[5] FVB/N mice were obtained from Harlan Sprague Dawley. The mice used in our experiments were either C57BL/6 or [C57BL/6XFVB] F1. Animal care and handling was conducted in accordance with established human guidelines and protocol approved by the University of Wisconsin, School of Medicine and Public Health Animal Care Committee.

Cell lines. Human prostate cell lines LNCaP, DU145, PC3, CW22rv1, and RWPE-1 were obtained from the American Type Culture Collection.

Histology. Prostate specimens were fixed for 1 h in 10% neutral buffered formalin, transferred to PBS (pH 7.4), and then embedded in paraffin. Sections (4 μm thick) of each specimen were cut for immunohistochemical study.

Western blot analysis. Indicated prostate specimens were homogenized in immunoprecipitation lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 200 mmol/L Na_3VO_4, 200 mmol/L NaF, 1 mmol/L EGTA]. The homogenate was centrifuged at 14,000 × *g* for 30 min at 4°C. Whole-cell lysate (25 μg) was fractionated on 10% or 15% Tris-glycine SDS-polyacrylamide gels for Western blot analysis as described before (16).

Determination of PKCε and Stat3 localization by immunofluorescence staining. Paraffin-fixed prostate samples and prostate cancer cell line DU145 were used to determine the localization of PKCε and Stat3. Tissue sections (5 μm thick) of prostate samples were cut to determine the localization of PKCε and Stat3 as described before (16).

PKCε immunocomplex kinase assay. Prostate tissue was placed in 0.5 mL of immunoprecipitation lysis buffer, homogenized using a glass Teflon tissue homogenizer, agitated for 30 min at 4°C, and centrifuged at 14,000 rpm in a microcentrifuge for 15 min at 4°C. The clear supernatant was used for immunoprecipitation using polyclonal antibody to PKCε. PKCε immunoprecipitate (25 μL) was assayed for kinase activity as described before (28).

PKCε siRNA transfection. Eighty percent confluent prostate cancer DU145 cells were starved by incubation for 18 to 24 h before assay in the serum-free medium. The transfection was done as per the manufacturer's instructions (Dharmacon). A set of four pooled nontargeting siRNAs (2 μmol/L/100-mm Petri dish) was used as a control. A set of four PKCε-specific siRNA (2 μmol/L/100-mm Petri dish) was used to silence PKCε. After 48 h of siRNA transfection, the cells were harvested and used for Western blot analysis, electrophoretic mobility shift assay (EMSA), and luciferase assay.

Electrophoretic mobility shift assay. Indicated amount of protein extract was incubated in a final volume of 20 μL of 10 mmol/L HEPES (pH 7.9), 80 mmol/L NaCl, 10% glycerol, 1 mmol/L DTT, 1 mmol/L EDTA, and 100 μg/mL poly(deoxyinosinic-deoxycytidylic acid) for 15 min. A γ-^{32}P–radiolabeled double-stranded Stat3 consensus binding motif 5′-GATCCTTCTGGGAATTCCTAGATC (Santa Cruz Biotechnology) probe was then added and incubated for 20 min at room temperature. The protein-DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel containing 2.5% glycerol in 0.25× Tris-borate EDTA at room temperature, and gels were dried and autoradiographed. Stat3 DNA-binding activities were determined.

Luciferase assay. To determine the influence of PKCε on Stat3-associated gene expression, a sensitive Dual-Glo luciferase reporter assay using plasmids pLucTKS3 and pTKRenillaLuc was used (pGEM-luc vector, Promega).

[4] http://www.foliobio.com

[5] http://jaxmice.jax.org/pubcgi/protocols/protocolssh?objtype=protocol&protocol_id=188

Cell invasion assay. The cell invasion was assayed using a 24-well Collagen-Based Cell Invasion Assay kit as per the manufacturer's instructions. Briefly, DU145 cells at 80% confluency were serum starved for 18 to 24 h before the assay. Cells were harvested and the pellet was gently resuspended in serum-free medium. In the upper chamber, 0.5×10^6 cells per well were plated in triplicates and incubated for 2 h at 37°C in a humidified incubator with 5% CO_2 before PKCε siRNA transfection. Both the insert and the holding well were subjected to the same medium composition with the exception of serum. The insert contained no serum, whereas the lower well contained 10% fetal bovine serum that served as a chemoattractant. PKCε siRNAs were obtained from Dharmacon. The transfection of siRNA was done as per the manufacturer's instructions. A set of four pooled nontargeting siRNAs (described earlier) was used as a control. A set of four PKCε siRNA (described earlier) was used to silence PKCε. Forty-eight hours after siRNA transfection, the cell invasion assay was done as per the manufacturer's instructions. The cells in the insert

