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Protein kinase C-η (PKCη) is involved in cell proliferation, differentiation and plays an anti-apoptotic role in various cancer models. The purpose of this dissertation is to understand the regulation of PKCη in cell death signaling in breast cancer cells.

Tumor promoting phorbol esters are potent activators of PKC and prolonged treatment with these activators leads to downregulation and desensitization of PKC signaling. PKCη resists phorbol ester-induced downregulation and upregulated in response to prolonged treatment. We have found that phosphorylation of PKCη at conserved serine/threonine sites is increased by phorbol ester treatment. Single mutations that prevent phosphorylation at these conserved sites resulted in downregulation of PKCη. These results suggest that phosphorylation of PKCη at conserved sites prevents its downregulation.

We have found that PKCη can be significantly downregulated by inhibition of two pathways: PKC and phosphoinositide 3-kinase (PI3K) using pharmacological inhibitors. Inhibitors of PKC and PI3K induce dephosphorylation of PKCη by a calyculin A-specific phosphatase. These inhibitors differ in their ability to degrade PKCη via the proteasome-mediated pathway. Downregulation of PKCη by PKC inhibitor is not mediated by the proteasome degradation pathway, whereas downregulation by PI3K inhibitor is dependent on the proteasome. These results demonstrate that dephosphorylation is important for PKCη downregulation and this occurs through two distinct mechanisms.

A comparison of PKC protein levels in a series of isogenic cell lines representing a breast cancer progression model revealed that PKCη protein levels increased from the normal, benign

stage to the pre-malignant and metastatic stages. These results indicate that PKCη plays a potential role in breast cancer progression. Further analysis indicated that overexpression of PKCη in the MCF-7 breast adenocarcinoma cell line conferred resistance to tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL)-induced cell death. Therefore, PKCη protects breast cancer cells from TRAIL-induced apoptosis. Depletion of PKCη by small interfering RNA (siRNA) resulted in increased dimerization of pro-apoptotic Bax and decreased induction of anti-apoptotic Mcl-1 by TRAIL. These results indicate that PKCη may exert its anti-apoptotic effect by targeting Bcl-2 proteins, Bax and Mcl-1.

REGULATION OF PROTEIN KINASE C ETA IN BREAST CANCER CELLS

DISSERTATION

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By

Shalini D. Persaud, M.S.

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CHAPTER I

LIST OF ABBREVIATIONS

A	Alanine
A-loop	activation loop
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2-associated X protein
BH	Bcl-2 homology
BHK	baby hamster kidney cells
BID	BH3-interacting domain death agonist
Bok	Bcl-2-related ovarian killer
Cal A	calyculin A
cIAP-2	cytoplasmic inhibitor of apoptosis protein-2
DAG	diacyclglycerol
DcR	decoy receptor
DD	death domain

DGK	diacylglycerol kinase
DISC	death-inducing signaling complex
DR	death receptor
E	glutamate
ERK	extracellular signal-related kinase
FADD	Fas-associated death domain
FRET	fluorescent resonance energy transfer
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
HEK 293	human embryonic kidney 293 cells
HM	hydrophobic motif
IκB	inhibitor of κB
IKKα	IκB kinase α
IKKβ	IκB kinase β
ILV	indolactam V
IP3	inositol-1,4,5-trisphosphate

JNK	c-Jun NH(2)-terminal kinase
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukemia sequence-1
mTOR	mammalian target of rapamycin
mTORC2	mammalian target of rapamycin complex 2
NF-κB	nuclear factor kappa-B
OA	okadaic acid
p70S6K	p70 ribosomal S6 kinase
PARP	poly(ADP)ribose polymerase
PDBu	phorbol 12, 13-dibutyrate
PDK1	phosphoinositide-dependent kinase-1
PHLPP	PH domain and leucin rich repeat protein phosphatase
PI3K	phosphoinositide 3-kinase
PIP_2	phosphotidylinositol bisphosphate

PIP_3	phosphotidylinositol (3,4,5)-trisphosphate
PKC	Protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PP1	protein phosphatase 1
PP2A	protein phosphase 2A
PP2B	protein phosphatase 2B
PS	phosphotidylserine
PTEN	phosphatase and tensin homologue deleted from chromosome-10
RACK	receptors for activated C kinase
S	serine
siRNA	small interfering RNA
T	threonine
TM	transmembrane domain

TM	turn motif
TNF	Tumor necrosis factor-α
TPA	12-*O*-tetradecanoyl-phorbol-13-acetate
TRAIL	TNF-related apoptosis-inducing ligand
WT	wild-type

CHAPTER I

INTRODUCTION

Breast Cancer

Breast cancer is the most common type of cancer among American women, with the exception of skin cancer. It is estimated that 1 in 8 women (12%) will develop invasive breast cancer at some point in their lives. The American Cancer Society reported an expected diagnosis of 192,370 new invasive breast cancer cases and 40,610 breast cancer-related deaths among women in the United States in 2009 (www.cancer.org). Since deregulation of cellular processes such as cell proliferation and cell death can contribute to the cancer, it is critical to identify and characterize proteins involved in regulating these cellular processes to improve drug therapy and patient outcome. Members of the protein kinase C (PKC) family play critical roles in regulating cell proliferation, cell survival and tumor progression. Consequently they are attractive targets for cancer therapy [1].

Protein Kinase C (PKC)

a. Molecular heterogeneity and structure

PKC was first identified by Nishizuka and colleagues as calcium-activated, phospholipid-dependent serine/threonine kinases [2, 3]. It is now established that PKC is a multigene family consisting of 10 isozymes differing in their structural characteristics and biochemical properties

[4-6]. All PKC family members share a common structural backbone mainly consisting of a C-terminal catalytic domain and an N-terminal regulatory domain (Figure 1) [7]. The catalytic domain contains motifs involved in ATP-binding, substrate binding and catalysis [8]. The regulatory domain maintains PKCs in an inactive conformation by interacting with the catalytic domain through an autoinhibitory pseudosubstrate sequence (resembling a PKC substrate but contains an alanine in the phospho-acceptor position) [9]. This interaction prevents substrate access to the catalytic site [10]. The catalytic and regulatory domains are connected by a hinge region (V3) which also serves as a site of proteolysis. PKC isozymes are divided into three subclasses based on their cofactor requirements and structural characteristics [11]. Conventional PKCs (cPKCα, -βI, βII, γ) contain the four conserved regions (C1-C4) and variable regions (V1-V5) and are dependent on both Ca^{2+} and diacyclglycerol (DAG) for activation. Novel PKCs (nPKCδ, -ε, -η, -θ) differ from conventional PKCs because their C2 domain (Ca^{2+}-binding domain) is replaced by a C2-like domain that does not depend on Ca^{2+} for activation. The C1 domain contains the two cysteine-rich zinc finger structures (C1A and C1B) involved in membrane targeting and binding to DAG [12] Phorbol esters are pharmacological activators of PKCs that mimic DAG and bind to the C1A and C1B domains [13]. Atypical PKCs (aPKCι/λ, -ζ) lack the calcium-sensitive C2 domain and contain an atypical C1 domain with only one cysteine-rich zinc finger structure that binds to PIP_3 and ceremide, but not DAG or phorbol esters. These distinct structural and biochemical properties of PKC isozymes provide a basis for their diverse functional roles as key signal transducers in a wide range of cellular processes

including proliferation, differentiation and cell death [14]. The activity and function of PKCs are controlled by three major mechanisms: 1) phosphorylation, 2) co-factor binding and membrane translocation and 3) desensitization mechanisms resulting in degradation of the enzyme and signal termination [15]. These mechanisms will be described in detail in the following sections.

b. Regulation by phosphorylation

It is generally accepted that PKCs are phosphorylated at three conserved phosphorylation sites which influence their maturation, activity and stability [16]. The three conserved sites of phosphorylation are located in the *activation loop* (near the active site), *turn motif* (at a turn in the structure) and *hydrophobic motif* (in the hydrophobic pocket of the kinase). Phosphoinositide-dependent kinase-1 (PDK1) has been shown to phosphorylate the activation loop of many proteins including PKC isozymes [17-21], Akt/PKB [22] and p70S6K [23]. The sequence of phosphorylation events has been well documented for conventional PKC isozymes; however, controversy remains regarding the phosphorylation events for novel and atypical PKCs. Newly synthesized PKCs are loosely tethered to the membrane in "open" conformation with the substrate binding and activation loop sites exposed [24]. PDK1 can phosphorylate the exposed activation loop site which triggers autophosphorylation events at the turn motif site and the hydrophobic site [25]. These phosphorylations stabilize the mature kinase in a conformation that is optimal for activity, not only for PKCs, but for many growth-factor activated AGC kinases [16, 26]. In addition to serine/threonine phosphorylation, conventional, novel and atypical PKCs can be phosphorylated at tyrosine sites [27]; however, most studies have been

centered on regulation of PKCδ by tyrosine phosphorylation [28–30]. The regulation of PKCη by phosphorylation is not well established and this will be addressed in the second and third chapters.

c. Regulation by co-factors, activators and inhibitors

PKCs are dependent on interaction with acidic phospholipids, such as phosphotidylserine (PS) at the plasma membrane for activity [31]. PKCs are generally inactive in the cytosol in a closed, auto-inhibited conformation that is resistant to proteolysis [24]. In response to activation by extracellular stimuli, PKCs are translocated from the cytosol to the membrane by interaction with receptors for activated C kinase (RACKS) [32]. Specific RACK binding sites on each PKC isozyme have been identified allowing for isozyme- specific localization and cellular responses [33, 34]. Once PKCs are in close proximity to the membrane, they bind with high affinity to co-factors such as DAG through their C1 and C2 domains [35]. This interaction provides the energy necessary to induce an open conformational change in the kinase that exposes the substrate-binding site leading to phosphorylation of downstream substrates [36] (Figure 2).

Another mode of activation involves proteolytic dissociation of the regulatory domain from the catalytic domain following membrane translocation. For cPKCs and nPKCs, proteolysis is achieved by Ca^{2+} activated proteases, calpains [37] which leads to downregulation via the proteasome-mediated pathway [38]. In response to apoptotic stimuli, PKCs undergo limited proteolysis by caspases, a family of cysteine proteases that cleave proteins after aspartate

residues [39-41]. The catalytic fragment generated can activate downstream signaling events in the absence of activators or cofactors.

Two classes of PKC activators have been identified: tumor-promoting and non-tumor-promoting. The tumor-promoting activators include phorbol esters [42], ingenols [43], teleocidins [44] and octylindolactam V [45]. Non-tumor-promoting activators include bryostatins and DAG derivatives to name a few [46]. Conventional and novel PKCs are intracellular receptors for phorbol esters that activate PKCs in a manner similar to DAG because they share a common binding site [47-50]. Bryostatins antagonize the effects of phorbol esters and have been exploited for anti-cancer therapy [6]. Because of the differential actions of PKC activators, they have become useful tools in studying the function of PKC isozymes in tumor-promotion. Chapter 2 will focus on the regulation of PKCη phosphorylation by tumor-promoting phorbol ester, phorbol 12, 13-dibutyrate (PDBu).

PKC inhibitors are classified based on their sites of action: catalytic site inhibitors and regulatory site inhibitors [6]. Catalytic site inhibitors such as bisindolymalemides and derivatives act as competitive inhibitors for the ATP-binding site of PKCs [51, 52]. Regulatory domain inhibitors such as calphostin C act on the phorbol ester/DAG binding site of PKCs and is highly specific to PKC, but inhibits all isozymes to the same extent [53]. Another class of PKC inhibitors called cofactor inhibitors include molecules such as tamoxifen and spingosine which contain lipophilic regions that can interfere with PKC binding to phospholipids and DAG [54, 55]. Recently, isozyme-specific inhibitors have been identified based on their unique RACK

binding sequence [56, 57]. These peptide inhibitors act as competitive inhibitors that bind to RACKs and prevent them from binding and activating PKCs [56]. The IC_{50} values for PKC inhibitors used in this study are described in Table 1.

d. Inactivation and termination of PKC signaling

Since localization to the membrane is important for PKC activation, termination of PKC signaling can be achieved by reversal of membrane translocation. PKCs are disengaged from the membrane by desensitization of G-protein coupled receptors and by their own autophosphorylation [58, 59]. PKC reporter assay studies indicate that the levels of active PKC correlate with the levels of Ca^{2+} and DAG [60]. Therefore, depletion of these second messengers can result in inactivation of PKCs. DAGs are metabolized by diacyclglycerol kinases (DGKs) that phosphorylate DAG to convert it to phosphatidic acid [61]. Thus, DGKs decrease the levels of DAG and thus inhibit activation of PKC.

Prolonged activation by phorbol esters leads to subsequent inactivation and degradation of PKCs [62]. This process is known as activation-induced downregulation. Downregulation occurs through an increased rate of degradation of PKCs rather than a decrease in protein synthesis [63]. However, there are controversies surrounding the mechanism of phorbol ester-induced downregulation of PKCs. It was first hypothesized that activation of PKCs results in a change in conformation that is more susceptible to proteolytic cleavage by proteases such as calpains [64]. It was later discovered that mutants lacking calpain cleavage sites or treatment with calpain inhibitors did not block downregulation of PKCs by phorbol esters indicating

calpains were not involved in this process [65-67]. The requirement of kinase activity of PKCs for downregulation by phorbol esters was also a subject of controversy. A mutation in the ATP-binding site rendered the PKC “catalytically inactive” and these mutants resisted downregulation by phorbol esters [68]. Other studies indicated that the catalytically inactive mutant could be downregulated in response to phorbol esters through the activity of other PKCs [69-71]. Furthermore, studies with specific PKC isozymes indicated kinase activity was required for PKCδ downregulation, but PKCε downregulation required the activity of another endogenous PKC [63]. Therefore differences exist among PKC isozymes with regards to their downregulation mechanisms.

The active conformation of PKC is not only susceptible to cleavage by proteases, but also to dephosphorylation by phosphatases. As explained in the previous section, phosphorylation at three conserved sites is important to maintain PKC in a mature and active form. For conventional PKCs, it generally known that dephosphorylation of these sites precedes degradation [72]. However, this mechanism is not well established for novel PKC isozymes, since it was shown that PKCδ requires phosphorylation at the activation loop site for phorbol ester-induced downregulation [73]. Furthermore, a recent report indicated that unphosphorylated kinase-inactive mutants of PKCε and PKCα resisted PMA-induced downregulation and kinase-inactive mutants that retained priming phosphorylation were susceptible to PMA-induced downregulation [74]. Therefore, conflicts exist in the literature with regards to the link between phosphorylation state of PKCs and downregulation by phorbol esters.

Several downregulation mechanisms have been described for PKCs. The first was via the ubiquitin/proteasome pathway, a process by which proteins are "tagged" with ubiquitin for degradation via the proteasome machinery [75]. Ubiquitination and degradation of PKC-α, -δ and -ε were shown to be physiological responses to activation by several activators such as TPA, PMA, bryostatin and DAG [38, 76]. Dephosphorylation of PKCε and PKCα seems to predispose them to ubiquitinylation [76]. A second PKC desensitization pathway bears similarity to receptor desensitization via internalization and endosome targeting. Several reports indicate that upon phorbol ester treatment, PKCs are targeted to endosomes for degradation [77-79]. Conventional PKCs (PKCα and PKCβII) accumulate in the juxtanuclear/perinuclear region upon phorbol ester stimulation [78, 80] which is associated with dephosphorylation [72] and trafficking to endosomal compartments via caveolae (in the case PKCα) [79]. The sequence of events for vesicle-mediated downregulation of novel PKC isozymes have not been fully elucidated, although there is evidence that novel PKCs are targeted to endosomes [77]. Interestingly, phorbol ester-induced accumulation of PKCs in the perinuclear region not only targets PKCs for degradation, but it also allows PKCs to perform their function to regulate internalization and membrane trafficking of receptors and other membrane-bound proteins [81]. The focus of chapter 3 is to elucidate the mechanisms of PKCη downregulation in breast cancer cells.

Since PKCs were first identified as signaling molecules involved in the tumor promoting effects of phorbol esters [48], they have become therapeutic targets for the treatment of cancer [82]. PKC isozymes are commonly deregulated in various cancer types including prostate,

breast, colon, pancreatic, liver and kidney [82]. PKC isozymes also have variable roles in tumor biology depending on the cell type, tissue specificity and subcellular localization. Because of these differential effects of PKC isozymes, several PKC inhibitors such as staurosporine, bryostatin 1, curcumin and resveratrol have not gained much success in clinical trials [82]. Therefore, further research must be conducted to elucidate isozyme-specific regulation of PKCs to improve the current anti-cancer therapies. PKCs can function as "molecular sensors" to promote cell survival or execute cell death, thereby playing an important role in regulating apoptosis [83].

Apoptosis

Activation of cell death pathways is a major mechanism by which anti-cancer drugs exert their cytotoxic effect. Apoptosis or programmed cell death is a physiological process by which an organism can maintain tissue homeostasis and development to promote cellular functions [84]. A deregulation in apoptosis can lead to disease states, such as Alzheimer's, Huntington's, glaucoma, autoimmune disorders and cancer. A defect in apoptotic signaling can also contribute to resistance to chemotherapeutic agents by tumors. Identifying and targeting key players that cause deregulation of apoptosis is an important strategy in developing effective therapeutics for these disease states.

The biochemical events leading to apoptosis can be characterized into two phases, the initial "commitment" phase and the execution phase. Caspases, a family of aspartate-specific cysteine

proteases, play a vital role in both the initiation and execution phases of apoptosis. Initiator caspase-8, -9, and -10 can activate executioner caspase-3, -6 and -7 to cleave intracellular substrates such as poly(ADP)ribose polymerase (PARP), lamins, PKCδ and other structural and regulatory proteins contributing to the apoptotic cascade [85]. Two major pathways involving the activation of caspases are the extrinsic (receptor-mediated) and intrinsic (mitochondrial) apoptotic pathways which will be described in the following sections (Figure 3).

TRAIL/TNF Signaling

The extrinsic pathway is mediated by death receptors which belong to the tumor necrosis factor-α (TNF) superfamily, including Fas ligand, TNF and TRAIL [86-88]. TRAIL signaling is mediated by interaction with two types of receptors: death receptors which trigger apoptosis or decoy receptors which prevent apoptosis (Figure 4). There are five human TRAIL receptors identified to date; DR4/TRAIL-R1 [89], DR5/TRAIL-R2/KILLER [90-93], TRID/DcR1/TRAIL-R3 [90, 91, 94], TRAIL-R4/DcR2 [95, 96] and soluble receptor osteoprotegrin [97]. The apoptotic machinery is activated by oligomerization of the death receptors (DR4, DR5) upon ligand binding which leads to recruitment of the Fas-associated death domain (FADD) and initiator caspase-8 or -10 to form the death-inducing signaling complex (DISC) [98-100]. FADD is not recruited upon binding of TRAIL with decoy receptors (DcR1, DcR2) and apoptosis is prevented. Caspase-8 or -10 is activated by proteolysis in the

DISC complex and further activates caspase-3 which cleaves downstream death substrates (Figure 3).

Different PKC isozymes may have pro- or anti-apoptotic functions in TRAIL-mediated cell death [101-107]. Our lab has previously shown that PKCε confers a protective role against TNF- or TRAIL-induced cell death in MCF-7 breast cancer cells [103, 108]. Additionally, phorbol-ester mediated down-modulation of PKCε can sensitize acute myeloid leukaemia (AML) cells to TRAIL [102]. Conversely, activation of PKCδ by phorbol esters induced apoptosis in androgen-dependent prostate cancer cells by releasing death receptor ligands such as TNF and TRAIL [109]. The levels of PKC isozymes can influence TRAIL sensitivity or resistance. Pretreatment of melanoma cells containing low levels of PKCε with phorbol 12-myristate 13-acetate (PMA) reversed sensitization of cells to TRAIL-induced apoptosis [101]. Introduction of PKCε into the cells containing low PKCε reversed the sensitization to TRAIL. Furthermore, introduction of dominant-negative PKCε sensitized cells to TRAIL-induced cell death [101]. Thus, the status of PKC is important in determining the outcome of TRAIL treatment in cancer.

Bcl-2 Family and regulation of apoptosis

The mitochondrial membrane integrity is regulated by Bcl-2 family members [110]. Bcl-2 family includes both pro- and anti-apoptotic members which share conserved Bcl-2 homology (BH) 3 domains (Figure 4). The anti-apoptotic or Bcl-2-like members contain three to four BH domains essential for their anti-apoptotic function [111]. These members include Bcl-2, Bcl-X_L,

Bcl-w, A1/Bfl-1 and Mcl-1[111]. Pro-apoptotic members include: Bax, Bak and BH3-only members: BIM, BID, PUMA, NOXA, BAD, BMF, HRK and BIK [112]. Activation of proapoptotic Bax and Bak leads to depolarization of the mitochondrial membrane and release of cytochrome c into the cytosol [113-115], whereas antiapoptotic members such as Bcl-2 and Bcl-X_L antagonizes the action of Bax and Bak [116]. The released cytochrome c binds to Apaf-1 to form the apoptosome to activate caspase-9 and downstream caspase -3, and -7 [117]. Cancer cells may act to inhibit the intrinsic mitochondrial pathway by increasing antiapoptotic or decreasing proapoptotic proteins, including those of the Bcl-2 family to prevent cell death [118]. Recently, another antiapoptotic member of the Bcl-2 family, Mcl-1 was shown to play an important role in mediating TRAIL resistance in cancer cells. TRAIL appears to be a promising cancer therapeutic agent since it can induce cytotoxicity in cancer cells without harming normal cells [119, 120]. TRAIL can also induce a synergistic effect with conventional radiotherapy or chemotherapy to potentiate cancer cell death [121-125]. Mcl-1 and antiapoptotic protein cIAP-2 were induced by TRAIL treatment in TRAIL-resistant cells and downregulation of Mcl-1 and cIAP-2 greatly sensitized cells to TRAIL-induced cell death [126-128]. Similarly, downregulation of antiapoptotic PKCε [104] and PKCη [106] sensitized cancer cells to cell death by TRAIL. Therefore, targeting specific antiapoptotic proteins of the PKC and Bcl2 family may be an effective strategy to combat TRAIL resistance in cancer cells.

