

ABSTRACT

Meagan A. McJilton. PROTEIN KINASE C ϵ PROMOTES THE SURVIVAL OF HUMAN PROSTATE CANCER CELLS. (Under the direction of Dr. David M. Terrian). Department of Biology, June 2002

Human prostate cancer (CaP) cells over-expressing protein kinase C epsilon (PKC ϵ) acquire a highly malignant phenotype that is androgen independent and autocrine in growth. Moreover, these CaP cells are highly tumorigenic in nude mice. These observations raise the question of whether CaP cells over-expressing PKC ϵ have also acquired the ability to survive in the presence of apoptotic signals, an important phenotypic characteristic of metastatic CaP cells. The LNCaP cell line provides an excellent model to study the effects of various known apoptotic stimuli, in particular the PKC agonist, phorbol-12-myristate-13-acetate (PMA). Several laboratories have independently shown that both PKC α and PKC δ are capable of inducing cell death through the intrinsic cell death pathway when LNCaP cells are exposed to PMA. Within 24 hours of PMA treatment more than 90% of the parental LNCaP cells undergo apoptosis, while LNCaP cells over-expressing PKC ϵ remain viable for at least 72 hours. Our studies demonstrate that signals transduced by PKC ϵ desensitize LNCaP cells to the pro-apoptotic effects of PMA by disrupting the intrinsic cell death pathway somewhere upstream of events leading to the release of cytochrome c from the intermembrane space of the mitochondria. In contrast, the expression of PKC ϵ did not alter the apoptotic response of LNCaP cells to tumor necrosis factor- α (TNF- α), a potent activator of the extrinsic cell death pathway. Upstream of the mitochondrion, PKC is known to suppress pro-apoptotic activity of the BH3-only protein, Bad, by promoting the heterodimerization

of Bad with the cytosolic protein 14-3-3 ζ . However, our data show that the levels of this heterodimer are not altered by the over-expression of PKC ϵ in LNCaP cells, indicating that such a mechanism is unlikely to be involved. Another such upstream of the mitochondria event that may be occurring to cause resistance to PMA-induced apoptosis of these LNCaP cells over-expressing PKC ϵ is Bax being sequestered in the cytosol. PKC ϵ may promote CaP cell survival through a direct or indirect interaction with Bax, another pro-apoptotic member of the Bcl-2 family that is directly responsible for the opening of the recently discovered mitochondrial apoptosis-induced channel (MAC). Because these studies were based on the forced expression of PKC ϵ , the CWR-R1 cell line was used to examine PKC ϵ 's endogenous role in PMA-induced apoptosis. It does appear that endogenous PKC ϵ is required to protect CaP cells from PMA-induced apoptosis.

PROTEIN KINASE C ϵ
PROMOTES THE SURVIVAL OF
HUMAN PROSTATE CANCER CELLS

A Thesis

Presented to
the Faculty of the Department of Biology
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In Partial Fulfillment
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by

Meagan McJilton

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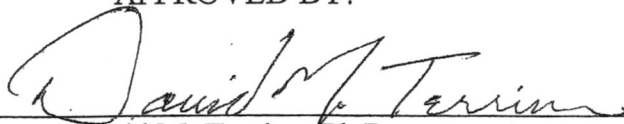
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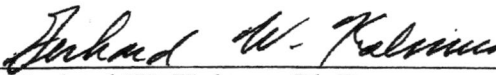
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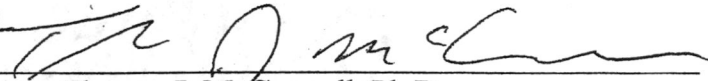
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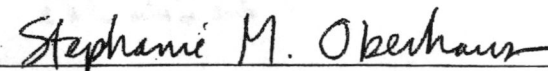
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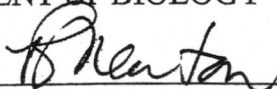
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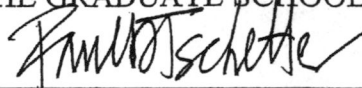
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LIST OF SYMBOLS

α alpha

β beta

δ delta

ε epsilon

γ gamma

μ mu

η eta

ι iota

θ theta

ζ zeta

LIST OF ABBREVIATIONS

ANT:	adenine nucleotide transporter
ATP:	adenosine triphosphate
AD:	androgen-dependent
AI:	androgen-independent
AR:	androgen receptor
ARE:	androgen response elements
AIF:	apoptosis inducing factor
Apaf-1:	apoptotic protease-activating factor
aPKC:	atypical protein kinase C
Bax:	Bcl-2-associated protein X
BH:	Bcl-2 homology
Ca ²⁺ :	calcium
CaMK:	calmodulin-dependent kinases
co-IPs:	co-immunoprecipitates
JNK:	c-Jun N-terminal kinase
C1-C4:	conserved regions
cDNA:	complementary deoxyribonucleic acid
cPKC:	conventional protein kinase C
CDKs:	cyclin dependent kinases
CKIs:	cyclin dependent kinase inhibitors
CAG:	cytosine adenine guanine

PDK:	DAG-dependent protein kinases
DAG:	diacylglycerol
DD:	death domain
DED:	death effector domain
DR4:	death receptor 4
DR5:	death receptor 5
DNA:	deoxyribonucleic acid
DRE:	digital rectal examination
DHT:	dihydrotestosterone
EGF:	epithelial growth factor
FasL:	Fas ligand
HPC:	hereditary prostate cancer
mRNA:	messenger ribonucleic acid
MAC:	mitochondrial apoptosis-induced channel
MAPK:	mitogen-activated protein kinase
nPKC:	novel protein kinase C
ODN:	oligodeoxynucleotide
PTP:	permeability transition pore
PI3K:	phosphatidylinositol 3'-kinase
PIP3:	phosphatidylinositol 3,4,5-trisphosphate
PS:	phosphatidylserine
PMA:	phorbol-12-myristate-13-acetate

CaP:	prostate cancer
PSA:	prostate specific antigen
PKC:	protein kinase C
PT:	permeability transition
PTEN:	phosphatase and tensin homologue deleted on chromosome 10
Rb:	retinoblastoma gene
pRb:	retinoblastoma protein
TRUS:	transrectal ultrasonography
TNF:	tumor necrosis factor
TNFR1:	tumor necrosis factor receptor 1
TNFR2:	tumor necrosis factor receptor 2
TRAIL:	TNF-related apoptosis-inducing ligand
2-D:	two-dimension
US:	United States
UV:	ultraviolet
VDAC:	voltage dependent anion channel

LITERATURE REVIEW

Cancer and the Progression to Metastasis

Cancer has been studied throughout the ages dating back to before the time when the Greek physician, Hippocrates, coined the terms “carcinos” and “carcinoma” to describe non-ulcer and ulcer forming tumors (Diamandopoulos, 1996). Today, after centuries of study, the mechanisms involved in this complex and deadly disease are still not known. However, our progress in tumor cell biology, treatment, and prevention has been phenomenal. All cancers result from the uncontrolled growth, survival, and spread of mutated cells, mutations that can be initiated by genetic, chemical, radiation, viral, or environmental factors (Diamandopoulos, 1996).

The spread of cancer cells from their origin is termed metastasis and involves several complex steps. First, after the initial transforming event, there must be progressive growth of the neoplastic cells followed by extensive vascularization by angiogenesis resulting in a neoplasm of multiple cell populations. Next these cells must be able to invade the host stroma, access circulation and subsequently detach and embolize in small aggregates. Circulating immune cells eliminate the vast majority of these cells; nevertheless, some survive to continue the metastasis. In order for metastasis to advance, the surviving cells must arrest in the capillary bed of an organ where they can penetrate and extravasate into the organ’s tissue. The cancer cells must survive and proliferate to form a secondary tumor in this foreign environment (Ellis and Fidler, 1996). This heterogeneous group of diseases (a variety of cancers) presents in various forms in various tissues but each has in common the uncontrolled cell survival and

proliferation that often leads to metastasis. Several factors act to promote tumorigenesis including proto-oncogenes and irregularly functioning tumor-suppressor genes, which will be discussed below. It is through oncogenic mutations that cancer cells acquire the ability to proliferate uncontrollably and survive various death promoting treatments.

Loss of Cell Cycle Control Promotes Cancer Progression

Altered cell cycle control may be a key factor in the progression of tumor cells where loss of, or aberrant function of, cell cycle regulators results in uncontrolled growth and proliferation. Cyclin dependent kinases (CDKs: CDK1, 2, 4, 5, 6, 7, 8, and 9) are serine/threonine kinases that function in promoting cell cycle progression when complexed to the regulatory subunit of a cyclin. Therefore, cyclins are CDK partners in catalytic action. Cyclin levels are mainly regulated at the transcriptional level and function to regulate cell passage through the cell cycle. The cyclins that are maximally expressed during the G₁ phase of cell growth include cyclins C, D1, D2, D3, E1, E2, F, and G (Sgambato et al., 2000; Sherr, 1996). The D-type cyclins are expressed early in this phase as cells enter the cell cycle from quiescence (Sherr, 1996). In late G₁ transition to S phase, A and E-type cyclins are maximally expressed (Sherr, 1996). Cyclin B1 and B2 function largely in the G₂/M period of the cell cycle. The cyclin H protein is expressed throughout the cell cycle (Sgambato et al., 2000). Of these cyclins, cyclin D1 is most frequently altered in human cancer by means of overexpression, mutation, and/or deregulation (Lu et al., 2000). CDK activity requires cyclin binding and depends on positive and negative phosphorylation (Sherr, 1996). These heterodimers are involved in activating other downstream substrates through phosphorylation. The CDK inhibitors

(CKIs) control the action of the cyclin-CDK complexes. The CKIs are currently known to be comprised of two families, Cip/Kip and INK4, that function in inhibiting the transition to S phase during the cell cycle. The Cip/Kip family of CKIs is composed of at least three proteins (p21, p27, and p57) that negatively regulate the cyclin D-, E-, and A-CDKs. As levels of p27 increase they serve as a barrier for transition from G₁ to S phase by binding and inhibiting the cyclin/CDK complex. Cancer cells display reduced expression of this key cell cycle inhibitor, resulting in a loss of control over cell cycle progression. There are currently four known INK4 proteins (p15, p16, p18, and p19) that function to inhibit complexes of cyclin D and CDK 4/6 (Sherr, 1996 and Tsihlias et al., 1999). While members of the Cip/Kip family are broad-spectrum inhibitors of CDKs, the INK4 proteins appear to specifically interact with the cyclinD-CDK4/6 heterodimers. A loss of p16 tumor suppressive activity is evident in a variety of cancer cells and considered a poor prognostic indicator (Tsihlias et al., 1999).

The retinoblastoma (Rb) family of tumor suppressor proteins (p107, p110, and p130) have been termed “master regulators” of cell cycle progression and control transit through the G₁ growth phase by sequestering/inhibiting various transcription factors such as E2F (Gorgoulis et al., 1999). Rb mutations were originally identified in child retinoblastoma tumors (Larminie et al., 1998). When protein Rb (pRb) is hyperphosphorylated by specific CDKs, it releases the bound E2F transcription factors and cells can then advance through the cell cycle. Hypophosphorylated pRb is active and bound to the E2F transcription factors, thereby preventing the transcription of key genes and regulators of cell cycle progression resulting in quiescence or apoptosis. Alterations

of pRb are common in various cancers resulting in an inactive allele or alleles, and aberration in the function of pRb resulting in uncontrolled proliferation, growth, and survival. It is the disruption of this pathway that is a frequent occurrence in advanced cancers (Sherr, 1996, Larminie et al., 1998, and Zhao et al., 1997).

Apoptosis

Along with loss of cell cycle control, anti-apoptotic signaling can increase survival of cancer cells. Apoptosis, or programmed cell death, is a vital mechanism in multicellular development. This essential physiological process is required to eliminate unwanted cells in normal development and for maintenance of tissue homeostasis in mammals (Antonsson, 2001). In the absence of certain “survival signals”, cells activate a suicide program ultimately resulting in the cell’s own demise. The term apoptosis is of Greek origin and means “dropping off” or “falling off”, as in leaves falling off a tree. Upon induction of apoptosis, the dying cell shrinks, the chromatin condenses, DNA fragments, the mitochondrial membrane depolarizes, and membrane-bound blebs, called apoptotic bodies, pinch off. Also, phosphatidylserine (PS) is translocated from its normal position on the inner cell membrane to the outer cell membrane. The PS on the outer membrane then serves as a marker for surrounding cells to phagocytose the apoptotic bodies with virtually no immune response. This process of dying is very different from necrosis where the dying cell swells and bursts, releasing intracellular contents resulting in inflammation. There are several proteins involved in the apoptotic pathway, which will be discussed below.

The Bcl-2 Family Members

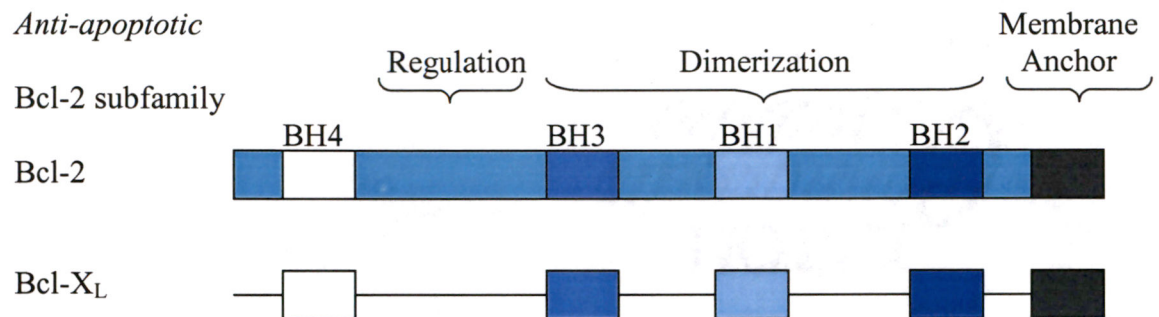
The Bcl-2 family of proteins has various anti-apoptotic members such as Bcl-2 and Bcl-x_L and pro-apoptotic members such as Bad, Bax, and Bid that are frequently altered in cancer cells (Figure 1). There are four Bcl-2 homology (BH) domains, BH1, BH2, BH3, and BH4. These motifs enable the different family members to form homo- or heterodimers in order to regulate their activities (Desagher and Martinou, 2000). The anti-apoptotic family members harbor at least three BH domains and are commonly referred to as the Bcl-2 subfamily. The pro-apoptotic family members are currently divided into two subfamilies, the Bax subfamily and the BH3 subfamily. The Bax subfamily consists of proteins with the BH1, BH2, and BH3 domains; they closely resemble the Bcl-2 subfamily members. The BH3-only subfamily of proteins contain only the BH3 domain and are commonly referred to as BH3-only proteins, the proteins Bid and Bad are in this subfamily. It is the BH3 domain that is necessary for the death promoting activity of the Bcl-2 family of proteins (Desagher and Martinou, 2000). Many Bcl-2 family members contain a C-terminal hydrophobic domain that targets them to the outer membrane of the mitochondria. Bcl-2 family members' activity at the mitochondrial membrane is a principle mechanism in regulating apoptosis (Desagher and Martinou, 2000).

Caspases are Executioners of Apoptosis

Caspases are cysteine proteases that cleave substrates at cysteine residues after specific aspartate residues and are considered the key effector proteins of apoptosis (Cryns and Yuan, 1998; Wolf and Green, 1999). All caspases contain an N-terminal

Figure 1. A schematic diagram of the Bcl-2 family of proteins as they pertain to this project. The Bcl-2 proteins are grouped into 3 subfamilies, the Bcl-2 subfamily, the Bax subfamily, and the BH3 only subfamily. The Bcl-2 proteins are grouped into these subfamilies based on the BH domain organization.

Anti-apoptotic



Pro-apoptotic

Bax subfamily



BH3 subfamily

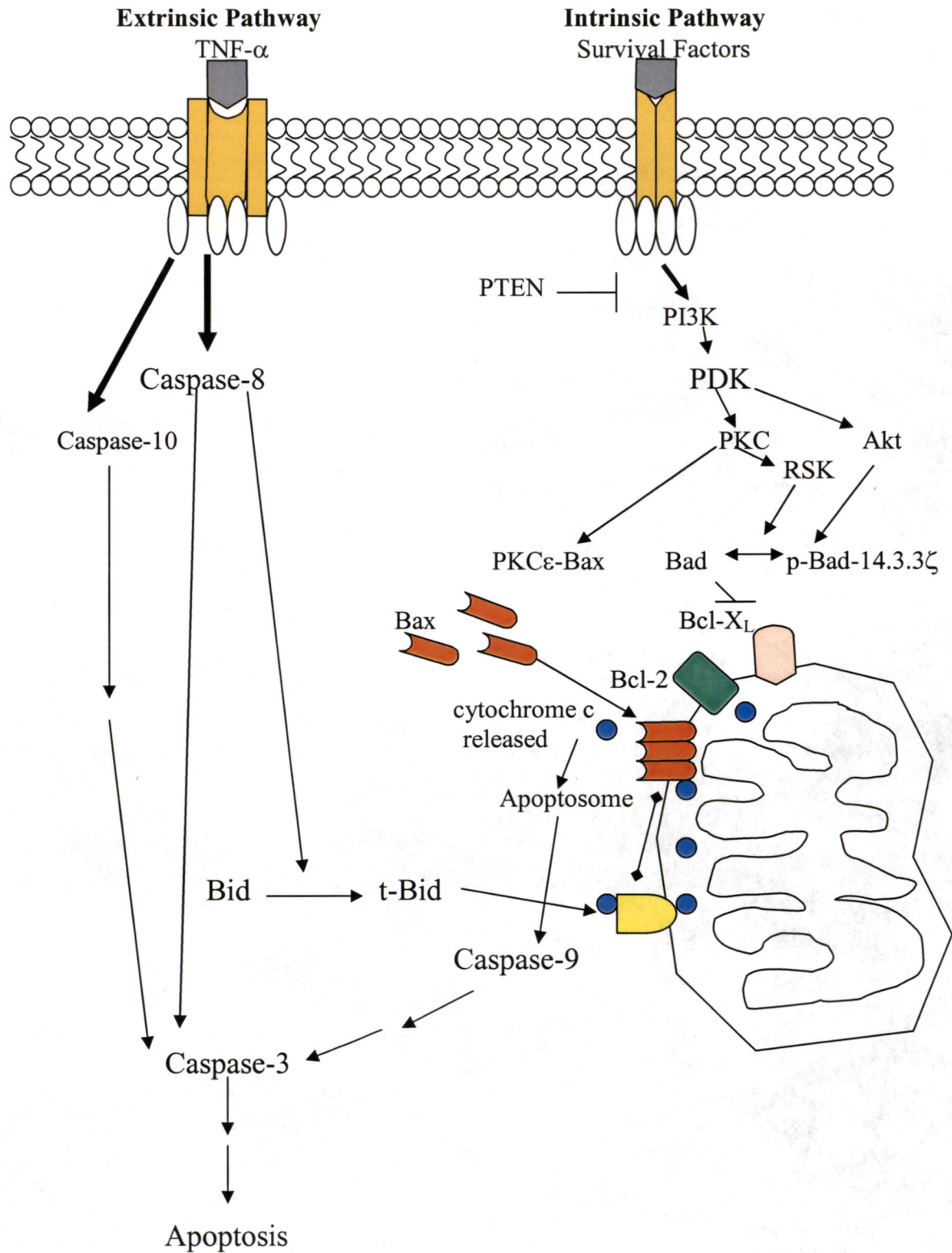


prodomain, a large subunit containing the active site cysteine within a conserved QACXG motif, and a C-terminal small subunit (Wolf and Green, 1999). Caspase prodomains vary in length and sequence (Wolf and Green, 1999). Caspases with long prodomains function as apoptosis initiators and some contain a sequence promoting their interaction with various adaptor proteins. The adaptor proteins have a death domain (DD) and a death effector domain (DED). The DD relays the death signal from the death receptor to the apoptosis initiator via the DED or in some cases a “caspase recruitment domain” (CARD). Caspases with short prodomains function as apoptosis executioners (Wolf and Green, 1999). The apoptosis initiators include caspase 2, 8, 9, and 10 and the apoptosis executioners include caspase 3, 6, and 7. The procaspases may be activated by autoactivation, transactivation, or proteolysis by other proteinases (Wolf and Green, 1999).