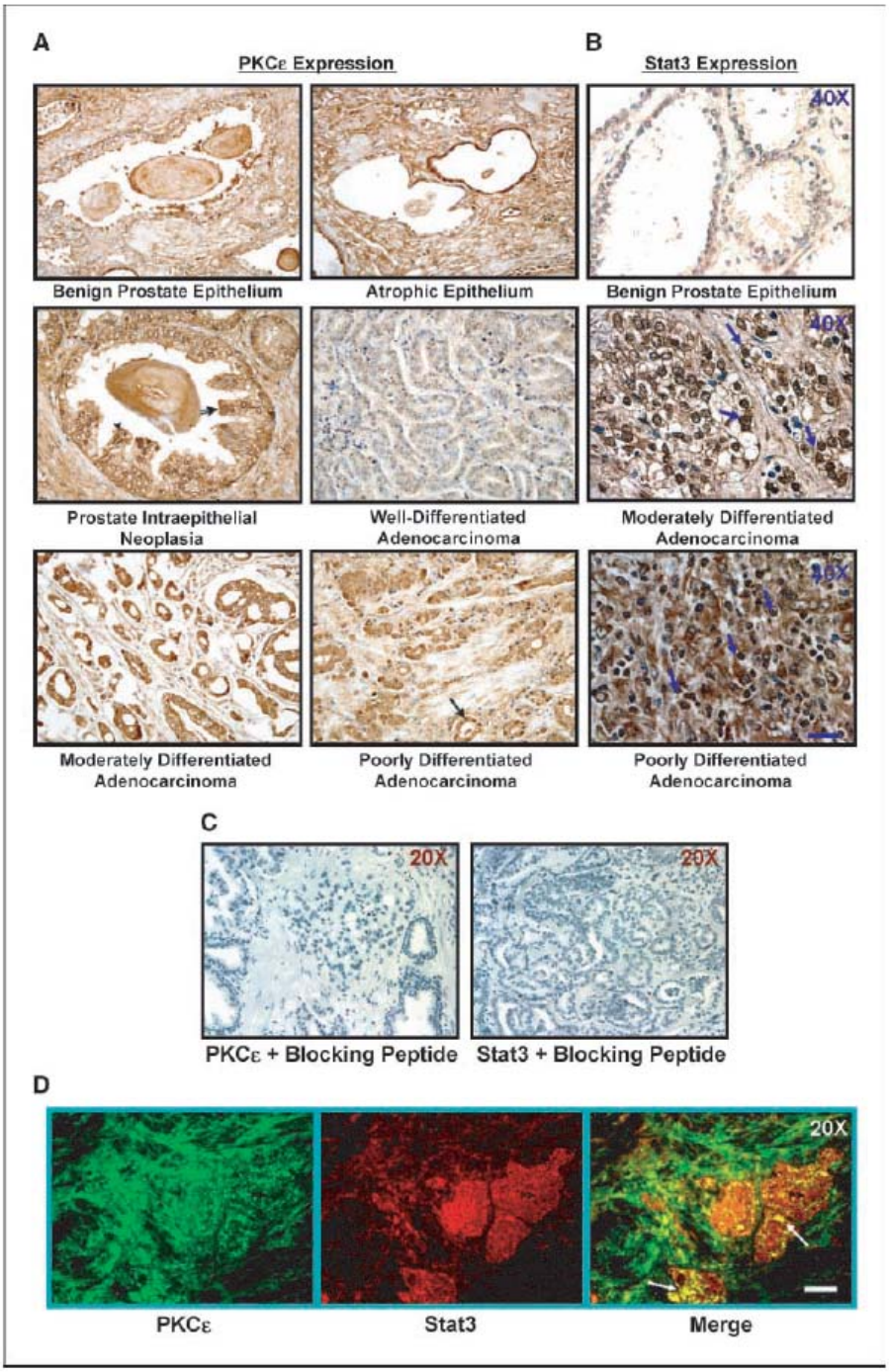

Figure 1. PKCε and Stat3 expression and colocalization in human prostate cancer. *A*, immunohistochemical staining for PKCε expression. Shown are benign prostate; prostate gland showing atrophic epithelium; area of gland showing prostate intraepithelial neoplasia (*double arrow*), light cytoplasmic staining (*arrowhead*), and nuclear staining; malignant glands of well-differentiated prostate adenocarcinoma (Gleason pattern 2); moderately differentiated prostate carcinoma (Gleason patterns 3–4); and poorly differentiated prostate adenocarcinoma (Gleason pattern 5; arrow pointing to nuclear staining). Magnification, ×20. *B*, immunohistochemical staining for Stat3 expression. Normal prostate epithelium showing low level of cytoplasmic Stat3 staining. *Arrow*, moderately differentiated prostate carcinoma (Gleason pattern 4) to poorly differentiated prostate adenocarcinoma (Gleason pattern 5) show moderate cytoplasmic and intense nuclear staining. Magnification, ×40. Gleason patterns were scored according to standard criteria (36). *Bar*, 50 μm. *C*, specificity of PKCε and Stat3 antibodies. Inclusion of PKCε or Stat3 blocking peptide before immunostaining of human SCC specimens completely prevented Stat3 staining, indicating that PKCε and Stat3 immunostaining were specific. *D*, colocalization of PKCε and Stat3. Immunofluorescence images of human prostate cancer illustrate localization of PKCε (*green*) and Stat3 (*red*). *White arrows*, colocalization is indicated by yellow fluorescence. Magnification, ×20. *Bar*, 25 μm.

Table 1. PKCε and Stat3 correlate with the aggressiveness of human prostate cancer

Prostate specimen	No. specimens	Weak (+)	Moderate (++)	Strong (+++)	P*
PKCε expression					
Benign prostate tissue	21	7 (33%)	12 (57%)	2 (10%)	—
Hyperplasia	9	3 (33%)	6 (67%)	0 (0%)	—
Prostate cancer grade 1	14	4 (29%)	7 (50%)	3 (21%)	<0.374
Prostate cancer grade 2	36	4 (11%)	16 (44%)	16 (45%)	<0.001
Prostate cancer grade 3	55	6 (11%)	21 (38%)	28 (51%)	<0.00004
Prostate cancer grade 4	25	2 (8%)	11 (44%)	12 (48%)	<0.002
Stat3 expression					
Benign prostate tissue	11	7 (64%)	3 (27%)	1 (9%)	—
Prostate cancer grade 1	14	8 (57%)	4 (29%)	2 (14%)	<0.699
Prostate cancer grade 2	36	6 (17%)	9 (25%)	21 (58%)	<0.0004
Prostate cancer grade 3	55	6 (11%)	19 (34%)	30 (55%)	<0.00005
Prostate cancer grade 4	25	2 (8%)	7 (28%)	16 (64%)	<0.0002

NOTE: Tissue microarrays were obtained from Folio Biosciences. The level of expression is denoted by the sign +. One "+" is the weak or faint staining and three "+" is the strong or intense staining. The slides were independently graded by M.H. Aziz and W. Zhong.
*Strong (+++) compared with benign prostate tissue.

were removed by wiping gently with a cotton swab. Migrated cells sticking to the bottom side of the insert were stained with Cell Stain. Invading cells on the bottom side of the membrane were photographed using a light inverted microscopy (Nikon Eclipse TS 100) at ×20 magnification. In addition, the number of cells migrated to the bottom side was estimated by colorimetric measurements at 560 nm according to assay instructions. Mean ± SE was calculated from three independent experiments.

Results

PKCε and Stat3 Expression and Their Colocalization in Human Prostate Cancer

Expression levels of PKCε and Stat3 and their *in vivo* colocalization were analyzed in human prostate cancer (Fig. 1). In the analysis illustrated in Fig. 1, prostate tumor specimens were obtained after prostatectomy and fixed in 10% neutral buffered formalin. Sections (5 μm thick) were cut for immunohistochemical staining of PKCε and Stat3. Small samples of prostate cancer were examined in a blinded fashion by two pathologists (T.D.O. and W.Z.) without previous knowledge of Gleason score and classified according to both Gleason pattern and WHO criteria (36, 37). Expression levels of PKCε in prostate are shown in Fig. 1*A*. Low levels of PKCε expression were detected in the cytoplasm of benign prostate epithelial cells. Atrophic epithelium showed trace to moderate cytoplasmic labeling for PKCε. Areas of prostate intraepithelial neoplasia showed light cytoplasmic (Fig. 1*A*, *double arrow*) and nuclear (Fig. 1*A*, *arrowhead*) expression levels of PKCε. Cytoplasm of the well-differentiated prostate adenocarcinoma showed only trace levels of PKCε staining. Cytoplasm of moderately differentiated prostate carcinoma showed moderate to strong expression levels of PKCε labeling. Poorly differentiated prostate adenocarcinoma showed moderate cytoplasmic and nuclear (*arrow*) PKCε expression levels (Fig. 1*A*). Immunohistochemical staining of human prostate carcinoma specimens showed increased nuclear staining of Stat3 in prostate cancer specimens compared with benign prostate specimens (Fig. 1*B*). Benign prostate epithelium showed low level of cytoplasmic Stat3. Moderately to poorly differentiated prostate adenocarcinoma showed moderate cytoplasmic and intense nuclear staining (Fig. 1*B*). Inclusion of appropriate blocking peptide before immunostaining of human SCC specimens completely prevented PKCε and Stat3 staining, indicating the immunostaining was specific (Fig. 1*C*).