PKCη

a. Structure, phosphorylation and activation

PKCη is a member of the novel PKC family. It is structurally distinct from other PKCs and displays the most homology with PKCε [129, 130]. PKCη contains three conserved phosphorylation sites at the activation loop (Thr513), turn motif (Thr655) and hydrophobic motif (Ser674) (Figure 5). Similar to other PKCs, PKCη is known to be phosphorylated by PDK1 at the activation loop *in vitro* [131]. Recently, it was reported that PKCλ phosphorylates PKCη at the Ser674 hydrophobic motif site, which allows PDK1 to dock and subsequently phosphorylate PKCη at its activation loop in A9 mouse fibroblasts in response to parvovirus infection. The Thr655 turn motif site of PKCη was autophosphorylated as a result of Thr513 and Ser674 phosphorylations [132]. Cholesterol sulfates and sulfatides can specifically activate PKCη [133, 134]. In the presence of cholesterol-3-sulfate, casein kinase I (CK-I) was shown to phosphorylate PKCη at threonine residues *in vitro* [135]. In response to chronic activation by tumor promoting phorbol esters, we and others have shown that in MCF-7 breast carcinoma cells [136], EL4 mouse thymoma cells [137], and glioblastoma cells [138], PKCη levels were upregulated whereas other PKCs were downregulated. This suggests that PKCη may play a role in tumor promotion in these cells.

b. Role in tumor promotion

PKCη has limited tissue distribution compared to other PKCs wherein it is specifically expressed in epithelial cells, predominantly in the skin, lung and heart [129, 130]. Depending on the cell type, PKCη plays different roles in regulating proliferation and differentiation which contributes to its function in tumor promotion. Several lines of evidence support the function of PKCη as a tumor promoter in breast cancer cells. First, PKCη plays a role in enhancing G1/S phase cell-cycle progression in MCF-7 cells [139]. Second, PKCη plays a positive role in mammary gland development and signaling and its expression is upregulated in response to estradiol in MCF-7 cells [140] and during branching in pregnancy [141]. Third, PKCη expression was increased in locally invasive breast tumors and were strongly associated with lymph node metastases in human breast tissue samples [142]. Therefore, PKCη can be a potential target for therapeutic intervention in breast cancer.

PKCη has been identified as a potential therapeutic target in glioblastoma multiform [143] Overexpression of PKCη promoted proliferation and resistance to irradiation-induced cell death in glioblastoma cells [144]. Glioblastoma cell proliferation is regulated by PKCη via extracellular signal-related kinase (ERK) and its downstream transcription factor target Elk-1 [145]. In response to phorbol ester activation, PKCη regulates glioblastoma cell proliferation by targeting the Akt and mammalian target of rapamycin (mTOR) pathways [146]. Furthermore, PKCη overexpressing glioblastoma cells exhibited a decrease in sensitivity to rapamycin and in

response to phorbol esters through the interaction of PKCη and p70S6K, a downstream target of mTOR to regulate cell proliferation [147].

In contrast, PKCη negatively regulates proliferation in keratinocytes. PKCη induces differentiation of keratinocytes to a squamous cell phenotype that is concomitant with growth arrest [148]. This growth arrest was only detected in normal human and mouse keratinocytes, but not in human or mouse fibroblasts [148]. Using a skin tumor mouse model, pretreatment with the PKCη activator, cholesterol sulfate was found to inhibit skin tumor formation induced by tumor promoters [134]. Moreover, PKCη null mice displayed an increased susceptibility to tumor formation in a two-stage skin carcinogenesis model using 7,12-dimethylbenz(a)anthracene (DMBA) for tumor initiation and TPA for tumor promotion [149]. Interestingly, in renal cell carcinoma, a 3-fold increase in PKCη levels was detected in grade 3 and 4 versus grade 1 and 2 tumors, whereas other PKC isozymes showed a modest difference [150] . Therefore, the negative role of PKCη in tumorigenesis is specific only to skin carcinoma, whereas PKCη promotes tumorigenesis in breast and renal carcinomas and glioblastomas.

c. Anti-apoptotic role of PKCη in response to chemotherapeutic agents

PKCη contributes to resistance to chemotherapeutics in various cancer types. We have reported that overexpression of PKCη in MCF-7 cells delayed the onset of TNF-induced cell death via attenuation of caspase activation [151]. Lung carcinoma cells were sensitized to vincristine and paclitaxel treatment through downregulation of PKCη by antisense

oligonucleotides [152]. In addition, prostate cancer cells were sensitized to TRAIL-induced cell death by PKCη downregulation [106]. Furthermore, downregulation of PKCη by small interfering RNA (siRNA) sensitized Hodgkin's Lymphoma cells to doxorubicin- and camptothecin-induced apoptosis. Based on these reports, targeting PKCη downregulation can improve the efficacy of chemotherapeutic agents.

Project Hypothesis and Specific Aims

Improving the effectiveness of anti-cancer drugs has been a challenge to scientists and researchers world-wide due to the development of resistance by the cancer cells to the therapy. Research focused on identifying possible targets involved in the pro-survival mechanisms of cancer cells can greatly improve the outcome of cancer therapy. PKCη is one potential target that plays a major role in cell proliferation and cell cycle control [139]. PKCη levels are upregulated in many cancers, especially breast cancer [140-142] . PKCη is associated with tumor progression in renal cells [150], glioblastoma [143, 144, 146] and breast cancer cells [139]. PKCη is also associated with resistance to apoptosis in a variety of cancer cell types including Hodgkin's lymphoma [153], human lung carcinoma [152] and prostate cancer [106]. Our laboratory has shown that PKCη functions as an anti-apoptotic protein because overexpression of PKCη prevents apoptosis induced by tumor necrosis factor-α (TNF) [151]. Furthermore, PKCη resists downregulation by persistent treatment with tumor-promoting phorbol esters, suggesting that PKCη plays a role in tumorigenesis [137]. Therefore, targeting PKCη downregulation in breast cancer may provide an important strategy in cancer therapy. Since the phosphorylation status of a protein can regulate its state of activation as well as stability, **we hypothesize that the downregulation and function of PKCη is regulated by phosphorylation**. We will test this hypothesis through the following specific aims:

Specific Aim 1: To determine if phosphorylation of PKCη is important for downregulation

Specific Aim 2: To determine the mechanism of PKCη downregulation

Specific Aim 3: To determine the functional significance of PKCη downregulation on cell death**.**

The hypothetical model for the project is illustrated in Figure 6.

Notes on Materials used in the Dissertation

Chapter II represents submitted manuscript (Persaud, S., Pal, D. and Basu, A, Biochemistry, 2009)

Figure 1. PKC structure and isozymes

The PKC family consists of 10 members that are classified into three groups based on their structure and biochemical properties. All PKCs have a regulatory domain at the N-terminus and a catalytic domain at the C-terminus separated by a hinge region. The regulatory domain contains a pseudosubstrate sequence that interacts with the substrate-binding site in the catalytic domain in an auto-inhibitory manner. The conventional PKCs are dependent on both DAG and Ca^{2+} for activity. Novel PKCs are Ca^{2+}-independent but DAG/phorbol ester dependent. Atypical PKCs depend only on phospholipids for activity. Modified from Parker, P.J. and Murray-Rust, J. PKC at a glance. 2004. Journal of Cell Science. 117:131-132.

Figure 1

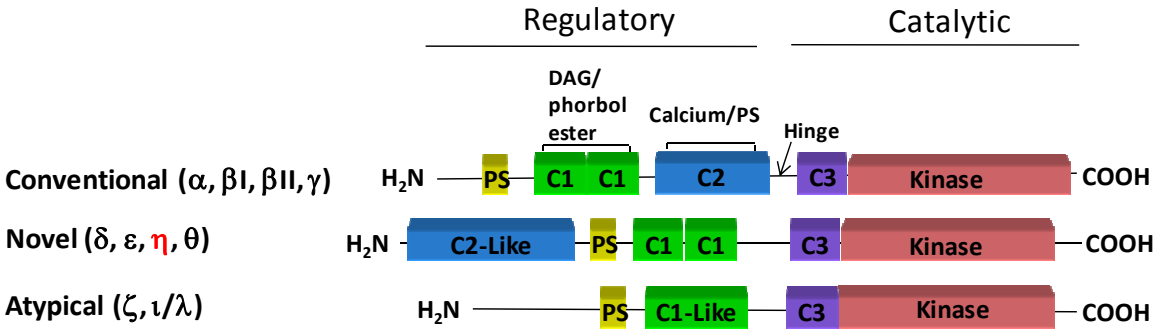
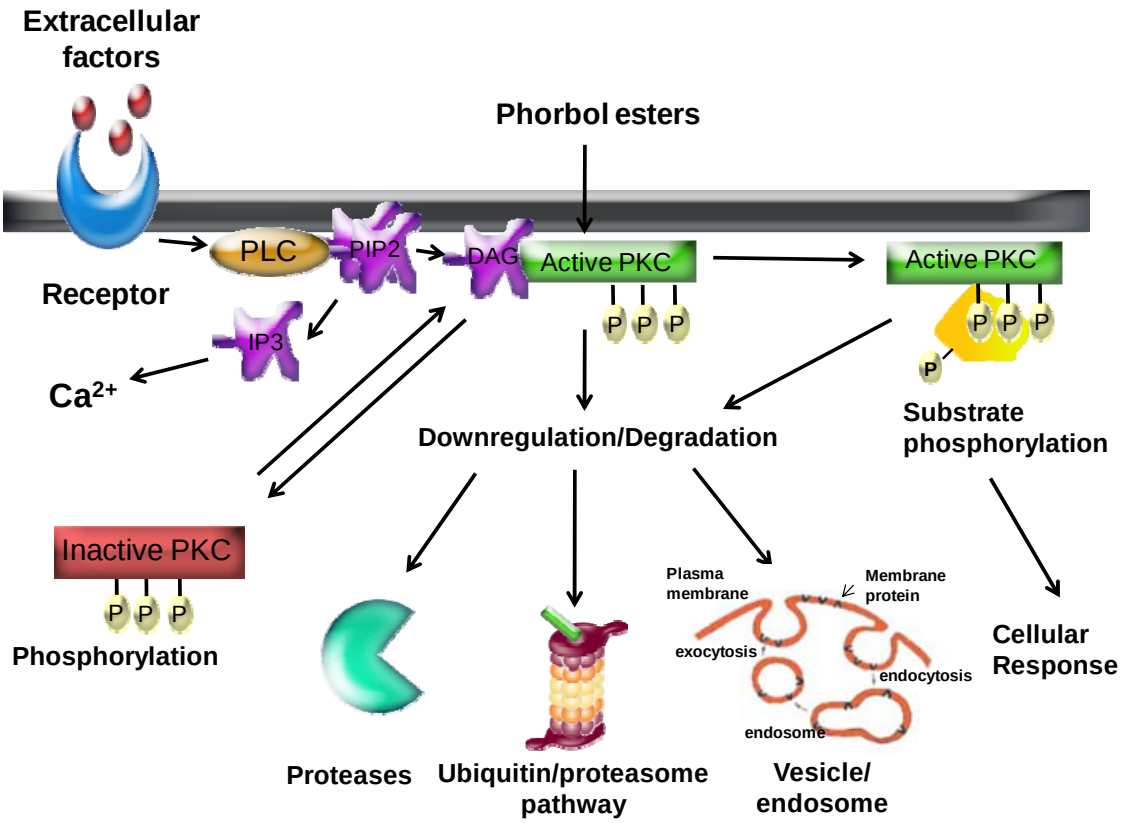

Regulatory
Catalytic
DAG/ phorbol ester
Calcium/PS
Hinge
Conventional (α, βI, βII, γ)
H2N
PS
C1
C1
C2
C3
Kinase
COOH
Novel (δ, ε, η, θ)
H2N
C2-Like
PS
C1
C1
C3
Kinase
COOH
Atypical (ζ, ι/λ)
H2N
PS
C1-Like
C3
Kinase
COOH

Figure 2. Regulation of PKCs

PKCs are inactive in the cytosol and are phosphorylated at three conserved sites in the activation loop, turn motif and hydrophobic motif which are important for the maturation, catalytic competence and stability of the kinase. Upon binding of extracellular factors to receptors on the plasma membrane, phospholipase C is activated to generate second messengers DAG (and IP3). IP3 acts to release Ca^{2+} from the endoplasmic reticulum. Ca^{2+} and DAG are physiological cofactors for PKC activation. PKCs are fully activated upon translocation to the plasma membrane which releases pseudosubtrate inhibition and allows PKCs to bind to downstream substrates to elicit a cellular response. Prolonged activation by pharmacological activators, such as phorbol esters triggers PKCs downregulation by protease-mediated, ubiquitin/proteasome-mediated or vesicle-mediated degradation, thereby terminating PKC signaling.

Figure 2



Adapted from Nature Reviews | Cancer

Figure 3: Apoptosis: Extrinsic and Intrinsic pathways

The hallmark of apoptosis or programmed cell death is the activation of caspases. Caspases can be either initiators (caspase-8, -9, -10) or executioners (caspase-3, -6, -7). Two major apoptotic pathways involving the activation of caspases are the extrinsic (receptor-mediated) and intrinsic (mitochondrial) pathways. The extrinsic pathway can be initiated by ligands such as TRAIL which bind to their receptors (DR4, DR5) containing death domains (DD) which trigger the recruitment of adaptor proteins such as FADD and caspase 8 forming the death-inducing signaling complex (DISC). Initiator procaspase 8 is processed into active caspase 8 which can activate executioner caspase 3 to trigger apoptosis. TRAIL can also bind to decoy receptors which do not have a functional death domain. The intrinsic pathway involves the Bcl-2 family of proteins which regulate the integrity of the mitochondrial membrane. Active caspase-8 can process the pro-apoptotic Bcl-2 family member Bid to form truncated Bid (t-Bid) that activates two other pro-apoptotic members Bax and Bak. Bax and Bak dimerize and bind to the mitochondrial membrane which depolarizes and destabilizes the membrane. Cytochrome C is released from the mitochondria and binds to Apaf1 forming the apoptosome which binds and activates initiator caspase-9. Active caspase-9 can activate caspase 3 and trigger apoptosis. Anti-apoptotic members such as Bcl-2 and Bcl-X_L antagonize Bax and Bak to prevent apoptosis. Modified from MacFarlane, M. TRAIL-induced signaling and apoptosis. Toxicology Letters 2003. 139: 89-97. [154]

Figure 3

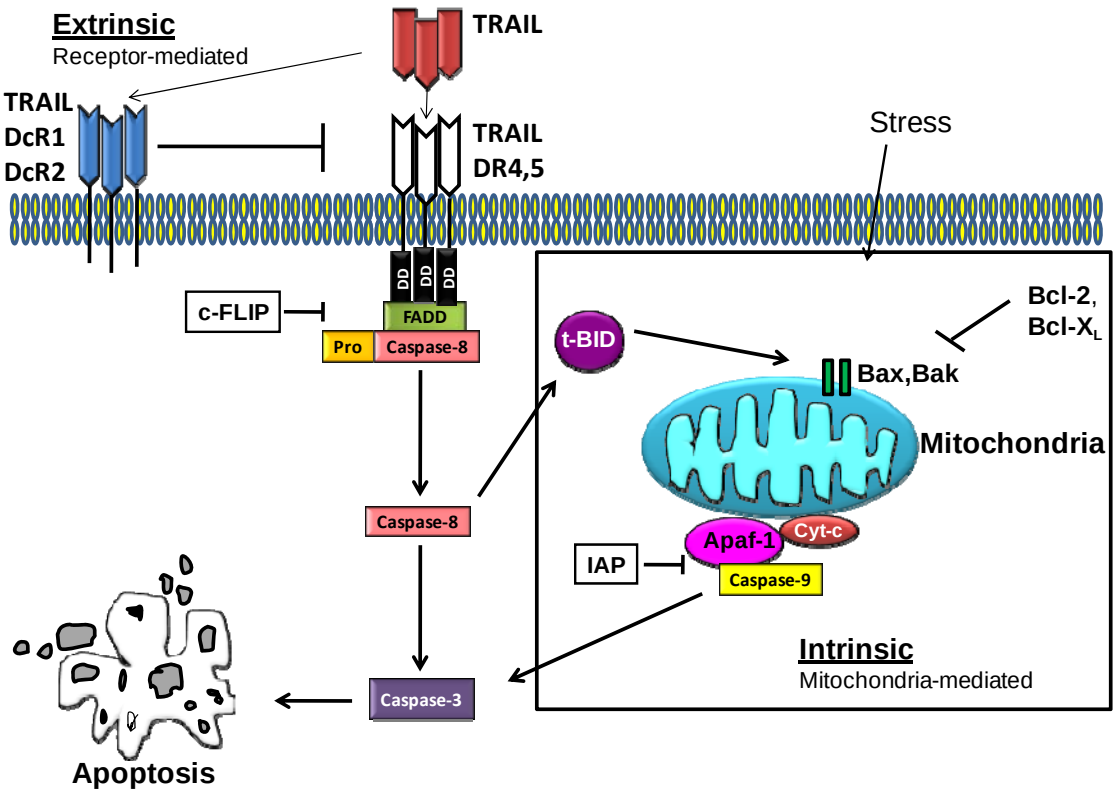

Extrinsic
Receptor-mediated
TRAIL
TRAIL
DcR1
DcR2
TRAIL
DR4,5
Stress
DD
DD
DD
FADD
c-FLIP
Pro
Caspase-8
t-BID
Bcl-2,
Bcl-X$_L$
Bax,Bak
Mitochondria
Caspase-8
Apaf-1
Cyt-c
IAP
Caspase-9
Intrinsic
Mitochondria-mediated
Caspase-3
Apoptosis

Figure 4: Bcl-2 protein family

The Bcl-2 family consists of anti-apoptotic and pro-apoptotic members. The multidomain members share four Bcl-2 homology (BH) domains which are important for their three-dimensional folding. BH3-interacting domain death agonist (BID) is the exception, since it displays similar folding as the multidomain members, but possesses only the BH3 domain. The transmembrane domain (TM) is important for interaction with the mitochondrial membrane. Bcl-2 antagonist/killer (Bak) and Bcl-2-associated X protein (Bax) are pro-apoptotic members that function to permeabilize the mitochondrial outer membrane. Bcl-2-related ovarian killer (Bok) is another pro-apoptotic membrane that is not well characterized. Anti-apoptotic members Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1 antagonize the activity of Bax and Bak in part by directly binding to them. The BH3 only domain members antagonize the activity of the anti-apoptotic members by inserting in their binding domain via the BH3 domain. Modified from Lessene, G. et. al. BCL-2 family antagonists for cancer therapy. Nature Reviews Drug Discovery 2008; 7: 989-1000

Figure 4.

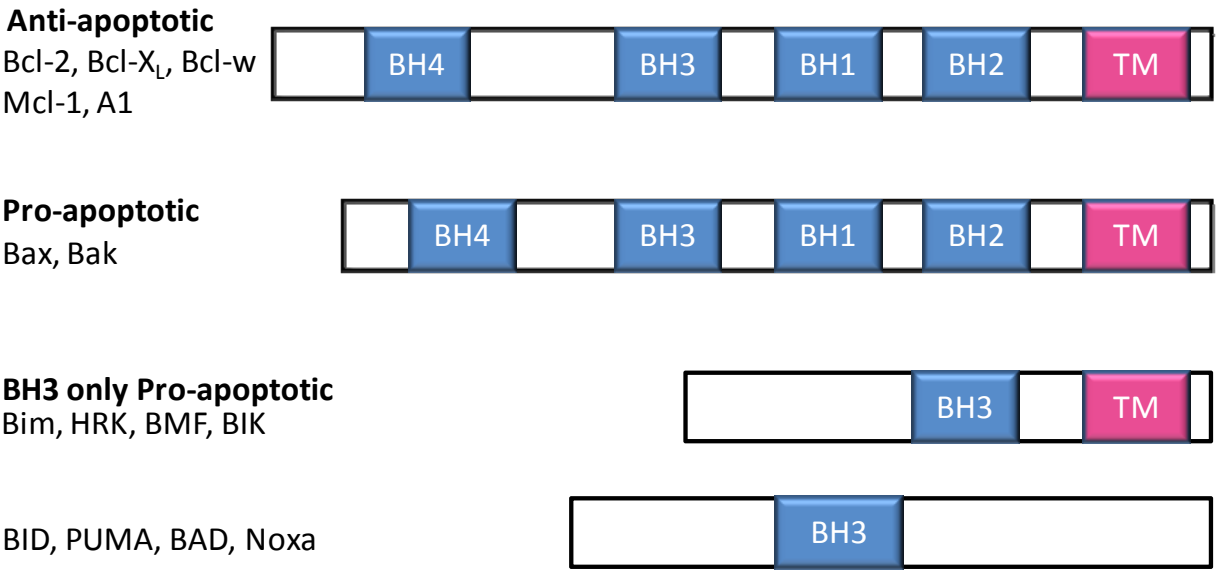

Anti-apoptotic
Bcl-2, Bcl-X_L, Bcl-w
Mcl-1, A1
BH4
BH3
BH1
BH2
TM
Pro-apoptotic
Bax, Bak
BH4
BH3
BH1
BH2
TM
BH3 only Pro-apoptotic
Bim, HRK, BMF, BIK
BH3
TM
BID, PUMA, BAD, Noxa
BH3

Figure 5: PKCη structure, phosphorylation, function

PKCη is a member of the novel group of PKCs. It is activated by DAG/phorbol ester binding through the C1 domain and its C2-like domain renders it insensitive to Ca^{2+}. PKCη, like other PKCs, contains three conserved sites of phosphorylation: activation loop (Thr513), turn motif (Thr655) and hydrophobic motif (Ser674); however, the regulation of PKCη by phosphorylation at these sites is not well understood. PKCη regulates several cellular functions, including, cell proliferation, differentiation and tumorigenesis. PKCη also functions as an anti-apoptotic protein and its overexpression can lead to resistance to apoptosis by chemotherapeutic agents. Depending on the cell-type, PKCη may have opposing effects in regulating proliferation and tumorigenesis.