Apoptotic Pathways: Mitochondrial and Death Receptor

Activation of the apoptotic machinery can occur through at least two pathways, the extrinsic cell death pathway (death receptor) and the intrinsic cell death pathway (mitochondrial) (Figure 2). The extrinsic cell death pathway involves activation of the apoptotic machinery through death receptors where apoptosis occurs independent of the mitochondria via caspase 10 or 8. However, activation of caspase 8 may serve as an amplification loop through the protein Bid, which will be discussed later. The engagement of the extrinsic pathway results when the ligation of a death receptor such as tumor necrosis factor receptor 1 (TNFR1), TNFR2, Fas, death receptor (DR)4, and DR5 with an activating molecule like TNF, FasL (ligand), and TNF-related apoptosis-inducing

Figure 2. A schematic diagram of the apoptotic pathway as pertaining to this project. The apoptotic pathway has two arms, the extrinsic and the intrinsic. The intrinsic arm of the apoptotic pathway ultimately leads to the release of cytochrome c from the mitochondrial inner membrane space resulting in apoptosis. Cytochrome c, along with other pro-apoptotic factors, is liberated from the mitochondrial inner membrane space through pores that allow its passage along with other molecules smaller in size. Although a formations of these pores does not cause the break down of the mitochondria, they do affect the mitochondrial membrane potential. The extrinsic arm involves death receptors found at the cell surface. Ligation of these death receptors activates a cascade of events that ultimately leads to apoptosis. Apoptosis through the extrinsic arm can be independent of the mitochondria or it may require amplification through the protein Bid.



ligand (TRAIL) occurs. Upon activation of the extrinsic pathway, molecules at the inner plasma membrane of cells recruit caspases to relay the death signal. It is presently understood that there are type I and type II cells involving the extrinsic cell death pathway. In a type I cell, stimulation of the extrinsic (death receptor) pathway leads to direct cleavage and activation of effector caspases via caspase 8 or caspase 10 (Antonsson, 2001; Kulik et al., 2001). In a type II cell, stimulation of the death receptor pathway leads to activation of caspase 8, which then cleaves the pro-apoptotic protein Bid (Antonsson, 2001; Kulik et al., 2001). The carboxy-terminal fragment of Bid, t-Bid, then translocates to the mitochondrial membrane enhancing cytochrome c release (Antonsson, 2001). This is necessary to amplify the apoptotic signal of the death receptor pathway.

The intrinsic cell death pathway (mitochondrial) involves the release of cytochrome c from the intermembrane space of the mitochondria resulting ultimately in the activation of caspase 3. The intrinsic cell death pathway is induced by removal of survival factors, stress, and some anti-cancer drugs (Kulik et al., 2001). The release of cytochrome c from the intermembrane space is controlled by the Bcl-2 family members and appears to be a result of the outer mitochondrial membrane becoming permeable to cytochrome c and other pro-apoptotic factors such as apoptosis inducing factor (AIF), SMAC/DIABLO and procaspases (Degterev et al., 2001; Desagher and Martinou, 2000).

Mitochondria play a key role in coordinating apoptosis. Under normal conditions, the mitochondrial outer membrane is only permeable to molecules 1.5 kDa and smaller by permeability transition (PT) (Degterev et al., 2001). Under certain conditions of

stress, where the cell initiates apoptosis, larger pores are formed permitting the release of pro-apoptotic proteins contained within the intermembrane space of the mitochondria. These pro-apoptotic proteins are larger than 1.5 kDa and include cytochrome c, AIF, and procaspases (Degterev et al., 2001). When cytochrome c is released from the intermembrane space it promotes oligomerization of apoptotic protease-activating factor (Apaf-1) in the presence of ATP (adenosine triphosphate), and recruitment of procaspase 9 (Antonsson, 2001; Crompton, 2000). This complex is commonly referred to as the apoptosome. This triggers autocleavage and the activation of caspase 9. Caspase 9 cleaves procaspase 3, activating caspase 3, which ultimately leads to the morphological changes associated with apoptotic death. Caspase 3 is often termed the executioner caspase in that it can cleave a broad range of cellular targets ultimately leading to cell death.

The Release of Cytochrome c from the Intermembrane Space of the Mitochondria

Several members of the Bcl-2 family share a protein fold, of several alpha helices, that is similar to pore-forming domains of certain bacterial toxins (Waterhouse and Green, 1999; Desagher and Martinou, 2000; Antonsson, 2001; Pavlov et al., 2001). Also, several studies have shown that some Bcl-2 family members form ion-channels or pores in synthetic lipid membranes (Antonsson, 2001; Degterev et al., 2001; Pavlov et al., 2001). Patch clamp analysis of the mitochondrial membrane reveals the existence of mitochondrial channels during apoptosis (Pavlov et al., 2001). Two opposing theories exist for the release of cytochrome c from the intermembrane space of the mitochondria. One suggests the mechanism for the release of proteins from the intermembrane space of

the mitochondria is through the PT pore interacting with the voltage-dependent anion channel (VDAC) on the outer membrane and the adenine nucleotide transporter (ANT) on the inner membrane to form the permeability transition pore (PTP) (Desagher and Martinou, 2000; Waterhouse and Green, 1999). The PTP is a high-conductance, unselective mitochondrial mega-channel that allows passage of molecules greater than 1.5 kDa. Opening of the PTP, possibly controlled by Bcl-2 family members, causes a sudden influx of molecules through the inner membrane causing osmotic swelling (Desagher and Martinou, 2000). This, in turn, results in outer membrane disruption, which allows the release of the apoptotic proteins from the intermembrane space (Desagher and Martinou, 2000; Degterev et al., 2001). However, data shows that this mechanism may not be how cytochrome c is released from the mitochondria during apoptosis (Desagher and Martinou, 2000). Another model suggests that certain pro-apoptotic Bcl-2 family members directly form pores in the outer membrane of the mitochondria (Degterev et al., 2001). Investigators believe these pro-apoptotic proteins can selectively control the release of cytochrome c without drastically affecting mitochondrial function (Degterev et al., 2001). The Bcl-2-associated protein X (Bax) can be activated by the binding of BH3-only proteins resulting in an oligomeric form of Bax that has an increased channel forming capacity (Degterev et al., 2001). Upon induction of apoptosis, Bax oligomerizes and translocates from the cytosol to the outer membrane of the mitochondrion (Pavlov et al., 2001; Suzuki et al., 2000). Investigators have found a mitochondria apoptosis-induced channel (MAC) that would allow passage of apoptotic proteins from within the intermembrane space and it is thought that Bax may be what

forms this channel (Degterev et al., 2001; Pavlov et al., 2001). Unlike the VDAC, MAC is not voltage-dependent (Pavlov et al., 2001). However, investigators have found that while Bax channels are similar to MAC, the Bax channels are much smaller (Degterev et al., 2001; Pavlov et al., 2001). Moreover, it is thought that Bax is involved in opening of the MAC after apoptosis induction (Degterev et al., 2001; Pavlov et al., 2001).

Anti-apoptotic Signaling Can Increase Cancer Cell Survival

Other mechanisms of anti-apoptotic signaling are also present in cancer cells. Often with progressive cancers, anti-apoptotic proteins are increased in expression and therefore can successfully antagonize pro-apoptotic signals; this survival mechanism is one way that growing cancer cells gain resistance to conventional therapies. The pro-apoptotic signals can become sequestered and the anti-apoptotic signal is dominant in providing survival to the cell. With many cancers the anti-apoptotic protein Bcl-2 is overexpressed allowing the cells to survive anticancer treatments. Another mechanism for the induction of anti-apoptotic signaling is accomplished through Akt by the phosphatidylinositol 3'-kinase (PI3K)- Akt/PKB "survival" pathway being constitutively active due to the loss of its regulator PTEN (phosphatase and tensin homolog deleted on chromosome ten). PTEN is a lipid phosphatase that will be discussed below. With the loss of PTEN there is a build up of the inositol lipid second messengers resulting in constant activation of Akt through DAG-dependent protein kinase (PKC). This pathway blocks apoptosis at a level prior to caspase 3 activation through the phosphorylation (inactivation) of Bad (Carson et al., 1999) (Figure 2). It is through these mechanisms,

and ones yet to be discovered, that cancer cells survive to form tumors that can in turn metastasize.

Prostate Cancer: Epidemiology

One of the most deadly metastatic forms of cancer is prostatic adenocarcinoma (CaP), which in the year 2001 claimed the lives of an estimated 31,500 United States (US) males (Hsing and Devesa, 2001). The incidence of CaP is the highest among black American males in the US and lowest among males in China suggesting that environment and/or diet play a role in the progression of the disease. This devastating disease represents the most common form of non-cutaneous cancer in western men and is the second leading cause of cancer death in males within the US (Hsing and Devesa, 2001). There are only a few established risk factors, which include age, race, and a family history of the disease (Hsing and Devesa, 2001). The high rate of mortality attributed to CaP is due to its transition from an androgen-dependent (AD) and localized tumor to androgen-independent (AI) and metastatic cancer that has no proven treatment or cure (Abate-Shen and Shen, 2000). This clinical progression of CaP has compelled researchers to investigate the many genes implicated in the development of AI CaP including transcriptional regulators, oncogenes, cell signaling molecules, and tumor suppressor genes.

Progression to Androgen-Independence: Adaptation vs. Clonal Selection

The growth and survival of normal prostate epithelial cells depends on androgens, however, prostatic adenocarcinomas are comprised of AD and AI cells. When androgens are removed, most of the AD cells undergo apoptosis, but a few remain along with the AI

cells (Tang et al., 1998). Today two theories are in place to explain the emergence of a hormone refractory CaP, the adaptive mechanism and the clonal selection model.

Understanding these mechanisms will help devise proper treatments to eliminate the aggressive tumors. According to the adaptation theory, cells *acquire* the ability to grow and survive in an androgen-deprived environment possibly through genetic alterations, which parlay the resistance to cell death genes and intensify tumorigenicity (Tso et al., 2000). With the clonal selection model, the already existing AI cells are selected for survival resulting in an aggressive AI tumor. It is conceivable that a merging of the two models is what results in a deadly hormone refractory cancer.

Prostate Cancer Detection and Treatment

Clinically, the prostate specific antigen (PSA) test is how physicians detect the onset of prostate cancer along with the digital rectal examination and transrectal ultrasonography; the best defense against this aggressive cancer is early detection (Miller and Torrko, 2001). Upon testing the serum PSA, the physician looks for a rise in the levels of this antigen, increases in PSA indicate a growing prostate tumor. Typically, the treatment entails maximal androgen ablation by pharmaceutical or surgical means along with removing the prostate and surrounding lymph nodes (Miller and Torrko, 2001). With androgen ablation therapy there should be a decrease in PSA levels, however, within two years the rate of recurrence for human CaP is 70%. In the unfortunate 70%, several years after treatment, PSA levels rise again and the emergence of an AI tumor is imminent. Moreover, a correlation between prognosis and the PSA levels in patients who have been treated with rounds of androgen deprivation has been established (Sadar et al.,

1999). Those whose PSA levels continually decrease after androgen ablation treatment often show good prognosis in that their cancer typically responds to the treatment. Conversely, when PSA levels plateau following a spontaneous recurrence this denotes progression to an AI CaP and poor prognosis (Sadar et al., 1999).

Biology of Prostate Cancer Cells: Human Cell Lines

Various human cell lines have been established to study the biology of CaP cells including the PC-3, DU-145, and LNCaP cells. The PC-3 and DU-145 models are AI in growth and lack androgen receptors (AR), PSA, and 5 α -reductase; and when injected into nude mice produce poorly differentiated xenograft tumors (Russell et al., 1998). The LNCaP cell line, which is used for the majority of this project, was originally isolated from the lymph node of a patient with advanced CaP that has remained AD for growth in culture and poorly tumorigenic when infected into nude mice (Russell et al., 1998). These characteristics permit one to examine the progression from AD to AI human CaP cell growth. Early investigators recognized that any event(s) capable of sensitizing the AR to agonistic ligands would provide a simple and direct means of allowing CaP cells to continue growing in the presence of the residual steroids that persist following androgen ablation therapies.

Androgen Receptor Mutations and Alterations

The AR, which is considered an oncogene, is an area of intense research in the progression of CaP; it is a phosphoprotein that mediates the actions of testosterone and dihydrotestosterone (DHT) by acting as a transcription factor. The AR is a member of the steroid receptor superfamily and is composed of three major domains: an N-terminal

transcriptional activation domain, a central DNA-binding domain, and a C-terminal steroid binding domain. The AR is phosphorylated upon ligand binding, dimerizes and translocates to the cell nucleus where it binds specific DNA elements, androgen response elements (ARE), regulating the expression of downstream genes containing AREs such as Jun, Fos, CDKs and CKIs (Gorgoulis et al., 1999; Grossmann et al., 2001). While most abundant in male sex tissue, the AR is found in many tissues of both sexes. In CaP cells, the AR often becomes mutated allowing it to be activated in an androgen-deprived environment possibly by peptide growth factors, other hormones such as progesterone and estrogenic steroids, and/or by crosstalk with other signal transduction pathways (Amantullah et al., 2000; Russell et al., 1998; Brinkmann and Trapman, 2000; Lin et al., 1999). The ligand-activated AR in CaP cells acquires mutations in the C-terminal steroid binding domain which in turn makes AI activation possible and reduces the efficiency of androgen ablation therapy (Brinkmann and Trapman, 2000; Amantullah et al., 2000). Also, possible amplification of the AR gene results in a larger amount of ARs in the cell, which may affect androgen ablation therapy in that the small number of leftover circulating androgen may be enough to activate of the AR (Sadar et al., 1999; Gorgoulis et al., 1999). Another genetic alteration in the AR that leads to aberration in its function is the varying lengths of the CAG trinucleotide repeat sequence (Abate-Shen and Shen, 2000). The first exon of the AR gene contains a polymorphic trinucleotide repeat region (CAG) that encodes a polyglutamine tract localized in the N-terminal domain of the AR protein that positively regulates AR transactivation activity (Krithivas et al., 1999). Shorter AR polyglutamine tracts have been associated with increased CaP risk (Krithivas

et al., 1999). However, it is the promiscuity of the AR caused by point mutations in the ligand-binding domain that may subserve androgen ablation therapy because the AR can then be activated by molecules other than androgen.

Tumor Suppressor Genes and Prostate Cancer

Mutations in tumor suppressor genes also lead to deadly metastatic CaP; a few of these genes are PTEN, p53, p27 and the metastasis suppressor gene, KAI1. As mentioned earlier, PTEN is a dual function phosphatase that acts as a negative regulator of the PI3-kinase/Akt “survival” pathway. PTEN has been implicated in severe prostate cancer. Often with advanced CaP there is a loss of PTEN function resulting from homozygous deletions, point mutations, and promoter methylation (Isaacs and Kainu, 2001). This causes the PI3-kinase/Akt “survival pathway” to be constitutively activated due to increased accumulation of the PTEN substrate phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Amantullah et al., 2000). Also, the constitutive activation of either PI3-kinase or Akt is known to induce cellular transformation and seems to be a key event in the progression of CaP (Amantullah et al., 2000).

The p53 tumor suppressor protein may have a vital role in the progression of CaP due to increased frequency of mutations when compared to a lack of mutations in early stage CaP (Burchardt et al., 2001). p53 functions as a key apoptosis regulator and was the first tumor suppressor gene linked to apoptosis. Mutations in the p53 gene have also been linked with advanced stage CaP, increased dedifferentiation of cells, and the transition from AD to hormone refractory CaP (Konishi et al., 1997).

Another tumor suppressor protein, p27, is implicated in advanced CaP that functions as a negative regulator of cyclin D, E, and A dependent kinases (Tsihlias et al., 1999). p27 expression increases during times of cell stress causing cells to quiesce. Studies indicate that reduced levels of p27 are associated with a more aggressive CaP phenotype (Isaac and Kainu, 2001). The loss of p27 may also serve as an adverse prognostic marker since the possibility of an AI tumor emerging in these cases is greater (Tsihlias et al., 1999).

KAI-1 is a metastasis suppressor gene for CaP mapped to human chromosome 11p11.2-13 (Dong et al., 1995). In normal prostate cells, the expression of KAI-1 is much greater when compared to expression in cell lines derived from metastatic deposits (Dong et al., 1995). This down-regulation of KAI-1 during the recurrence of CaP does not involve mutation or allelic loss of the KAI-1 gene (Akita et al., 2000). Also, the downregulation of KAI-1 seems to play an important role in determining the clinical behavior of the CaP as well as prognosis due to the reduced efficiency of metastasis suppression (Akita et al., 2000).

Oncogenes and Prostate Cancer

There are several oncogenes that play a role in the progression of CaP including c-myc, Bcl-2, and AR and Rb, which have already been discussed. The c-myc oncogene is mapped to the 8q24 region on the human chromosome (Isaac and Kainu, 2000). The c-myc protein functions as a transcription factor regulating cellular proliferation, differentiation, and apoptosis. c-myc has been found overexpressed in recurrent CaP, but not in benign prostatic hyperplasia suggesting c-myc involvement in the progression of

the disease (Konishi et al., 1997). Bcl-2 is another established oncogene with implications in the progression of CaP. This anti-apoptotic gene is mapped to 18q21.3 on the human chromosome (Isaac and Kainu, 2001). Bcl-2 is located in the outer membrane of the mitochondrion and functions in blocking apoptosis by preventing the release of cytochrome c. Bcl-2 can protect cells against multiple cell death signals including TNF- α mediated cytotoxicity, growth factor withdrawal, androgen withdrawal, and γ - and UV-radiation (Herrmann et al., 1997). The overexpression of Bcl-2 has been observed in recurrent, AI tumors (Gao et al., 1999). Also, the forced overexpression of Bcl-2 in LNCaP cells by stable transfection leads to resistance to apoptosis and AI growth (Raffo et al., 1995). Because of these characteristics, Bcl-2 may be a good target for anti-cancer therapy (Isaac and Kainu, 2001).