The localization of PKCε with Stat3 was determined by double immunofluorescence staining. In this experiment (Fig. 1*D*), 5-μm-thick sections from paraffin-fixed human prostate cancer specimen were used. The sections were incubated with a mixture of PKCε (goat polyclonal, 1:50) and Stat3 (rabbit polyclonal, 1:50) primary antibodies. The sections were subsequently incubated with the mixture of two secondary antibodies (donkey anti-goat IgG-FITC for PKCε and donkey anti-rabbit IgG-rhodamine for Stat3). The stained sections were examined using an Olympus microscope. PKCε and Stat3 localization are indicated by the presence of green and red fluorescence, respectively. The yellow fluorescence indicates colocalization of both PKCε and Stat3 (Fig. 1*D*). Merged images clearly illustrate significant colocalization of PKCε and Stat3 (Fig. 1*D*, *white arrow*).

In a more detailed analysis, PKCε and Stat3 levels were determined by immunohistochemistry of human prostate tissue microarrays prepared from paraffin-fixed benign, hyperplastic, and carcinoma (grade 1–4) specimens (Table 1). For each section, staining was assessed as weak or faint (+), moderate or intermediate (++), and strong or intensive (+++) staining when present in >50% of total cells evaluated in each tissue core. As compared with benign prostate tissue, PKCε expression level in prostate cancer grade 2 to 4 was significantly ($P < 0.001$) increased. The level of expression of PKCε seemed to correlate with aggressiveness of prostate cancer (Table 1).

Stat3 staining was moderate to strong in human prostate cancer specimens compared with benign prostate specimens, which exhibited very weak staining (Table 1). Cytoplasmic as well as nuclear Stat3 staining was found in prostate cancer tissue specimens. Lower-grade or well-differentiated human prostate cancer specimens had more cytoplasmic Stat3 staining, whereas

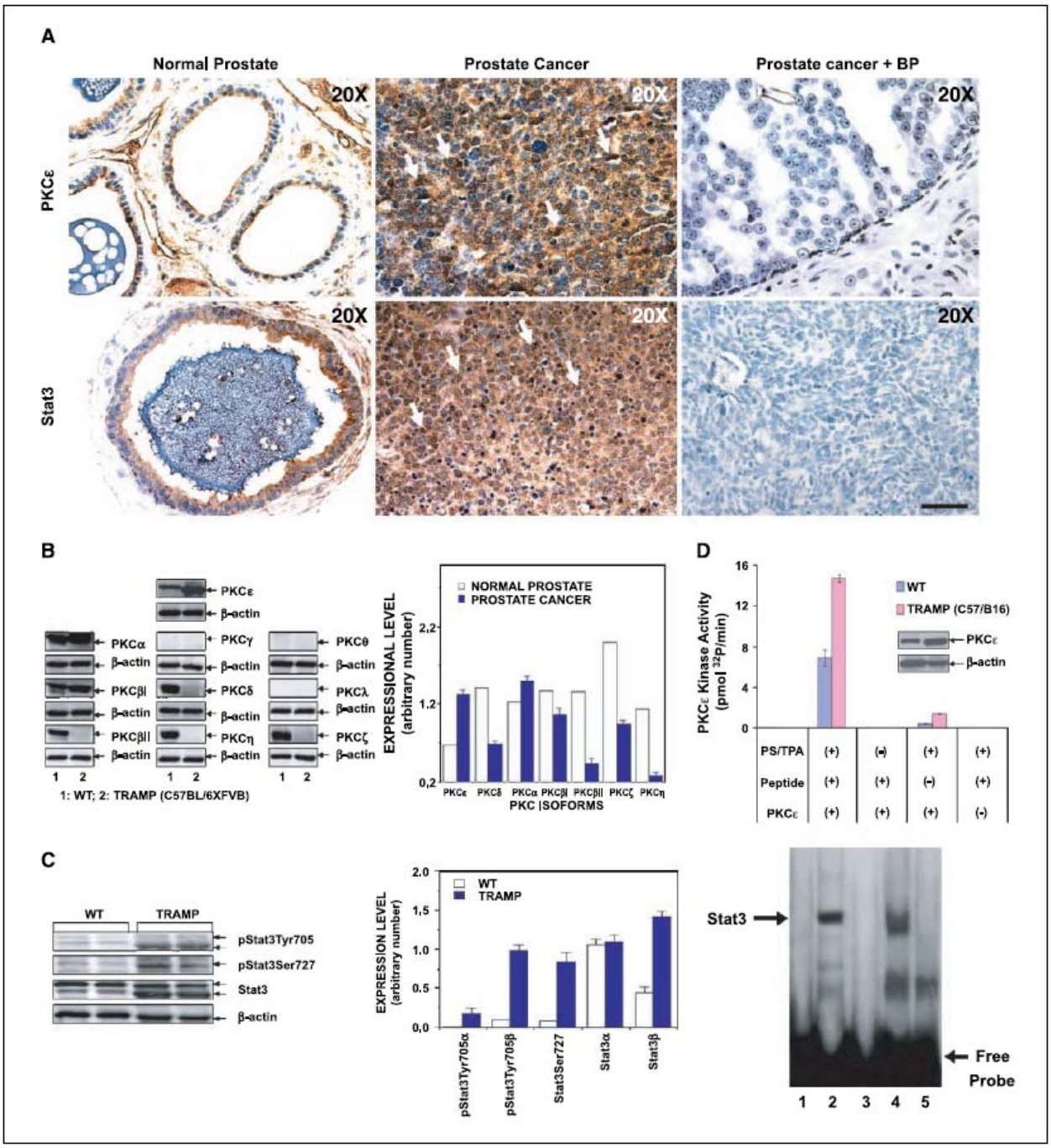

Figure 2. PKCε and Stat3 expression in prostate cancer from TRAMP mice. *A*, immunohistochemical staining for PKCε and Stat3 expression. WT and TRAMP [C57BL/6XFVB] F1 male mice ($n = 13$) were sacrificed at 26 wk of age and normal prostate and prostate cancer were excised. Prostate specimens were fixed in formalin for immunohistochemical study. *White arrows*, expression of PKCε and Stat3 in a typical prostate cancer specimen. Specificity of PKCε and Stat3 antibodies was determined using respective blocking peptides (*BP*). Magnification, ×20. *Bar*, 25 μm. *B* and *C*, PKC isoforms and Stat3 expression levels. The normal prostate from nontransgenic and prostate cancer from male TRAMP mice [C57BL/6 TRAMP × FVB] F1 (26 wk old; $n = 3$) were homogenized in lysis buffer to extract proteins for Western blot analysis. The protein extracts (25 μg protein) were immunoblotted for analyses of PKC isoforms (*B*) and pStat3Tyr705, pStat3Ser727, and Stat3 (*C*). *D*, PKCε activity. *Top*, normal prostate from 42-week-old WT mice and prostate cancer from age-matched TRAMP mice (C57BL/6) were excised and total protein extract was prepared in immunoprecipitation lysis buffer for PKC activity using an immunocomplex kinase assay as described in Materials and Methods; *bottom*, Stat3 DNA-binding activities. Normal prostate from 42-week-old WT mice and prostate cancer from age-matched TRAMP mice (C57BL/6) was excised. Prostate tissues and DU145 cells were homogenized in the lysis buffer to extract total proteins. The whole-cell extract (50 μg protein) was subjected to EMSA as described in Materials and Methods using a γ-^{32}P–labeled oligonucleotide probe containing the Stat3-binding motif following the manufacturer's protocol (Santa Cruz Biotechnology). *Lane 1*, oligo only; *lane 2*, DU145; *lane 3*, normal prostate sample from nontransgenic mice; *lane 4*, prostate cancer sample from TRAMP mice; *lane 5*, prostate cancer sample + 200× excess cold oligo.