Figure 5:

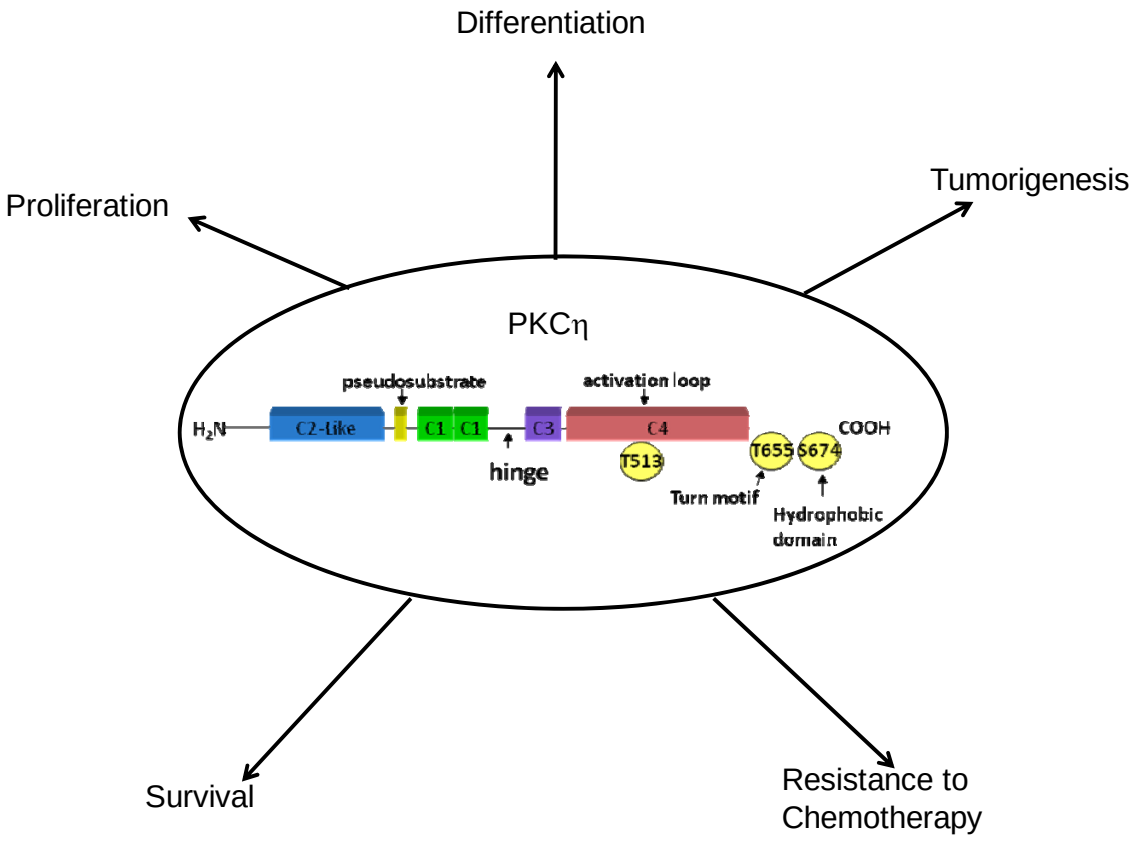

Differentiation
Proliferation
Tumorigenesis
PKCη
pseudosubstrate
activation loop
H2N
C2-Like
C1
C1
C3
C4
COOH
hinge
T513
T655
S674
Turn motif
Hydrophobic domain
Survival
Resistance to Chemotherapy

Figure 6. Hypothetical model of the project

Since PKCη plays a major role in regulating signaling events leading to survival and proliferation, it is worthwhile to study the mechanisms of PKCη downregulation so that it can be exploited for therapeutic intervention. In this study, we will use a breast cancer model to investigate how PKCη is regulated by phosphorylation and if mutations at the conserved phosphorylation sites can alter its expression (Aim 1). The second aim focuses on elucidating the mechanism(s) of PKCη downregulation. The third aim will evaluate the functional significance of PKCη downregulation on cell death via the extrinsic pathway through TRAIL-mediated apoptosis or the intrinsic pathway by regulating members of the Bcl-2 family. We hypothesize that the downregulation and function of PKCη is regulated by phosphorylation.

Figure 6.

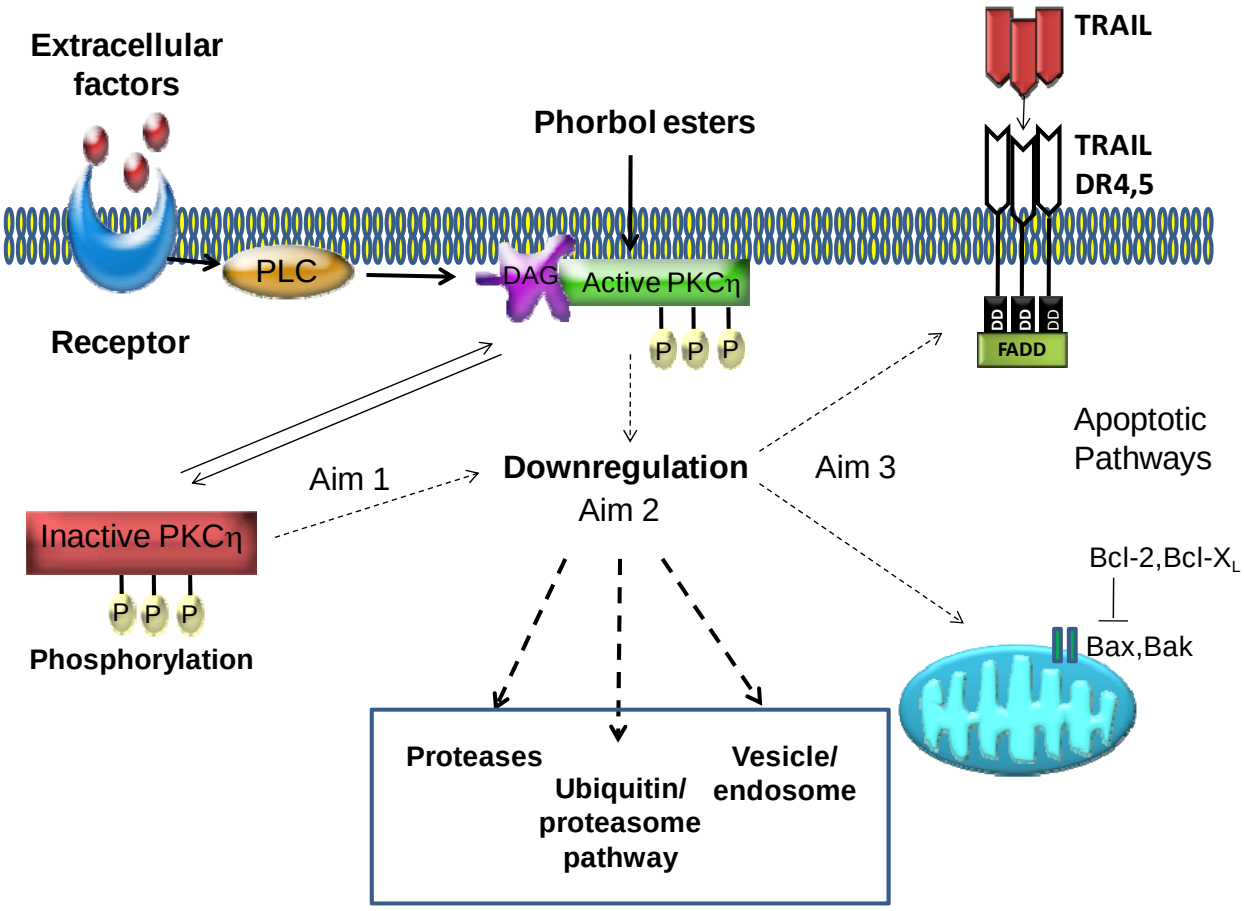

Extracellular factors
Receptor
PLC
Phorbol esters
DAG
Active PKCη
P P P
TRAIL
TRAIL DR4,5
DD DD DD
FADD
Apoptotic Pathways
Aim 1
Downregulation
Aim 2
Aim 3
Inactive PKCη
P P P
Phosphorylation
Bcl-2,Bcl-X$_L$
Bax,Bak
Proteases
Ubiquitin/ proteasome pathway
Vesicle/ endosome

Table 1. PKC inhibitors used in this study

Inhibitors				
Bisindolylmalemide 1 **GF-109203X**	Catalytic ATP-binding	cPKC nPKC aPKC	8-20 200-700 5800	Toullec, D., *et al*. 1991
Gö 6983	Catalytic ATP-binding	cPKC nPKC aPKC	6-7 10 60	Gschwendt, M., et al. 1996.
Gö 6976	Catalytic ATP-binding	cPKC nPKC aPKCs	2-6 (rat brain) No inhibition No inibition	Martiny-Baron, G., *et al*. 1993

REFERENCES

1. Griner, E.M. and M.G. Kazanietz, *Protein kinase C and other diacylglycerol effectors in cancer.* Nat Rev Cancer, 2007. **7**(4): p. 281-94.
2. Takai, Y., et al., *Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum.* J Biol Chem, 1977. **252**(21): p. 7603-9.
3. Inoue, M., et al., *Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain.* J Biol Chem, 1977. **252**(21): p. 7610-6.
4. Parker, P.J. and S.J. Parkinson, *AGC protein kinase phosphorylation and protein kinase C.* Biochem Soc Trans, 2001. **29**(Pt 6): p. 860-3.
5. Nishizuka, Y., *Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C.* Science, 1992. **258**(5082): p. 607-14.
6. Basu, A., *The potential of protein kinase C as a target for anticancer treatment.* Pharmacol Ther, 1993. **59**(3): p. 257-80.
7. Newton, A.C., *Protein kinase C. Seeing two domains.* Curr Biol, 1995. **5**(9): p. 973-6.
8. Taylor, S.S. and E. Radzio-Andzelm, *Three protein kinase structures define a common motif.* Structure, 1994. **2**(5): p. 345-55.
9. House, C. and B.E. Kemp, *Protein kinase C contains a pseudosubstrate prototope in its regulatory domain.* Science, 1987. **238**(4834): p. 1726-8.
10. Soderling, T.R., *Protein kinases. Regulation by autoinhibitory domains.* J Biol Chem, 1990. **265**(4): p. 1823-6.
11. Stabel, S. and P.J. Parker, *Protein kinase C.* Pharmacol Ther, 1991. **51**(1): p. 71-95.
12. Bell, R.M. and D.J. Burns, *Lipid activation of protein kinase C.* J Biol Chem, 1991. **266**(8): p. 4661-4.

13. Burns, D.J. and R.M. Bell, *Protein kinase C contains two phorbol ester binding domains.* J Biol Chem, 1991. **266**(27): p. 18330-8.
14. Dempsey, E.C., et al., *Protein kinase C isozymes and the regulation of diverse cell responses.* Am J Physiol Lung Cell Mol Physiol, 2000. **279**(3): p. L429-38.
15. Gould, C.M. and A.C. Newton, *The life and death of protein kinase C.* Curr Drug Targets, 2008. **9**(8): p. 614-25.
16. Newton, A.C., *Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm.* Biochem J, 2003. **370**(Pt 2): p. 361-71.
17. Cenni, V., et al., *Regulation of novel protein kinase C epsilon by phosphorylation.* Biochem J, 2002. **363**(Pt 3): p. 537-45.
18. Le Good, J.A., et al., *Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1.* Science, 1998. **281**(5385): p. 2042-5.
19. Balendran, A., et al., *Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms.* FEBS Lett, 2000. **484**(3): p. 217-23.
20. Dutil, E.M., A. Toker, and A.C. Newton, *Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1).* Curr Biol, 1998. **8**(25): p. 1366-75.
21. Chou, M.M., et al., *Regulation of protein kinase C zeta by PI 3-kinase and PDK-1.* Curr Biol, 1998. **8**(19): p. 1069-77.
22. Alessi, D.R., et al., *Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha.* Curr Biol, 1997. **7**(4): p. 261-9.
23. Pullen, N., et al., *Phosphorylation and activation of p70s6k by PDK1.* Science, 1998. **279**(5351): p. 707-10.

24. Dutil, E.M. and A.C. Newton, *Dual role of pseudosubstrate in the coordinated regulation of protein kinase C by phosphorylation and diacylglycerol.* J Biol Chem, 2000. **275**(14): p. 10697-701.
25. Behn-Krappa, A. and A.C. Newton, *The hydrophobic phosphorylation motif of conventional protein kinase C is regulated by autophosphorylation.* Curr Biol, 1999. **9**(14): p. 728-37.
26. Hauge, C., et al., *Mechanism for activation of the growth factor-activated AGC kinases by turn motif phosphorylation.* Embo J, 2007. **26**(9): p. 2251-61.
27. Konishi, H., et al., *Activation of protein kinase C by tyrosine phosphorylation in response to H2O2.* Proc Natl Acad Sci U S A, 1997. **94**(21): p. 11233-7.
28. Hall, K.J., M.L. Jones, and A.W. Poole, *Coincident regulation of PKCdelta in human platelets by phosphorylation of Tyr311 and Tyr565 and phospholipase C signalling.* Biochem J, 2007. **406**(3): p. 501-9.
29. Benes, C. and S.P. Soltoff, *Modulation of PKCdelta tyrosine phosphorylation and activity in salivary and PC-12 cells by Src kinases.* Am J Physiol Cell Physiol, 2001. **280**(6): p. C1498-510.
30. Li, W., et al., *Tyrosine phosphorylation of protein kinase C-delta in response to its activation.* J Biol Chem, 1994. **269**(4): p. 2349-52.
31. Nishizuka, Y., *Studies and perspectives of protein kinase C.* Science, 1986. **233**(4761): p. 305-12.
32. Mochly-Rosen, D., H. Khaner, and J. Lopez, *Identification of intracellular receptor proteins for activated protein kinase C.* Proc Natl Acad Sci U S A, 1991. **88**(9): p. 3997-4000.
33. Mochly-Rosen, D., et al., *Intracellular receptors for activated protein kinase C. Identification of a binding site for the enzyme.* J Biol Chem, 1991. **266**(23): p. 14866-8.
34. Schechtman, D. and D. Mochly-Rosen, *Adaptor proteins in protein kinase C-mediated signal transduction.* Oncogene, 2001. **20**(44): p. 6339-47.

35. Cho, W., *Membrane targeting by C1 and C2 domains.* J Biol Chem, 2001. **276**(35): p. 32407-10.
36. Johnson, J.E., J. Giorgione, and A.C. Newton, *The C1 and C2 domains of protein kinase C are independent membrane targeting modules, with specificity for phosphatidylserine conferred by the C1 domain.* Biochemistry, 2000. **39**(37): p. 11360-9.
37. Kishimoto, A., et al., *Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain).* J Biol Chem, 1989. **264**(7): p. 4088-92.
38. Lu, Z., et al., *Activation of protein kinase C triggers its ubiquitination and degradation.* Mol Cell Biol, 1998. **18**(2): p. 839-45.
39. Datta, R., et al., *Caspase-3-mediated cleavage of protein kinase C theta in induction of apoptosis.* J Biol Chem, 1997. **272**(33): p. 20317-20.
40. Basu, A., et al., *Proteolytic activation of protein kinase C-epsilon by caspase-mediated processing and transduction of antiapoptotic signals.* J Biol Chem, 2002. **277**(44): p. 41850-6.
41. Emoto, Y., et al., *Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells.* Embo J, 1995. **14**(24): p. 6148-56.
42. Blumberg, P.M., *In vitro studies on the mode of action of the phorbol esters, potent tumor promoters: part 1.* Crit Rev Toxicol, 1980. **8**(2): p. 153-97.
43. Kedei, N., et al., *Characterization of the interaction of ingenol 3-angelate with protein kinase C.* Cancer Res, 2004. **64**(9): p. 3243-55.
44. Fujiki, H. and T. Sugimura, *New classes of tumor promoters: teleocidin, aplysiatoxin, and palytoxin.* Adv Cancer Res, 1987. **49**: p. 223-64.
45. Irie, K., Y. Nakagawa, and H. Ohigashi, *Indolactam and benzolactam compounds as new medicinal leads with binding selectivity for C1 domains of protein kinase C isozymes.* Curr Pharm Des, 2004. **10**(12): p. 1371-85.
46. Nelson, T.J. and D.L. Alkon, *Neuroprotective versus tumorigenic protein kinase C activators.* Trends Biochem Sci, 2009. **34**(3): p. 136-45.

47. Castagna, M., et al., *Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters.* J Biol Chem, 1982. **257**(13): p. 7847-51.
48. Kikkawa, U., et al., *Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters.* J Biol Chem, 1983. **258**(19): p. 11442-5.
49. Leach, K.L., M.L. James, and P.M. Blumberg, *Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol.* Proc Natl Acad Sci U S A, 1983. **80**(14): p. 4208-12.
50. Sharkey, N.A., K.L. Leach, and P.M. Blumberg, *Competitive inhibition by diacylglycerol of specific phorbol ester binding.* Proc Natl Acad Sci U S A, 1984. **81**(2): p. 607-10.
51. Toullec, D., et al., *The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C.* J Biol Chem, 1991. **266**(24): p. 15771-81.
52. Davis, P.D., et al., *Potent selective inhibitors of protein kinase C.* FEBS Lett, 1989. **259**(1): p. 61-3.
53. Kobayashi, E., et al., *Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C.* Biochem Biophys Res Commun, 1989. **159**(2): p. 548-53.
54. O'Brian, C.A., et al., *Inhibition of protein kinase C by tamoxifen.* Cancer Res, 1985. **45**(6): p. 2462-5.
55. Hannun, Y.A., et al., *Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets.* J Biol Chem, 1986. **261**(27): p. 12604-9.
56. Souroujon, M.C. and D. Mochly-Rosen, *Peptide modulators of protein-protein interactions in intracellular signaling.* Nat Biotechnol, 1998. **16**(10): p. 919-24.
57. Churchill, E.N., N. Qvit, and D. Mochly-Rosen, *Rationally designed peptide regulators of protein kinase C.* Trends Endocrinol Metab, 2009. **20**(1): p. 25-33.

58. Feng, X., et al., *Regulation of receptor-mediated protein kinase C membrane trafficking by autophosphorylation.* J Biol Chem, 2000. **275**(22): p. 17024-34.
59. Feng, X. and Y.A. Hannun, *An essential role for autophosphorylation in the dissociation of activated protein kinase C from the plasma membrane.* J Biol Chem, 1998. **273**(41): p. 26870-4.
60. Gallegos, L.L., M.T. Kunkel, and A.C. Newton, *Targeting protein kinase C activity reporter to discrete intracellular regions reveals spatiotemporal differences in agonist-dependent signaling.* J Biol Chem, 2006. **281**(41): p. 30947-56.
61. Luo, B., et al., *Diacylglycerol kinases.* Cell Signal, 2004. **16**(9): p. 983-9.
62. Young, S., et al., *Down-regulation of protein kinase C is due to an increased rate of degradation.* Biochem J, 1987. **244**(3): p. 775-9.
63. Parker, P.J., et al., *Protein kinase C (PKC)-induced PKC degradation: a model for down-regulation.* Biochem Soc Trans, 1995. **23**(1): p. 153-5.
64. Pontremoli, S., et al., *Effects of a monoclonal anti-calpain antibody on responses of stimulated human neutrophils. Evidence for a role for proteolytically modified protein kinase C.* J Biol Chem, 1988. **263**(4): p. 1915-9.
65. Hong, D.H., et al., *Protein kinase C isoforms in muscle cells and their regulation by phorbol ester and calpain.* Biochim Biophys Acta, 1995. **1267**(1): p. 45-54.
66. Junco, M., et al., *Protein kinase C V3 domain mutants with differential sensitivities to m-calpain are not resistant to phorbol-ester-induced down-regulation.* Eur J Biochem, 1994. **223**(1): p. 259-63.
67. Junoy, B., et al., *Proteasome implication in phorbol ester- and GnRH-induced selective down-regulation of PKC (alpha, epsilon, zeta) in alpha T(3)-1 and L beta T(2) gonadotrope cell lines.* Endocrinology, 2002. **143**(4): p. 1386-403.
68. Ohno, S., et al., *A point mutation at the putative ATP-binding site of protein kinase C alpha abolishes the kinase activity and renders it down-regulation-insensitive. A*

molecular link between autophosphorylation and down-regulation. J Biol Chem, 1990. **265**(11): p. 6296-300.