Familial Clustering of Prostate Cancer

A family history of CaP implies higher risk of developing the disease. Therefore, investigators are looking for genes that may be involved in familial clustering of CaP. Segregation analysis suggests the existence of at least one rare dominant susceptibility gene or dominantly acting allele in hereditary prostate cancer (HPC) (Wu et al., 2001; Wang et al., 2001; Carpten et al., 2002). Studies have implicated several HPC susceptibility loci including HPC1 on chromosome 1 localized to 1q24-25, PCAP mapped to 1q42.2-43, CAPB on chromosome 1p36, HPCX localized to Xq27-28, HPC20 on chromosome 20, and most recently HPC2/ELAC2 on chromosome 17p (Wu et al., 2001; Wang et al., 2001). Studies using CaP kindred have focused on HPC2/ELAC2 and HPC1. With HPC2/ELAC2, two common polymorphisms (Ser217Leu and Ala541Thr)

exist along with germline mutations that may increase the risk for HPC (Rebbeck et al., 2000). Wang et al. (2001) screened 300 CaP patients from 150 families with HPC for germline mutations and examined the frequency of the two common polymorphisms. They detected a nonsense mutation in the HPC2/ELAC2 gene, however, their data suggest that germline mutations are rare in HPC and that the variants Leu217 and Thr541 do not appear to influence the risk of HPC (Wang et al., 2001). Wu and colleagues (2001) are focusing on the possibility of HPC2/ELAC2 functioning as a tumor suppressor gene where loss of heterozygosity (LOH) would increase the risk of HPC. These investigators hypothesize that deletion of 17p may be observed in some HPC cases, however, their data suggest that inactivation of the HPC2/ELAC2 gene via LOH is a relatively uncommon event in HPC (Wu et al., 2001).

In another study, dealing with HPC1, recombination mapping and candidate gene analysis was used to examine the critical region of HPC1, including the RNase L gene. RNase L protein mediates the antiviral and pro-apoptotic activities of the interferon-inducible 2'-5'-oligo (A) synthetase system (Carpten et al., 2002). Carpten and colleagues (2002) found germline mutations in microdissected tumors that showed LOH and loss of RNase L protein suggesting that this may be of diagnostic value in screening HPC patients.

Early Prognostic Indicators

While alterations in the expression and/or activity of several tumor suppressor genes and oncogenes have now been associated with the recurrence of AI CaP, relatively few such changes have been found to provide a reliable prognostic indicator of disease

progression in early stage adenocarcinoma. The potential clinical value of such molecular markers is abundantly clear. For example, with the biochemical signature of CaP cells that are equipped to survive in an AI manner, clinicians would be prepared to identify those patients most likely to benefit from androgen ablation therapies. Patients bearing primary tumors rich in CaP cells possessing these markers for AI survival may, ironically, be placed at an even greater risk if such therapies are employed. This is because androgen withdrawal would naturally lead to the clonal selection and propagation of the pre-existing, marker positive, AI CaP cells that are known to be responsible for the metastatic spread of this disease. While working in this laboratory, we identified such an early prognostic marker that reliably predicts the recurrence of AI CaP, the oncogenic protein, PKC ϵ .

Protein Kinase C Isoforms

PKC isozymes belong to the protein kinase “superfamily”, in which genes are related by virtue of their homologous kinase domains consisting of ~250-300 amino acid residues (Hanks and Hunter, 1995). While the PKC gene family codes for structurally and functionally diverse proteins, they do share a common origin. The PKC family of serine/threonine protein kinases consists of at least 10 isoforms acting in transmembrane signal transduction pathways. The family is subdivided into three main categories (conventional, novel, and atypical) based on their cofactor requirements. The conventional PKCs (cPKC; α , β_1 , β_2 , and γ) require negatively charged phospholipids, calcium (Ca²⁺), and diacylglycerol (DAG) or phorbol esters (*in vitro*) for activation (Cartee and Kucera, 2000). The novel PKCs (nPKC; δ , ϵ , θ , and η) also require

phospholipids and DAG for activation, but are Ca^{2+} independent (Cartel and Kucera, 2000). The activity of atypical PKCs (aPKC; ζ and λ/ι) is stimulated by PS; however, these kinases are Ca^{2+} and DAG independent (Cartel and Kucera, 2000). The isoforms PKC μ and ν were formerly thought to be a distinct class of PKC, however, the kinase core is most similar to calmodulin-dependent kinases (CaMK) so it was often included with the CaMK. However, recent evidence suggests that there is a distinct class of DAG-dependent protein kinases, PKD, which is composed of PKD μ , PKD ν , and PKD2 (Sturany et al., 2001).

Domain Organization of PKC Isozymes

The general structure of a PKC molecule consists of a single polypeptide chain that has a catalytic and a regulatory domain (Newton, 1997; Webb et al., 2000). The amino-terminal regulatory domain is around 20-70 kDa and the carboxy-terminal catalytic domain is approximately 45 kDa (Newton, 1997). The PKC molecule is composed of 4 conserved regions (C1-C4) with all isoforms having in common the C3 and C4 region in the catalytic domain. However, the organization of the regulatory domain differs between subcategories (Figure 3). The C1 domain binds DAG or phorbol esters (Newton, 1997). The C2 domain binds lipids and Ca^{2+} in the relevant isoforms (Newton, 1997). The C3 and C4 domains form the kinase domain and bind ATP and substrate, respectively (Webb et al., 2000; Newton, 1997).

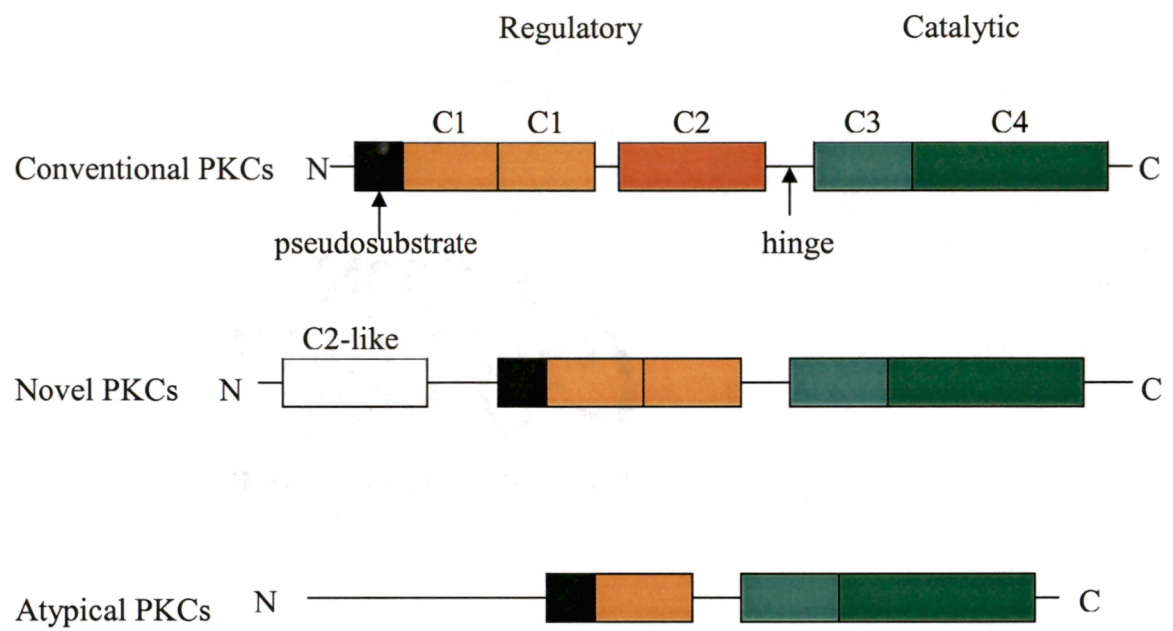
Patterns of PKC Expression in Prostatic Adenocarcinoma

The PKC isoforms α , β_1 , β_2 , δ , and ϵ , for the most part, are ubiquitously expressed throughout normal mammalian cells and tissues (Webb et al., 2000). However,

this pattern of expression can become altered upon transformation. What role PKCs play in prostatic carcinoma is of much interest due to the fact PKC activity is essential for the growth of AI CaP cells and causes apoptosis in AD CaP cells (Cornford et al., 1999). Cornford and colleagues (1999) hypothesize that during prostatic oncogenesis there is a loss or down-regulation of specific intracellular homeostatic mechanisms likely to involve particular isozymes within the PKC family. They found PKC δ expressed by smooth muscle and by dendritic reticulum cells in both benign and malignant tissues but not expressed in the epithelium in either case. PKC γ was also expressed in the stroma by nerve cells and their ganglia but not in the epithelium. Prostatic adenocarcinomas are derived from the glandular epithelial cells lining the ducts of this organ. Therefore, it is the PKC isozymes expressed by these cells that are of particular interest. The isoforms PKC ι and PKC μ , now PKD μ , were found to be expressed in the epithelia in both benign and malignant tissue but not in the stromal tissue of the prostate (Cornford et al., 1999). PKC θ was weakly expressed by invasive cells in three cases of CaP, but not found in any benign epithelium (Cornford et al., 1999). PKC δ was found ubiquitously expressed throughout benign and malignant tissues (Cornford et al., 1999). PKC β was strongly and uniformly expressed throughout the epithelium of the benign prostatic tissue samples (Cornford et al., 1999). However, this isoenzyme was only strongly expressed in three of the malignant cancer cases and was not detected in twelve other malignant cancer cases (Cornford et al., 1999). PKC ϵ was not expressed by any benign epithelia; however, it was expressed by glandular epithelial cells in 22 of the 23 samples of early stage CaP examined (Cornford et al., 1999).



Figure 3. A schematic diagram of the PKC family of proteins. The PKC isozymes are separated in the three distinct groups based on their requirements for activation. The cPKCs are Ca^{2+} , phospholipids, and DAG dependent; also, cPKCs contain 4 of the conserved regions. The nPKCs require phospholipids and DAG for activation and are Ca^{2+} -independent. nPKCs contain 3 of the 4 conserved regions, however nPKCs do have a C2-like domain. The aPKCs activation is independent of both DAG and Ca^{2+} and aPKCs contain 3 of the 4 conserved regions.



The Pro-Apoptotic Effects of Phorbol Esters in LNCaP cells are Mediated by PKC α and δ

Several laboratories have studied the role of the PKC α isozyme in LNCaP human CaP cells and found the isozyme to have a pro-apoptotic effect (Gschwend et al., 2000; Garci-Bermejo et al., 2002). PKC δ has also been implicated in phorbol-12-myristate-13-acetate (PMA)-induced apoptosis of LNCaP cells (Lorenzo et al., 1997; Fujii et al., 2000; Majumder et al., 2000). These investigators conclude that it is the membrane translocation of the PKC α and δ isozymes that results in LNCaP cell death (Lorenzo et al., 1997; Fujii et al., 2000; Majumder et al., 2000). Gschwend et al. (2000) found that prolonged activation of PKC α is necessary for the induction of apoptosis in LNCaP cells by PKC activators such as PMA. Investigators have established that PKC α is a mediator of PMA-induced apoptosis in LNCaP cells (Powell et al., 1996; Gschwend et al., 2000). Also, data suggests that the prolonged activation of PKC α along with the prolonged activation of the mitogen-activated protein kinase (MAPK) pathway may be required for induction of apoptosis in LNCaP cells (Powell et al., 1996; Gschwend et al., 2000). Investigators have also found PKC α and PKC δ to be downregulated in PMA-resistant LNCaP cells; a cell line established by continuous passage in culture of cells in presence of 10nM PMA (Powell et al., 1996). Fujii and colleagues (2000) have demonstrated that overexpression of PKC δ induces apoptosis in LNCaP cells. Another study of PKC δ demonstrated that, upon PMA treatment, PKC δ translocates to the mitochondria, where it appeared to be involved in cytochrome c release (Majumder et al., 2000). In human U-937 cells (lymphoma, histiocytic cells) and MCF-7 cells (breast cancer cells), PMA

treatment results in the translocation of PKC δ from the cytosol to the mitochondria (Majumder et al., 2000). Treatment of U-937 cells with rottlerin, an inhibitor of PKC δ activity, resulted in attenuation of PMA-induced apoptosis (Majumder et al., 2000). Furthermore, rottlerin significantly blocked cytochrome c release in PMA treated U-937 cells (Majumder et al., 2000). All of these studies taken together suggest that multiple PKC isozymes play a role in the induction of apoptotic cell death.

PKC ϵ : An Oncogenic Protein Upregulated in Early Prostatic Tumors

An oncogene by definition is a gene with the capacity of inducing oncogenic transformation. PKC ϵ is found to be naturally upregulated in early stage intraepithelial neoplasm of the prostate (Cornford et al., 1999). PKC ϵ appears to function as a dominant oncogene in LNCaP cells, in that this protein alone is sufficient to initiate AI growth (Wu et al., 2002). Also, LNCaP cells overexpressing PKC ϵ rapidly form xenograft tumors when injected sub-cutaneously into male nude mice, whereas the parental cell line is non-tumorigenic (Wu et al., 2002). Moreover, LNCaP cells overexpressing PKC ϵ form tumors more rapidly in castrated than in intact male nude mice (Wu et al., 2002). Our lab has found that PKC ϵ co-immunoprecipitates with caveolin-1 and Raf-1 while indirectly promoting ERK activation and a deregulation of cell cycle progression (Wu et al., 2002). We have now established that PKC ϵ overexpression in LNCaP cells results in the expulsion of caveolin-1 from cells, an anti-apoptotic protein, and hyperphosphorylation of protein kinases in the MAPK signaling cascade (Wu et al., 2002).

Thesis Statement and Hypothesis

The present study focuses upon the role of a known oncogenic protein in the advancement of CaP. The overall goal of this work was to test the hypothesis that the oncogenic protein, known as PKC ϵ , has the potential to actively oppose the pro-apoptotic effects of signals transduced by PKC α and δ in CaP cells and to promote the AI survival of these cells in the presence of apoptotic stimuli.

MATERIALS AND METHODS

Materials

All the materials used were of the highest grade available and were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. Antibodies purchased from Santa Cruz (Santa Cruz, CA) were raised against Bax (P-19): sc-526, Bcl-2 (100): sc-509, c-myc (C-19): sc-788, caspase-8 p20 (H134): sc-7890, and 14-3-3 ζ (C-16): sc-1019. The secondary antibodies used were conjugated donkey-anti-goat IgG-horseradish peroxidase purchased from Santa Cruz and anti-rabbit IgG-horseradish peroxidase and anti-mouse IgG-horseradish peroxidase from Amersham (Piscataway, NJ). All antibodies were specific for human. An anti-mouse IgG (whole molecule) was purchased from Sigma Chemical Co. The caspase 3 assay kit was purchased from Pharmingen (San Diego, CA). A mitochondrial membrane potential dye (JC-1) was purchased from Molecular Probes (Eugene, OR). TNF- α (recombinant protein expressed in *E.coli*) was purchased from Upstate Biotechnology (Lake Placid, NY). Antisense ODN for Bax and PKC ϵ were purchased from Invitrogen Life Technologies (Rockville, MD). Sequences used were: antisense Bax, 5'-TCG ATC CTG GAT GAA ACC CT-3' and 5'-TCC CCC CCC ATT CGC CCT GC-3'; and scrambled Bax, 5'-TCA GTC CTG GTA GAA CAC CT-3' and 5'-CTC CCC CCA CTT CGC CTC GC-3' (Dibbert et al., 1999 and Kim et al., 2001). The PKC ϵ sequences used were as exactly specified (Wu et al., 2002).

Cell Lines and Culture Conditions

The LNCaP cell line was obtained from the American Type Culture Collection (Rockville, MD; ATCC CRL-1740). All tissue culture reagents were purchased from Invitrogen Life Technologies (Rockville, MD). LNCaP cells were maintained as adherent cells in culture in RPMI-1640 media 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% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin, which will be referred to as complete media. The cells were kept at low passage and were split 1:3 by trypsinization before reaching monolayer to prevent unintended mutations. All cells were housed in a 37°C 5% CO₂ incubator in culture.

Expression Plasmid and Transfection into LNCaP Cells

LNCaP cells were infected with pLXSN recombinant retrovirus alone (LNCaP/v) or pLXSN harboring the gene for p3/PKC ϵ (LNCaP/ ϵ) and subcloned (LNCaP/ ϵ 3) as previously described (Wu et al., 2002). Cells were selected and subcloned for stable expression by limiting dilution in 500 μ g/ml Geneticin® (Gibco). Subclones were screened for PKC ϵ protein expression and *in vitro* kinase activity as reported earlier (Wu et al., 2002).

Co-immunoprecipitation Assays

LNCaP variants (LNCaP, LNCaP/v, LNCaP/ ϵ , and LNCaP/ ϵ 3) were grown to 70% confluency on 150 mm tissue culture plates in complete media [two plates per cell line per condition]). Growth media was then aspirated off and replaced with either complete media or complete media containing 100 nM PMA. Cells were incubated for a

specified time before the media containing cells that have detached from the plate, were transferred to labeled 50 ml tubes. The plates were washed twice with 15 ml cold phosphate buffered saline (PBS); (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.1 mM KH₂PO₄). The PBS was transferred to the corresponding 50 ml tube containing anoikis cells. The detached cells were sedimented at ¼ speed on an IEC HN-SII centrifuge (clinical centrifuge) for 5 minutes. The supernatant was aspirated off and the pellet was resuspended in 2.2 ml of cold immunolysis buffer (ILB); (150 mM NaCl, 1% IGEPAL CA-630, 20 mM Tris pH 8.0, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mg/ml aprotinin, 1 mg/ml leupeptin, and 2 µM pepstatin). Next, the pellet resuspended in ILB was transferred to the corresponding 150 mm tissue culture plate containing attached cells. The 150mm tissue culture plates were placed on an orbital shaker at 4 °C for 30 min. Next, the lysed cells were pooled at the bottom edge of the plate using a cell scraper and were triturated ten times using a 25-g needle to assure all cells were lysed. Following this, 1 ml aliquots of lysate were transferred to labeled microtubes and spun at 4 °C for 10 min at 12,000 rpm. The supernatants (lysates) were pooled into labeled 15 ml tubes and the lysates were precleared of proteins by adding 60 µl of protein A agarose (Gibco Life Technologies). Each lysates was incubated on a spinning wheel in the cold room for 10 min before centrifugation on a IEC HN-SII centrifuge at ¾ speed for 5 min. Next, 1 ml aliquots of the supernatant were transferred to prechilled microtubes with screw tops. Three micrograms of primary antibody were added to each sample and the controls received 3 µg of anti mouse IgG. Samples were incubated on the wheel in the cold room overnight. The next day, 50 µl of protein A agarose was added to each sample

and the precleared lysates were incubated on a wheel in the cold room for 2 h. The samples were then spun on a microcentrifuge kept at 4°C at 12,000 rpm for 5 min and the supernatants discarded. Aliquots (1 ml) of ILB (minus leupeptin) were added to the immunoprecipitates (IP) and the samples were vortexed to resuspend the agarose. The samples were spun for 5 min in the cold room at 12,000 rpm and the supernatant discarded. This resuspension and spinning (washing) of the samples was repeated two more times. The IPs were resuspended in 50 µl of hot 2 x Western stop: 60m M Tris HCL, pH 6.8 7.5% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 100 µM dithiothretol (DTT) and 2% (w/v) SDS. The samples were boiled for 3 min using a heat block at 110 °C, then, pelleted for 1 min at 14,000 rpm on a Micro14 microcentrifuge (Fisher Scientific). The supernatants (IPs) were transferred to a fresh tube and the pellet discarded.