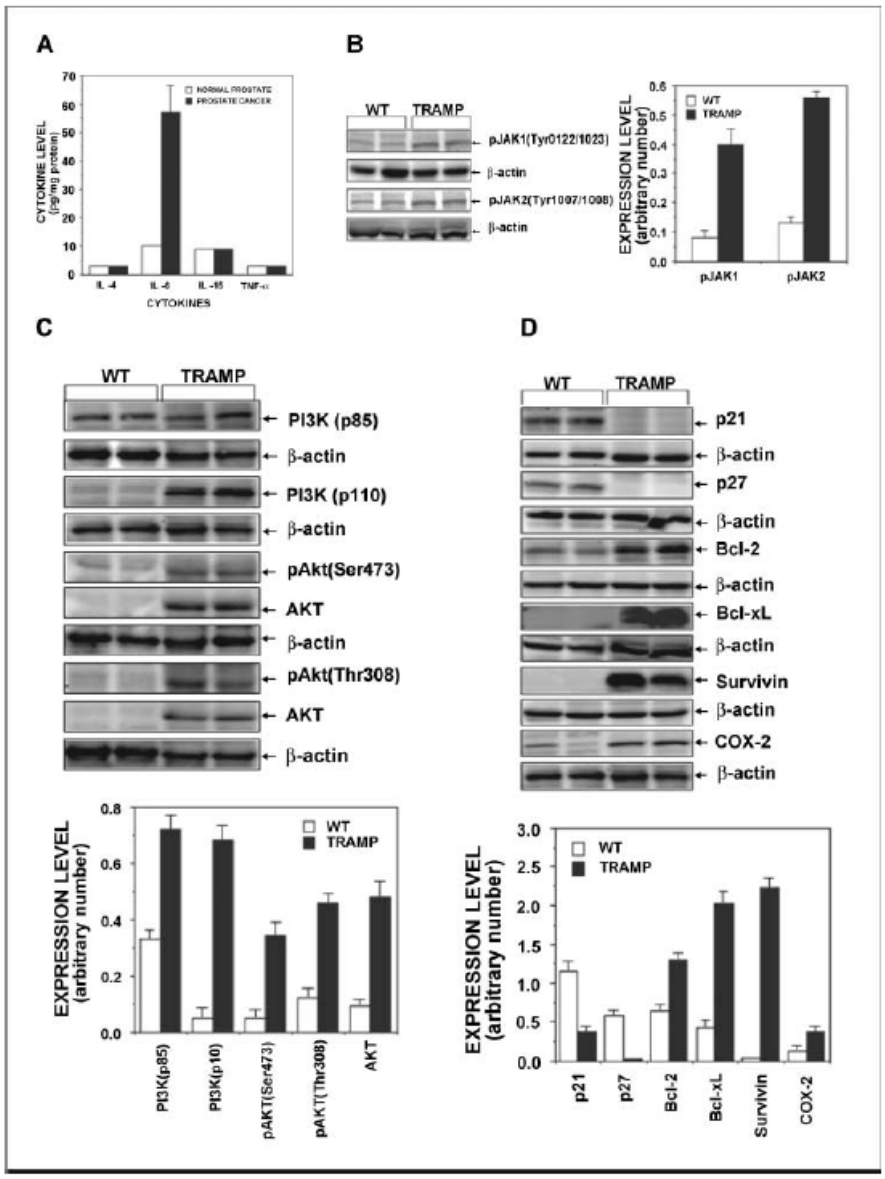

Figure 3. Expression level of cytokines and Stat3 signaling components in prostate cancer from TRAMP mice. *A*, analysis of cytokines. Normal prostate from WT and prostate cancer from TRAMP mice [C57BL/6 TRAMP × FVB] F1 were excised at 26 wk of age. Whole tissue lysate was prepared for analysis of cytokines by Linco Diagnostics. *Columns*, mean of determinations from samples from four separate mice; *bars*, SE. *B*, pJAK expression levels. *C* and *D*, expression of proliferative, cell cycle, and antiapoptotic proteins. Normal prostate from WT and prostate cancer from TRAMP mice [C57BL/6 TRAMP × FVB] F1 ($n = 2$) were excised at 26 wk of age. Prostate tissue protein extracts (25 μg) were subjected to 10% or 15% Tris-glycine SDS-PAGE followed by immunoblot analysis using antibodies specific for the indicated proteins. *Columns*, mean of three samples; *bars*, SE.

higher-grade or poorly differentiated human prostate cancer specimens showed more nuclear staining. As compared with benign prostate tissue, Stat3 expression level in prostate cancer grade 2 to 4 was significantly ($P < 0.001$) elevated (Table 1).

Expression Levels of PKCε and Stat3 and Their Related Signaling Components and Characterization of PKCε Interaction with Stat3 in Prostate Cancer from TRAMP Mice

To precisely determine the modulation of PKCε, Stat3, and related signaling components in prostate cancer, we used prostate cancer from TRAMP mice. The TRAMP mice spontaneously develop progressive prostate cancer that is invasive and capable of metastatic spread to distant sites, primarily in pelvic lymph nodes and the lungs (35, 38, 39). It has been reported that TRAMP mice castrated at 12 weeks of age develop androgen-independent prostate cancer (38). In addition, TRAMP mice develop spontaneous mutations in the androgen receptor (AR; ref. 39). Thus, prostate cancer in TRAMP mice closely mimics clinical prostate cancer with respect to progression, androgen independence, and biochemical profiles (35, 38, 39). The mouse genetic background makes a difference in prostate cancer progression (39). Prostate cancer progression in TRAMP mice on a mixed C57BL/6XFVB background is different from that of a C57BL/6 background. The C57BL/6 TRAMP mice can survive longer (~52 weeks) than [C57BL/6 TRAMP × FVB] F1 mice (~32 weeks; ref. 35). In addition, prostate cancer in C57BL/6 TRAMP mice often invades into seminal vesicles. Like the human prostate cancer disease, prostate cancer in TRAMP mice is also multifocal and heterogenous. In the experiments described here,

both C57BL/6 and [C57BL/6 TRAMP × FVB] F1 male TRAMP mice were used.

PKC isoforms and Stat3 expression levels. In this experiment (Fig. 2), [C57BL/6 TRAMP × FVB] F1 mice ($n = 13$) were sacrificed at 26 weeks of age and prostate cancer was excised. A portion of the prostate cancer specimen was fixed in formalin, whereas another part was homogenized in lysis buffer to extract total protein for analysis of protein expression levels of PKC isoforms, Stat3, pStat3Tyr705, and pStat3Ser727 as well as PKCε activity and Stat3 DNA-binding activity. Normal prostate tissue specimens from nontransgenic mice were processed in parallel.