69. Freisewinkel, I., D. Riethmacher, and S. Stabel, *Downregulation of protein kinase C-gamma is independent of a functional kinase domain.* FEBS Lett, 1991. **280**(2): p. 262-6.
70. Pears, C. and P.J. Parker, *Down-regulation of a kinase defective PKC-alpha.* FEBS Lett, 1991. **284**(1): p. 120-2.
71. Lindner, D., M. Gschwendt, and F. Marks, *Down-regulation of protein kinase C in Swiss 3T3 fibroblasts is independent of its phosphorylating activity.* Biochem Biophys Res Commun, 1991. **176**(3): p. 1227-31.
72. Hansra, G., et al., *Multisite dephosphorylation and desensitization of conventional protein kinase C isotypes.* Biochem J, 1999. **342 (Pt 2)**: p. 337-44.
73. Srivastava, J., et al., *Phosphorylation is required for PMA- and cell-cycle-induced degradation of protein kinase Cdelta.* Biochem J, 2002. **368**(Pt 1): p. 349-55.
74. Cameron, A.J., et al., *PKC maturation is promoted by nucleotide pocket occupation independently of intrinsic kinase activity.* Nat Struct Mol Biol, 2009.
75. Ciechanover, A., *The ubiquitin-mediated proteolytic pathway: mechanisms of action and cellular physiology.* Biol Chem Hoppe Seyler, 1994. **375**(9): p. 565-81.
76. Lee, H.W., et al., *Bryostatin 1 and phorbol ester down-modulate protein kinase C-alpha and -epsilon via the ubiquitin/proteasome pathway in human fibroblasts.* Mol Pharmacol, 1997. **51**(3): p. 439-47.
77. Goode, N.T., M.A. Hajibagheri, and P.J. Parker, *Protein kinase C (PKC)-induced PKC down-regulation. Association with up-regulation of vesicle traffic.* J Biol Chem, 1995. **270**(6): p. 2669-73.
78. Becker, K.P. and Y.A. Hannun, *cPKC-dependent sequestration of membrane-recycling components in a subset of recycling endosomes.* J Biol Chem, 2003. **278**(52): p. 52747-54.

79. Prevostel, C., et al., *Protein kinase C(alpha) actively downregulates through caveolae-dependent traffic to an endosomal compartment.* J Cell Sci, 2000. **113 (Pt 14)**: p. 2575-84.

80. Becker, K.P. and Y.A. Hannun, *Isoenzyme-specific translocation of protein kinase C (PKC)betaII and not PKCbetaI to a juxtanuclear subset of recycling endosomes: involvement of phospholipase D.* J Biol Chem, 2004. **279**(27): p. 28251-6.

81. Alvi, F., et al., *Regulation of membrane trafficking and endocytosis by protein kinase C: emerging role of the pericentrion, a novel protein kinase C-dependent subset of recycling endosomes.* Cell Mol Life Sci, 2007. **64**(3): p. 263-70.

82. Ali, A.S., et al., *Exploitation of protein kinase C: a useful target for cancer therapy.* Cancer Treat Rev, 2009. **35**(1): p. 1-8.

83. Reyland, M.E., *Protein Kinase C and Apoptosis,* in *Apoptosis, Cell Signaling, and Human Diseases Molecular Mechanisms, Volume 2* R. Srivastava, Editor. 2007, Humana Press. p. 31-55.

84. Waring, P., F.J. Kos, and A. Mullbacher, *Apoptosis or programmed cell death.* Med Res Rev, 1991. **11**(2): p. 219-36.

85. Cohen, G.M., *Caspases: the executioners of apoptosis.* Biochem J, 1997. **326 (Pt 1)**: p. 1-16.

86. Baker, S.J. and E.P. Reddy, *Transducers of life and death: TNF receptor superfamily and associated proteins.* Oncogene, 1996. **12**(1): p. 1-9.

87. Cosman, D., *A family of ligands for the TNF receptor superfamily.* Stem Cells, 1994. **12**(5): p. 440-55.

88. Song, C. and B. Jin, *TRAIL (CD253), a new member of the TNF superfamily.* J Biol Regul Homeost Agents, 2005. **19**(1-2): p. 73-7.

89. Pan, G., et al., *The receptor for the cytotoxic ligand TRAIL.* Science, 1997. **276**(5309): p. 111-3.

90. Pan, G., et al., *An antagonist decoy receptor and a death domain-containing receptor for TRAIL.* Science, 1997. **277**(5327): p. 815-8.
91. Sheridan, J.P., et al., *Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors.* Science, 1997. **277**(5327): p. 818-21.
92. Walczak, H., et al., *TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL.* Embo J, 1997. **16**(17): p. 5386-97.
93. Wu, G.S., et al., *KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene.* Nat Genet, 1997. **17**(2): p. 141-3.
94. Degli-Esposti, M.A., et al., *Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family.* J Exp Med, 1997. **186**(7): p. 1165-70.
95. Degli-Esposti, M.A., et al., *The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain.* Immunity, 1997. **7**(6): p. 813-20.
96. Marsters, S.A., et al., *A novel receptor for Apo2L/TRAIL contains a truncated death domain.* Curr Biol, 1997. **7**(12): p. 1003-6.
97. Emery, J.G., et al., *Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL.* J Biol Chem, 1998. **273**(23): p. 14363-7.
98. Bodmer, J.L., et al., *TRAIL receptor-2 signals apoptosis through FADD and caspase-8.* Nat Cell Biol, 2000. **2**(4): p. 241-3.
99. Kischkel, F.C., et al., *Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5.* Immunity, 2000. **12**(6): p. 611-20.
100. Sprick, M.R., et al., *FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2.* Immunity, 2000. **12**(6): p. 599-609.
101. Gillespie, S., X.D. Zhang, and P. Hersey, *Variable expression of protein kinase C epsilon in human melanoma cells regulates sensitivity to TRAIL-induced apoptosis.* Mol Cancer Ther, 2005. **4**(4): p. 668-76.

102. Gobbi, G., et al., *Phorbol ester-induced PKC{epsilon} down-modulation sensitizes AML cells to TRAIL-induced apoptosis and cell differentiation.* Blood, 2008.
103. Shankar, E., U. Sivaprasad, and A. Basu, *Protein kinase C epsilon confers resistance of MCF-7 cells to TRAIL by Akt-dependent activation of Hdm2 and downregulation of p53.* Oncogene, 2008. **27**(28): p. 3957-66.
104. Felber, M., J. Sonnemann, and J.F. Beck, *Inhibition of novel protein kinase C-epsilon augments TRAIL-induced cell death in A549 lung cancer cells.* Pathol Oncol Res, 2007. **13**(4): p. 295-301.
105. Shinohara, H., et al., *A protective role of PKCepsilon against TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in glioma cells.* Biochem Biophys Res Commun, 2001. **284**(5): p. 1162-7.
106. Sonnemann, J., et al., *Down-regulation of protein kinase Ceta potentiates the cytotoxic effects of exogenous tumor necrosis factor-related apoptosis-inducing ligand in PC-3 prostate cancer cells.* Mol Cancer Ther, 2004. **3**(7): p. 773-81.
107. Farrow, B., et al., *Activation of conventional PKC isoforms increases expression of the pro-apoptotic protein Bad and TRAIL receptors.* Int J Gastrointest Cancer, 2002. **32**(2-3): p. 63-72.
108. Lu, D., J. Huang, and A. Basu, *Protein kinase Cepsilon activates protein kinase B/Akt via DNA-PK to protect against tumor necrosis factor-alpha-induced cell death.* J Biol Chem, 2006. **281**(32): p. 22799-807.
109. Gonzalez-Guerrico, A.M. and M.G. Kazanietz, *Phorbol ester-induced apoptosis in prostate cancer cells via autocrine activation of the extrinsic apoptotic cascade: a key role for protein kinase C delta.* J Biol Chem, 2005. **280**(47): p. 38982-91.
110. Borner, C., *The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions.* Mol Immunol, 2003. **39**(11): p. 615-47.
111. Adams, J.M. and S. Cory, *The Bcl-2 protein family: arbiters of cell survival.* Science, 1998. **281**(5381): p. 1322-6.

112. Chao, D.T. and S.J. Korsmeyer, *BCL-2 family: regulators of cell death.* Annu Rev Immunol, 1998. **16**: p. 395-419.
113. Er, E., et al., *Mitochondria as the target of the pro-apoptotic protein Bax.* Biochim Biophys Acta, 2006. **1757**(9-10): p. 1301-11.
114. Degli Esposti, M. and C. Dive, *Mitochondrial membrane permeabilisation by Bax/Bak.* Biochem Biophys Res Commun, 2003. **304**(3): p. 455-61.
115. Scorrano, L. and S.J. Korsmeyer, *Mechanisms of cytochrome c release by proapoptotic BCL-2 family members.* Biochem Biophys Res Commun, 2003. **304**(3): p. 437-44.
116. Cheng, E.H., et al., *BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis.* Mol Cell, 2001. **8**(3): p. 705-11.
117. Zou, H., et al., *Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3.* Cell, 1997. **90**(3): p. 405-13.
118. van Loo, G., et al., *The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet.* Cell Death Differ, 2002. **9**(10): p. 1031-42.
119. Ashkenazi, A., et al., *Safety and antitumor activity of recombinant soluble Apo2 ligand.* J Clin Invest, 1999. **104**(2): p. 155-62.
120. Walczak, H., et al., *Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo.* Nat Med, 1999. **5**(2): p. 157-63.
121. Xu, L.H., et al., *Synergistic antitumor effect of TRAIL and doxorubicin on colon cancer cell line SW480.* World J Gastroenterol, 2003. **9**(6): p. 1241-5.
122. Maduro, J.H., et al., *Targeting pro-apoptotic trail receptors sensitizes HeLa cervical cancer cells to irradiation-induced apoptosis.* Int J Radiat Oncol Biol Phys, 2008. **72**(2): p. 543-52.
123. Xu, L.H., et al., *[Synergistic effect of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and doxorubicin on human colon cancer SW480 cells].* Ai Zheng, 2003. **22**(8): p. 816-20.

124. Mizutani, Y., et al., *Potentiation of the sensitivity of renal cell carcinoma cells to TRAIL-mediated apoptosis by subtoxic concentrations of 5-fluorouracil.* Eur J Cancer, 2002. **38**(1): p. 167-76.
125. Nagane, M., et al., *Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand in vitro and in vivo.* Cancer Res, 2000. **60**(4): p. 847-53.
126. Ricci, M.S., et al., *Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death.* Cancer Cell, 2007. **12**(1): p. 66-80.
127. Wirth, T., et al., *Telomerase-dependent virotherapy overcomes resistance of hepatocellular carcinomas against chemotherapy and tumor necrosis factor-related apoptosis-inducing ligand by elimination of Mcl-1.* Cancer Res, 2005. **65**(16): p. 7393-402.
128. Han, J., et al., *Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis.* J Biol Chem, 2006. **281**(15): p. 10153-63.
129. Bacher, N., et al., *Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin, and heart.* Mol Cell Biol, 1991. **11**(1): p. 126-33.
130. Osada, S., et al., *A phorbol ester receptor/protein kinase, nPKC eta, a new member of the protein kinase C family predominantly expressed in lung and skin.* J Biol Chem, 1990. **265**(36): p. 22434-40.
131. Brandlin, I., et al., *Protein kinase C(mu) regulation of the JNK pathway is triggered via phosphoinositide-dependent kinase 1 and protein kinase C(epsilon).* J Biol Chem, 2002. **277**(47): p. 45451-7.
132. Lachmann, S., et al., *Parvovirus interference with intracellular signalling: mechanism of PKCeta activation in MVM-infected A9 fibroblasts.* Cell Microbiol, 2008. **10**(3): p. 755-69.

133. Kuroki, T., et al., *Cholesterol sulfate, an activator of protein kinase C mediating squamous cell differentiation: a review.* Mutat Res, 2000. **462**(2-3): p. 189-95.
134. Chida, K., et al., *Cholesterol sulfate, a second messenger for the eta isoform of protein kinase C, inhibits promotional phase in mouse skin carcinogenesis.* Cancer Res, 1995. **55**(21): p. 4865-9.
135. Okano, M., et al., *Activation of C-kinase eta through its cholesterol-3-sulfate-dependent phosphorylation by casein kinase I in vitro.* Biol Pharm Bull, 2004. **27**(1): p. 109-12.
136. Basu, A., *The involvement of novel protein kinase C isozymes in influencing sensitivity of breast cancer MCF-7 cells to tumor necrosis factor-alpha.* Mol Pharmacol, 1998. **53**(1): p. 105-11.
137. Resnick, M.S., et al., *Selective up-regulation of protein kinase C eta in phorbol ester-sensitive versus -resistant EL4 mouse thymoma cells.* Cancer Res, 1997. **57**(11): p. 2209-15.
138. Hussaini, I.M., et al., *Phorbol 12-myristate 13-acetate induces protein kinase ceta-specific proliferative response in astrocytic tumor cells.* J Biol Chem, 2000. **275**(29): p. 22348-54.
139. Fima, E., et al., *PKCeta enhances cell cycle progression, the expression of G1 cyclins and p21 in MCF-7 cells.* Oncogene, 2001. **20**(46): p. 6794-804.
140. Karp, G., A. Maissel, and E. Livneh, *Hormonal regulation of PKC: estrogen up-regulates PKCeta expression in estrogen-responsive breast cancer cells.* Cancer Lett, 2007. **246**(1-2): p. 173-81.
141. Masso-Welch, P.A., et al., *Protein kinase C eta upregulation and secretion during postnatal rat mammary gland differentiation.* Eur J Cell Biol, 1998. **77**(1): p. 48-59.
142. Masso-Welch, P.A., et al., *Altered expression and localization of PKC eta in human breast tumors.* Breast Cancer Res Treat, 2001. **68**(3): p. 211-23.
143. Martin, P.M. and I.M. Hussaini, *PKC eta as a therapeutic target in glioblastoma multiforme.* Expert Opin Ther Targets, 2005. **9**(2): p. 299-313.

144. Hussaini, I.M., et al., *Protein kinase C-eta regulates resistance to UV- and gamma-irradiation-induced apoptosis in glioblastoma cells by preventing caspase-9 activation.* Neuro Oncol, 2002. **4**(1): p. 9-21.
145. Uht, R.M., et al., *The protein kinase C-eta isoform induces proliferation in glioblastoma cell lines through an ERK/Elk-1 pathway.* Oncogene, 2007. **26**(20): p. 2885-93.
146. Aeder, S.E., et al., *PKC-eta mediates glioblastoma cell proliferation through the Akt and mTOR signaling pathways.* Oncogene, 2004. **23**(56): p. 9062-9.
147. Martin, P.M., et al., *Phorbol 12-myristate 13-acetate and serum synergize to promote rapamycin-insensitive cell proliferation via protein kinase C-eta.* Oncogene, 2007. **26**(3): p. 407-14.
148. Ohba, M., et al., *Induction of differentiation in normal human keratinocytes by adenovirus-mediated introduction of the eta and delta isoforms of protein kinase C.* Mol Cell Biol, 1998. **18**(9): p. 5199-207.
149. Chida, K., et al., *Disruption of protein kinase Ceta results in impairment of wound healing and enhancement of tumor formation in mouse skin carcinogenesis.* Cancer Res, 2003. **63**(10): p. 2404-8.
150. Brenner, W., et al., *Protein kinase C eta is associated with progression of renal cell carcinoma (RCC).* Anticancer Res, 2003. **23**(5A): p. 4001-6.
151. Akkaraju, G.R. and A. Basu, *Overexpression of protein kinase C-eta attenuates caspase activation and tumor necrosis factor-alpha-induced cell death.* Biochem Biophys Res Commun, 2000. **279**(1): p. 103-7.
152. Sonnemann, J., et al., *Down-regulation of protein kinase Ceta by antisense oligonucleotides sensitises A549 lung cancer cells to vincristine and paclitaxel.* Cancer Lett, 2004. **209**(2): p. 177-85.
153. Abu-Ghanem, S., et al., *PKCeta expression contributes to the resistance of Hodgkin's lymphoma cell lines to apoptosis.* Cancer Biol Ther, 2007. **6**(9): p. 1375-80.

154. MacFarlane, M., *TRAIL-induced signalling and apoptosis.* Toxicol Lett, 2003. **139**(2-3): p. 89-97.

CHAPTER II

PHOSPHORYLATION OF PROTEIN KINASE Cη AT CONSERVED SITES PREVENTS ITS DOWNREGULATION

SUMMARY

Tumor promoting phorbol esters are potent activators of conventional and novel PKCs, but persistent treatment with phorbol esters leads to downregulation of these PKCs. Interestingly, PKCη, a novel PKC isozyme, resists downregulation by tumor-promoting phorbol esters. In the present study, we have investigated if the phosphorylation status of PKCη regulates its stability. Prolonged treatment with several PKC activators, including phorbol 12, 13-dibutyrate, 12-*O*-tetradecanoylphorbol-13-acetate and indolactam V caused upregulation of PKCη whereas the general PKC inhibitor Gö 6983 led to the downregulation of PKCη in MCF-7 cells. The conventional PKC inhibitor Gö 6976 had little effect on PKCη downregulation. Treatment of MCF-7 cells with the protein phosphatase inhibitor calyculin A prevented PKCη downregulation by the PKC inhibitor Gö 6983. Mutation of PKCη at the activation loop (Thr513), turn-motif (Thr655) or hydrophobic motif (Ser674) to Ala but not Glu decreased PKCη level compared to wild-type PKCη. Gö 6983 had no additional effect on the dowregulation of PKCη in these Ala mutants. Thus, phosphorylation of PKCη at the conserved serine/threonine sites is important for its stability.

INTRODUCTION

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases involved in regulating various cellular processes including cell proliferation, differentiation and cell death [1, 2]. To date, there are at least ten PKC isozymes identified which differ in their structure, function and biochemical properties [3-7]. Conventional PKCα, -βI, -βII and -γ require both calcium and diacylglycerol (DAG) for activation. Novel PKCδ, -ε, -η and -θ are calcium-independent but DAG-dependent whereas atypical PKCι/λ and -ζ require neither calcium nor DAG for their activity. PKCs are receptors for tumor promoting phorbol esters which are potent activators of conventional and novel PKCs and can substitute for DAG [8, 9]. Interaction of PKC with DAG is transient, whereas phorbol esters bind to PKC with higher affinity leading to persistent activation [10]. Prolonged treatment with tumor promoting phorbol esters leads to the degradation or downregulation of phorbol-ester sensitive PKCs.

In addition to cofactors, PKCs are also regulated by phosphorylation at three conserved sites: the activation loop (A-loop), turn motif (TM) and hydrophobic motif (HM) [11]. Phosphorylation of PKCs at these sites primes them for activation and maintains them in a closed protease/phosphatase resistant form [11, 12]. While the A-loop phosphorylation of PKCs and other AGC kinases has been shown to regulate their activity [11], the phosphorylation at HM has been implicated in regulating stability and phosphatase resistance [13-16]. The phosphorylation at TM site has been reported to regulate both kinase activity and stability [14, 15, 17].

Since the levels of PKCs can determine their function, it is important to understand the mechanisms regulating PKC level. The novel PKCη was upregulated in breast cancer tissues [18] and overexpression of PKCη was associated with resistance to many chemotherapeutic agents [19-23]. We and others have shown that PKCη resists downregulation by phorbol ester in various cell types [24-27]. PKCη also contains three conserved phosphorylation sites at the A-loop (Thr513), TM (Thr655) and HM (Ser674) [28]. It is, however, not known how phosphorylation of PKCη at conserved sites regulates PKCη downregulation. In the present study, we have examined if phosphorylation of PKCη protects it against phorbol ester-mediated downregulation. Our results show that PKC activators upregulate PKCη whereas general PKC inhibitors trigger its downregulation. In addition, PKC inhibitor-mediated downregulation of PKCη is blocked by protein phosphatase inhibitor calyculin A. Furthermore, mutation of PKCη at the A-loop, TM and HM to Ala attenuates PKCη level. Thus, our results suggest that phosphorylation of PKCη at the A-loop (Thr513), TM (Thr655) and HM (Ser674) is important for its stability.

EXPERIMENTAL PROCEDURES

Materials

PDBu, TPA and ILV were purchased from Alexis Biochemicals (San Diego, CA). Gö 6983, Gö 6976, calyculin A and okadaic acid were purchased from Calbiochem (San Diego, CA). Polyclonal antibodies to PKCη, PKCδ, PKCε and monoclonal antibody against GAPDH were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody to PKCα was obtained from Upstate Bioechnology (Lake Placid, NY) and monoclonal antibody to PKCι was obtained from Transduction Laboratories (San Jose, CA). Polyclonal antibody to phospho-PKCγ (Thr514) was purchased from Cell Signaling Technology (Beverly, MA). Polyclonal antibodies to phospho-PKCη were purchased from Biosource/Invitrogen (Carlsbad, CA). Monoclonal antibody against actin was obtained from Sigma (St. Louis, MO). Horseradish-peroxidase-conjugated donkey anti-rabbit and goat anti-mouse secondary antibodies were purchased from JacksonImmunoResearch Lab, Inc. (West Grove, PA). Poly(vinylidenedifluoride) membrane was obtained from Millipore (Bedford, MA). Enhanced chemiluminescence detection kit was purchased from Amersham (Arlington Heights, IL).