Western Blot Analysis

Co-immunoprecipitates (co-IPs) and cell lysates were loaded onto 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Protein concentrations of samples, except IPs, were determined using the Bradford protein assay (Bio-Rad, Hercules, CA) to assure equal loading (equal amounts of protein) of the lanes. Equal loading for IPs was assessed by comparing the heavy chain bands. The gel was electrophoresed at 200 volts for 1 to 1.5 h in 4 °C reservoir buffer (25 mM Trizma base, 197 mM glycine, and 3.5 mM SDS, pH 8.3). The proteins were then transferred to a nitrocellulose membrane in 4 °C transfer buffer (25 mM Trizma base, 192 mM glycine and, 20% (v/v) methanol) at 100 volts for 1 hour. The nitrocellulose was placed in TBS-

BLOTTO (5% powdered milk in TBS: 20 ml 1M Tris-HCl and 18 g NaCl in 2 L dH₂O) for blocking, 1 h at 4 °C. Next, the blocking solution was poured off and replaced with primary antibody solution (recommended antibody concentration in TBS-BLOTTO). The nitrocellulose was incubated for at least 18 h at 4 °C on an orbital shaker. Following the incubation, the nitrocellulose was washed in TBS and TBS-tween 20 (500 µl of polyoxyethylenesorbitan monolaurate in 1 L TBS). Next, the nitrocellulose was placed in a fresh box and incubated with secondary antibody (recommended antibody concentration in TBS-BLOTTO) for at least 1 h. The nitrocellulose was washed with TBS and TBS-tween 20 again, then, placed in fresh TBS to carry to the dark room. Proteins were detected on film using the ECL detection solution (0.1 M Tris-HCl, 0.68 mM p-coumaric acid, 100 mg/ml luminol in DMSO, and 3% hydrogen peroxide added in the dark room just before detection).

Cellular Proliferation Assays

Cellular proliferation was detected using the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT was dissolved at a concentration of 5 mg/ml in RPMI-1640 without phenol red as a stock solution. The LNCaP variants were seeded at 500 cells/well in RPMI containing 10% charcoal-dextran treated (CDT)-FBS (three wells per cell line skipping rows between different cell lines, five plates, one for each day measured) Cells were allowed to attach for 72 h at 37 °C in a 5% CO₂ incubator before taking measurements. At the specified time point (day 3 through day 7), 10 µl of MTT stock solution was added to each well (1/10 volume in the well and incubated at 37 °C in a 5% CO₂ incubator for 3.5 h). MTT is converted to an insoluble

purple formazan product by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes. Following the incubation, the media was carefully aspirated not to remove any of the purple product. The purple product was solubilized with 50 μ l of acidic isopropanol. The plates were shaken vigorously for 2 min on an orbital shaker. The optical density of each sample was measured using a Power wave 200 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT) with a primary wavelength of 570 nm and a secondary wavelength of 690 nm. This was calculated as the percent change from the 0 day plate.

Cellular Viability Assays

LNCaP variants were seeded on 100 mm plates at 50% confluency (3 plates for control for each cell line and 3 plates for treated cells for each cell line). Cells were allowed to attach for 24 h at 37 °C in a 5% CO₂ incubator. Following this, the growth media was aspirated off and replaced with complete media or complete media containing 100 nM wortmannin or 100 nM PMA or 10 nM bryostatin-1 or a combination of 10 nM bryostatin-1 and 100 nM PMA. Cells were incubated for 24 hours in the treatment solutions or complete media (for controls). Following the incubation, cells were tested for viability using trypan blue exclusion. The viable cells were cells that excluded the trypan blue dye and, therefore, remained clear. Dead cells took up the trypan blue and appeared blue in color. Cells were counted using a hemocytometer, counting the four corner squares and the inside square of the hemocytometer to give the number of dead and viable cells per ml.

Acridine Orange/Ethidium Bromide Assays

LNCaP variants and CWR-R1 cells were seeded at $3.0\text{--}4.1 \times 10^4$ cells/well on 24 well plates in complete media. Cells were allowed to attach for 24 h at 37 °C in a 5% CO₂ incubator. The media was aspirated off and replaced with complete media or complete media containing 100 nM thymeleatoxin or 100 nM PMA and the cells were incubated for 24h. Following the incubation, the media containing anoikis cells was transferred to labeled microtubes. The wells were washed with PBS and the PBS was transferred to the tube containing the corresponding anoikis. Next, the attached cells were trypsinized with 300 µl of 1X warm trypsin (10 ml trypsin, 90 ml PBS) for 5 min or until cells detached. The trypsinized cells were transferred to the corresponding tube with the detached cells. The wells were washed again with PBS to assure all cells had been transferred to the tubes. The cells were sedimented for 10 min at 1,000 rpm using a Sorvall RT6000B refrigerated centrifuge set at 4 °C. The supernatants were poured off and the tubes were touched to a paper towel leaving about 50 µl of backwash. The cells were resuspended using the backwash. Then, 2 µl of working dye solution was added to each tube. The working dye solution is 0.1 mg/ml acridine orange and 0.1 mg/ml ethidium bromide dissolved in PBS and stored at 4°C covered with foil. The samples were placed on ice and covered to protect from light until ready to view. When ready to view, 10 µl of sample was dispensed on to glass microscope slides and covered with a square glass coverslip, two samples per slide. Samples were viewed using a Nikon Microphot-FX microscope equipped with Nikon super high pressure mercury lamp power

supply model HB 10101AF using a fluorescein isothiocyanate (FITC) filter. The cells were counted as either apoptotic or non-apoptotic, counting a total of 100 cells per sample.

In another experiment using this staining method, the LNCaP variants were seeded at 50-60% confluency in complete media on 24-well tissue culture plates. The cells were allowed to attach for two days at 37 °C in a 5% CO₂ incubator. Following this incubation, the media was aspirated off and replaced with serum-free (0.5% FBS) RPMI. The cells were incubated with these conditions for 24 h at 37 °C in a 5% CO₂ incubator. The following day the cells were treated with 3 µg/ml cyclohexamide for 30 min. Next, 10 ng/ml of TNF-α or 25 µM LY294002 was added to the designated wells and the plates were incubated for 12 h at 37 °C in a 5% CO₂ incubator. Following this treatment, the cells were analyzed using the acridine orange/ethidium bromide staining method.

Caspase 3 Proteolytic Activity Assays

LNCaP variants were seeded at 30-50% (about 25,000 cells) confluency on 100 mm plates and allowed to attach for 24 h at 37 °C in a 5% CO₂ incubator. The media was aspirated off and replaced with either complete media or complete media containing 100nM PMA and incubated for 6 h. Following the incubation, the media containing the detached cells was transferred to a labeled 15 ml tube and sedimented for 5 min on a clinical centrifuge. While sedimenting the anoikis, the attached cells were trypsinized with 1ml warm 1x trypsin for 5 minutes and no longer than 10 minutes. While waiting for the cells to detach, the supernatants were aspirated off the sedimented anoikis pellets and the tubes were placed on ice. The trypsin was diluted on the attached cells by pipetting 1 ml

of complete media around the plate. The cell suspension was then transferred to the corresponding tube containing the anoikis. The suspension was pipetted gently up and down to resuspend the anoikis pellet into the cell suspension. Next, a cell count was taken and 1×10^6 cells were transferred to labeled microtubes. The cells were sedimented at $200 \times g$ for 10 min at 4°C and the supernatant discarded. Using the caspase 3-assay kit (PharMingen), caspase 3 proteolytic activity was measured. The cells were resuspended in $50\ \mu\text{l}$ of the chilled Cell Lysis Buffer and the cells were incubated on ice for 10 min. Next, the cell lysates were centrifuged in a cold room at $12,000\ \text{rpm}$ for 3 min and the supernatants were transferred to fresh microtubes. Aliquots ($50\ \mu\text{l}$) of the 2X Reaction Buffer containing DTT (7 mM) was added to each sample. Then, $7.5\ \mu\text{l}$ of the 1 mM conjugated substrate was added to each tube and the tubes were incubated for 1 h in a 37°C water bath. A negative control was set up containing just the reaction mixture and no cell lysate. Samples were read using a 96-well plate reader with a excitation wavelength of 400 nm and emission wavelength of 505 nm.

Assay of Mitochondrial Membrane Potential in Individual Cells

LNCaP variants were seeded at 70% confluency on 100 mm plates and allowed to attach for 24 h in a $\text{CO}_2\ 37^\circ\text{C}$ incubator. Media was then aspirated off and replaced with complete media or complete media containing 100 nM PMA and incubated for 3 h in a $\text{CO}_2\ 37^\circ\text{C}$ incubator. Following the incubation, the media containing detached cells was transferred to labeled 15 ml tubes. The detached cells were sedimented for 5 min at $200 \times g$ and the supernatant discarded. While sedimenting the anoikis, the attached cells were trypsinized with 1 ml warm 1X trypsin for 5 min and no longer than 10 min. Next,

the trypsin was diluted with 1 ml of complete media pipetted around the plate. The cells were transferred to corresponding tube containing the anoikis and pipetted gently up and down to resuspend the anoikis cells. The cells were sedimented to remove the trypsin and the cells were resuspended in $\frac{1}{2}$ volume complete media. The tubes were placed on ice. Cell counts were performed on each sample and 0.5×10^6 cells/ml were transferred to microtubes, the volume was adjusted to 1 ml using complete media. Next, 1.3 μ l of the 7.7 mM JC-1 stock was added to each sample for a final concentration of 10 μ M. The samples were incubated in the dark for 30 min at room temperature. The cells were then washed twice in cold PBS and resuspend in a total volume of 400 μ l PBS and transferred to flow cytometry tubes. Cells were analyzed for mitochondrial depolarization using a Becton Dickinson FACScan flow cytometer machine and the CELL Quest 3.2.1 analysis program. Cells were not gated on due to spread out cell population and 10,000 cells were counted per sample and samples were counted in triplicate.

Antisense Oligodeoxynucleotide Assay to Block Protein Expression

LNCaP cells and CWR-R1 cells were cultured in complete media until 80% confluent. Growth media was aspirated off and attached cells were trypsinized for 5 minutes. The trypsin was diluted with an equal volume of complete media, then, the cell suspensions were transferred to 15 ml tube. At this point, a cell count was obtained as described earlier. 4×10^4 cells/0.5 ml were transferred to a 24-well tissue culture plate. The cells were allowed to attach for two d. Next, the medium was aspirated off and replaced with 0.5% FBS RPMI media. The cells were serum starved overnight. The following day, the wells were washed with OPTI-MEM I media at 37 °C, then, 1 ml of

ODN/lipofectin (Invitrogen Life Technologies, Rockville, MD) mixture was added to the designated wells. The phosphorothioate Bax ODNs were at a final concentration of 5 μ M, the phosphorothioate PKC ϵ ODN was at a concentration of 1 μ M and the lipofectin (in both cases) was at a concentration of 2 μ g/ml. Sequences for the antisense Bax and corresponding scrambled control ODNs were specified above. Sequences for the antisense PKC ϵ ODN and corresponding scrambled control ODN were exactly as specified (Traub et al, 1997). The cells were incubated in this mixture for 6 h at 37 °C. Next, the ODN/lipofectin mixture was aspirated out of the wells and the wells were washed with OPTI-MEM I media. Then, complete medium was added to each well along with 5 μ M ODN with no lipofectin. The cells were incubated for 18 h at 37 °C. The next day, additional ODN (5 μ M) was added to each well and the cells were incubated at 37 °C over the weekend. Following this incubation, the designated wells were treated with PMA for 24 h. The cells were then analyzed for apoptosis using the acridine orange/ethidium bromide staining method.

RESULTS AND DISCUSSION

Hormone refractory prostatic adenocarcinomas typically arise following a prolonged two-step process that is precipitated by androgen ablation therapy. During the initial transition phase, androgen withdrawal leads to the apoptosis of secretory epithelial cells due to their strict requirement for androgens, while basal epithelial cells become growth arrested (Craft et al., 1999). Tumor regression during this dormant period is often pronounced. There is now evidence that preexisting AI CaP cells are gradually selected for in the transition phase as a result of their unique ability to continue to proliferate in the absence of circulating testicular androgens (Craft et al, 1999). The second “recurrent” phase of tumor progression may arise from this process of clonal selection, leading to the regrowth of AI tumors that are no longer susceptible to androgen ablation or conventional chemotherapies.

Our preliminary analysis of LNCaP cells that were forced to stably overexpress PKC ϵ indicates that this oncoprotein is capable of transforming an AD CaP cell line into an AI variant that recapitulates many of the hallmark features of a recurrent CaP cell (Wu et al., 2002).

PKC ϵ confers AI proliferation in LNCaP cells.

The MTT assay was used to track the rate of cellular proliferation in the absence and presence of steroid hormones by substituting charcoal-dextran treated fetal bovine serum (CDT-FBS) for untreated FBS. The effects of PKC ϵ overexpression were evaluated by comparing the proliferation rates of the parental LNCaP cell line to that of the geneticin-resistant LNCaP cells harboring either an empty pLXSN retroviral vector

(LNCaP/v) or one containing the full-length PKC ϵ cDNA (LNCaP/ ϵ). Because the LNCaP cell line was originally derived from a metastatic lesion it is inherently heterogeneous and, thus, so is the LNCaP/ ϵ derivative. Therefore, a subclone of the LNCaP/ ϵ line was established by limiting dilution and is designated LNCaP/ ϵ 3. Upon removal of steroid hormones, LNCaP and LNCaP/v cells became quiescent and no longer proliferated (Figure 4). However, LNCaP cells overexpressing PKC ϵ (LNCaP/ ϵ and LNCaP/ ϵ 3) continued to grow and proliferate in this condition (Figure 4). These data indicate that PKC ϵ enabled LNCaP cells to proliferate in an AI manner. Two hallmark features of most cancer cells are an uncontrolled proliferation that is coupled with enhanced survival. Together, these phenotypic changes lead to the rapid growth of tumors *in vivo*. Because these data confirmed that PKC ϵ conferred AI proliferation, the next question was whether PKC ϵ had also enhanced the survival of LNCaP cells.

PKC ϵ enhances the survival of LNCaP cells.

A trypan blue exclusion assay was used to count viable and dead cells. PMA, a phorbol ester, is a PKC agonist in that it mimics the effects of DAG and activates novel and conventional PKCs. Within 24 h, over 80% of the parental (LNCaP) or vector control (LNCaP/v) cells gradually became non-viable (dead) upon treatment with PMA (Figure 5). However, LNCaP/ ϵ and LNCaP/ ϵ 3 cells effectively resisted the death promoting effects that PMA had on LNCaP cells (Figure 5). These preliminary trypan blue exclusion assays provided a simple measure of cell viability, but did not distinguish between apoptotic and necrotic causes of cell death. Several laboratories have

Figure 4. PKC ϵ overexpression results in AI growth of LNCaP cells. LNCaP ($\circ$), LNCaP/v ($\square$), LNCaP/ ϵ ($\bullet$), and LNCaP/ ϵ 3 ($\blacksquare$) were grown in CDT 10% FBS RPMI media for seven days. Cell proliferation was measured starting at day 3 using the MTT assay. Data are the means ($\pm$ SE) of triplicate determinations of three independent experiments.

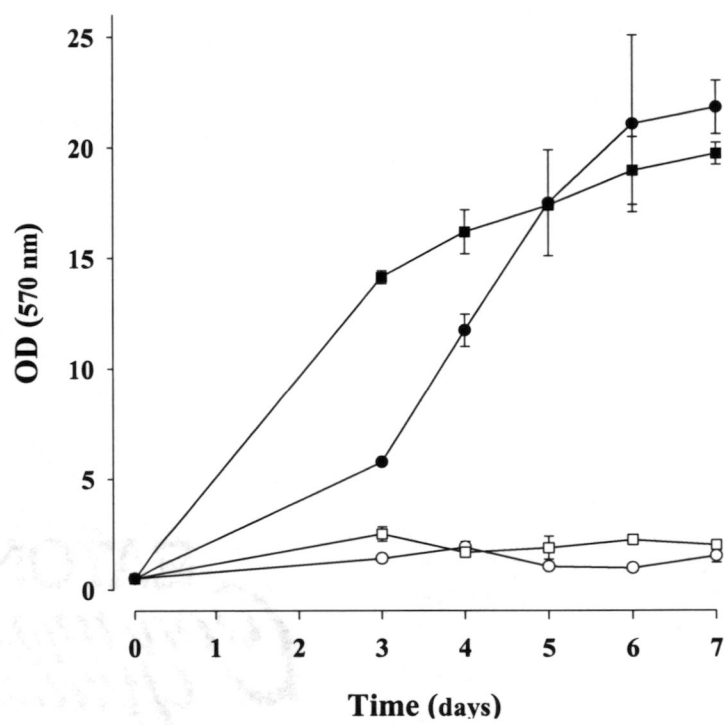
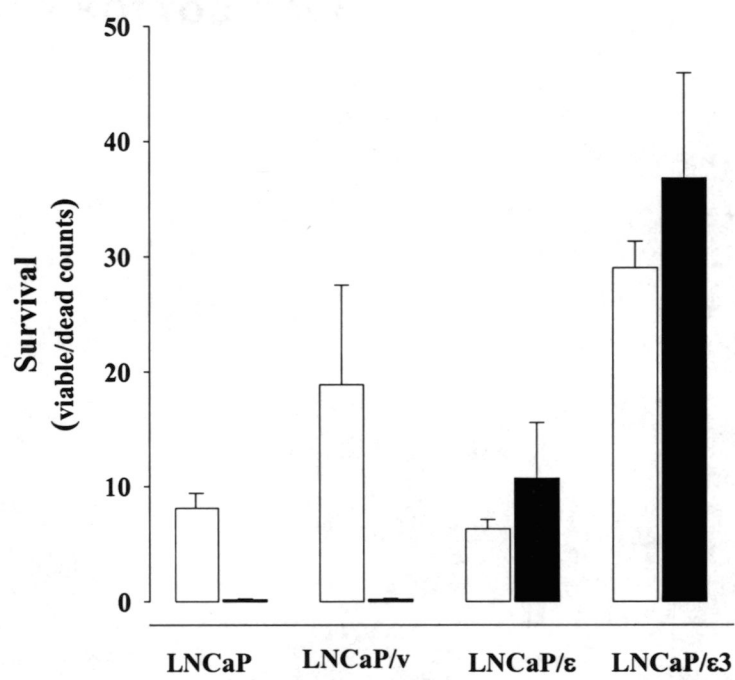


Figure 5. LNCaP cells overexpressing PKC ϵ have increased survival when compared to parental cells. LNCaP, LNCaP/v, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells were grown in complete medium to 80% confluency. Growth media was aspirated off and replaced with either RPMI-1640 10% FBS (open bars) or RPMI-1640 10% FBS containing 100nM PMA (closed bars) and cells were incubated for 24 h in a CO₂ 37°C incubator. Viable cells were counted on a hemocytometer using trypan blue exclusion and data are the means ($\pm$ SE) of triplicate determinations in three independent experiments.



independently confirmed that PMA induces apoptosis in LNCaP cells (Powell et al., 1996; Lorenzo et al., 1997; Fujii et al., 2000; Majumder et al., 2000). It, therefore, seemed reasonable to anticipate that the enhanced survival of LNCaP/ε and LNCaP/ε3 cells observed in the presence of this apoptogenic agent could be attributed to an escape from this cell fate. To directly address this important issue, we reevaluated the effects of PMA on LNCaP survival using a well established assay of apoptosis.