Typical PKCε and Stat3 immunostaining results are illustrated in Fig. 2*A*. Increased protein expression levels of PKCε in prostate cancer (Fig. 2*A*) correlated with increased PKCε activity (Fig. 2*D*). In addition, constitutively activated Stat3 in prostate cancer (Fig. 2*A*) seems to have increased DNA-binding activity (Fig. 2*D*). We also determined the level of expression of various PKC isoforms in prostate cancer from TRAMP mice. Western blot analysis of PKC isoforms in the normal prostate from nontransgenic mice and prostate cancer from 26-week-old TRAMP mice is shown in Fig. 2*B*. PKCγ, PKCμ, and PKCλ were not detected in either normal or prostate cancer samples. PKCε expression was elevated in prostate cancer with reciprocal decreases in the expression levels of PKCδ, PKCβII, PKCη, and PKCζ. PKCα and PKCβI remain unaltered in prostate cancer (Fig. 2*B*). It is noteworthy that these results are consistent with the expression of PKC isoforms in human prostate adenocarcinomas as reported by Cornford et al. (33). In human prostate cancer specimens, the level of PKCε was elevated, whereas the levels of expression of PKCδ and PKCβII were decreased or undetected (33).

Western blot analysis revealed increased expression levels of pStat3Tyr705, pStat3Ser727, and Stat3β in prostate cancer from 26-week-old TRAMP mice compared with normal prostate from age-matched nontransgenic mice (Fig. 2*C*).

Expression level of cytokines and Stat3 signaling components in prostate cancer from TRAMP mice. Several cancer-promoting agents induce the release of proinflammatory cytokines [interleukin (IL)-1, IL-6, IL-7, IL-15, granulocyte macrophage colony-stimulating factor, and tumor necrosis factor-α (TNF-α)], chemotactic cytokines (IL-1 and TNF-α), and cytokines regulating immunity (IL-10, IL-12, and IL-18; ref. 40). Overwhelming evidence indicates a relationship between cytokines and the development and progression of many cancers. Elevated serum levels of specific cytokines have been reported in prostate cancer specimens and in prostate cancer cell lines (41, 42). Several reports indicate high concentrations of IL-6 in men with hormone-refractory prostate cancer compared with normal controls (43, 44). We also found elevated levels of IL-6 in prostate cancer from [C57BL/6 TRAMP × FVB] F1 mice (Fig. 3*A*). Increased IL-6 expression levels accompanied increased expression of pJAK1 and pJAK2 (Fig. 3*B*).

To find clues of the kinase cascade(s) involved with increased IL-6 expression in prostate cancer, we examined the expression of various signaling kinases and found increased expression of PI3K (p85), PI3K (p110), phosphorylated AKTSer473, phosphorylated AKTThr308, and AKT (Fig. 3*C*).

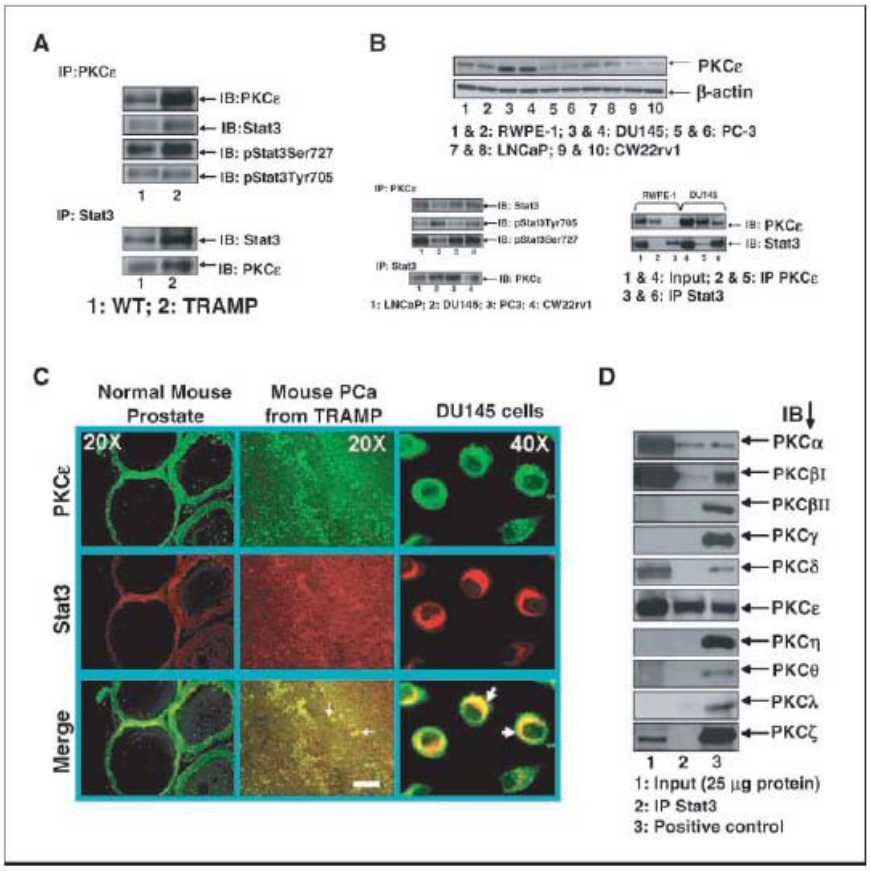

Figure 4. PKCε interacts with Stat3. *A*, WT and TRAMP [C57BL/6] mice ($n = 3$) were sacrificed at 42 wk and the prostate tissues were homogenized in immunoprecipitation (*IP*) lysis buffer as described in Materials and Methods. The homogenate was centrifuged at 14,000 × *g* for 30 min at 4°C. The whole-cell lysates were pooled (50 μg protein) and used for immunoprecipitation with the indicated antibodies. Immunoprecipitated samples were analyzed by Western blot (*IB*) using the indicated antibodies. *B*, association of PKCε with Stat3 in human prostate cancer cell lines LNCaP, DU145, PC3, CW22rv1, and RWPE-1. Whole-cell lysate (50 μg protein) from the human prostate cancer cell lines was used for immunoprecipitation with the indicated antibodies. The immunoprecipitated samples were analyzed by Western blot using the indicated antibodies. *C*, double immunofluorescence localization of PKCε and Stat3 in normal mouse prostate, prostate cancer (*PCa*) from TRAMP [C57BL/6], and DU145 cells. Localization of PKCε and Stat3 is shown by green and red fluorescence, respectively. *Arrows*, colocalization of PKCε with Stat3 is shown by yellow fluorescence. Magnification, ×20 magnification. *Bar*, 25 μm. *D*, PKC isoform interaction with Stat3. WT and TRAMP [C57BL/6] mice were sacrificed at age 42 wk and the tissue extracts were prepared for reciprocal immunoprecipitation/blotting experiments using antibodies specific for PKCε and Stat3.