Cell culture and transfection

MCF-7 breast cancer cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. Human embryonic kidney (HEK) 293 cells

were maintained in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were kept in a humidified incubator at 37°C with 95% air and 5% CO_2. HEK293 cells were plated one day before transfection. Cells were transfected with WT-PKCη and phosphorylation site mutant constructs using Lipofectamine reagent (Invitrogen, Carlsbad, CA) and the manufacturer's protocol. One day post-transfection, the cells were re-plated for treatment with PDBu and Gö 6983.

Site-directed mutagenesis

PKCη was sub-cloned into pcDNA3 by PCR. Serine or threonine phosphorylation sites of PKCη at T513, T655 and S674 were mutated to alanine or glutamate using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following manufacturer's protocol. Mutations were confirmed by DNA sequencing.

Reverse Transcriptase PCR

MCF-7 cells were treated with PDBu, ILV and Gö 6983 for 16 h. Total RNA was extracted using TRI Reagent from Molecular Research Center, Inc. (Cincinnati, OH). cDNA was synthesized using random primers and Improm II reverse transcriptase from Promega (Madison, WI). PCR amplification of cDNA was performed using Promega PCR Master Mix (Madison, WI), PKCη and β-actin primers. The sequences of forward and reverse PKCη primers were 5'-ATGCGGTGGAACTTGCCA-3' and 5'-CGTGACCACAGAGCATCTCATAGA-3' respectively. The sequences of the forward and reverse β-actin primers were 5'-

ACCCAGCACAATGAAGATCA-3' and 5'-GCGCAAGTTAGGTTTTGTCA-3'. After PCR cycling, a 750 bp product for PKCη and 800-bp product for β-Actin was detected by gel electrophoresis.

Immunoblot Analysis

Cells were lysed in extraction buffer containing 1 mM DTT, protease inhibitors and phosphatase inhibitors. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on PVDF membranes. Western blot analysis was performed as described before [20].

Immunoprecipitation

Cells were harvested and lysed in buffer containing 20 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1% Nonidet P-40, 25 mM β-glycerophosphate, protease inhibitor cocktail, 10 mM NaF, and 0.1 mM sodium vanadate. After collecting total cell lysate (input), samples were immunoprecipated with PKCη-agarose conjugated polyclonal antibody or control rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocomplexes were processed as described previously [29] and subjected to SDS-PAGE and Western blotting.

RESULTS

Effects of PKC activators and inhibitors on PKCη level

We have previously shown that prolonged treatment with phorbol 12,13-dibutyrate (PDBu) caused upregulation of PKCη in MCF-7 breast cancer cells [25]. In the present study, we compared the ability of several PKC activators to upregulate PKCη in MCF-7 cells (Figure 1). While PDBu and TPA belong to the same class of compounds, indolactam V (ILV) is structurally distinct from phorbol esters [30]. All three PKC activators caused substantial upregulation of PKCη (Figure 1a). Based on the densitometric scanning of three separate experiments, PDBu, ILV and TPA caused 5-, 5- and 4-fold increase in PKCη level, respectively (Figure 1b). In contrast, conventional PKCα and novel PKCδ were downregulated after prolonged treatment with PDBu and TPA (Figure 1a). ILV had little effect on PKCα downregulation. As expected, the phorbol ester-insensitive atypical PKCι remained unaffected by the treatment with PKC activators. PDBu caused a time-dependent increase in downregulation of PKCα and PKCδ whereas treatment of MCF-7 cells with PDBu for 1 h was sufficient to increase PKCη level, and it remained elevated even when cells were treated with PDBu for 8 h (Figure 1c). We also treated cells with different concentrations of PDBu. Figure 1d shows that a 12 h treatment with PDBu caused a concentration-dependent increase in PKCη

level whereas PKCδ was downregulated with increasing PDBu concentrations. Thus, the regulation of PKCη is unique in comparison to other novel and conventional PKCs.

Since PKC activators caused upregulation of PKCη, we examined if treatment with PKC inhibitors would lead to the downregulation of PKCη. We examined the effects of the general PKC inhibitor Gö 6983 and conventional PKC inhibitor Gö 6976 on PKCη downregulation. Gö 6983 but not Gö 6976 caused substantial downregulation of PKCη (Figures 2a and 2b). The levels of PKC-α, -δ and -ε were not decreased by Gö 6983 treatment (Figure 2a). Thus, the effect of Gö 6983 on PKCη downregulation was specific to this isozyme.

To determine if PKC activators and inhibitors alter PKCη expression at the mRNA level, we treated MCF-7 cells with PDBu, ILV and Gö 6983 and analyzed mRNA expression by reverse-transcriptase PCR. As shown in Figure 3, treatment of MCF-7 cells with PKC activators and inhibitors did not alter PKCη mRNA expression. Taken together, these results demonstrate that PKCη level is altered post-transcriptionally following treatment with PKC activators and inhibitors.

Phosphatase inhibitor calyculin A prevents PKCη downregulation by Gö 6983.

To determine if phosphorylation of PKCη confers PKCη stability, we examined the effects of Ser/Thr phosphatase inhibitors okadaic acid and calyculin A on PKC inhibitor-induced downregulation of PKCη. Calyculin A inhibits protein phosphase 1 (PP1) and protein phosphatase 2A (PP2A), whereas okadaic acid preferentially inhibits PP2A [31]. As shown in

Figure 4, treatment of MCF-7 cells with calyculin A alone was sufficient to increase PKCη level. Moreover, calyculin A but not okadaic acid prevented downregulation of PKCη by Gö 6983. These results suggest that dephosphorylation of PKCη by PP1 is important for its downregulation.

Phosphorylation of PKCη at the conserved Ser/Thr sites regulates its stability.

To determine if phospho-PKCη resists PKCη downregulation, we wanted to monitor PKCη phosphorylation at all three conserved sites A-loop (Thr513), TM (Thr655) and HM (Ser674). There is no commercially available phospho-specific antibody against Thr513 at the A-loop of PKCη. Although phospho-specific antibody against Ser674 at the HM is commercially available it also recognizes equivalent phosphorylation sites in conventional PKCs. We therefore monitored PKCη phosphorylation following immunoprecipitation with PKCη antibody. We have used phospho-specific antibody against Thr514 of PKCγ, which also recognizes Thr513 site in PKCη, to monitor A-loop phosphorylation. TM phosphorylation was monitored using phospho-specific antibody against Thr655 of PKCη. Figure 5 shows that PDBu caused an increase in PKCη level and this was associated with an increase in PKCη phosphorylation at the A-loop, TM and HM sites.

To determine the importance of the A-loop (T513), TM (T655) and HM (S674) site phosphorylation in regulating PKCη level, we substituted Ser/Thr residues for alanine (A) to prevent phosphorylation or glutamate (E) to mimic phosphorylation. We introduced wild-type

(WT) and mutant PKCη constructs in HEK293 cells since these cells contain little endogenous PKCη and they are easy to transfect. PDBu caused substantial upregulation of PKCη in cells expressing WT PKCη, and Gö 6983 caused a modest decrease in PKCη (Figures 6a-c). Mutation of Thr513, Thr655 and Ser674 to Glu had little effect on PKCη level (Figures 6a-c). However, the constitutive PKCη level was substantially reduced in cells expressing T513A, T655A or S674A mutants (Figure 6a-c). Consequently, Gö 6983 did not have an additional effect in decreasing PKCη level. Mutation of PKCη at Thr513, Thr655 or Ser674 to Ala did not completely prevent upregulation of PKCη by PDBu, although the level of PKCη following PDBu treatment was much less in Ala mutant expressing cells compared to cells expressing WT or Glu mutants (Figures 6a-c). These results indicate that the phosphorylation at A-loop, TM and HM sites are important for the stability of PKCη.

DISCUSSION

The results of our present study demonstrate that the regulation of PKCη is unique compared to other phorbol ester-sensitive PKC isozymes. Although tumor-promoting phorbol esters are potent activators of conventional and novel PKCs [8, 10], persistent treatment with phorbol esters leads to the downergulation and termination of PKC signaling [32]. In contrast, we have shown that treatment of cells with PKC activators induced upregulation of PKCη whereas PKC-specific inhibitors triggered downregulation of PKCη. Furthermore, phosphorylation of PKCη at the conserved Ser/Thr sites were important for the stability of PKCη.

It is generally believed that treatment with PKC activators leads to their membrane translocation followed by dephosphorylation [33]. The dephosphorylated PKCs are subject to downregulation by proteases [34, 35]. However, fully phosphorylated PKCα was shown to be downregulated at the plasma membrane via the proteasome-mediated pathway [36]. A recent report indicated that the fully phosphorylated and primed form of PKCε was downregulated by phorbol ester treatment independent of its intrinsic kinase activity [37]. Although prolonged treatment with PDBu triggered downregulation of PKCα and PKCδ in MCF-7 cells, it caused upregulation of PKCη (Figure 1a). Our results suggest that phosphorylation of PKCη regulates its stability. First, treatment with several PKC activators, such as TPA, PDBu and ILV induced upregulation of PKCη in MCF-7 cells (Figure 1a). Second, PDBu not only increased PKCη

level, but the increase in PKCη was associated with an increase in its phosphorylation status. Third, PKC-specific inhibitors Gö 6983 (Figure 2a, b) and bisindolylmaleimide (data not shown) induced downregulation of PKCη. Finally, phosphatase inhibitor calyculin A blocked PKCη downregulation by Gö 6983 and increased constitutive level of PKCη.

It has been, however, reported that active conformation of PKCη is necessary for PKCη downregulation since mutation of Ala161 at the pseudosubstrate domain of PKCη to Glu (A161E) resulted in proteolysis of PKCη in baby hamster kidney (BHK) cells whereas wild-type PKCη was stable inside cells [38]. It is conceivable that the regulation of PKCη depends on the cellular context. We have, however, found that the upregulation of PKCη by PKC activator and downregulation by PKC inhibitor was not unique to MCF-7 cells, and was observed in other cell types, including HEK293 cells (Figures 5 and 6) and several other breast cancer cells, including BT-20, CAMA-1, SKBR-3 and HCC1806 (data not shown). Interestingly, the expression of A161E mutant in BHK cells resulted in distinct cleavage of full-length PKCη, rather than its disappearance which usually follows downregulation of PKCs. Furthermore, the disappearance of the smaller cleavage products but not the full-length A161E PKCη could be blocked by proteasome inhibitors, suggesting that the initial proteolysis of A161E PKCη was proteasome-independent. In this study, mechanisms for PKCη degradation were studied in the absence of phorbol ester treatment and the effects of PKC inhibitors were not tested. One possibility is that a change in conformation of PKCη caused by mutation at the pseudosubstrate domain resulted in

a protease-mediated cleavage of PKCη followed by further proteolysis via the ubiquitin proteasome-mediated pathway.

It is well established that the activity, maturation and stability of PKCs are regulated by phosphorylation and dephosphorylation events [11, 12, 39, 40]. It is generally believed that phosphorylation of PKCs at the activation loop primes them for activation whereas phosphorylation at the C-terminal hydrophobic motif confers stability. Phosphoinositide-dependent kinase-1 (PDK1) has been shown to phosphorylate PKCs at their activation loop sites [39, 41-44]. PDK1 has also been implicated in phosphorylating PKCη at its Thr513 A-loop site. A recent report indicated that in mouse A9 fibroblasts infected with parvovirus, atypical PKCλ (usually referred to as PKCι) phosphorylates PKCη at the Ser674 hydrophobic site, allowing PDK1 to subsequently phosphorylate PKCη at its activation loop site (42). However, knockdown of neither PDK1 nor PKCι prevented upregulation of PKCη by PDBu in MCF-7 cells (data not shown). We have found that mutation at the A-loop (Thr513), TM (Thr655) or the HM (Ser674) site to Ala was sufficient to decrease basal PKCη level, suggesting the importance of PKCη phosphorylation at these sites in regulating PKCη level. Although the level of PKCη in the PDBu-treated cells was much less in Ala mutant expressing cells compared to WT PKCη expressing cells, single mutation at the A-loop, TM or HM sites was not sufficient to obliterate the effect of PDBu on PKCη upregulation. Similarly, mutation of Ser/Thr to Glu did not block the effects of PDBu on PKCη upregulation. It appears that although

dephosphorylation at a single conserved phosphorylation site in PKCη is enough to destabilize it, phosphorylation at multiple sites by PDBu can result in its upregulation.

In summary, we have made several novel observations that have important implications. The observation that PKCη is the only PKC isozyme upregulated by tumor-promoting phorbol esters suggests that PKCη may play an important role in tumorigenesis. We have also demonstrated that phosphorylations at the A-loop, TM and HM determine PKCη level. Since PKCη is often upregulated in breast cancer and overexpression of PKCη confers resistance to chemotherapeutic drugs, future studies should determine how phosphorylation of PKCη at these sites is regulated.

Figure 1: Effects of PKC activators on upregulation of PKCη.

(a) MCF-7 cells were treated with 1 μM PDBu, 10 μM ILV and 1 μM TPA for 15 h. (b) Densitometric quantification of PKCη protein level from 3 separate experiments corrected for loading. Data represents the mean +/- s.e.m. The asterisk (*) indicates significant difference from control ($P<0.01$) using Student's t-test. (c) MCF-7 cells were treated with 1 μM PDBu for the indicated time periods and (d) indicated concentrations for 12 h. Western blot analysis was performed with total cell extract and probed with the indicated antibodies. Actin was used as a loading control.

Figure 1.

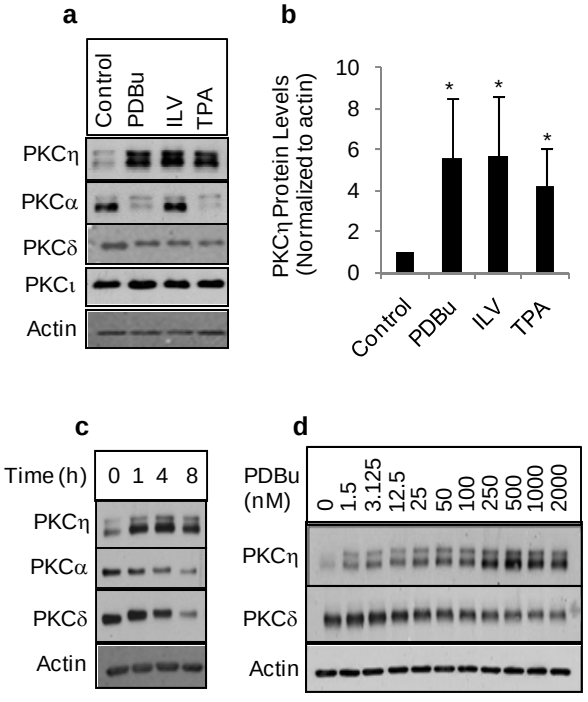

a
Control
PDBu
ILV
TPA
PKCη
PKCα
PKCδ
PKCι
Actin
b
PKCη Protein Levels (Normalized to actin)
10
8
6
4
2
0
*
*
*
Control
PDBu
ILV
TPA
c
Time (h)
0 1 4 8
PKCη
PKCα
PKCδ
Actin
d
PDBu (nM)
0
1.5
3.125
12.5
25
50
100
250
500
1000
2000
PKCη
PKCδ
Actin

Figure 2: Downregulation of PKCη was mediated by the general PKC inhibitor Gö 6983. (a) MCF-7 cells were treated with 1 μM Gö 6983 or 1 μM Gö 6976 for 15 h. Total cell lysates were subjected to SDS-PAGE and western blot analysis was performed using the indicated antibodies. Actin was used as a loading control. (b) Densitometric quantification of PKCη protein expression from 3 separate experiments corrected for loading. Data represents the mean +/- s.e.m. The asterisk (*) indicates significant difference from the control ($P<0.01$) using Student's t-test.

Figure 2.

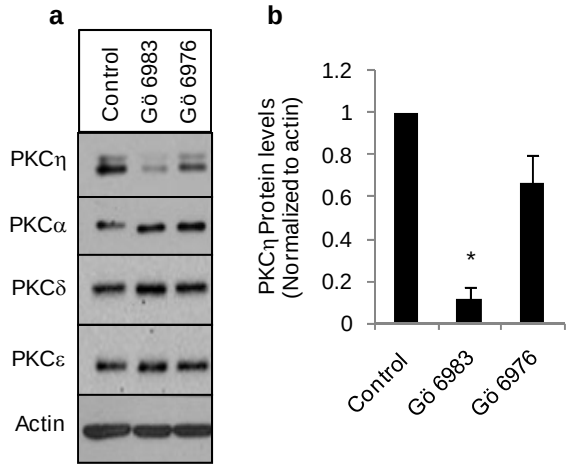

a
Control
Gö 6983
Gö 6976
PKCη
PKCα
PKCδ
PKCε
Actin
b
PKCη Protein levels (Normalized to actin)
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Gö 6983
Gö 6976

Figure 3: Effects of PKC activators and inhibitors on PKCη mRNA expression.

MCF-7 cells were treated with 1 μM PDBu, 10 μM ILV and 1 μM Gö 6983 for 16 h. Total RNA was extracted and cDNA was synthesized by reverse transcriptase reaction. PKCη and β-actin was amplified by PCR and electrophoresed. Results were representative of at least 2 independent experiments.

Figure 3.

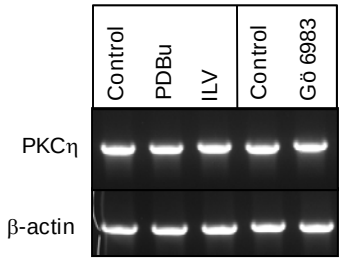


Figure 4: Effects of phosphatase inhibitors on PKCη level.

MCF-7 cells were pretreated with 10 nM calyculin A or 100 nM okadaic acid (OA) for 15 min and then treated with 1 μM Gö 6983 for 3 h. Western blot analysis was performed with the indicated antibodies. Actin was used as a loading control. Results were representative of at least 2 independent experiments.

Figure 4.

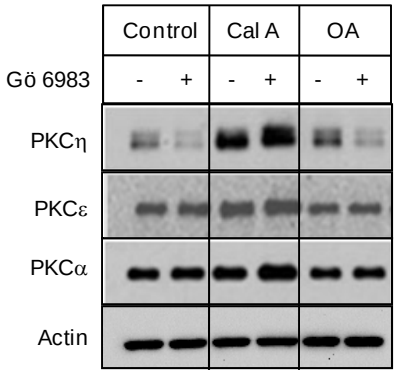


Figure 5: Effect of PDBu on PKCη phosphorylation.

HEK293 cells transfected with WT-PKCη were treated with 1 μM PDBu for 8 h or left unstimulated. Total cell lysates (input) were prepared and immunoprecipitated with PKCη (IP) or control Rabbit IgG (IgG) antibodies overnight. Western blot analyses were performed using indicated antibodies.

Figure 5.

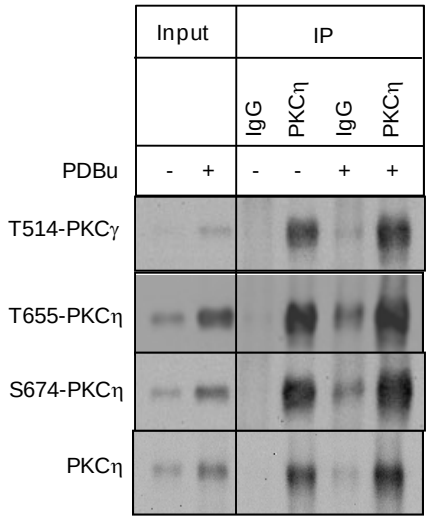
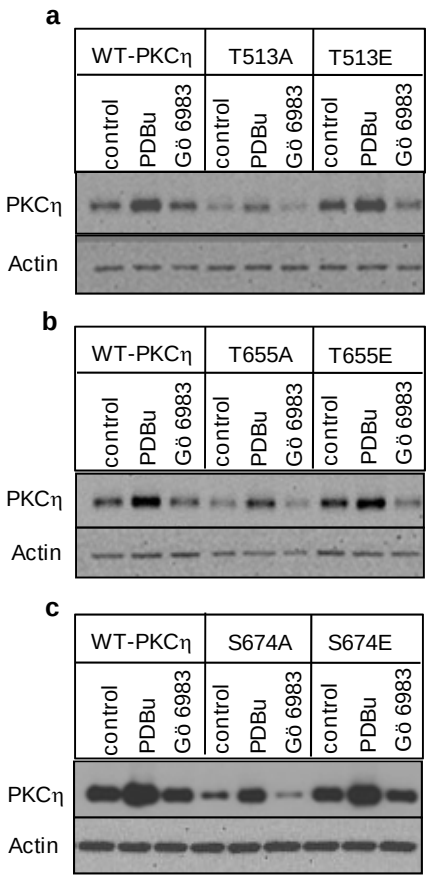

Input
IP
IgG
PKCη
IgG
PKCη
PDBu
- + - - + +
T514-PKCγ
T655-PKCη
S674-PKCη
PKCη

Figure 6: Effects of mutation of PKCη at the A-loop, TM or HM on PKCη level.

HEK293 cells were transfected with WT-PKCη or mutant PKCη and treated with 1 μM PDBu or 1 μM Gö 6983 for 15 h or left unstimulated. (a) T513A or T513E; (b) T655A or T655E; (c) S674A or S674E. Western blot analysis was performed with indicated antibodies. Actin was used as a loading control. Results were representative of at least 2 independent experiments.