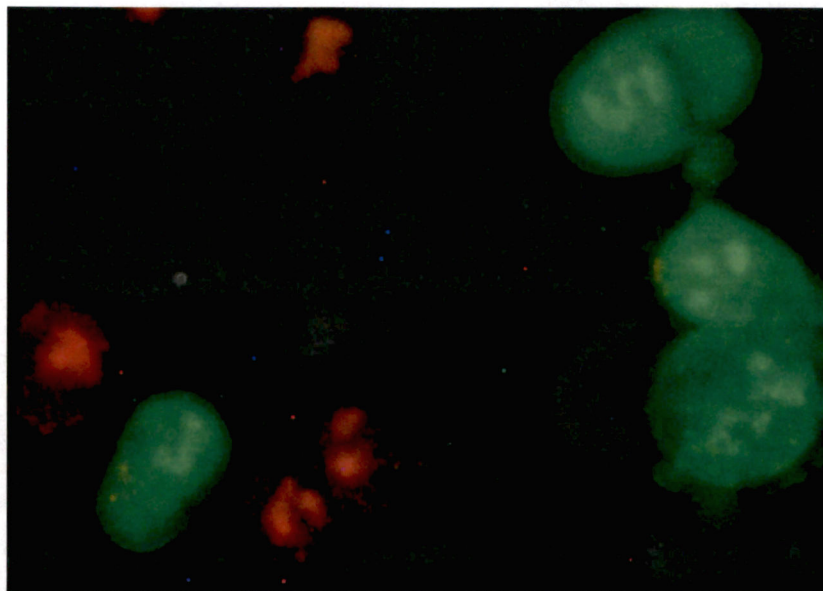
PKCε blocks the PMA-induced apoptosis of LNCaP cells.

Apoptotic morphology can be reliably detected using the DNA binding fluorochromes acridine orange and ethidium bromide (AO/EtBr). A live normal cell stains green with a uniform round nucleus and darker staining of the nucleosomes; a dead normal cell displays the same appearance but stains orange (Figure 6A). An apoptotic LNCaP cell has an irregularly shaped nucleus with chromatin condensed on the edge or the cell appears to have dark, condensed staining in the center along with being irregular in shape (Figure 6B). Apoptotic cells are often smaller than normal cells (S.M. Oberhaus, personal communication).

When treated with PMA for 24 hours, the vast majority of LNCaP cells display this apoptotic morphology, with about 75% of the parental cells becoming apoptotic when compared to untreated cells (Figure 7). This observation was consistent with earlier reports (Fujii et al., 2000; Majumder et al., 2000). Here we report that PKCε completely blocked the apoptotic effect that PMA has on LNCaP cells. LNCaP/ε and LNCaP/ε3 cells were significantly more resistant to apoptosis than the parental cells (Figure 7). Moreover, these cells rarely became apoptotic in response to PMA

Figure 6. (A) AO/EtBr staining of normal live (green) and dead (orange) LNCaP cells maintained in complete media. (B) AO/EtBr staining of apoptotic live (green) and apoptotic dead (orange) LNCaP cells following a 24 h incubation in complete media containing 100 nM PMA. 200X magnification.

A.



B.

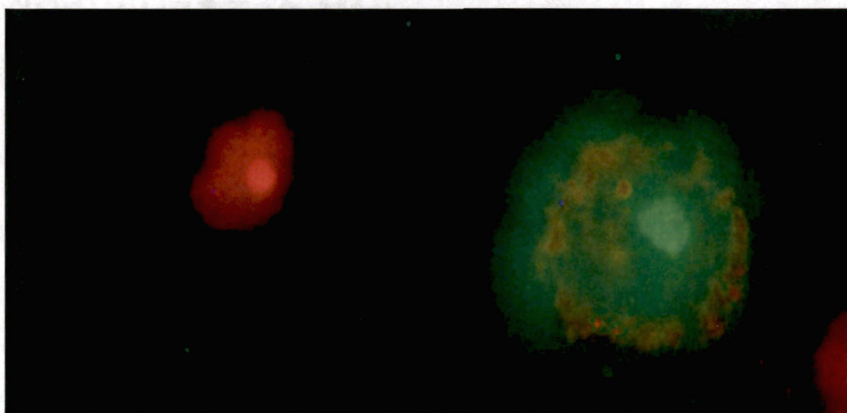
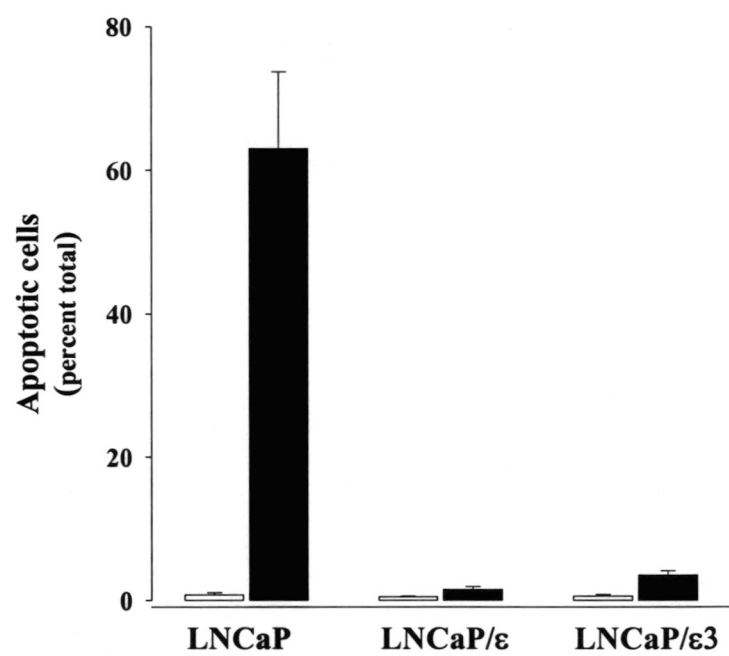


Figure 7. LNCaP cells overexpressing PKC ϵ resist PMA-induced apoptosis. LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells were grown to 80% confluency in complete media. Cells were trypsinized and 3.0×10^4 cells/0.5 ml were transferred to 24-well tissue culture plate. Cells were allowed to attach for 24 hours in complete media. Following attachment, media was aspirated off and replaced with complete media (open bars) or complete media containing 100 nM PMA (closed bars) and cells were incubated at 37 °C for 24 h. Cells were analyzed using a AO/EtBr staining method. 100 cells per sample were counted and the data are means ($\pm$ SE) of triplicate determinations of three independent experiments.



treatment (Figure 8). Thus, PKC ϵ is the first member of this multi-gene family that has been shown to be capable of imparting PMA-resistance to the LNCaP cell line.

Thymeleatoxin is a diterpene derivative of mezerein that preferentially activates PKC α , β , and γ over PKC δ and ϵ . Several studies have implicated PKC α in mediating the pro-apoptotic effects of PMA in LNCaP cells (Gschwend et al., 2000; Powell et al., 1996; Garcia-Bermejo et al., 2002). Our studies have confirmed that thymeleatoxin induces an apoptotic response in LNCaP cells (Figure 9). In contrast, LNCaP/ ϵ and LNCaP/ ϵ 3 cells were resistant to the apoptosis induced by thymeleatoxin as detected by AO/EtBr staining (Figure 9). These data indicate that PKC ϵ may be playing a more dominant role than PKC α in determining the fate of the LNCaP cell line.

PKC ϵ blocks the pro-apoptotic effects of PI3K inhibitors on LNCaP cells.

In addition to the observation that PKC ϵ overexpression had led to the AI growth of LNCaP cells, our preliminary studies indicated that PKC ϵ played a pivotal role in increasing CaP cell survival by blocking apoptosis. It is presently understood that the induction of the apoptotic response may arise by activating an extrinsic cell death pathway, which will be discussed later, and/or an intrinsic cell death pathway. The intrinsic cell death pathway leads to activation of effector caspases via the release of cytochrome c from the mitochondrial intermembrane space. This pathway is often engaged by stress, some anticancer drugs, and withdrawal of survival factors (Kulik et al., 2001).

Figure 8. AO/EtBr staining of LNCaP/ε cells that had been treated with 100 nM PMA for 36 h, note the arrow pointing to one apoptotic cell among several live normal cells. 200X magnification.

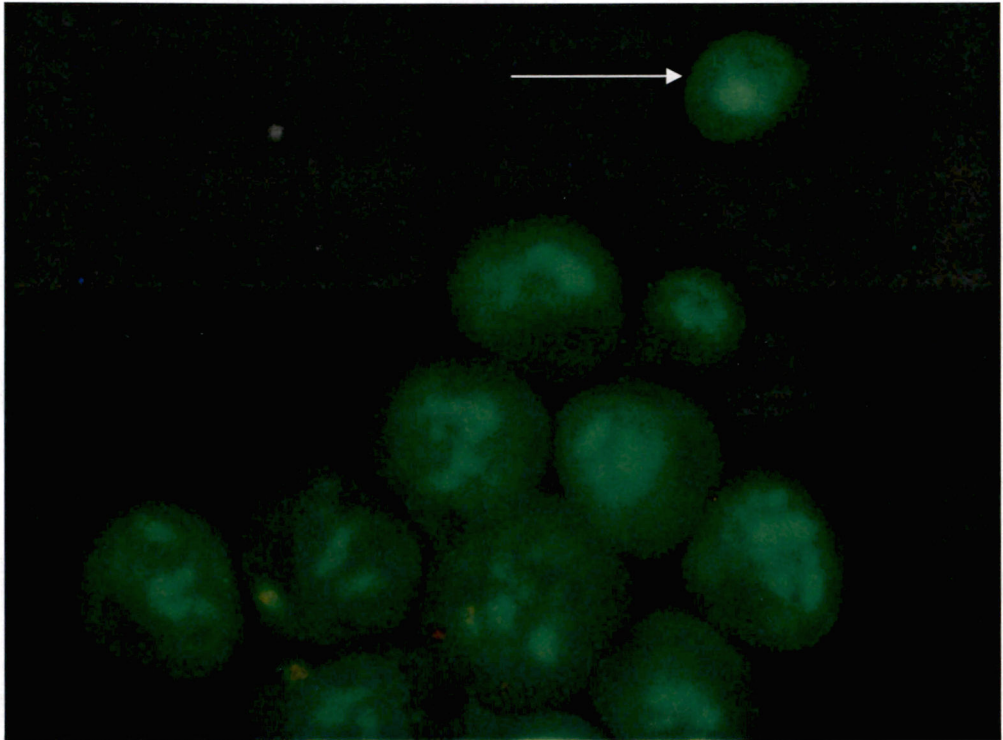
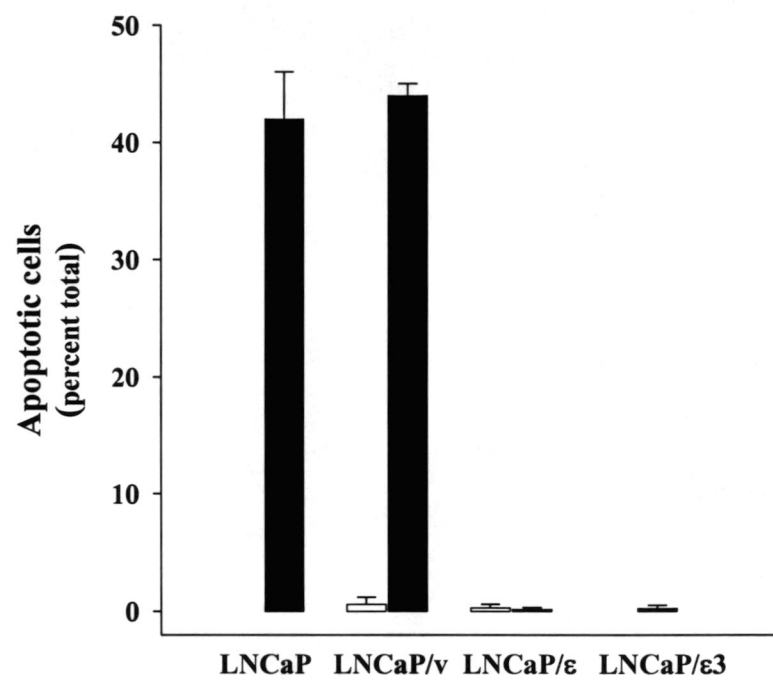
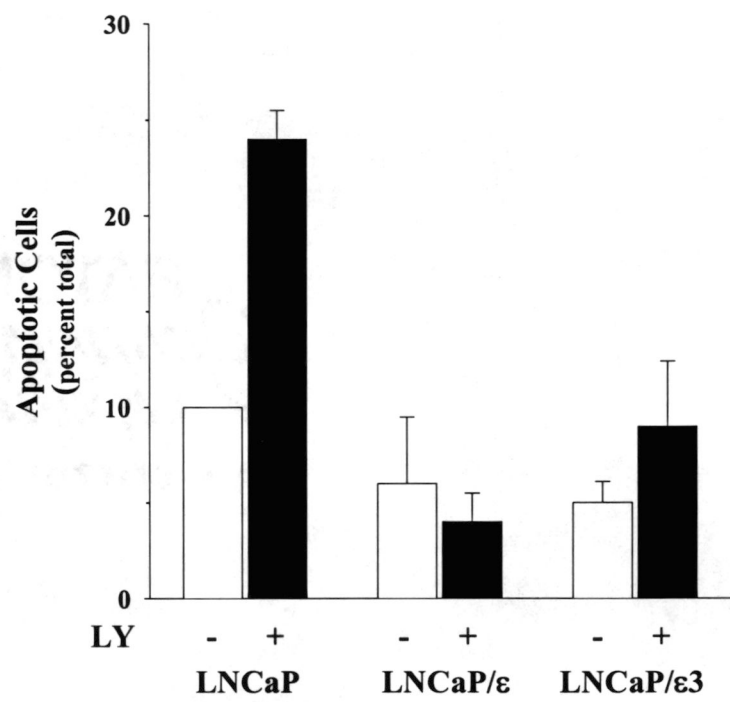


Figure 9. The overexpression of PKC ϵ protects LNCaP cells from thymeleatoxin-induced apoptosis. LNCaP, LNCaP/v, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells were grown to 80% in complete media. Growth media was aspirated off and replaced with either complete media (open bars) or complete media containing 100 nM thymeleatoxin (closed bars) for 24 h at 37 °C. Cells were then analyzed using AO/EtBr staining. 100 cells per sample were counted and the data are means ($\pm$ SE) of triplicate determinations of three independent experiments.



It has been established that PMA induces an apoptotic response in LNCaP cells by activating PKC isozymes and the intrinsic arm of this pathway. The PKC pathway is endogenously regulated by production of phospholipids and DAG where PI3K is a key upstream regulator of this pathway (Figure 2). Inhibitors of PI3K have been reported to induce an apoptotic response in LNCaP cells by activating the intrinsic, caspase-9 dependent, pathway (Kulik et al., 2001). Therefore, if PKC ϵ is operating downstream of PI3K to block an intrinsic response to apoptogenic signals, it must be hypothesized that the overexpression of PKC ϵ would be capable of protecting LNCaP cells from the apoptosis that is induced by LY294002, an inhibitor of PI3K. Under the conditions used in this experiment, LY294002 is known to produce an apoptotic response in ~20% of the exposed LNCaP cells (Kulik et al., 2001), a result that was confirmed in our lab (Figure 10). However, LNCaP/ ϵ and LNCaP/ ϵ 3 cells were insensitive to the pro-apoptotic effects LY294002 had on the intrinsic cell death pathway (Figure 10). While it is curious that both direct agonists of PKC and an inhibitor of the PI3K-PDK-PKC pathway induce an apoptotic response in LNCaP cells, our data indicate that PKC ϵ disrupts an important step in the intrinsic cell death pathway that is activated by these apoptogenic agents. Activators of the extrinsic arm of the apoptotic pathway (e.g., TNF- α) also engage mitochondria in the amplification of caspase proteolytic activity. Could PKC ϵ be capable of disrupting an event in the cell death pathway that is common to both an intrinsic and extrinsic program for apoptosis?

Figure 10. PKC ϵ blocks the pro-apoptotic effects of LY294002 in LNCaP cells. LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells were grown to 60% confluency in complete media on 24-well tissue culture plates and cells were allowed to attach for 2 d. Growth media was aspirated off and replaced with RPMI containing 0.5% FBS and incubated 24 h at 37 °C. Next, 3 μ g/ml of cycloheximide was added to each well and the cells were incubated for 30 min at 37 °C. Following this incubation, LY294002 was added to the designated wells at a final concentration of 25 μ M. Cells were then analyzed after a 12 h exposure using AO/EtBr staining. 100 cells per sample were counted and the data are means ($\pm$ SE) of triplicate determinations.



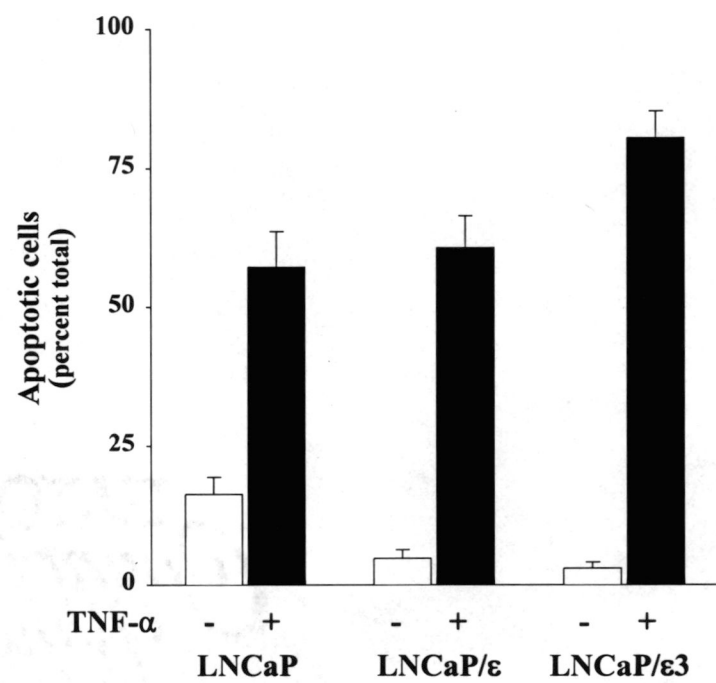
PKC ϵ does not alter the apoptogenic effects of TNF- α in LNCaP cells.