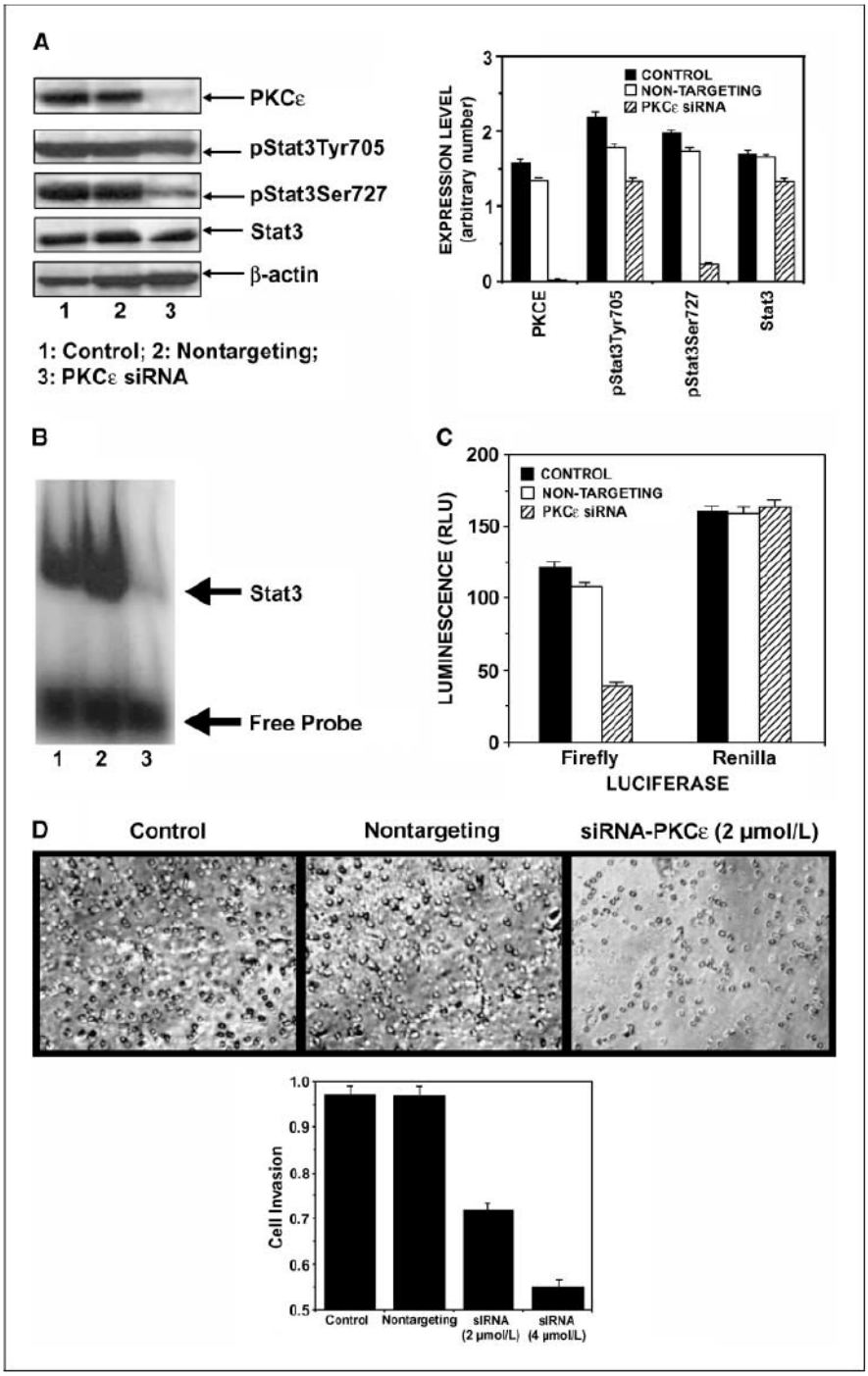

Figure 5. PKCε is linked to phosphorylation of Stat3Ser727, Stat3 DNA-binding activity, Stat3 transcriptional activity, and prostate cancer progression. *A,* PKCε and Stat3 expression. DU145 cells were untransfected (*lane 1*) or transfected with nontargeting siRNA (*lane 2*) or PKCε-specific siRNA (*lane 3*), and whole-cell lysates were prepared as described in Materials and Methods. The protein extracts (25 μg protein) were immunoblotted and indicated protein expression levels were detected with the appropriate antibodies. β-Actin was used as a control for gel loading variations. The quantification of proteins (normalized to β-actin) was done as described in Materials and Methods. *Columns,* mean of two independent experiments; *bars,* SE. *B,* EMSA. DU145 total cells were suspended in buffer A [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L $MagCl_2$, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L PMSF]. After 15 min of incubation on ice, the cells were pelleted and resuspended in buffer B [20 mmol/L HEPES (pH 7.9), 20 mmol/L NaF, 1.5 mmol/L $MgCl_2$, 1 mmol/L Na_3VO_4, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 420 mmol/L NaCl, 20% glycerol, 1 μg/mL leupeptin, 1 μg/mL aprotinin]. The samples were then centrifuged and the clear supernatant was used for EMSA as described in Materials and Methods. *Lane 1,* control; *lane 2,* nontargeting; *lane 3,* PKCε siRNA. *C,* Stat3 transcriptional activity. DU145 cells were transfected with Stat3 reporter plasmid, pLucTKS3, and pLucRenillaLuc with or without PKCε-specific siRNA (Dharmacon) using LipofectAMINE. Cells were lysed in passive lysis buffer and firefly luciferase (pLucTKS3) and *Renilla* luciferase activities (transfection control) were measured using a luciferase assay system (Promega) as outlined in the manufacturer's instructions. Luciferase activities were normalized to total protein. *Columns,* mean of triplicate assays; *bars,* SE. *D,* DU145 cell invasion. DU145 cells were transfected with PKCε siRNA and cell invasion was determined as described in Materials and Methods. *Top,* photographs of invading cells. Magnification, ×20. *Bottom,* the number of invading cells was estimated by colorimetric measurements at 560 nm according to assay instructions (Chemicon International). *Columns,* mean of three separate wells; *bars,* SE.

Activation of the PKCε and Stat3 pathway has been shown to inhibit apoptosis and promote survival of prostate cancer cells (7, 13, 34). We determined the expression of genes associated with apoptosis (*Bcl-2, Bcl-xL,* and *survivin*), cell cycle regulation (*p21* and *p27*), and proliferation (*COX-2*) in prostate cancer from TRAMP mice. As shown in Fig. 3*D*, increased expression of PKCε and Stat3 in prostate cancer from TRAMP mice accompanied decreased expression of p21 and p27 and increased expression of Bcl-xL, Bcl-2, survivin, and COX-2.