Figure 6.


a
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PDBu
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PDBu
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PDBu
Gö 6983
PKCη
Actin

REFERENCES

1. Dempsey, E.C., et al., *Protein kinase C isozymes and the regulation of diverse cell responses.* Am J Physiol Lung Cell Mol Physiol, 2000. **279**(3): p. L429-38.
2. Stabel, S. and P.J. Parker, *Protein kinase C.* Pharmacol Ther, 1991. **51**(1): p. 71-95.
3. Nishizuka, Y., *Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C.* Science, 1992. **258**(5082): p. 607-14.
4. Nelsestuen, G.L. and M.D. Bazzi, *Activation and regulation of protein kinase C enzymes.* J Bioenerg Biomembr, 1991. **23**(1): p. 43-61.
5. Mellor, H. and P.J. Parker, *The extended protein kinase C superfamily.* Biochem J, 1998. **332 (Pt 2)**: p. 281-92.
6. Newton, A.C., *Protein kinase C: structure, function, and regulation.* J Biol Chem, 1995. **270**(48): p. 28495-8.
7. Steinberg, S.F., *Structural basis of protein kinase C isoform function.* Physiol Rev, 2008. **88**(4): p. 1341-78.
8. Blumberg, P.M., *Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture.* Cancer Res, 1988. **48**(1): p. 1-8.
9. Nishizuka, Y., *The role of protein kinase C in cell surface signal transduction and tumour promotion.* Nature, 1984. **308**(5961): p. 693-8.
10. Mosior, M. and A.C. Newton, *Mechanism of interaction of protein kinase C with phorbol esters. Reversibility and nature of membrane association.* J Biol Chem, 1995. **270**(43): p. 25526-33.
11. Newton, A.C., *Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm.* Biochem J, 2003. **370**(Pt 2): p. 361-71.
12. Parekh, D.B., W. Ziegler, and P.J. Parker, *Multiple pathways control protein kinase C phosphorylation.* Embo J, 2000. **19**(4): p. 496-503.

13. Bornancin, F. and P.J. Parker, *Phosphorylation of protein kinase C-alpha on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state.* J Biol Chem, 1997. **272**(6): p. 3544-9.
14. Ikenoue, T., et al., *Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling.* Embo J, 2008. **27**(14): p. 1919-31.
15. Gao, T. and A.C. Newton, *Invariant Leu preceding turn motif phosphorylation site controls the interaction of protein kinase C with Hsp70.* J Biol Chem, 2006. **281**(43): p. 32461-8.
16. Lim, T. and W.S. Sossin, *Phosphorylation at the hydrophobic site of protein kinase C Apl II is increased during intermediate term facilitation.* Neuroscience, 2006. **141**(1): p. 277-85.
17. Hauge, C., et al., *Mechanism for activation of the growth factor-activated AGC kinases by turn motif phosphorylation.* Embo J, 2007. **26**(9): p. 2251-61.
18. Masso-Welch, P.A., et al., *Altered expression and localization of PKC eta in human breast tumors.* Breast Cancer Res Treat, 2001. **68**(3): p. 211-23.
19. Sonnemann, J., et al., *Down-regulation of protein kinase Ceta by antisense oligonucleotides sensitises A549 lung cancer cells to vincristine and paclitaxel.* Cancer Lett, 2004. **209**(2): p. 177-85.
20. Akkaraju, G.R. and A. Basu, *Overexpression of protein kinase C-eta attenuates caspase activation and tumor necrosis factor-alpha-induced cell death.* Biochem Biophys Res Commun, 2000. **279**(1): p. 103-7.
21. Martin, P.M. and I.M. Hussaini, *PKC eta as a therapeutic target in glioblastoma multiforme.* Expert Opin Ther Targets, 2005. **9**(2): p. 299-313.
22. Sonnemann, J., et al., *Down-regulation of protein kinase Ceta potentiates the cytotoxic effects of exogenous tumor necrosis factor-related apoptosis-inducing ligand in PC-3 prostate cancer cells.* Mol Cancer Ther, 2004. **3**(7): p. 773-81.
23. Abu-Ghanem, S., et al., *PKCeta expression contributes to the resistance of Hodgkin's lymphoma cell lines to apoptosis.* Cancer Biol Ther, 2007. **6**(9): p. 1375-80.

24. Resnick, M.S., et al., *Selective up-regulation of protein kinase C eta in phorbol ester-sensitive versus -resistant EL4 mouse thymoma cells.* Cancer Res, 1997. **57**(11): p. 2209-15.
25. Basu, A., *The involvement of novel protein kinase C isozymes in influencing sensitivity of breast cancer MCF-7 cells to tumor necrosis factor-alpha.* Mol Pharmacol, 1998. **53**(1): p. 105-11.
26. Chen, C.C., J.K. Wang, and W.C. Chen, *TPA induces translocation but not down-regulation of new PKC isoform eta in macrophages, MDCK cells and astrocytes.* FEBS Lett, 1997. **412**(1): p. 30-4.
27. Murakami, A., et al., *Absence of down-regulation and translocation of the eta isoform of protein kinase C in normal human keratinocytes.* J Invest Dermatol, 1996. **106**(4): p. 790-4.
28. Bacher, N., et al., *Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin, and heart.* Mol Cell Biol, 1991. **11**(1): p. 126-33.
29. Lu, D., J. Huang, and A. Basu, *Protein kinase Cepsilon activates protein kinase B/Akt via DNA-PK to protect against tumor necrosis factor-alpha-induced cell death.* J Biol Chem, 2006. **281**(32): p. 22799-807.
30. Basu, A., A.P. Kozikowski, and J.S. Lazo, *Structural requirements of lyngbyatoxin A for activation and downregulation of protein kinase C.* Biochemistry, 1992. **31**(15): p. 3824-30.
31. Ishihara, H., et al., *Calyculin A and okadaic acid: inhibitors of protein phosphatase activity.* Biochem Biophys Res Commun, 1989. **159**(3): p. 871-7.
32. Hansra, G., et al., *Multisite dephosphorylation and desensitization of conventional protein kinase C isotypes.* Biochem J, 1999. **342 (Pt 2)**: p. 337-44.
33. Lee, H.W., et al., *Bryostatin 1 and phorbol ester down-modulate protein kinase C-alpha and -epsilon via the ubiquitin/proteasome pathway in human fibroblasts.* Mol Pharmacol, 1997. **51**(3): p. 439-47.

34. Prevostel, C., et al., *Protein kinase C(alpha) actively downregulates through caveolae-dependent traffic to an endosomal compartment.* J Cell Sci, 2000. **113 (Pt 14)**: p. 2575-84.

35. Brognard, J. and A.C. Newton, *PHLiPPing the switch on Akt and protein kinase C signaling.* Trends Endocrinol Metab, 2008. **19**(6): p. 223-30.

36. Leontieva, O.V. and J.D. Black, *Identification of two distinct pathways of protein kinase Calpha down-regulation in intestinal epithelial cells.* J Biol Chem, 2004. **279**(7): p. 5788-801.

37. Cameron, A.J., et al., *PKC maturation is promoted by nucleotide pocket occupation independently of intrinsic kinase activity.* Nat Struct Mol Biol, 2009.

38. Kang, B.S., et al., *Activation-dependent degradation of protein kinase C eta.* Oncogene, 2000. **19**(37): p. 4263-72.

39. Cenni, V., et al., *Regulation of novel protein kinase C epsilon by phosphorylation.* Biochem J, 2002. **363**(Pt 3): p. 537-45.

40. Shirai, Y. and N. Saito, *Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting.* J Biochem, 2002. **132**(5): p. 663-8.

41. Le Good, J.A., et al., *Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1.* Science, 1998. **281**(5385): p. 2042-5.

42. Balendran, A., et al., *Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms.* FEBS Lett, 2000. **484**(3): p. 217-23.

43. Dutil, E.M., A. Toker, and A.C. Newton, *Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1).* Curr Biol, 1998. **8**(25): p. 1366-75.

44. Chou, M.M., et al., *Regulation of protein kinase C zeta by PI 3-kinase and PDK-1.* Curr Biol, 1998. **8**(19): p. 1069-77.

CHAPTER III

DOWNREGULATION OF PROTEIN KINASE Cη OCCURS VIA TWO DISTINCT MECHANISMS

SUMMARY

Protein kinase C (PKC) is a family of serine/threonine kinases that are key signaling proteins involved in regulating various cellular processes. PKCη, a member of the novel group of PKC isozymes, plays an important role in regulating cell proliferation and differentiation. PKCη is also upregulated in several cancers including breast cancer and overexpression of PKCη is associated with resistance to chemotherapeutic agents. Persistent activation of PKCs by tumor-promoting phorbol esters leads to their degradation or downregulation. However, PKCη resists activation-induced downregulation, suggesting that PKCη may play a role in tumorigenesis. Targeting the downregulation of PKCη may be an important strategy in cancer therapy. We have identified two pathways of PKCη downregulation involving PKC inhibition and phosphoinositide 3-kinase (PI3K) inhibition by pharmacological inhibitors Gö 6983 and Ly294002, respectively. Both Gö 6983 and Ly294003-mediated downregulation of PKCη depends on dephosphorylation by a calyculin A-sensitive phosphatase. However, only Ly294002-mediated downregulation was blocked by proteasome inhibitor, MG-132 and phorbol

12, 13 dibutyrate (PDBu). Therefore, dephosphorylation of PKCη may induce its downregulation. In addition, downregulation of PKCη is mediated via both proteasome-dependent and -independent pathways.

INTRODUCTION

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases involved in multiple cellular functions including proliferation, differentiation and cell survival [1, 2]. The PKC family consists of 10 isozymes that differ in structure, function and biochemical properties [3-7]. The classical PKCs (cPKCα, -βI, βII, and γ) are activated by physiological second messengers Ca^{2+} and diacylglycerol (DAG), novel PKCs (nPKCδ, -ε, -η, and -θ) respond to DAG only and the atypical PKCs (aPKCι/λ and -ζ) respond to neither. Classical and novel PKCs are cellular receptors for phorbol esters which are pharmacological PKC agonists analogous to DAG [8, 9]. Phorbol esters promote membrane translocation and sustained activation of PKCs [10]. As a consequence of sustained activation, PKCs are downregulated resulting in signal termination [11]. Contrary to the other phorbol-ester sensitive PKCs, PKCη resists phorbol ester-induced downregulation [12-14] suggesting that the termination of PKCη signal occurs via a different mechanism.

Several degradation/downregulation pathways have been described for PKCs. Following membrane translocation, Ca^{2+} - activated neutral proteases (calpains) have been shown to downregulate cPKCs [15]. PKCα, -δ and -ε were shown to be degraded via the ubiquitin/proteasome-mediated pathway [11, 16, 17]. Vesicular trafficking can localize PKCs to lysosomal or membrane compartments, such as lipid rafts or caveolae where they can be degraded by proteases [18, 19]. It is generally believed that activated PKCs are not only sensitive

to protease-mediated degradation, but also to dephosphorylation by phosphatases [20]. The classical model of PKC downregulation involved chronic activation of PKC resulting in dephosphorylation, inactivation and subsequent degradation by accumulation in a cytoskeletal fraction of the cell [21]. Recent studies pose challenges to the classical model. Fully phosphorylated PKCα was shown to be downregulated at the plasma membrane via the proteasome-mediated pathway [17]. Moreover, PKCα was shown to be downregulated by two distinct mechanisms in the same cell type: 1) proteasome-dependent and 2) caveolar trafficking-dependent pathway [19]. These studies highlight the complexity of PKC downregulation mechanisms.

It has been reported that full-length PKCη expressed in BHK cells was resistant to phorbol ester-induced downregulation and transfection of constitutively-active mutant at the pseudosubstrate site (A161EPKCη) resulted in the appearance of PKCη degradation fragments [14]. Proteasome inhibitors had a modest effect in preventing downregulation of wild-type PKCη, whereas they increased the abundance of PKCη fragments. These results indicate that activation of PKCη is necessary for downregulation by the proteasome-mediated pathway. These results could not be extended to other cell types since the A161EPKCη constitutively-active mutant could not be expressed in HEK 293 cells [14]. Therefore, the exact mechanism of PKCη downregulation remains unclear.

The purpose of the present study was to understand the mechanism of PKCη downregulation in the breast cancer MCF-7 cell line. We report for the first time that PKCη can be downregulated by PKC as well as phosphoinositide-3-kinase (PI3K) inhibitors. PKCη downregulation mediated by PKC and PI3K inhibition occurs via dephosphorylation by a calyculin A-sensitive phosphatase..PKCη downregulation by PI3K inhibition was proteasome-dependent whereas downregulation by Gö 6983 was proteasome-independent.These studies indicate that PKCη can be downregulated by inhibition of both PI3K and PKC and the downregulation occurs by two distinct pathways.

EXPERIMENTAL PROCEDURES

Materials

Gö 6983, Ly294002, calyculin A, okadaic acid, and calpeptin A were purchased from Calbiochem (San Diego, CA). PDBu was purchased from Alexis Biochemicals (San Diego, CA). Polyclonal antibodies to PKCη, PKCδ and PKCε, and monoclonal antibody to GAPDH were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody to PKCα was from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal antibody to actin was obtained from Sigma (St. Louis, MO). Non-targeting siRNA and siRNA SMARTpool against PTEN and PHLPP were obtained from Dharmacon (Lafayette, CO). Horseradish peroxidase conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from JacksonImmunoResearch Lab. Inc. (West Grove, PA). Poly(vinylidenedifluoride) membrane was from Millipore (Bedford, MA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL).

Cell Culture and Transfection

MCF-7 breast cancer cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. Cells were kept in a humidified incubator at 37°C with 95% air and 5% CO_2. Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were kept in a humidified incubator at 37°C with 95% air and 5% CO_2.

HEK293 cells were grown on glass coverslips one day before transfection. Cells were transfected with 0.3 μg PKCη–GFP using Lipofectamine reagent (Invitrogen, Carlsbad, CA) and the manufacturer's protocol. One day post-transfection, the cells were treated with PDBu, Gö 6983 and Ly294002.

Immunoblot Analysis

Cells were lysed in extraction buffer containing 1 mM DTT, protease inhibitors and phosphatase inhibitors. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on PVDF membranes. Western blot analysis was performed as described previously [22] .

siRNA transfection

Control non-targeting siRNA or SMARTpool siRNA against PDK1, PTEN and PHLPP were introduced into MCF-7 cells using Lipofectamine 2000 (Invitrogen) and manufacturer's protocol. Briefly, cells were seeded one day before transfection. 48 h following siRNA transfection, cells were treated as indicated in the text and processed for Western blot analysis as described before [23].

Confocal microscopy

HEK 293 cells were grown on glass coverslips and transfected with 0.3 μg PKCη-GFP construct. One day, post-transfection, cells were treated with 1 μM PDBu, 1 μM Gö 6983 and 25

μM Ly294002 for 8 h. Cells were fixed and permeabilized with methanol and triton-X and nuclei were stained with DAPI (Molecular Probes, Eugene, OR). Coverslips were mounted on glass slides and cells were visualized on a Zeiss LSM 410 confocal microscope. Images were captured using LSM 4 software (Carl Zeiss Microimaging).

RESULTS

PI3K and PKC inhibitors induce PKCη downregulation

We have previously reported that PKCη resists phorbol ester-induced downregulation in MCF-7 breast cancer cells [12]. Therefore, persistent activation by phorbol esters does not lead to its downregulation. To determine if inhibition of PKCη activity influences its downregulation, we compared the effect of the general PKC inhibitor (Gö 6983) and activation loop kinase, PI3K/phosphoinositide-dependent protein kinase-1 (PDK1) inhibitor (Ly294002) on PKCη protein levels. Figure 1a, b shows that both Gö 6983 and Ly294002 caused a substantial decrease in PKCη protein levels. The mitogen-activated protein kinase inhibitor U0126 did not alter PKCη protein levels suggesting MAPK does not contribute to PKCη stability. Moreover, the downregulation by Gö 6983 and Ly294002 was specific to PKCη since the protein levels of other PKCs such as PKCε, –δ and -α were not affected. In Figure 2 a, b, PKCη downregulation by both inhibitors was time-dependent. These results suggest that both PKC and PI3K pathways are involved in PKCη downregulation.

PKCη downregulation is mediated by two distinct mechanisms

Since inactivation of PKCη by Gö 6983 and Ly294002 leads to its downregulation, we next determined if PKC activators can reverse the effect of inhibitor-mediated downregulation of PKCη. In Figure 3, pretreatment with phorbol 12, 13 dibutyrate, PDBu had a modest effect on blocking Gö 6983-mediated downregulation of PKCη; however, PDBu completely reversed the

effect of Ly294002-mediated downregulation. This suggests that activation of PKCη by PDBu can protect PKCη from downregulation by PI3K-inhibition but not PKC-inhibition. Therefore the downregulation of PKCη by Gö 6983 and Ly294002 is mediated by different mechanisms.

It has been reported that PKCα, -δ, and -ε are downregulated by the ubiquitin/proteasome mediated pathway in response to phorbol ester treatment [11, 16, 17]. Therefore, we explored the possibility of PKCη downregulation by Gö 6983 and Ly294002 via the ubiquitin/proteasome pathway. Interestingly, Ly294002-mediated but not Gö 6983-mediated downregulation was significantly blocked by the proteasome inhibitor, MG-132 (Figure 4a, b). Since PKCs can also be degraded by proteases such as calpains [15], we investigated the ability of calpains to degrade PKCη using the calpain inhibitor, calpeptin. Figure 5 shows that inhibition of calpains did not affect downregulation of PKCη by Gö 6983 or Ly294002 indicating PKCη downregulation does not depend on calpains. These data further confirm two distinct pathways of PKCη downregulation: proteasome-dependent downstream of PI3K inhibition and proteasome-independent in response to PKC inhibition.

Calyculin A sensitive phosphatase regulates downregulation of PKCη by PKC and PI3K inhibitors

It is generally believed that dephosphorylation precedes downregulation of PKCs [20]. To determine the involvement of phosphatases in PKCη downregulation we depleted by siRNA two phosphatases involved in PKC signaling: phosphatase and tensin homologue deleted from

chromosome-10 (PTEN) and PH domain and leucine rich repeat protein phosphatase (PHLPP). PTEN opposes the activity of PI3K to negatively regulate Akt and PKC [24]. PHLPP has been reported to dephosphorylate both Akt and PKC at the hydrophobic motif to inactivate Akt and degrade PKC [25, 26]. Figure 6 shows that neither PTEN nor PHLPP depletion blocks PKCη downregulation by Gö 6983 or Ly294002. Therefore, downregulation of PKCη by Gö 6983 or Ly294002 is not mediated by PTEN or PHLPP dephosphorylation.

We next looked at the effect of Ser/Thr phosphatases on Gö 6983- and Ly294002-mediated downregulation of PKCη by treating cells with Ser/Thr phosphatase inhibitors calyculin A which inhibits PP1 and PP2A and okadaic acid which inhibits PP2A at the 100 nM concentration tested. Figure 7 shows complete reversal of Gö 6983- and Ly294002-mediated downregulation of PKCη with calyculin A, whereas okadaic acid had no apparent effect on PKCη downregulation. This suggests that PP1 may be playing a role in regulating PKCη dephosphorylation in the presence of Gö 6983 and Ly294002. Calyculin A also increased the levels of PKCα and PKCε basally and in the presence of Gö 6983 indicating that PKCα and PKCε may be dephosphorylated by PP1, but this dephosphorylation does not lead to degradation of PKCα and PKCε as it does with PKCη. This data indicates that dephosphorylation by a calyculin A-sensitive phosphatase is an important step in the downregulation of PKCη by both Gö 6983 and Ly294002.

Treatment with PDBu, Gö 6983 and Ly294002 affects subcellular localization of PKCη.

Since we have seen that PDBu upregulates PKCη levels and both Gö 6983 or Ly294002 downregulates PKCη levels, we next determined if the localization of PKCη is altered in response to these treatments. We transfected HEK 293 cells with PKCη-GFP construct and monitored localization by confocal microscopy after treatment with PDBu, Gö 6983 and Ly294002 for 8 h. As shown in In Figure 8, PKCη−GFP is concentrated in the perinuclear region in the control cells. Treatment with PDBu caused a dramatic increase in PKCη−GFP expression with localization mostly in the cytosol, but also can be detected in the nucleus. Treatment with either Gö 6983 or Ly294002 caused a decrease in PKCη-GFP expression and the localization was more dispersed throughout the cell with less concentration in the perinuclear region and more expression throughout the nucleus and cytoplasm. Therefore, the expression and localization of PKCη is altered in response to treatment with PDBu, Gö 6983 and Ly294002.

DISCUSSION

PKCη upregulation has been linked to several cancer types including breast [12], renal [27] and glioblastoma [28]. In addition, overexpression of PKCη has been linked to resistance to chemotherapeutic agents in breast cancer [22], prostate cancer [29], lung cancer [30] and Hodgkin's lymphoma [31]. Furthermore, PKCη resists downregulation by chronic activation of tumor promoting phorbol esters [12, 13, 28], suggesting PKCη plays a role in tumorigenesis. Therefore, elucidating the mechanism of PKCη downregulation and desensitization is critical to improve cancer therapy. In the present study, we report that PKCη is downregulated by two different inhibitors, the general PKC inhibitor Gö 6983 and PI3K inhibitor, Ly294002 rather than by prolonged activation as other PKCs (Figure 1a, b). Therefore, the inhibition of PKCη kinase activity leads to downregulation.