The extrinsic cell death pathway is activated by ligands of death receptors resulting in the recruitment of initiator caspases and ultimately leading to the activation of effector caspases (Kulik et al., 2001). Activation of caspase 8 through the extrinsic pathway serves as an amplification loop in that caspase 8 can cleave the pro-apoptotic protein Bid, which can then promote the release of cytochrome c from mitochondria (Kulik et al., 2000). One of several death receptors on the cell surface is TNFR1, which can be stimulated using TNF- α , a ligand of this death receptor. TNF- α is a polypeptide cytokine that has been reported to induce apoptosis in LNCaP cells (Kulik et al., 2001). Upon TNF- α ligation, a signaling cascade of initiator caspases (caspase 8 and/or caspase 10) ultimately leads to the activation of effector caspases and apoptosis.

TNF- α was used to activate the extrinsic (death receptor) cell death pathway in the LNCaP variants to determine if PKC ϵ has any protective effects in this arm of the apoptotic pathway. The parental (LNCaP) cells underwent apoptosis when treated with TNF- α (Figure 11). An equivalent apoptotic response to TNF- α was also observed in the LNCaP/ ϵ and the LNCaP/ ϵ 3 cells (Figure 11). Therefore, PKC ϵ appeared to selectively block the activity of the intrinsic cell death pathway through a mechanism that is independent of the extrinsic cell death pathway in LNCaP cells.

Apoptosis via the intrinsic cell death pathway is a multi-step process that ultimately leads to the activation of the terminator caspase, caspase 3. The activation of caspase 3 occurs downstream of the mitochondria via caspase 9, which is engaged by an

Figure 11. PKC ϵ does not block the pro-apoptotic effects of TNF- α . LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells were grown to 60% confluency in complete media on 24-well tissue culture plates and cells were allowed to attach for 2 days. Growth media was aspirated off and replaced with RPMI containing 0.5% FBS and incubated 24 h at 37 °C. Next, 3 μ g/ml of cycloheximide was added to each well and the cells were incubated for 30 min at 37 °C. Following this incubation, TNF- α was added to the designated wells at a concentration of 10 ng/ml. Cells were then analyzed after a 12 h exposure using AO/EtBr staining. 100 cells per sample were counted and the data are means ($\pm$ SE) of triplicate determinations.



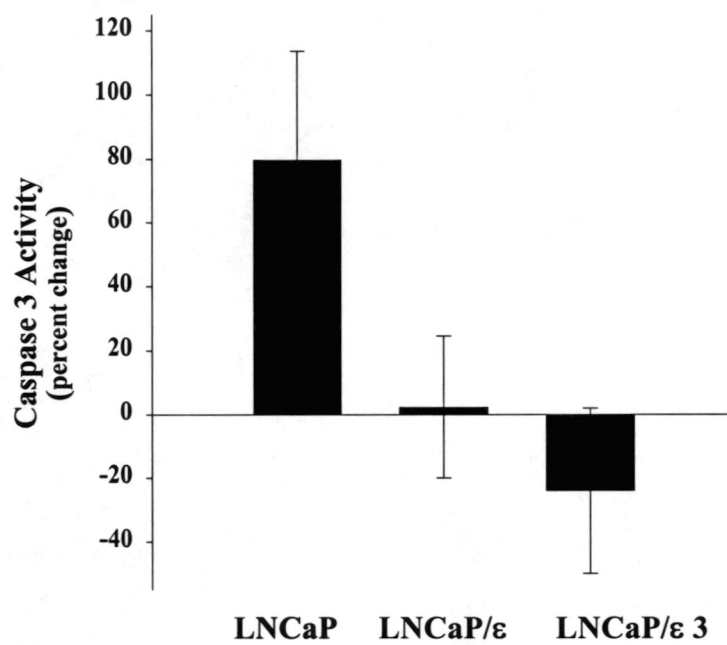
active apoptosome complex. Activation of the apoptosome complex occurs when cytochrome c is released from the intermembrane space of the mitochondria into the cytosol, where this protein binds Apaf-1. The release of cytochrome c has been associated with a loss of mitochondrial membrane potential and the Bcl-2 family of proteins is known to prevent this collapse of the mitochondrial membrane potential ($\Delta\psi_m$). To determine where PKC ϵ may be providing protection from PMA-induced apoptosis, we conducted a systematic analysis of these critical steps in the intrinsic cell death pathway.

The protective effect of PKC ϵ on the intrinsic cell pathway is upstream of caspase 3 activation.

Caspase 3 is an effector caspase that, once activated, can cleave a broad range of cellular substrates required for cell survival (Winter et al., 2001). Therefore, activation of caspase 3 is a terminal event in the apoptotic process. A caspase 3 assay kit (PharMingen) was used to detect the proteolytic activity of caspase 3 in the absence or presence of PMA. Earlier studies reported that caspase 3 activation could not be detected in LNCaP cells treated with PMA (Winter et al., 2001). However, in the present study caspase 3 activity is increased by more than 75% when LNCaP cells were exposed to 100 nM PMA for 6 h (Figure 12). In contrast, the level of caspase 3 activity was not significantly altered in LNCaP/ ϵ and LNCaP/ ϵ 3 cells (Figure 12). Therefore, PKC ϵ appears to act upstream of caspase 3 activation to provide protection from PMA-induced apoptosis in LNCaP cells. Beginning with this terminal event in the intrinsic cell death pathway, we next moved up the apoptotic pathway to a mitochondrial event.



Figure 12. PKC ϵ blocked caspase 3 activation following PMA treatment. LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 were grown to 80% confluency. Caspase 3 proteolytic activity was measured after a 6 h incubation in the presence or absence of 100nM PMA using the Caspase 3 assay kit from PharMingen. Data are expressed as the percent change in proteolytic activity resulting from exposure to 100 nM PMA and are the means ($\pm$ SE) of triplicate determinations in three independent experiments.

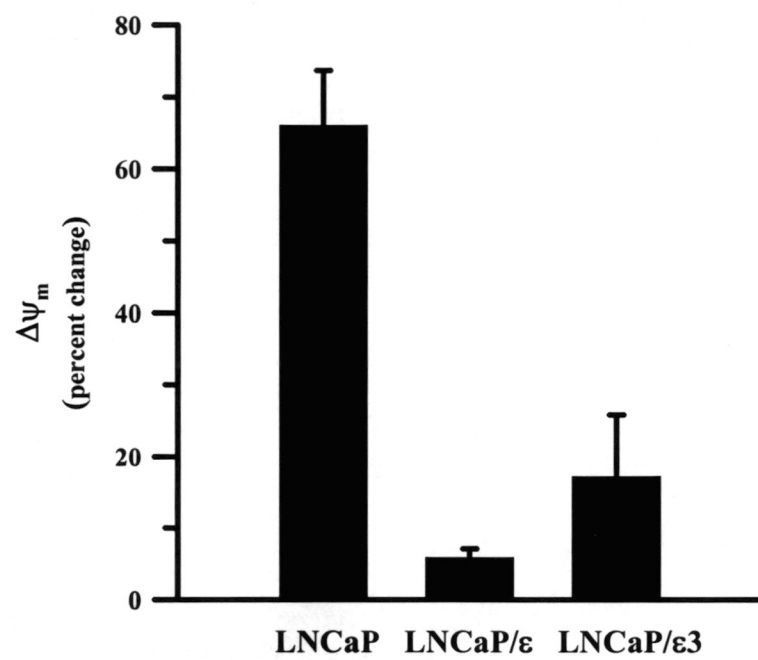


PKC ϵ prevents the loss of mitochondrial membrane potential induced by PMA in LNCaP cells.

Some of the Bcl-2 family members possess hydrophobic grooves that are capable of forming pores in the mitochondrial membrane that are large enough to permit cytochrome c to be released into the cytosol, where this protein starts the chain of events that lead to caspase 3 activation (Antonsson, 2001; Crompton, 2000). While the molecular composition of these pores remains controversial, it has been established that the PMA-induced apoptosis of LNCaP cells is associated with the liberation of mitochondrial cytochrome c and the subsequent loss of mitochondrial membrane potential (Waterhouse and Green, 1999; Desagher and Martinou, 2000; Antonsson, 2001; Pavlov et al., 2001). Therefore, it would be informative to know whether PKC ϵ was capable of blocking either of these mitochondrial responses to PMA.

Changes in the mitochondrial membrane potential were monitored using the JC-1 dye compound and were measured in individual cells using flow cytometry. JC-1 is a fluorescent cationic dye which emits fluorescence in the red spectrum when sequestered in polarized mitochondria and is shifted to green with a loss of the mitochondrial membrane potential (Eilon et al., 2000). As expected, this potentiometric dye reported a significant loss of mitochondrial membrane potential in LNCaP cells exposed to PMA (Figure 13). However, LNCaP/ ϵ and LNCaP/ ϵ 3 cells retained their a mitochondrial membrane integrity (Figure 13). These data suggest that the anti-apoptotic effect that PKC ϵ has on the intrinsic cell death pathway operates upstream of the mitochondria to block the release of cytochrome c.

Figure 13. PKC ϵ prevents PMA-induced depolarization of the mitochondrial membrane. LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 were grown to 80% confluency. Changes in the mitochondrial membrane potential were determined using the JC-1 dye method following a 6 h incubation in the presence or absence of 100nM PMA. Data are expressed as the percent change in the mitochondrial membrane potential of 10,000 individual cells, detected with FACS, resulting from exposure to PMA and are the means ($\pm$ SE) of triplicate determinations in three independent experiments.



Upstream of the mitochondria, the Bcl-2 gene family plays an important role in regulating the intrinsic cell death pathway. One such gene product is the pro-apoptotic protein, Bad, which when phosphorylated is rendered inactive and unable to interact with mitochondrial membrane.

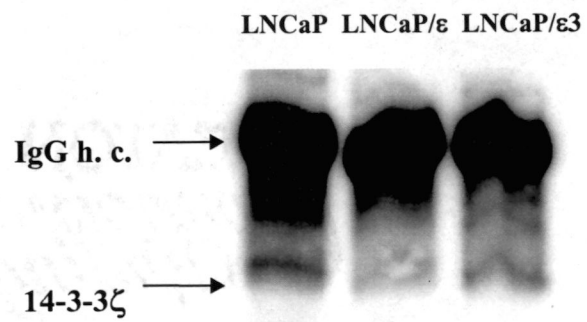
The pro-apoptotic protein Bad co-immunoprecipitates with 14-3-3 ζ in lysates of PMA-treated LNCaP cells.

Phosphorylation of Bad enhances the affinity of this pro-apoptotic protein for 14-3-3 ζ in the cytosol, inhibiting Bad from interacting with the anti-apoptotic proteins Bcl-2 and Bcl-X_L (Tan et al., 1999). Bad is a substrate for at least two known protein kinases. One mechanism for Bad phosphorylation is the classic PI3K-Akt survival pathway in which Akt phosphorylates Bad at serine¹³⁶ (Tan et al., 1999; Bertolotto et al., 2000; Tan et al., 2000). However, Bad can also be phosphorylated at serine¹¹², which may occur through PKC activation (Tan et al, 1999; Bertolotto et al., 2000). Activation of PKC by PMA can in turn result in the activation of the serine/threonine kinase p90^{RSK} (RSK-1). RSK-1 can directly phosphorylate Bad at serine¹¹² *in vitro* (Tan et al, 1999). Tan and colleagues (2000) have also demonstrated that the phosphorylation of Bad at serine¹⁵⁵ results in Bad binding to Bcl-X_L causing a pro-apoptotic chain of events. Because RSK-1 is known to phosphorylate Bad in response to PKC activation, we tested the hypothesis that PKC ϵ might protect LNCaP cells from PMA-induced apoptosis by promoting the association between phospho-Bad and 14-3-3 ζ . Co-immunoprecipitation experiments were performed to test this hypothesis by immunoprecipitating Bad and then probing from changes in the amount of the associated 14-3-3 ζ protein. LNCaP variants were

treated with 100 nM PMA for 0, 3, and 24 h prior to cell lysis in a ILB buffer. The 14-3-3 ζ protein was not detected in BAD IPs of cells incubated in the absence of PMA or following a 3 h exposure to this agent (data not shown). Importantly, however, 14-3-3 ζ was detected in the BAD IPs of LNCaP cells undergoing apoptosis after a 24 h exposure to PMA (Figure 14). 14-3-3 ζ was not detected in any of the BAD IPs prepared using LNCaP/ ϵ or LNCaP/ ϵ 3 cell lysates. These data imply that the activation of a PKC ϵ /RSK-1/BAD pathway is not responsible for protecting LNCaP cells from PMA-induced apoptosis, by promoting the sequestration of BAD in cytosolic 14-3-3 ζ heterodimers. *The pro-apoptotic protein, Bax, is required for PMA-induced apoptosis.*

In a parallel project using proteomics to detect proteins that interact with PKC ϵ in human prostate xenograft tumors, my colleague (Van Sikes) has recently obtained evidence of a possible interaction between PKC ϵ and Bax. PKC ϵ immunoprecipitates were prepared from the AD and AI variants of CWR22 tumor tissue, generously provided by Dr. Christopher Gregory (University of North Carolina, Chapel Hill, NC). PKC ϵ IP lysates were analyzed using two-dimensional (2-D) gel electrophoresis to separate the PKC ϵ -associated proteins and a computer algorithm was used to tentatively identify the protein spots found on the gel. A 2-D gel allows researchers to separate proteins based on their isoelectric point in one dimension and their molecular weight in the second dimension. Based on the coordinates (x and y) of a given spot, the computer program generates a prioritized list of candidate proteins that may correspond to a given spot. This project revealed a possible association of the pro-apoptotic protein, Bax, and PKC ϵ .

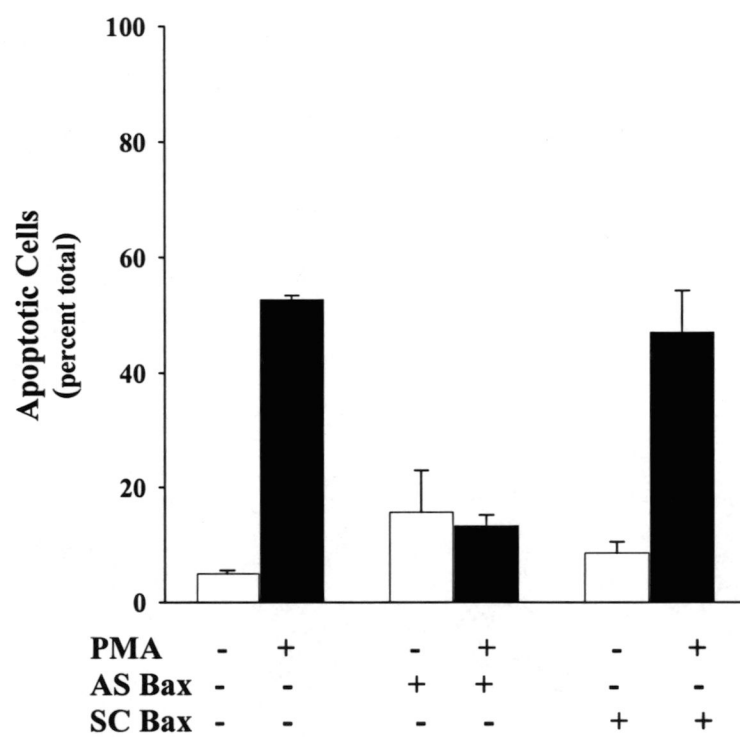
Figure 14. Bad phosphorylation is shown here by coimmunoprecipitation Bad and probing by western blot for 14-3-3 ζ . Bad was immunoprecipitated from lysates of LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells following a 24 h exposure to 100 nM PMA. Western Blot analysis of the Bad immunoprecipitate revealed that 14-3-3 ζ was associated with this pro-apoptotic protein in LNCaP cells undergoing apoptosis (see Figure), while 14-3-3 ζ was only detected by a very faint band in LNCaP/ ϵ or LNCaP/ ϵ 3 cells. The pronounced bands are the IgG heavy chain.



This was an unexpected and novel finding that suggested a potential mechanism for PKC ϵ blocking the pro-apoptotic effects of the intrinsic cell death pathway. Because a recent patch-clamp analysis has suggested that Bax is capable of forming pores in the mitochondrial membrane large enough for the release of cytochrome c (Pavlov et al, 2000), we wanted to see if Bax was required for PMA-induced apoptosis.

Bax is a pro-apoptotic member of the Bcl-2 sub-family of Bcl-2 proteins (Figure 1). Under normal conditions, Bax resides in the cytosol. Upon induction of apoptosis, Bax forms oligomers and translocates to the mitochondrial membrane where it is involved in the release of cytochrome c. To determine whether Bax played an important role in PMA-induced apoptosis, antisense Bax ODN were employed. Literature suggests that a combination of two Bax ODNs is required to knock down Bax translation (Dibbert et al., 1999 and Kim et al., 2001). Therefore, inhibition of Bax protein expression was achieved by using a mixture of two antisense ODN at a final concentration of 5 μ M for each. LNCaP cells were transfected with the antisense ODN in the presence of lipofectin. A pair of scrambled (SC) ODN were used, at an equimolar concentration (5 μ M), as a control for the sequence-specific effects of the antisense ODNs. To date, we have been unable to detect the Bax protein in lysates from untreated LNCaP cells. Completion of this goal will be required to determine whether or not the combination of antisense Bax ODN used in this experiment actually decreased Bax expression in this cell line. Importantly, however, we have shown that Bax ODN pretreatment completely blocked the apoptogenic effects of PMA in LNCaP cells (Figure 15). Moreover, this effect of the

Figure 15. Bax is required for PMA-induced apoptosis in LNCaP cells. LNCaP cells were grown to 80% confluency. The cells were trypsinized and 4×10^4 cells/0.5 ml were transferred to 24-well tissue culture plates. Cells were allowed to attach for 2 d at 37 °C. Following the attachment, the cells were serum starved overnight. Next, the cells were transfected with a combination of 2 ODNs (5 μ M) by lipofectin (2 μ g/ml) and incubated for 6 h at 37 °C. The media was aspirated off and replaced with complete media along with the ODNs (no lipofectin) and cells were incubated at 37 °C for 18 h. Then, fresh ODN was added for 72 h. Next, the media was aspirated off and replaced with either complete media or complete media containing 100 nM PMA and the cells were incubated for 24 h. Following this incubation, the cells were analyzed using the AO/EtBr staining. 100 cells per sample were counted and the data are means ($\pm$ SE) of triplicate determinations.



Bax ODN was sequence-specific. These data provide the first evidence that the pro-apoptotic protein Bax may be required for PMA-induced apoptosis in LNCaP cells.

Having identified Bax as a key component in this cell death pathway, we speculated that a novel association between PKC ϵ and Bax may contribute to the anti-apoptotic effects of PKC ϵ overexpression in LNCaP cells.