PKCε Interacts with Stat3 in Prostate Cancer

To determine whether PKCε associates with Stat3, reciprocal immunoprecipitation/blotting experiments were done using

prostate tissue specimens from wild-type (WT) and TRAMP mice (Fig. 4*A*) and human prostate cancer cell lines (Fig. 4*B*). In these experiments, the protein extract was immunoprecipitated with antibodies against PKCε or Stat3. The immunoprecipitated samples were subjected to immunoblot analysis using antibodies against PKCε, Stat3, pStat3Tyr705, and pStat3Ser727. The results of PKCε interaction with Stat3 in prostate tissue are shown in Fig. 4*A*. Stat3, pStat3Tyr705, and pStat3Ser727 coimmunoprecipitated with PKCε. In the reciprocal immunoprecipitation/blotting experiments, PKCε coimmunoprecipitated with Stat3 (Fig. 4*A*). PKCε interaction with pStat3Ser727 was more dramatic in prostate cancer from TRAMP mice than normal prostate tissue from WT mice. PKCε interaction with Stat3 in human prostate cancer cell lines is shown in Fig. 4*B*. Analysis of steady-state expression levels of PKCε revealed increased PKCε expression level in DU145 cells (Fig. 4*B, top*). PKCε interaction with Stat3 was observed in LNCaP, DU145, PC3, and CW22rv1 cell lines. However, PKCε interaction with Stat3 was not detectable in RWPE-1 cell line. The co-localization of PKCε with Stat3 in prostate cancer from TRAMP mice (Fig. 4*C, left*) and in DU145 cells (Fig. 4*C, right*) was confirmed by double immunofluorescence staining.

The interaction of Stat3 with other PKC isoforms was also determined. In this experiment (Fig. 4*D*), prostate cancer protein extract from TRAMP mice was immunoprecipitated with Stat3 antibody. The immunoprecipitated samples were subjected to immunoblot analysis using antibodies against various PKC isoforms. PKCε coimmunoprecipitated with Stat3. However, other PKC isoforms showed little (α and βI) or no (βII, γ, δ, λ, ζ, η, and *u*) association with Stat3 (Fig. 4*D*).

PKCε Mediates Stat3Ser727 Phosphorylation and Transcriptional Activity of Stat3

To determine whether PKCε is linked to Stat3Ser727 phosphorylation and whether this phosphorylation is essential for the DNA-binding and transcriptional activity of Stat3, we used siRNAs to silence PKCε in DU145 cells (Fig. 5*A* and *B*). A pool of four specific siRNA oligonucleotides directed against PKCε mRNA was transfected into DU145 cells to inhibit PKCε synthesis (Fig. 5*A*). Inhibition of PKCε expression resulted in dramatic inhibition of Stat3Ser727 phosphorylation but minimally inhibited Stat3Tyr705 phosphorylation and total Stat3 levels (Fig. 5*A*). Furthermore, siRNA-mediated inhibition of PKCε inhibited Stat3 DNA-binding activity as determined by EMSA (Fig. 5*B, left*). In addition, siRNA-mediated PKCε deficiency in DU145 cells inhibited Stat3-regulated gene expression as determined using the luciferase reporter gene assay (Fig. 5*C, right*).

PKCε Is Linked to DU145 Cell Invasion

The possibility was explored whether PKCε is linked to prostate cancer progression. In this experiment (Fig. 5*D*), PKCε expression was inhibited using PKCε siRNA. PKCε inhibition, which accompanies inhibition of Stat3Ser727 phosphorylation and Stat3 DNA-binding and transcriptional activities, decreased the invasive ability of DU145 cells (Fig. 5*D, top*). siRNA-mediated inhibition of DU145 cells invasion was dependent on the dose of siRNA (Fig. 5*D, bottom*).

Discussion

We report here that both PKCε and constitutively activated Stat3 are overexpressed in prostate cancer and their expression levels correlate with prostate cancer aggressiveness. PKCε overexpression in prostate cancer accompanied (*a*) increased in the levels of IL-6 and pJAK1 and pJAK2, (*b*) increased phosphorylation of PI3K and AKT, (*c*) decreased expression of cyclin-dependent protein kinase inhibitors (p21 and p27), and (*d*) up-regulation of antiapoptotic (Bcl-2, Bcl-xL, and survivin) and proliferative (COX-2) markers. PKCε interacts with Stat3 and phosphorylates Stat3Ser727. PKCε-mediated Stat3Ser727 phosphorylation correlated with Stat3 DNA-binding and transcriptional activities as well as prostate cancer progression.

PKCε was overexpressed in human prostate cancer and prostate cancer developed either in C57BL/6 or [C57BL/6 TRAMP × FVB] F1 TRAMP mice (Figs. 1 and 2). The fact that PKCε expression is

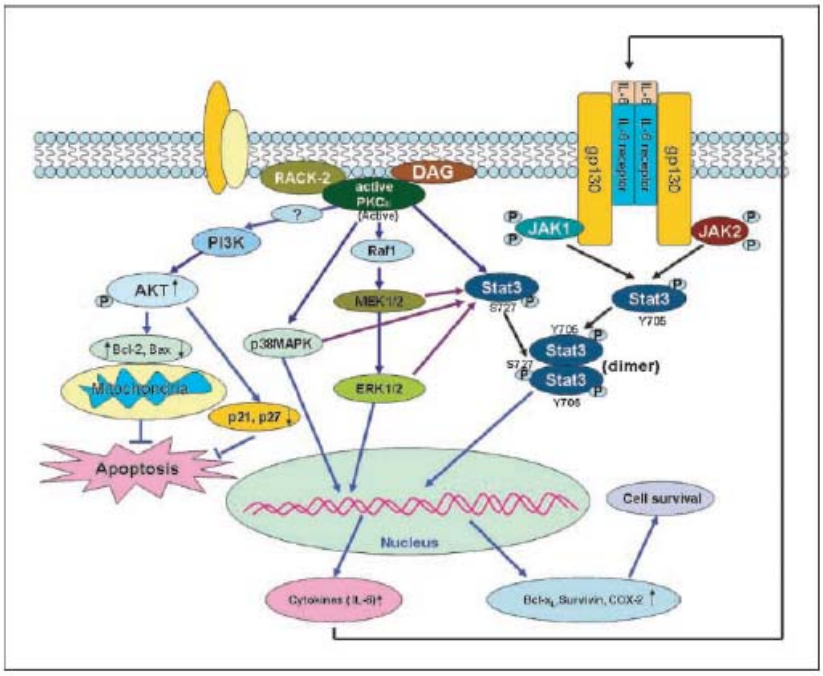

Figure 6. Proposed model illustrating how PKCε is linked to constitutive activation of Stat3 in prostate cancer. Both PKCε and Stat3 are constitutively activated in prostate cancer. Stat3 is activated by phosphorylation at both Tyr^{705} and Ser^{727} residues. PKCε may mediate, through activation of the PI3K-AKT pathway, induction of cytokines (e.g., IL-6). IL-6, either via autocrine or paracrine mechanism and through the JAK-Stat pathway, phosphorylates Stat3Tyr705. PKCε either directly or indirectly via association with other protein kinases [e.g., Raf1, MAPK/ERK 1/2 (*MEK1/2*), ERK1/2, and p38MAPK] phosphorylates Stat3Ser727. Constitutive activation of PKCε and Stat3 correlates with the aggressiveness of prostate cancer. This model assumes that PKCε and Stat3, the proteins with oncogenic traits, are essential components of development and maintenance of androgen-independent prostate cancer.

significantly elevated in prostate cancer and correlates with prostate cancer aggressiveness (Figs. 1 and 2; Table 1) implies that PKCε is probably linked to the maintenance of androgen-independent prostate cancer. In this context, the pioneering work of Terrian and his associates on the role of PKCε in prostate carcinogenesis, using prostate cancer–derived cell lines, is noteworthy (5, 34, 45). In their reports, PKCε overexpression transformed androgen-dependent LNCaP tumor cells to androgen-independent cells (5). The transformation of androgen-dependent LNCaP cells to an androgen-independent variant was associated with increased cell proliferation and resistance to apoptosis. Antisense experiments established that endogenous PKCε plays an important role in regulating the growth and survival of androgen-independent prostate cancer cells, suggesting that PKCε expression may be sufficient to maintain prostate cancer growth and survival after androgen ablation (5).