We have developed a model of two distinct pathways of PKCη downregulation (Figure 9). One pathway is proteasome-independent which mediates Gö 6983 induced downregulation and the other pathway is proteasome-dependent which mediates Ly294002 induced downregulation (Figure 4). Both pathways involve dephosphorylation by a calyculin A-sensitive phosphatase (Figure 7). The pathways differ in response to phorbol ester PDBu. Since Gö 6983 is a general PKC inhibitor, the kinase involved in phosphorylating and protecting PKCη against downregulation is most likely a PKC. Since PDBu had no effect on PKCη downregulation by Gö 6983 (Figure 3), the PKC involved may either be insensitive to phorbol esters, such as the

atypical PKCs or downregulated by PDBu treatment such as the conventional or novel PKCδ, -ε or θ. PKCη has been shown to directly phosphorylate PKCμ/PKD [32, 33] and PKCδ and PKCε have been shown to phosphorylate each other on their activation loop or hydrophobic motif [34]. PKCη has been reported to be phosphorylated at the hydrophobic motif by atypical PKCι which stabilizes PKCη and allows PDK1 to dock and phosphorylate the activation loop [35]. Preliminary results using PDK1 siRNA suggest that depletion of PDK1 can destabilize PKCη basal levels (data not shown) indicating PDK1 may also be the potential target of Gö 6983 or Ly294002 in the downregulation of PKC. Furthermore, mammalian target of rapamycin complex 2 (mTORC2) was reported to phosphorylate and stabilize PKCε at the turn motif and hydrophobic motif [36]. Recently, we have shown that depletion of PKCε and mTORC2 enhanced downregulation of PKCδ [37]. Based on these reports, PKCε, –δ or -ι, PDK1 or mTORC2 could be potential kinases involved in the Gö 6983 and Ly294002-mediated downregulation of PKCη.

PHLPP has been shown to dephosphorylate and destabilize PKCα and PKCε through removal of the hydrophobic motif phosphate, but not PKCη [25, 26]. In this study, PHLPP depletion had no effect on blocking Gö 6983 or Ly294002 downregulation of PKCη (Figure 6). However, since calyculin A but not okadaic acid blocked the downregulation of PKCη by Gö 6983 and Ly294002, it is likely that PP1 is the potential phosphatase involved in

dephosphorylating PKCη. Future studies should test the direct interaction of PP1 with PKCη and the specific site of dephosphorylation of PKCη.

The dual mechanisms of PKC downregulation was also reported for PKCα in response to downregulation by two different activators bryostatin and phorbol 12-myristate 13-acetate (PMA) [19]. One pathway involves ubiquitination of the fully phosphorylated, mature PKCα at the plasma membrane followed by proteasome-mediated degradation. In contrast, the second pathway involved caveolae-mediated trafficking to the perinuclear compartment, dephosphorylation and degradation via a proteasome-independent pathway. Therefore, both PKCη and PKCα are downregulated via proteasome-dependent and independent mechanisms. For PKCη, the proteasome-independent pathway does not involve calpains (Figure 5) nor does it involve cathepsins or lysosomal-mediated degradation since inhibitors of these mechanism had no effect on PKCη downregulation by Gö 6983 or Ly294002 (data not shown). Therefore, further investigation is necessary to elucidate the proteasome-independent mechanism of PKCη downregulation by Gö 6983.

We have shown that the localization of PKCη is altered in response to treatment with PDBu, Gö 6983, or Ly294002 (Figure 8). It has been reported that PKCη is localized in the Golgi, ER and nuclear envelop and translocates to the nuclear envelope upon treatment with PMA [38]. We have detected a striking upregulation of PKCη in response to PDBu treatment with localization in the cytoplasm, nucleus and nuclear membrane. Similarly, treatment with inhibitors Gö 6983 and Ly294002 caused more nuclear and cytoplasmic localization of PKCη and less perinuclear

concentration compared to the control unstimulated cells. It is possible that PKCη is stabilized in the perinuclear region and translocation out of this region can lead to it destabilization and degradation.

In this study, we have identified two distinct mechanism of PKCη downregulation by two different inhibitors. Since overexpression of PKCη has been associated with resistance to chemotherapeutic agents [31], Gö 6983 or Ly294002, which we have shown in this report leads to PKCη-specific downregulation and not other PKCs, can be used in combination with chemotherapeutic agents to overcome chemotherapeutic-resistance associated with PKCη.

Figure 1. Effect of PKC and P13K inhibitors on PKCη protein levels.

(a) MCF-7 cells were treated with 1 μM Gö 6983, 25 μM Ly294002 and 10 μM U0126 for 15h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. Actin was used as a loading control. (b) Densitometric quantification of PKCη protein levels from 3 separate experiments corrected for loading. Data represents the mean +/- s.e.m. The asterisk (*) indicates significant difference from control ($P<0.01$) using Student's *t*-test.

Figure 1.

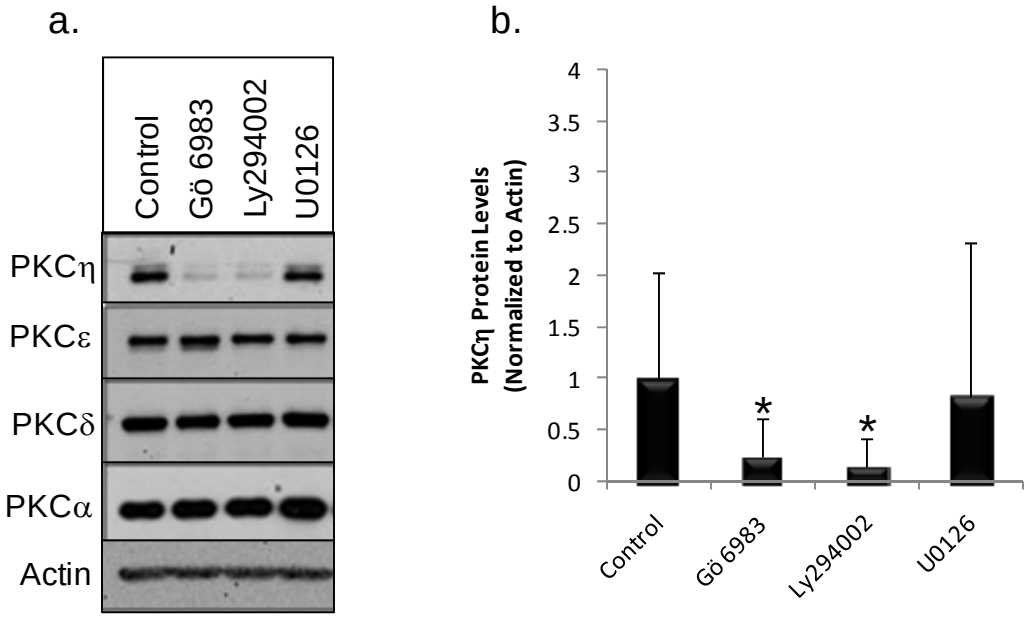

a.
Control
Gö 6983
Ly294002
U0126
PKCη
PKCε
PKCδ
PKCα
Actin
b.
PKCη Protein Levels
(Normalized to Actin)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
Control
Gö 6983
Ly294002
U0126

Figure 2. Time-course comparison of PKC and P13K inhibitors on PKCη protein levels. MCF-7 cells were treated with (a) 1 μM Gö 6983 or (b) 25 μM Ly294002 for the indicated time points. Western blot analysis was performed with total cellular extracts using the indicated antibodies. Actin was used as a loading control. Results were representative of at least 2 independent experiments.

Figure 2.

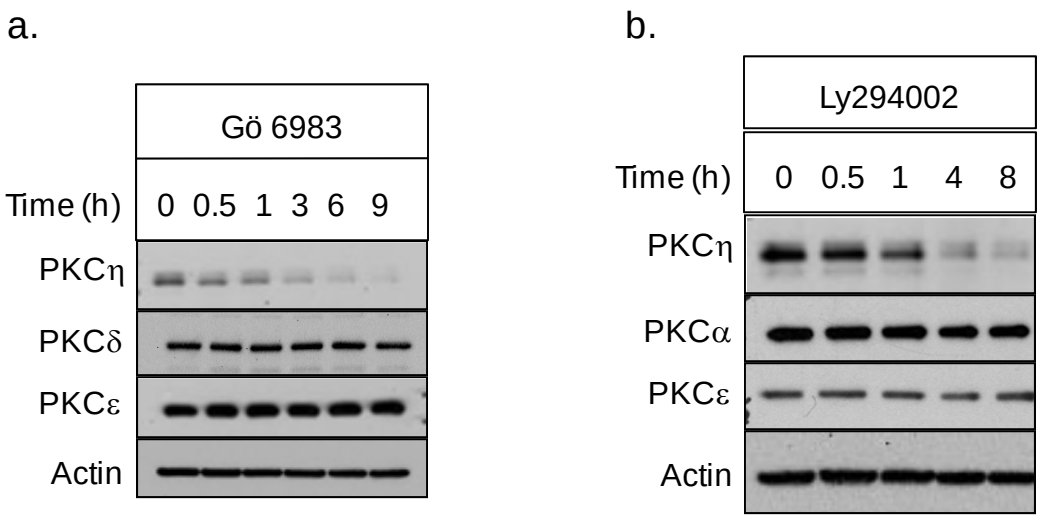

a.
Gö 6983
Time (h)
0 0.5 1 3 6 9
PKCη
PKCδ
PKCε
Actin
b.
Ly294002
Time (h)
0 0.5 1 4 8
PKCη
PKCα
PKCε
Actin

Figure 3. Effect of PDBu on Gö 6983- or Ly294002-mediated downregulation of PKCη.

MCF-7 cells were pre-treated with 1 μM PDBu for 15 min, followed by treatment with 1 μM Gö 6983 or 25 μM Ly294002 for 15h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. Actin was used as a loading control. Results were representative of 2 independent experiments.

Figure 3.

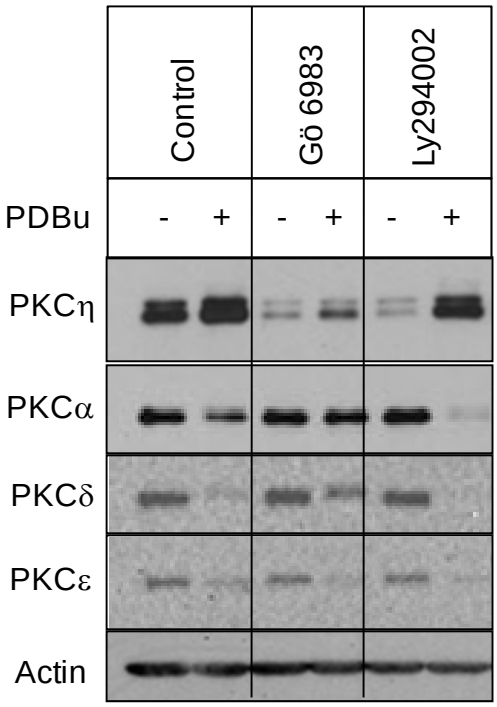


Figure 4. Effect of proteasome inhibitor, MG-132 on PKCη downregulation by Gö 6983 or Ly294002.

(a) MCF-7 cells were pretreated with 10 μM MG-132 for 30 min, followed by treatment with 1 μM Gö 6983 or 25 μM Ly294002 for 12 h.Western blot analysis was performed with total cellular extracts using the indicated antibodies. GAPDH was used as a loading control. (b) Densitometric quantification of PKCη protein levels from 3 separate experiments corrected for loading. Data represents the mean +/- s.e.m. The asterisk (*) indicates significant difference of MG-132 treated cells from control or treatment with the inhibitors alone ($P<0.01$) using Student's *t*-test.

Figure 4.

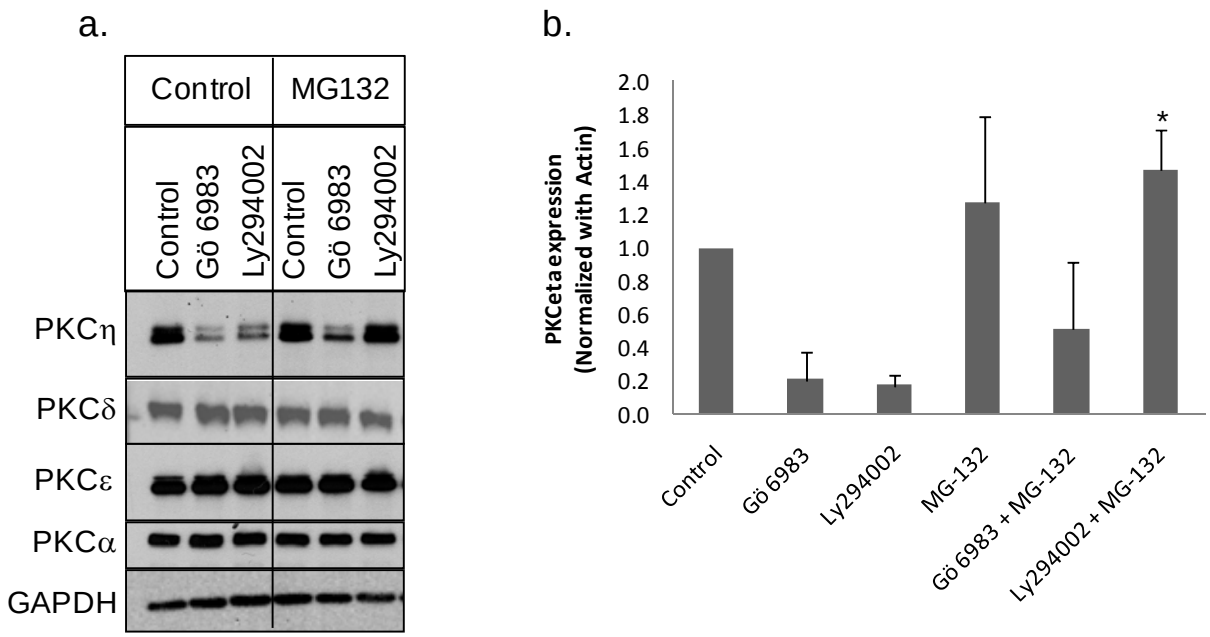

a.
Control
MG132
Control
Gö 6983
Ly294002
Control
Gö 6983
Ly294002
PKCη
PKCδ
PKCε
PKCα
GAPDH
b.
PKCeta expression
(Normalized with Actin)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Gö 6983
Ly294002
MG-132
Gö 6983 + MG-132
Ly294002 + MG-132

Figure 5. Effect of calpain inhibitor, Calpeptin on Gö 6983- or Ly294002-mediated downregulation of PKCη.

MCF-7 cells were pretreated with 50 μM Calpeptin for 30 min, followed by treatment with 1 μM Gö 6983 or 25 μM Ly294002 for 12 h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. Actin was used as a loading control. Results were representative of 2 independent experiments.

Figure 5.

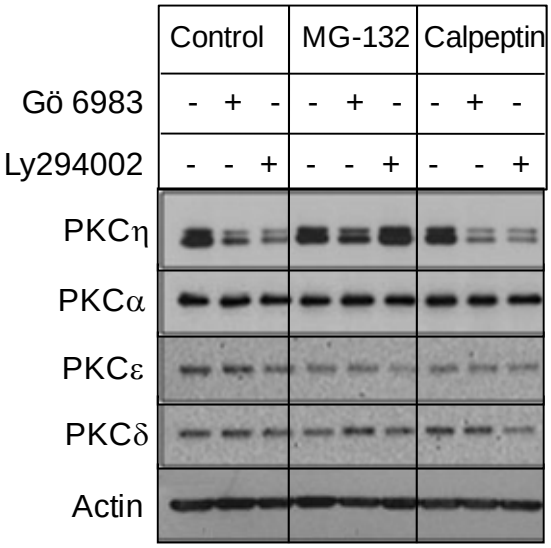

Control
MG-132
Calpeptin
Gö 6983
- + - - + - - + -
Ly294002
- - + - - + - - +
PKCη
PKCα
PKCε
PKCδ
Actin

Figure 6. Effect of PHLPP or PTEN phosphatase depletion on Gö 6983- or Ly294002-mediated downregulation of PKCη.

MCF-7 cells were transfected with non-targeting siRNA (Control) or siRNA against PTEN or PHLPP. Cells were treated with 1 μM Gö 6983 or 25 μM Ly294002 for 15 h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. GAPDH was used as a loading control. Results were representative of 2 independent experiments.

Figure 6.

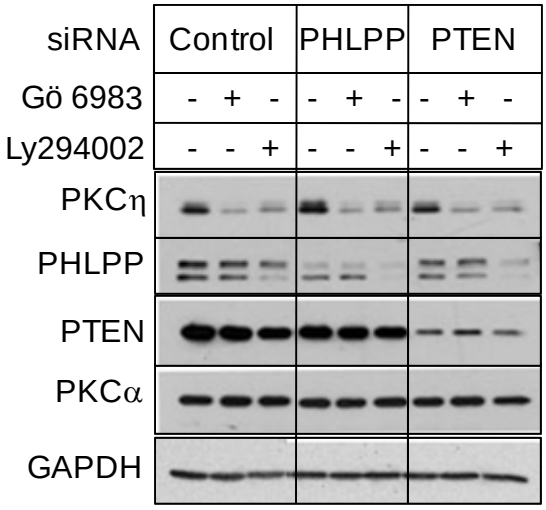

siRNA
Control
PHLPP
PTEN
Gö 6983
- + - - + - - + -
Ly294002
- - + - - + - - +
PKCη
PHLPP
PTEN
PKCα
GAPDH

Figure 7. Effect of phosphatase inhibitors, calyculin A and okadaic acid on Gö 6983- or Ly294002-mediated downregulation of PKCη.

MCF-7 cells were pretreated with 10 nM calyculin A (Caly A) or 100 nM okadaic acid (OA) for 15 min, followed by treatment with 1 μM Gö 6983 or 25 μM Ly294002 for 3 h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. GAPDH was used as a loading control. Results were representative of 2 independent experiments.

Figure 7.

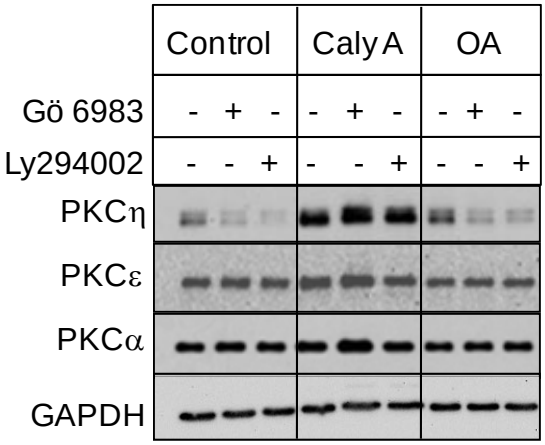


Figure 8. Effect of PDBu, Gö 6983 or Ly294002 treatment on localization of PKCη.

PKCη-GFP construct was transiently transfected in HEK 293 cells and treated with 1 μM PDBu, 1 μM Gö 6983 and 25 μM Ly294002 for 8 h. Cells were fixed and imaged with confocal microscopy. PKCη-GFP was visualized as green fluorescent signals mostly in the perinuclear, cytoplasmic and nuclear regions. Nuclei staining with DAPI were visualized in blue.

Figure 8.