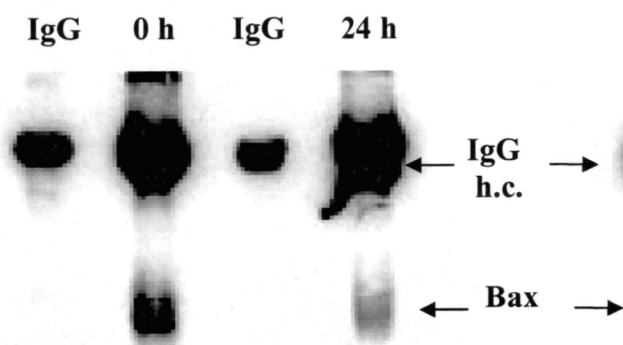
PKC ϵ appears to sequester Bax in the cytosol of LNCaP cells exposed to PMA.

A co-immunoprecipitation assay was performed using LNCaP and LNCaP/ ϵ cells, immunoprecipitating PKC ϵ in these cell lysates. The resultant PKC ϵ IPs were probed for Bax by western blotting. In LNCaP/ ϵ cells, the interaction between Bax and PKC ϵ was not affected by treatment with PMA (Figure 16B). However, in LNCaP cells, the association between Bax and PKC ϵ was effectively disrupted by this treatment (Figure 16A). These data suggest that Bax may oligomerize and translocate to the mitochondrial membrane to cause the release of cytochrome c in LNCaP cells but, due to the level of PKC ϵ overexpression, Bax may remain sequestered in the cytosol of LNCaP/ ϵ cells. An inspection of the subcellular distribution of Bax and cytochrome c in LNCaP variants, using confocal microscopy, supports this hypothesis (data not shown). Based on these, and other, observations we suggest that PKC ϵ directly or indirectly interacts with Bax in a manner that prevents the Bax from inserting itself into the outer mitochondrial membrane.

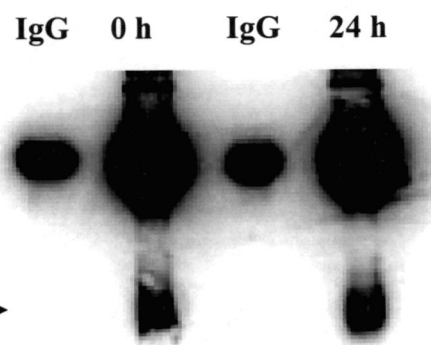


Figure 16. PKC ϵ is associated with the pro-apoptotic protein Bax in LNCaP cells. LNCaP (A) and LNCaP/ ϵ (B) cells were grown to 80% confluency on four 150mm plates. The growth media was aspirated and replaced with complete media on two of the plates and complete media containing 100 nM PMA on the other two plates. PKC ϵ was immunoprecipitated from the cell lysates after a 24 h exposure. The samples were analyzed by western blotting for Bax. The IgG lanes are a control for non-specific binding. The pronounced higher molecular weight bands are the IgG heavy chain.

A.



B.



CWR-R1 cells rely on endogenous PKC ϵ to resist PMA-induced apoptosis.

The CWR-R1 cell line is considered a clinically relevant model for studying the recurrence of CaP (Gregory et al., 2001). CWR-R1 cells were isolated from a recurrent xenograft prostate tumor and retain many of features of hormone refractory CaP. Also, the CWR-R1 cells express high levels of endogenous PKC ϵ and provided a unique opportunity to test the hypothesis that CaP cells rely on endogenous PKC ϵ to escape the apoptogenic effects of PMA. AO/EtBr staining of CWR-R1 cells, following PMA treatment, confirmed that CWR-R1 cells were insensitive to the PKC agonist PMA (Figure 17).

To investigate the role of endogenous PKC ϵ in the survival of this cell line, CWR-R1 cells were transfected with antisense PKC ϵ ODN (1 μ M) using lipofectin to selectively downregulate the expression of this isozyme. Daquing Wu, a research associate in our lab that has mastered this procedure and performed this transfection. Cell lysates were analyzed by immunoblotting to assure that the translation of PKC ϵ mRNA had selectively and effectively been downregulated (Wu et al., 2002). The number of apoptotic CWR-R1 cells greatly increased in response to PMA treatment upon transfecting with antisense PKC ϵ when compared to untreated cells (Figure 18). These data indicate that endogenous PKC ϵ plays an important role in regulating the survival of advanced, AI, CaP cells.

Figure 17. Endogenous PKC ϵ protects CWR-R1 cells from PMA-induced apoptosis. CWR-R1 were grown to 80% confluency in complete media. Cells were trypsinized and 3.0×10^4 cells/0.5 ml were transferred to 24-well tissue culture plate. Cells were allowed to attach for 24 hours in complete media. Following attachment, media was aspirated off and replaced with complete media (open bars) or complete media containing 100 nM PMA (closed bars) and cells were incubated at 37 °C for 24 h. Cells were analyzed using a AO/EtBr staining method. 100 cells per sample were counted and the data are means ($\pm$ SE) of triplicate determinations.

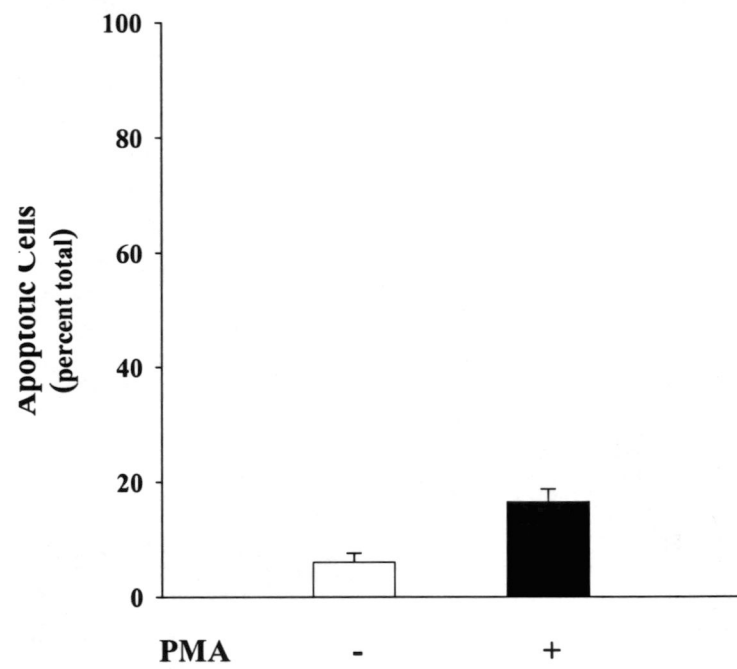
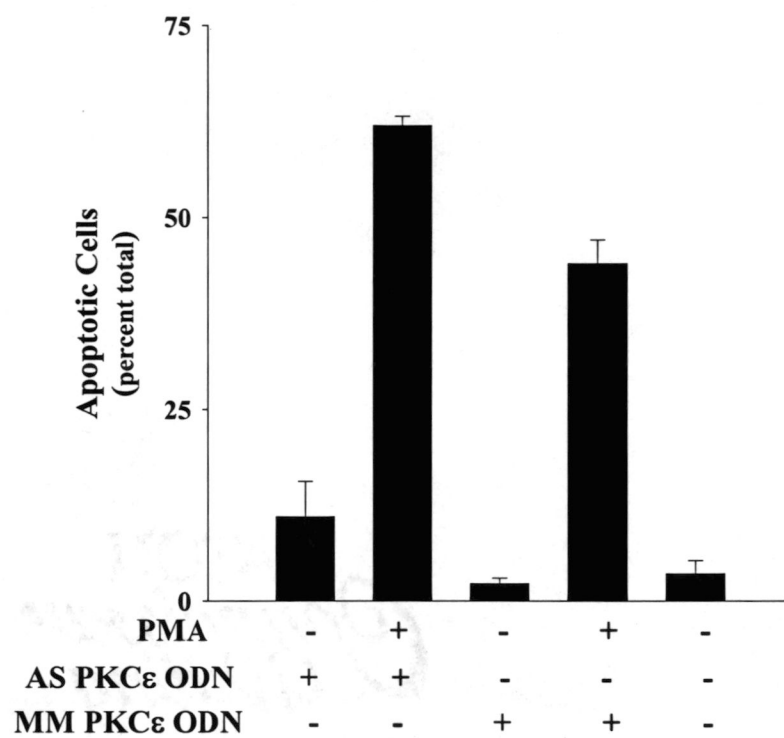




Figure 18. PKC ϵ is required for protection from PMA-induced apoptosis. CWR-R1 cells were grown to 80% confluency. The cells were trypsinized and 4×10^4 cells/0.5 ml were transferred to 24-well tissue culture plates. Cells were allowed to attach for 2 days at 37 °C. Following the attachment, the cells were serum starved overnight. The cells were transfected with PKC ϵ antisense ODN (1 μ M) using lipofectin (2 μ g/ml) for 48 h or a PKC ϵ mismatch ODN. The cells were then analyzed using AO/Et/Br staining method. 100 cells per sample were counted and the data are means ($\pm$ SE) of triplicate determinations.



GENERAL DISCUSSION

The major findings of the present study are that PKC ϵ protects CaP cells from the effects of androgen withdrawal and apoptogenic agents acting through an intrinsic cell death pathway, but not those acting through an extrinsic cell death pathway. This protection from activation of the intrinsic cell death pathway is through a Bax-dependent mechanism involving the mitochondria and efflux of cytochrome c. PKC ϵ is able to protect LNCaP cells from PMA-induced apoptosis prior to the release of cytochrome c and the subsequent following events leading to activation of effector caspases. While we have found PKC ϵ to inhibit apoptosis induced through the intrinsic cell death pathway, it has been reported that PKC α and PKC δ induce apoptosis through this pathway. Several laboratories have studied the effects of PKC α and PKC δ in the LNCaP cell line and it is evident that the different PKC isozymes play antagonistic roles in transducing the apoptotic signals that agents, such as PMA and LY294002, activate (Powell et al., 1996; Fujii et al., 2000; Gschwend et al., 2000; Majumder et al., 2000; Garcia-Bermejo et al., 2002; Sumitomo et al., 2002). The present study also investigated the effects of PKC ϵ in the extrinsic cell death pathway by using the peptide TNF- α . LNCaP, LNCaP/ ϵ , and LNCaP/ ϵ 3 cells all became apoptotic upon TNF- α -induced apoptosis and this was consistent with findings that TNF- α -induced apoptosis bypasses anti-apoptotic signaling in LNCaP cells (Kulik et al., 2001). Accordingly, our studies were directed toward the intrinsic cell death pathway.

Liberation of cytochrome c from the intermembrane space of mitochondria is critical in induction of apoptosis through the intrinsic cell death pathway. PMA-induced

apoptosis in LNCaP cells results in depolarization of the mitochondria (Majumder et al, 2000). This depolarization of the mitochondria results from a disruption of the mitochondrial membrane that permits the release of several pro-apoptotic factors, including cytochrome c. The effect of cytochrome c in the cytosol is exerted downstream in the intrinsic cell death pathway and indirectly leads to the activation of caspase 3. Contrary to earlier reports (Winter et al., 2001), the present studies indicate that PMA-induced apoptosis of LNCaP cells was associated with an increase in caspase 3 proteolytic activity. Importantly, PKC ϵ was able to effectively block this chain of events. Mitochondria of PMA-treated LNCaP/ ϵ and LNCaP/ ϵ 3 cells did not exhibit a loss of membrane potential nor an increase in caspase 3 activity. There are several events upstream of the mitochondria that aid in the depolarization of this organelle. Although the mechanism of PMA-induced apoptosis in LNCaP cells is not yet clear, these studies indicate that actions upstream of the mitochondria determine the fate of CaP cells.

Bax protein is one such upstream negotiator that appears to be involved in PMA-induced apoptosis of LNCaP cells. When Bax expression was blocked by using antisense Bax ODNs, the percent of apoptotic LNCaP cells was significantly decreased following a 24 h treatment with 100 nM PMA. Therefore, in LNCaP cells, Bax is required for PMA-induced apoptosis. However, it is likely that several other proteins regulate/execute this pathway, including PKC ϵ .

Under certain conditions, Bax oligomerizes and inserts into the outer membrane of mitochondria forming a channel large enough for the release of cytochrome c. In an effort to understand Bax regulation, Suzuki and colleagues (2000) determined the

solution structure of Bax crystals. Bax consists of nine α helices and the overall fold closely resembles Bcl-X_L. Bcl-X_L contains eight amphipathic α helices clustered around one central hydrophobic α helix. In Bax, a portion of the helix of α 1 is hidden in the cytosolic form and becomes exposed upon induction of apoptosis (Suzuki et al., 2000). The hydrophobic portion of Bax contains the α 9 helix and movement of the α 9 helix out of the hydrophobic pocket is also important in apoptosis induction (Suzuki et al., 2000). The BH3 domain of Bax is in the α 2 helix, this is the portion required for dimer formation. In this region are several hydrophobic residues that are oriented toward the center hydrophobic core and this orientation is shifted upon apoptosis induction. Bax binds the mitochondria via its C-terminal helix and this only becomes exposed upon apoptosis induction. Therefore, Bax cannot form oligomers unless apoptosis is induced and conformational changes occur to produce an active Bax. For that reason, we are investigating PKC ϵ 's role in holding Bax in its cytosolic and inactive conformation. In LNCaP cells, it appears that Bax is activated and inserted into the mitochondrial membrane due to the downstream events observed in this cell line. Given what is known regarding the crystal structure of AGC Group II protein kinase domains (Johnson et al., 1996) and the N-terminus of PKC ϵ (Hanks and Hunter, 1995), it seems unlikely that PKC ϵ physically interacts with the BH3 domain of the α 2 helix in Bax. Alternatively, PKC ϵ may interact with a Bax-Bcl-X_L complex in a manner that stabilizes these inactive Bax heterodimers. Two lines of evidence support this speculation. First, both Bax and Bcl-2 co-IPs with PKC ϵ in CWR-R1 cell lysates (Van Sikes, personal communication). Second, the present study has demonstrated that the PMA-induced apoptosis of LNCaP

cells was associated with a disruption of the interactions between PKC ϵ and Bax in LNCaP cells. Moreover, this apoptogenic agent did not alter the co-immunoprecipitation of PKC ϵ and Bax in PMA-resistant LNCaP/ ϵ cells.

Apoptotic pathways have evolved into a complex web of interdependent pathways. One such pathway in the LNCaP cell line is the c-Jun N-terminal kinase (JNK) pathway. It has been reported that PKC ϵ phosphorylates the activation loop of PKD μ , resulting in an association with JNK (Waldron et al., 1999; Waldron et al., 2001; Hurd et al., 2002; Yuan et al., 2002). This association prevents the downstream phosphorylation of c-Jun, a key factor in the apoptotic effects of PMA on LNCaP cells (Bossy-Wetzel et al., 1997). Therefore, this is yet another way PKC ϵ may provide protection from PMA-induced apoptosis. The importance of JNK activity in the PMA-induced apoptosis of LNCaP cells was emphasized by the finding that dominant negative JNK blocks PMA-induced apoptosis in this CaP cell line (Engedal et al., 2002). We have independently confirmed that PMA stimulates the phosphorylation (activation) of JNK in LNCaP cells and have extended this analysis by demonstrating that PKC ϵ is capable of blocking this effect of PMA on JNK phosphorylation (Ginger Wescott, personal communication). Therefore, PKC ϵ appears to be acting in an alternate, parallel PKC ϵ -PDK-JNK-c-Jun pathway through its kinase domain to inhibit PMA-induced apoptosis in the LNCaP cell line.

In another ongoing project, it was found through the use of confocal microscopy that Bax translocates into the nucleus in LNCaP cells following treatment with PMA. The explanation for this phenomenon is still unclear, but has previously been observed

(Raffo et al., 2000). A nuclear import of Bax was not observed in the LNCaP/ε cells supporting the theory that PKCε may be sequestering Bax in the cytosol and preventing the pro-apoptotic effect of this protein. The localization of Bax to the nucleus has not been previously reported in CaP cells.

Because the work done on this project was based on gene transfer studies, some preliminary analyses were implemented to study endogenous PKCε function. The CWR-R1 prostate cancer cell line expresses endogenous PKCε and provides a more clinically relevant model in that it retains many of the features of recurrent prostate cancer. The endogenous PKCε in these cells was sufficient to block PMA-induced apoptosis. Moreover, treatment with antisense PKCε ODNs rendered this cell line susceptible to PMA-induced apoptosis. The gene transfer experiments were useful in demonstrating that PKCε is capable of transforming LNCaP cells into a cell line with increased survival under certain death promoting conditions and this proposal was strengthened with the work done to manipulate the expression of endogenous PKCε in CWR-R1 cells.

Now that there is direct evidence that PKCε is capable of blocking apoptogenic agents acting through the intrinsic cell death pathway, the signaling mechanisms that transduce this resistance should be rigorously investigated. PKCε is definitely involved in preventing a Bax-dependent mechanism of mitochondrial depolarization. However, it may not be through a direct PKCε-Bax interaction. Therefore, further investigation into this complex must be performed. Although many important details need to be further

investigated, this study clearly demonstrated that PKC ϵ has an anti-apoptotic role in CaP cell survival.

REFERENCES

- Abate-Shen, C., and Shen, M. M. (2000) "Molecular Genetics of Prostate Cancer". *Genes and Development* 14: 2410-2434.
- Akita, H., Izuka, A., Hashimoto, Y., Kohri, K., Ikeda, K., and Nakanishi, M. (2000) "Induction of KAI-1 expression in metastatic cancer cells by phorbol esters". *Cancer Letters* 153 : 79-83.
- Amanatullah, D.F., Reutens A.T., Zafonte, B.T., Fu, M., Mani, S., and Pestell, R.G. (2000). "Cell-cycle Dysregulation and the Molecular Mechanisms of Prostate Cancer". *Frontiers in Bioscience* 5: 372-390.
- Antonsson, B. (2001) "Bax and other pro-apoptotic Bcl-2 family "killer-proteins" and their victim, the mitochondrion". *Cell Tissue Research* 306 : 347-361.
- Bertolotto, C., Maulon, L., Filippa, N., Baier, G., and Auburger, P. (2000) "Protein kinase C θ and ϵ promote T-cell survival by a Rsk-dependent phosphorylation and inactivation of BAD". *The Journal of Biological Chemistry* 275 : 37246-37250.
- Bossy-Wetzel, E., Bakiri, L., and Yaniv, M. (1997) "Induction of apoptosis by the transcription factor c-Jun". *The EMBO Journal* 16 : 1695-1709.
- Brinkmann, A. O., and Trapman, J. (2000) "Prostate Cancer Schemens for Androgen Escape". *Nature Medicine* 6: 628-629.
- Burchardt, M., Burchardt, T., Shabsigh, A., Ghafar, M., Chen, M.W., Anastasiadis, A., de la Taille, A., and Buttyan, R. (2001) "Reduction of wild type p53 function confers a hormone resistant phenotype on LNCaP prostate cancer cells". *Prostate* 48 : 225-230.
- Carpten, J., Nupponen, N., Isaacs, S., Sood, R., Robbins, S., Xu, J., Faruque, M., Moses, T., Ewing, C., Gillanders, E., Hu, P., Bujnovszky, P., Makalowska, I., Baffoe-Bonnie, A., Faith, D., Smith, J., Stephan, D., Wiley, K., Brownstein, M., Gildea, D., Kelly, B., Jenkins, R., Hostetter, G., Matikainen, M., Schleutker, J., Klinger, K., Connors, T., Xiang, Y., Wang, Z., De Marzo, A., Papadopoulos, N., Kallioniemi, O.-P., Burk, R., Meyers, D., Gronberg, H., Meltzer, P., Silverman, R., Bailey-Wilson, J., Walsh, P., Isaacs, W., and Trent, J. (2002) "Germline mutations in the ribonuclease L gene in families showing linkage with HPC1". *Nature Genetics* 30 : 181-184.
- Carson, J.P., Kulik, G., and Weber, M.J. (1999) "Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3'-kinase and Akt/protein kinase B" *Cancer Research* 59 : 1449-1453.