PKCε, which is overexpressed in prostate cancer, seems to be constitutively active. This conclusion is supported by two lines of evidence. First, increased PKCε expression correlated with PKCε activity in prostate cancer from TRAMP mice (Fig. 2), and second, in an immunohistochemical analysis, PKCε was observed to translocate to both plasma and nuclear membranes (Figs. 1 and 2). The mechanism by which PKCε remained constitutively active during progression of prostate cancer can be speculated. The generally accepted paradigm for PKC activation (22) consists of two major biological events. The initial event is a well-ordered sequential phosphorylation followed by allosteric activation mediated by two lipid signals. Priming phosphorylation on the activation loop residue threonine by phospholipid-dependent kinase 1 initiates autophosphorylation on the turn motif threonine residue and hydrophobic motif serine residues. Most PKC in unstimulated cells is in the triple-phosphorylated mature form. The mature, phosphorylated species are released to the cytosol and the pseudosubstrate gains access to the active site, thus maintaining the enzyme in an autoinhibited state (22). As the mature PKC positions near the membrane for rapid access to DAG, the translocation of the mature enzyme from the cytosolic soluble fraction to particulate plasma membrane fraction is mediated by the binding of a lipid second messenger. Subsequent association with the membrane allows PKC to carry out phosphorylation of target substrates. The constitutive activation of PKCε is perhaps due to dysregulation of PKCε synthesis, activation, and/or degradation.

Constitutively activated PKCε regulates activation of the signaling network linked to cell survival essential for the maintenance of androgen-independent prostate cancer (5). As observed in prostate cancer from TRAMP mice, PKCε expression accompanied up-regulation of phosphorylated PI3K and AKT (Fig. 3), major components of the cell survival pathway (Fig. 6). Consistent with these findings, using CWR22 xenografts, it was shown by proteomic analysis that the association of PKCε with Bax may neutralize apoptotic signals propagated through the mitochondrial death signaling pathway. In addition, integrin signaling links PKCε to the protein kinase B/AKT survival pathway in recurrent prostate cancer cells (34). Our results (Fig. 3), using the *in vivo* TRAMP mouse model, are consistent with these findings (34). PKCε overexpression, which correlates with prostate cancer aggressiveness (Table 1), accompanied an increase in proteins that modulate apoptosis (survivin, Bcl-2, and Bcl-xL) and cell survival (Stat3; Fig. 3).

Several lines of evidence support the link between PKCε and Stat3. First, both PKCε and Stat3 are overexpressed in prostate cancer (Figs. 1 and 2; Table 1). Second, using reciprocal immunoprecipitation experiments, it was observed that PKCε associates with Stat3 (Figs. 4 and 5). Finally, double immunofluorescence studies confirm their colocalization (Figs. 1 and 4). PKCε depletion prevents PKCε association with Stat3 and Stat3Ser727 phosphorylation. Together, these biochemical, immunologic, and enzymology studies indicate that PKCε interacts with Stat3 and is linked to Stat3Ser727 phosphorylation.

Depending on the cellular context, Stat3 is a substrate for several protein kinases (46). Stat3 is an *in vitro* substrate for mitogen-activated protein kinase (MAPK) and/or extracellular signal-regulated kinases (ERK; ref. 47). Pioneering research from Dr. Zigang Dong's laboratory (Hormel Institute, University of Minnesota, Austin, MN) has shown that UV-induced Stat1 and Stat3 (Ser^{727}) phosphorylation involves the integration of multiple kinase pathways (48, 49). Recently, we have reported that PKCε interacts with Stat3 in mouse skin. PKCε interaction with Stat3 was increased after exposure of skin with UVR (16). We also observed *in vivo* using PKCε knockout mice and *in vitro* in an immunocomplex kinase assay that PKCε phosphorylated Stat3 at Ser^{727} residue, implying that PKCε is a Stat3Ser727 kinase (16). Our results (Figs. 4 and 5) indicate that PKCε is also an initial signal that is linked to phosphorylation of Stat3Ser727 in prostate cancer. Dependent on the cellular context and stimulus, Stat3 may be a direct or indirect substrate of PKCε.

The functional specificity of each PKC isoform is determined in part by the differential localization of the isozyme-specific RACKS. RACKS, which anchor activated PKC isozymes in close proximity to its selective substrates, are not PKC substrates. Proteins that interact with PKC include proteins localizing PKC to a specific intracellular site (e.g., RACKS and RICKS), protein kinases (e.g., Raf1, Btk, S6 kinase, PKD, PDK1, PKF, and Fyn), substrate proteins (e.g., MARCKS and STICKS), and other proteins with unique functions (25, 50–54). Several proteins have specifically been shown to interact with PKCε (51, 54). Our results (Figs. 4 and 5) indicate that PKCε interacts with Stat3 and phosphorylates Stat3Ser727. PKCε-mediated phosphorylation of Stat3Ser727 may be an essential component of constitutive activation of Stat3 in a wide variety of human cancers (14–18).

The mechanism by which Stat3 is constitutively activated in prostate cancer can be possibly explained as shown in Fig. 6. Stat3 activation, which involves dimerization, nuclear translocation, DNA binding, and transactivation of transcription, requires phosphorylation of both Tyr^{705} and Ser^{727} (8–12). Stat3Tyr705 phosphorylation is mediated by a wide variety of growth factors (e.g., IL-6). IL-6 has been characterized as a prostate exocrine gene product that interacts with its receptors, activates AR, and regulates prostate cancer cell proliferation. IL-6 signaling is mediated through JAK, Stat3, as well as MAPK, which induces AR-mediated gene activation in prostate cancer (12, 13). JAK-Stat is the classic pathway that has been shown to mediate cellular responses to a variety of cytokines, including IL-6. In response to IL-6, Stat3 is transiently associated with gp130 and subsequently phosphorylated by JAKs on Tyr^{705} of Stat3. Stat3 represents an important regulator of IL-6–targeted gene expression. PKCε-mediated Stat3 Ser^{727} phosphorylation is essential for both optimal DNA-binding and transcriptional activities of Stat3 (Fig. 6).

In summary, PKCε and constitutively activated Stat3 are key components in the mechanisms involved in transition of androgen-dependent prostate cancer to androgen-independent prostate cancer (45). We have made the novel observation that PKCε associates with Stat3 and regulates Stat3 activation (Figs. 1, 4,

and 5). PKCε activation transduces multiple signals involving inhibition of apoptotic pathways and promotion of cell survival pathways (Figs. 3 and 6). PKCε-mediated cell survival pathway involves constitutive activation of Stat3. PKCε is an initial signal that regulates activation of Stat3. PKCε and Stat3, the proteins with oncogenic traits, should be explored as potential targets for prevention of androgen-independent prostate cancer.

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