- Cartee, L. and Kucera, G.L. (2000) "Protein kinase C modulation and anticancer drug response". *Cancer Investigation* 18 : 731-739.
- Cato, A.C.B. and Mink, S. (2001) "BAG-1 family of co-chaperones in the modulation of nuclear receptor action". *Journal of Steroid Biochemistry and Molecular Biology* 78 : 379-388.
- Cornford, P., Evans, J, Dodson, A., Parsons, K., Woolfenden, A., Neoptolemos, J., and Foster C.S. (1999) "Protein kinase C isoenzyme patterns characteristically modulated in early prostate cancer". *American Journal of Pathology* 154 : 137-144.
- Craft, N., Chhor, C., Tran, C., Beldegrun, A., DeKernion, J., Witte, O.N., Said, J., Reiter, R.E., and Sawyers, C.L. (1999) "Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process". *Cancer Research* 59 : 5030-5036.
- Crompton, M. (2000) "Bax, Bid and the permeabilization of the mitochondrial outer membrane in apoptosis". *Current Opinion in Cell Biology* 12 : 414-419.
- Cryns, V. and Yuan, J. (1998) "Proteases to die for". *Genes and Development* 12 : 1551-1570.
- Degterev, A., Boyce, M., and Yuan, J. (2001) "The channel of death". *Journal of Cell Biology* 155 : 695-697.
- Desagher, S. and Martinou, J-C. (2000) "Mitochondria as the central control point of apoptosis". *Trends in Cell Biology* 10 : 369-377.
- Diamandopoulos, G. T. (1996) "Cancer: An Historical Perspective". *Anticancer Research* 16: 1595-1602.
- Dibbert, B., Wever, M., Nikolaizik, W.H., Bogt, P., Shoni, M.H., Blaser, K., and Simon, H.-U. (1999) "Cytokine-mediated Bax deficiency and consequent delayed neutrophil apoptosis: a general mechanism to accumulate effector cells in inflammation". *Proceedings of the National Academy of Sciences* 96 : 13330-13335.
- Dong, J.T., Lamb, P.W., Rinker-Schaeffer, C.W., Vukanovic, J., Ichikawa, T., Isaac, J.T., and Barrett, J.C. (1995) "KAI, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2". *Science* 268 : 884-886.
- Eilon, G.F., Gu, J., Slater, L.M., Hara, K., and Jacobs, J.W. (2000) "Tumor apoptosis induced by epoxide-containing piperazines, a new class of anti-cancer agents". *Cancer Chemotherapy Pharmacology* 45 : 183-181.

Ellis, L.M., and Fidler, I.J. (1996) "Angiogenesis and Metastasis". *European Journal of Cancer* 32A: 2451-2460.

Engedal, N., Korkmaz, C.G., and Saatcioglu, F. (2002) "C-Jun N-terminal kinase is required for phorbol ester- and thapsigargin-induced apoptosis in the androgen responsive prostate cancer cell line LNCaP". *Oncogene* 21 : 1017-1027.

Froesch, B.A., Takayama, S., and Reed, J.C. (1998) "BAG-1L protein enhances androgen receptor function". *The Journal of Biological Chemistry* 273 : 11660-11666.

Fujii, T., Garcia-Bermejo, M.L., Bernabo, J.L., Caamano, J., Ohba, M., Kuroki, T., Li, L., Yuspa, S.H., and Kazanietz, M.G. (2000) "Involvement of Protein Kinase C δ (PKC δ) in Phorbol Ester-induced Apoptosis in LNCaP Prostate Cancer Cells". *Journal of Biological Chemistry* 275: 7574-7582.

Gao, M., Ossowski, L., and Ferrari, A.C. (1999) "Activation of Rb and decline in androgen receptor protein precede retinoic acid-induced apoptosis in androgen-dependent LNCaP cells and their androgen-independent derivative". *Journal of Cellular Physiology* 179 : 336-346.

Garcia-Bermejo, M.L., Leskow, F.C., Fujii, T., Wang, Q., Blumberg, P.M., Ohba, M., Kuroki, T., Han, K.C., Lee, J., Marquez, V.E., and Kazanietz, M.G. (2002) "Diacylglycerol (DAG)-lactones, a new class of protein kinase C agonists, induce apoptosis in LNCaP prostate cancer cells by selective activation of PKC α ". *The Journal of Biological Chemistry* 277 : 645-655.

Gorgoulis, V. G., Tiniakos, D. G., Mariatos, G., Manolis, E. N., Mitropoulos, D., and Kittas, C. (1999) "Recent Advances in Early Prostatic Cancer". *Anticancer Research* 19: 2327-2348.

Gregory, C.W., Johnson, Jr., R.T., Mohler, J.L., French, F.S., and Wilson, E.M. (2001) "Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen". *Cancer Research* 61 : 2892-2898.

Grossmann, M.E., Huang, H., and Tindall, D.J. (2001) "Androgen Receptor Signaling in Androgen-Refractory Prostate Cancer". *Journal of the National Cancer Institute* 93: 1687-1697.

Gschwend, J.E., Fair, W.R., and Powell, C.T. (2000) "Bryostatin 1 Induces Prolonged Activation of Extracellular Regulated Protein Kinases in and Apoptosis of LNCaP Human Prostate Cancer Cells Overexpressing Protein Kinase C α ". *Molecular Pharmacology* 57: 1224-1234.

Gutman, M., and Fidler, I.J. (1995) "Biology of Human Colon Cancer Metastasis". *World Journal of Surgery* 19: 226-234.

Hanks, S.K. and Hunter, T. (1995) "The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification". *The FASEB Journal* 9 : 576-596.

Herrmann, J.L., Menter, D.G., Beham, A., von Eschenbach, A., and McDonnell, T.J. (1997) "Regulation of lipid signaling pathways for cell survival and apoptosis by bcl-2 in prostate carcinoma cells". *Experimental Cell Research* 234 : 442-451.

Hsing, A.W., and Devesa, S.S. (2001) "Trends and Patterns of Prostate Cancer: What Do They Suggest?". *Epidemiologic Reviews* 23: 3-13.

Hurd, C., Waldron, R.T., and Rozengurt, E. (2002) "Protein kinase D complexes with C-Jun N-terminal kinase via activation loop phosphorylation and phosphorylates the C-Jun N-terminus". *Oncogene* 21 : 2154-2160.

Isaac, W., and Kainu, T. (2001) "Oncogenes and Tumor Suppressor Genes in Prostate Cancer". *Epidemiologic Reviews* 23: 36-40.

Johson, L.N., Noble, M.E.M., and Owen, D.J. (1996) "Active and inactive protein kinases: structural basis for regulation". *Cell* 85 : 149-158.

Kim, H.J., Mun, J.Y., Chun, Y.J., Choi, K.H, and Kim, M.Y. (2001) "Bax-dependent apoptosis induced by ceramide in HL-60 cells". *Federation of European Biochemical Societies Letters* 505 : 264-268.

Konishi, N., Sho, M., Yamaoto, K., and Hiasa, Y. (1997) "Genetic changes in prostate cancer". *Pathology International* 47 : 735-747.

Krithivas, K., Yurgalevitch, S.M., Mohr, B.A., Wilcox, C.J., Batter, S.J., Brown, M., Longcope, C., McKinlay, J.B., and Kantoff, P.W. (1999) "Evidence that the CAG repeat in the androgen receptor gene is associated with the age-related decline in serum androgen levels in men". *Journal of Endocrinology* 162 : 137-142.

Kulik, G., Carson, J.P., Vomastek, T., Overman, K., Gooch, B.D., Srinivasula, S., Alnemri, E., Nunez, G., and Weber, M.J. (2001) "Tumor necrosis factor α induces BID cleavage and bypasses antiapoptotic signals in prostate cancer LNCaP cells" *Cancer Research* 61 : 2713-2719.

Larminie, C.G.C., Alzuherri, H.M., Cairns, C.A., McLees, A., and White, R.J. (1998) "Transcription by RNA polymerases I and III: a potential link between cell growth, protein synthesis and the retinoblastoma protein". *Journal of Molecular Medicine* 76 : 94-103.

Lin, J., Adam, R.M., Santiestevan, E., and Freeman, M.R. (1999) "The phosphatidylinositol 3'-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells". *Cancer Research* 59 : 2891-2897.

Lorenzo, P.S., Bogi, K., Acs, P., Pettit, G.R., and Blumberg, P.M. (1997) "The catalytic domain of protein kinase C δ confers protection from down-regulation induced by bryostatin 1". *The Journal of Biological Chemistry* 272 : 33338-33343.

Lu, K., Shih, C., and Teicher, B.A. (2000) "Expression of pRB, cyclin/cyclin-dependent kinases and E2F1/DP-1 in human tumor lines in cell culture and in xenograft tissues and response to cell cycle agents". *Cancer Chemotherapeutic Pharmacology* 46 : 293-304.

Majumder, P.K., Pandey, P., Sun, X., Cheng, K., Datta, R., Saxena, S., Kharbanda, S., and Kufe, D. (2000) "Mitochondrial translocation of protein kinase C δ in phorbol ester-induced cytochrome *c* release and apoptosis". *Journal of Biological Chemistry* 275 : 21793-21796.

Miller, G. J., and Torkko, K. C. (2001) "Natural History of Prostate Cancer-Epidemiologic Considerations". *Epidemiologic Reviews* 23: 14-18.

Newton, A.C. (1997) "Regulation of protein kinase C". *Current Opinion in Cell Biology* 9 : 161-167.

O'Brian, C.A. (1998) "Protein kinase C- α : a novel target for the therapy of androgen-independent prostate cancer? (review-hypothesis)". 5 : 305-309.

Pavlov, E.V., Priault, M., Pietkiewixz, D., Cheng, E.H-Y., Antonsson, B., Manon, S., Korsmeyer, S.J., Mannella, C.A., and Kinnally, K.W. (2001) "A novel, high conductance channel of mitochondria linked to apoptosis in mammalian cells and Bax expression in yeast". *Journal of Cell Biology* 155 : 725-731.

Powell, C.T., Brittis, N.J., Stec, D., Hug, H., Heston, W.D.W., and Fair, W.R. (1996) "Persistent membrane translocation of protein kinase C α during 12-o-tetradecanoylphorbol-13-acetate-induced apoptosis of LNCaP human prostate cancer cells". *Cell Growth and Differentiation* 7 : 419-428.

Raffo, A.J., Kim, A.L., and Fine, R.L. (2000) "Formation of nuclear Bax/p53 complexes is associated with chemotherapy induced apoptosis". *Oncogene* 19 : 6216-6228.

Raffo, A.J., Perlman, H., Chen, M-W., Day, M.L., Streitman, J.S., and Buttyan, R. (1995) "Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo". *Cancer Research* 55 : 4438-4445.

- Rebbeck, T.R., Walker, A.H., Zeigler-Johnson, C., Weisburg, S., Martin, A.M., Nathanson, K.L., Wein, A.J., and Malkowicz, S.B. (2000) "Association of HPC2/ELAC2 genotypes and prostate cancer". *American Journal of Human Genetics* 67 : 1014-1019.
- Russell, P. J., Bennett, S., and Stricker, P. (1998) "Growth Factor Involvement in Progression of Prostate Cancer". *Clinical Chemistry* 44: 705-723.
- Sadar, M.D., Hussain, M., and Bruchovsky, N. (1999) "Prostate Cancer: Molecular Biology of Early Progression to Androgen Independence". *Endocrine-Related Cancer* 6: 487-502.
- Saito, M., Korsmeyer, S.J., and Schlesinger, P.H. (2000) "Bax-dependent transport of cytochrome c reconstituted in pure liposomes". *Nature Cell Biology* 2 : 553-555.
- Sgambato, A., Cittadini, A., Faraglia, B., and Weinstein, I.B. (2000). "Multiple functions of p27^{kip1} and its alterations in tumor cells: a review". *Journal of Cellular Physiology* 183 : 18-27.
- Sherr, C.J. (1996) "Cancer Cell Cycles". *Science* 274 : 1672-1677.
- Spitaler, M., Wiesenhofer, B., Biedermann, V., Seppi, T., Zimmermann, J., Grunicke, H., and Hofmann, J. (1999) "The Involvement of Protein Kinase C Isoenzymes α , ϵ and ζ in the Sensitivity to Antitumor Treatment and Apoptosis Induction". *Anticancer Research* 19: 3969-3976.
- Sturany, S., Lint, J.V., Muller, F., Wilda, M., Hameister, H., Hocker, M., Brey, A., Gern, U., Vandenheede, J., Gress, T., Adler, G., and Seufferlein, T. (2001) "Molecular cloning and characterization of the human protein kinase D2". *The Journal of Biological Chemistry* 276 : 3310-3318.
- Sumitomo, M., Ohba, M., Asakuma, J., Asano, T., Kuroki, T., Asano, T., and Hayakawa, M. (2002) "Protein kinase C δ amplifies ceramide formation via mitochondrial signaling in prostate cancer cells". *The Journal of Clinical Investigation* 109 : 827-836.
- Suzuki, M., Youle, R.J., and Tjandra, N. (2000) "Structure of Bax: coregulation of dimer formation and intracellular localization". *Cell* 103 : 645-654.
- Tan, Y., Demeter, M.R., Ruan, H., and Comb, M.J. (2000) "BAD Ser-155 phosphorylation regulates BAD/Bcl-XL interaction and cell survival". *The Journal of Biological Chemistry* 275 : 25865-25869.

Tan, Y., Ruan, H., Demeter, M.R., and Comb, M.J. (1999) "p90^{rsk} blocks Bad-mediated cell death via a protein kinase C-dependent pathway". *The Journal of Biological Chemistry* 274 : 34859-34867.

Tang, D.G., Li, L., Chopra, D.P., and Porter, A.T. (1998). "Extended Survivability of Prostate Cancer Cells in the Absence of Trophic Factors: Increased Proliferation, Evasion of Apoptosis, and the Role of Apoptosis Proteins". *Cancer Research* 58: 3466-3479.

Traub, O., Monia, B.P., Dean, N.M., and Berk, B.C. (1997) "PKC ϵ is required for mechano-sensitive activation of ERK1/2 in endothelial cells". *Journal of Biological Chemistry* 273 : 3125-3127.

Tsihlias, J., Kapusta, L., and Slingerland, J. (1999) "The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer". *Annual Reviews Medicine* 50 : 401-423.

Tso, C., McBride, W.H., Sun, J., Patel, B., Tsui, K., Paik, S.H., Gitlitz, B., Caliliw, R., van Ophoven, A., Wu, L., deKernion, J., and Belldgrun, A. (2000). "Androgen Deprivation Induces Selective Outgrowth of Aggressive Hormone-Refractory Prostate Cancer Clones Expressing Distinct Cellular and Molecular Properties Not Present in Parental Androgen-Dependent Cancer Cells". *The Cancer Journal* 6: 220-233.

Waldron, R.T., Iglesias, T., and Rozenfurt, E. (1999) "The pleckstrin homology domain of protein kinase D interacts preferentially with the η isoforms of protein kinase C". *The Journal of Biological Chemistry* 274 : 9224-9230.

Waldron, R.T., Rey, O., Iglesias, T., Tugal, T., Cantrell, D., and Rozengurt, E. (2001) "Activation loop Ser⁷⁴⁴ and Ser⁷⁴⁸ in protein kinase D are transphosphorylated *in vivo*". *The Journal of Biological Chemistry* 276 : 32606-32615.

Wang, H-G., Takayama, S., Rapp, U.R., and Reed, J.C. (1996) "Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1". *Proceedings of the National Academy of Sciences* 93 : 7063-7068.

Wang, L., McDonnell, S.K., Elkins, D.A., Slager, S.L., Christensen, E., Marks, A.F., Cunningham, J.M., Peterson, B.J., Jacobsen, S.J., Cerhan, J.R., Blute, M.L., Schaid, D.J., and Thibodeau, S.N. (2001) "Role of HPC2/ELAC2 in hereditary prostate cancer". *Cancer Research* 61 : 6494-6499.

Waterhouse, N.J. and Green, D.R. (1999) "Mitochondria and apoptosis: HQ or high-security prison?" *Journal of Clinical Immunology* 19 : 378-387.

- Webb, B.L.J., Hirst, S.J., and Giembycz, M.A. (2000) "Protein kinase C isoenzymes: a review of their structure, regulation and role in regulating airways smooth muscle tone and mitogenesis" *British Journal of Pharmacology* 130 : 1433-1452.
- Winter, R.N., Kramer, A., Borkowski, A., and Kyprianou, N. (2001) "Loss of caspase-1 and caspase-3 expression in human prostate cancer". *Cancer Research* 61 : 1227-1232.
- Wolf, B.B. and Green, D.R. (1999) "Suicidal tendencies: apoptotic cell death by caspase family proteinases". *The Journal of Biological Chemistry* 274 : 200049-200052.
- Wu, D., Foremann, T.L., Gregory, C.W., McJilton, M.A., Wescott, G.G., Ford, O.H., Alvey, R.F., Mohler, J.L., and Terrian, D.M. (2002) "Protein kinase C ϵ has the potential to advance the recurrence of human prostate cancer". *Cancer Research* 62 : 2423-2429.
- Wu, Y.-Q., Chen, H., Rubin, M.A., Wojno, K.J., and Cooney, K.A. (2001) "Loss of heterozygosity of the putative prostate cancer susceptibility gene HPC2/ELAC2 is uncommon in sporadic and familial prostate cancer". *Cancer Research* 61 : 8651-8653.
- Yuan, J., Bae, D., Cantrell, D., Nel, A.E., and Rozengurt, E. (2002) "Protein kinase D is a downstream target of protein kinase C θ ". *Biochemical and Biophysical Research Communications* 291 : 444-452.
- Zhao, X., Gschwend, J.E., Powell, C.T., Foster, R.G., Day, K.C., and Day, M.L. (1997) "Retinoblastoma protein-dependent growth signal conflict and caspase activity are required for protein kinase C-signaled apoptosis of prostate epithelial cells". *Journal of Biological Chemistry* 272 : 22751-22757.