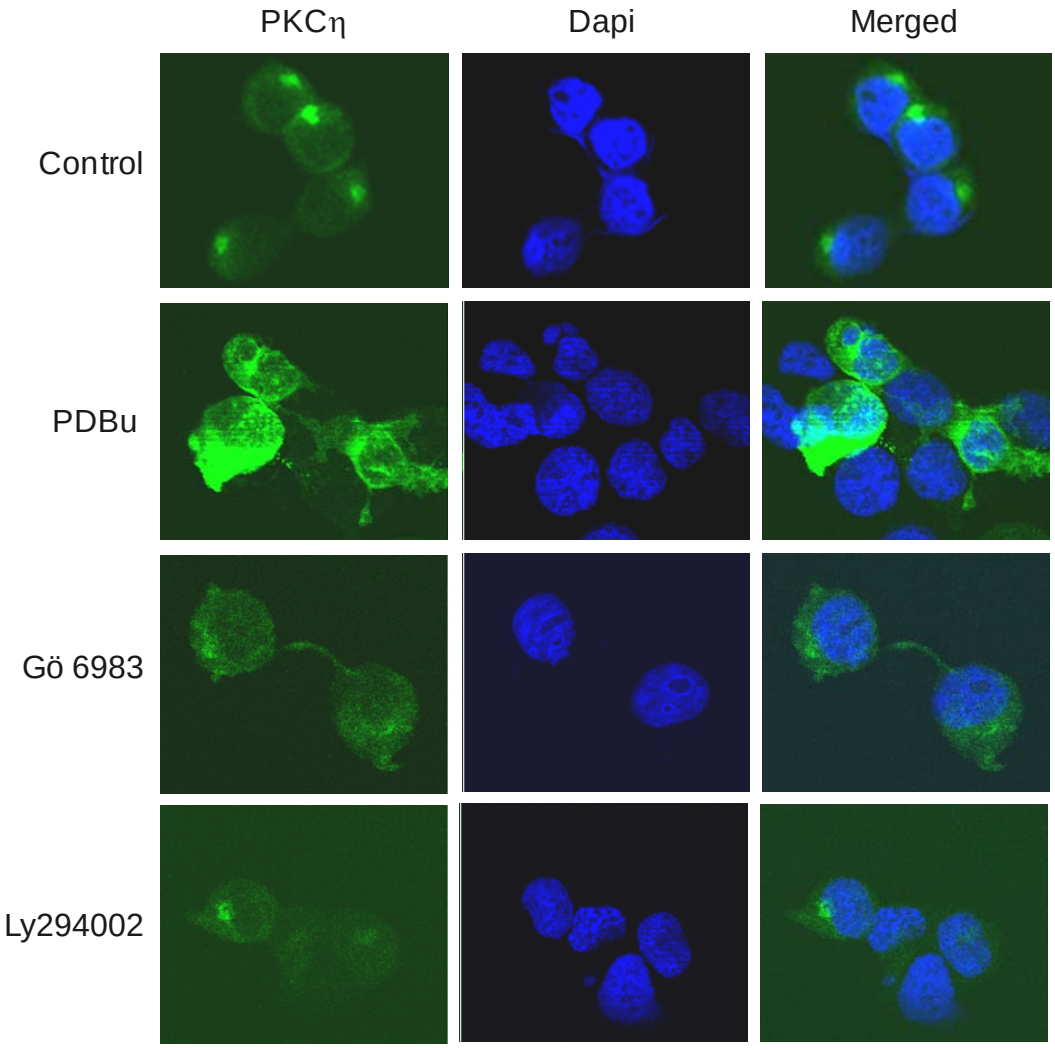
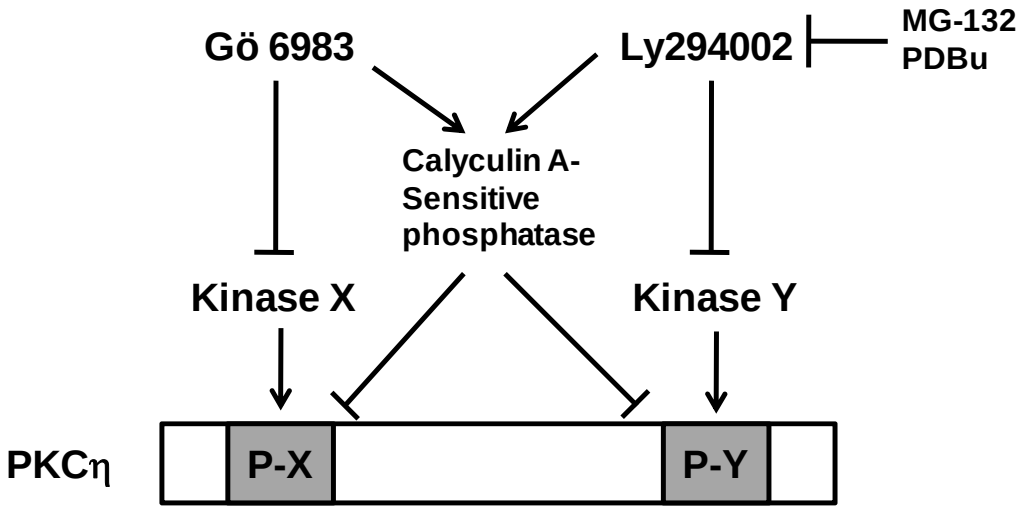


Figure 9. Model of two distinct pathways of PKCη downregulation by Gö 6983 or Ly294002.

PKCη can be downregulated by treatment with the PKC inhibitor, Gö 6983 or PI3K inhibitor, Ly294002. Gö 6983 inhibits hypothetical (Kinase X) and Ly294002 inhibits hypothetical (Kinase Y) which can phosphorylate and stabilize PKCη possibly at two different sites (P-X or P-Y). Activation of a calyculin A-sensitive phosphatase by both inhibitors can dephosphorylate and downregulate PKCη. Ly294002 mediated downregulation of PKCη is blocked by proteasome inhibitor, MG-132 or PKC activator PDBu indicating proteasome-dependent downregulation. Downregulation of PKCη by Gö 6983 occurs independently of the proteasome degradation pathway.

Figure 9.


Gö 6983
Ly294002
MG-132
PDBu
Calyculin A-
Sensitive
phosphatase
Kinase X
Kinase Y
PKCη
P-X
P-Y

REFERENCES

1. Dempsey, E.C., et al., *Protein kinase C isozymes and the regulation of diverse cell responses.* Am J Physiol Lung Cell Mol Physiol, 2000. **279**(3): p. L429-38.
2. Stabel, S. and P.J. Parker, *Protein kinase C.* Pharmacol Ther, 1991. **51**(1): p. 71-95.
3. Steinberg, S.F., *Structural basis of protein kinase C isoform function.* Physiol Rev, 2008. **88**(4): p. 1341-78.
4. Mellor, H. and P.J. Parker, *The extended protein kinase C superfamily.* Biochem J, 1998. **332 (Pt 2)**: p. 281-92.
5. Nishizuka, Y., *Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C.* Science, 1992. **258**(5082): p. 607-14.
6. Nelsestuen, G.L. and M.D. Bazzi, *Activation and regulation of protein kinase C enzymes.* J Bioenerg Biomembr, 1991. **23**(1): p. 43-61.
7. Newton, A.C., *Protein kinase C: structure, function, and regulation.* J Biol Chem, 1995. **270**(48): p. 28495-8.
8. Mosior, M. and A.C. Newton, *Mechanism of interaction of protein kinase C with phorbol esters. Reversibility and nature of membrane association.* J Biol Chem, 1995. **270**(43): p. 25526-33.
9. Blumberg, P.M., *Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture.* Cancer Res, 1988. **48**(1): p. 1-8.
10. Nishizuka, Y., *The role of protein kinase C in cell surface signal transduction and tumour promotion.* Nature, 1984. **308**(5961): p. 693-8.
11. Lu, Z., et al., *Activation of protein kinase C triggers its ubiquitination and degradation.* Mol Cell Biol, 1998. **18**(2): p. 839-45.
12. Basu, A., *The involvement of novel protein kinase C isozymes in influencing sensitivity of breast cancer MCF-7 cells to tumor necrosis factor-alpha.* Mol Pharmacol, 1998. **53**(1): p. 105-11.

13. Resnick, M.S., et al., *Selective up-regulation of protein kinase C eta in phorbol ester-sensitive versus -resistant EL4 mouse thymoma cells.* Cancer Res, 1997. **57**(11): p. 2209-15.
14. Kang, B.S., et al., *Activation-dependent degradation of protein kinase C eta.* Oncogene, 2000. **19**(37): p. 4263-72.
15. Pontremoli, S., et al., *Effects of a monoclonal anti-calpain antibody on responses of stimulated human neutrophils. Evidence for a role for proteolytically modified protein kinase C.* J Biol Chem, 1988. **263**(4): p. 1915-9.
16. Srivastava, J., et al., *Phosphorylation is required for PMA- and cell-cycle-induced degradation of protein kinase Cdelta.* Biochem J, 2002. **368**(Pt 1): p. 349-55.
17. Lee, H.W., et al., *Bryostatin 1 and phorbol ester down-modulate protein kinase C-alpha and -epsilon via the ubiquitin/proteasome pathway in human fibroblasts.* Mol Pharmacol, 1997. **51**(3): p. 439-47.
18. Prevostel, C., et al., *Protein kinase C(alpha) actively downregulates through caveolae-dependent traffic to an endosomal compartment.* J Cell Sci, 2000. **113 (Pt 14)**: p. 2575-84.
19. Leontieva, O.V. and J.D. Black, *Identification of two distinct pathways of protein kinase Calpha down-regulation in intestinal epithelial cells.* J Biol Chem, 2004. **279**(7): p. 5788-801.
20. Hansra, G., et al., *Multisite dephosphorylation and desensitization of conventional protein kinase C isotypes.* Biochem J, 1999. **342 (Pt 2)**: p. 337-44.
21. Bornancin, F. and P.J. Parker, *Phosphorylation of protein kinase C-alpha on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state.* J Biol Chem, 1997. **272**(6): p. 3544-9.
22. Akkaraju, G.R. and A. Basu, *Overexpression of protein kinase C-eta attenuates caspase activation and tumor necrosis factor-alpha-induced cell death.* Biochem Biophys Res Commun, 2000. **279**(1): p. 103-7.

23. Lu, D., J. Huang, and A. Basu, *Protein kinase Cepsilon activates protein kinase B/Akt via DNA-PK to protect against tumor necrosis factor-alpha-induced cell death.* J Biol Chem, 2006. **281**(32): p. 22799-807.
24. Maehama, T. and J.E. Dixon, *The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate.* J Biol Chem, 1998. **273**(22): p. 13375-8.
25. Gao, T., J. Brognard, and A.C. Newton, *The phosphatase PHLPP controls the cellular levels of protein kinase C.* J Biol Chem, 2008. **283**(10): p. 6300-11.
26. Gao, T., F. Furnari, and A.C. Newton, *PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth.* Mol Cell, 2005. **18**(1): p. 13-24.
27. Brenner, W., et al., *Protein kinase C eta is associated with progression of renal cell carcinoma (RCC).* Anticancer Res, 2003. **23**(5A): p. 4001-6.
28. Hussaini, I.M., et al., *Phorbol 12-myristate 13-acetate induces protein kinase ceta-specific proliferative response in astrocytic tumor cells.* J Biol Chem, 2000. **275**(29): p. 22348-54.
29. Sonnemann, J., et al., *Down-regulation of protein kinase Ceta potentiates the cytotoxic effects of exogenous tumor necrosis factor-related apoptosis-inducing ligand in PC-3 prostate cancer cells.* Mol Cancer Ther, 2004. **3**(7): p. 773-81.
30. Sonnemann, J., et al., *Down-regulation of protein kinase Ceta by antisense oligonucleotides sensitises A549 lung cancer cells to vincristine and paclitaxel.* Cancer Lett, 2004. **209**(2): p. 177-85.
31. Abu-Ghanem, S., et al., *PKCeta expression contributes to the resistance of Hodgkin's lymphoma cell lines to apoptosis.* Cancer Biol Ther, 2007. **6**(9): p. 1375-80.
32. Waldron, R.T., T. Iglesias, and E. Rozengurt, *The pleckstrin homology domain of protein kinase D interacts preferentially with the eta isoform of protein kinase C.* J Biol Chem, 1999. **274**(14): p. 9224-30.

33. Brandlin, I., et al., *Protein kinase C (PKC)eta-mediated PKC mu activation modulates ERK and JNK signal pathways.* J Biol Chem, 2002. **277**(8): p. 6490-6.
34. Rybin, V.O., et al., *Cross-regulation of novel protein kinase C (PKC) isoform function in cardiomyocytes. Role of PKC epsilon in activation loop phosphorylations and PKC delta in hydrophobic motif phosphorylations.* J Biol Chem, 2003. **278**(16): p. 14555-64.
35. Lachmann, S., et al., *Parvovirus interference with intracellular signalling: mechanism of PKCeta activation in MVM-infected A9 fibroblasts.* Cell Microbiol, 2008. **10**(3): p. 755-69.
36. Ikenoue, T., et al., *Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling.* Embo J, 2008. **27**(14): p. 1919-31.
37. Basu, A., S. Sridharan, and S. Persaud, *Regulation of protein kinase Cdelta downregulation by protein kinase Cepsilon and mammalian target of rapamycin complex 2.* Cell Signal, 2009.
38. Maissel, A., et al., *PKCeta is localized in the Golgi, ER and nuclear envelope and translocates to the nuclear envelope upon PMA activation and serum-starvation: C1b domain and the pseudosubstrate containing fragment target PKCeta to the Golgi and the nuclear envelope.* Cell Signal, 2006. **18**(8): p. 1127-39.

CHAPTER IV

FUNCTIONAL SIGNIFICANCE OF PROTEIN KINASE Cη DOWNREGULATION

SUMMARY

A deregulation in apoptosis or programmed cell death can contribute to cancer progression and resistance to chemotherapeutic agents. The protein kinase C (PKC) family plays an important role in regulating apoptosis. Several anti-apoptotic PKC isozymes such as PKCε and PKCη have been shown to be overexpressed in cancers and contribute to resistance against TNF or TRAIL-induced cell death. In this study, we have investigated the anti-apoptotic function of PKCη in breast cancer cells and report that increased PKCη protein level plays a role in breast cancer progression and confers resistance to TRAIL-induced cell death. Depletion of PKCη altered the protein levels of two Bcl-2 family members, pro-apoptotic Bax and anti-apoptotic Mcl-1. PKCη depletion increased the dimerization of Bax and decreased the induction of Mcl-1 in response to TRAIL. MCF-7 breast cancer cells were sensitized to TRAIL with PKCη or Mcl-1 depletion. This study highlights the potential role of PKCη in regulating proteins involved in the intrinsic mitochondrial pathway of TRAIL-induced cell death.

INTRODUCTION

Apoptosis or programmed cell death plays an important role in the development and homeostasis of normal tissues [1, 2]. An imbalance between cell proliferation and cell death has been linked to several human diseases including cancer, autoimmune disease and neurodegenerative disorders [3-6]. Current cancer therapies are based on their ability to induce cell death in rapidly growing cells [7]. Therefore, deciphering the mechanisms of signaling pathways involved in apoptosis is crucial to develop more efficient and specific cancer therapeutics. Two major apoptotic signaling pathways have been described which are the extrinsic (death receptor-mediated) and intrinsic (mitochondrial-mediated) pathways [8] (Figure 3 from Introduction). The extrinsic pathway involves death receptors belonging to the tumor necrosis factor-alpha receptor (TNFR) superfamily. Ligands such as TNF or TNF-related apoptosis-inducing ligand (TRAIL) can bind to death receptors and recruit adaptor molecules to form the death-inducing signaling complex (DISC). The second pathway, intrinsic pathway is mediated by Bcl-2 family of proteins which regulate mitochondrial membrane integrity in response to apoptotic stimuli such as DNA damage or other cytotoxic insults [9]. Both pathways further recruit cysteine aspartic acid specific proteases (caspases) to activate apoptosis [8, 10, 11].

Several signaling pathways controlling apoptosis are commonly altered in cancer [12]. The protein kinase C (PKC) family of isozymes play a major role in regulating cell proliferation and cell death processes [13]. Anti-apoptotic PKC isozymes such as PKCε and PKCη have been

reported to be associated with resistance to TNF or TRAIL-induced cell death [14-18]. Overexpression of anti-apoptotic Bcl-2 family members is also found in many types of human cancers [12]. Therefore, both PKC and Bcl-2 protein families can be exploited as potential targets for cancer therapy [19-21]. We have reported that PKCε exerts its anti-apoptotic effect by downregulating the pro-apoptotic Bcl-2 member Bid and upregulating anti-apoptotic Bcl-2 to confer resistance to TRAIL [17]. Others have found that PKC isozymes can regulate pro- or anti-apoptotic members of the Bcl-2 family to influence TRAIL resistance [22, 23]. For instance, activation of PKC by phorbol-12-myristate-13-acetate (PMA) induced the expression of pro-apoptotic Bad and anti-apoptotic Mcl-1 in the pancreatic cancer cell line PANC-1 [23] . Furthermore, the activation of Bad was attributed to conventional and not novel or atypical PKC isozymes since pretreatment with conventional PKC inhibitors prevented Bad induction [23]. Activation of PKC also increased the expression of TRAIL receptors to provide a potential mechanism to sensitize pancreatic cancer cells to TRAIL [23]. The mechanism by which PKCη exerts its anti-apoptotic effect in response to TRAIL is not well established.

In this study, we report that increased protein levels of PKCη is associated with breast tumor progression using an isogenic model of breast epithelial cells that progress from benign to pre-malignant to metastatic stages. Overexpression of PKCη was associated with resistance to TRAIL-induced cell death in breast adenocarcinoma MCF-7 cells. Knockdown of PKCη by itself did not sensitize cells to TRAIL-induced apoptosis via cleavage of caspase substrate poly (ADP-ribose) polymerase (PARP), but enhanced the dimerization of pro-apoptotic Bax and

blocked the induction of anti-apoptotic Mcl-1. These data suggest that PKCη is an upstream effector of the Bcl-2 family in response to TRAIL. Mcl-1 depletion by itself caused cell death in MCF-7 cells and further enhanced TRAIL-induced cell death. PKCη depletion resulted in a modest increase in cell death suggesting there may be a compensatory effect of other anti-apoptotic proteins due to PKCη depletion. These results suggest that PKCη may exert its anti-apoptotic effect by regulating Bcl-2 family proteins upstream of the mitochondria-mediated cell death pathway.

EXPERIMENTAL PROCEDURES

Materials

Human recombinant TRAIL was purchased from R&D systems (Minneapolis, MN). Polyclonal antibodies to PKCη, PKCδ and PKCε, and monoclonal antibodies to glyceraldehydes-3-phosphate dehydrogenase (GAPDH), Bcl-2 and Bax (6A7) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody to Bid and monoclonal antibodies to PARP and caspase-7 were purchased from Pharmingen (San Diego, CA). Polyclonal antibody to Mcl-1 was purchased from Cell Signaling Technologies (Beverly, MA). Monoclonal antibody to PKCα was from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal antibody to actin was obtained from Sigma (St. Louis, MO). Non-targeting siRNA and siRNA SMARTpool against PKCη and Mcl-1 were obtained from Dharmacon (Lafayette, CO). YO-PRO-1 dye was obtained from Molecular Probes (Eugene, OR). Horseradish peroxidase conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from JacksonImmunoResearch Lab. Inc. (West Grove, PA). Poly(vinylidenedifluoride) membrane was from Millipore (Bedford, MA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL).

Cell Culture

MCF10A and MCF10AT1 cells were maintained in DMEM /F12 medium supplemented with 5% horse serum, 15 mM HEPES buffer, 10 mg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin and 0.5 mg/ml hydrocortisone. MCF10CA1d and MCF10CA1a cells were

maintained in DMEM/ F12 medium supplemented with 5% horse serum. MCF-7 breast cancer cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. Cells were kept in a humidified incubator at 37°C with 95% air and 5% CO_2.

Transfection

MCF-7 cells were transfected with 1 μg of pcDNA3 or vector containing wild-type PKCη (pcDNA3-PKCη) using FuGENE 6 (Roche, NJ). Briefly, cells were seeded 1 day before transfection. 24 h following transfection, cells were treated with Geneticin (G418) (Gibco) and resistant cells were pooled and re-plated after 3 days. Cells were treated with the indicated concentrations of TRAIL and processed for immunoblot analysis.

Immunoblot Analysis

Cells were lysed in extraction buffer containing 1 mM DTT, protease inhibitors and phosphatase inhibitors. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on PVDF membranes. Western blot analysis was performed as described previously [24] .

siRNA

Control non-targeting siRNA or SMARTpool siRNA against PKCη or Mcl-1 were introduced into MCF-7 cells using Lipofectamine 2000 (Invitrogen) and manufacturer's protocol. Briefly, cells were seeded one day before transfection. 48 h following siRNA

transfection, cells were treated as indicated in the text and processed for Western blot analysis as described before [25].

Assessment of Cell Death by YO-PRO-1 Assay

MCF-7 cells transfected with non-targeting control, PKCη or Mcl-1 siRNA. Two days after transfection, cells were treated with TRAIL for 12 h and analyzed for the number of apoptotic and dead cells using YO-PRO-1 staining. Live cells were stained with 0.5 μM YO-PRO-1 for 15 min in the dark. Fluorescent staining was then visualized using a Zeiss Axiovert 40 inverted microscope with the AxioVision Rel 4.6 software (Zeiss, Göttingen, Germany). Cells staining positively for YO-PRO-1 binding (green fluorescence) were scored as apoptotic, since YO-PRO-1 permeates early apoptotic cells. [26].

RESULTS

Overexpression of PKCη confers resistance to TRAIL-induced cell death in MCF-7 cells.

Since we have previously reported that overexpression of PKCη attenuated TNF-induced cell death in MCF-7 cells [24], we examined if overexpression of PKCη can also confer resistance to TRAIL. MCF-7 cells overexpressing PKCη was previously generated [24]. Apoptotic cell death was monitored by processing of caspase 7 and cleavage of caspase 7 substrate, poly (ADP-ribose) polymerase (PARP). PARP cleavage and decrease in full-length caspase 7 was detected in MCF-7 cells transfected with empty vector in response to TRAIL in a concentration dependent manner (Figure 1). In contrast, PKCη overexpressing cells were completely resistant to TRAIL-induced cell death. This data suggests that PKCη is involved in resistance to TRAIL-induced cell death in MCF-7 cells.

Depletion of PKCη blocks TRAIL-induced Mcl-1 expression.

Since it was reported that overexpression of PKCε conferred resistance to TRAIL-induced cell death by regulating members of the Bcl-2 family [17], we wanted to determine if PKCη also exerts its protective effect via the Bcl-2 family. We depleted PKCη by siRNA using non-targeting siRNA as a control and treated MCF-7 cells with TRAIL. As shown in Figure 2a, PKCη siRNA successfully depleted PKCη protein levels without affecting the levels of PKCδ, PKCε and PKCα. In Figure 2b, PKCη knockdown increased Bax dimerization indicating PKCη

may play a role in the intrinsic apoptotic pathway. PKCη depletion also blocked the induction of Mcl-1, an anti-apoptotic Bcl-2 family member by TRAIL. PKCη depletion had no detectable affect on Bcl-2 or Bid protein levels. This data suggests that PKCη may exert its anti-apoptotic affect by targeting Bax and Mcl-1.

Mcl-1 knockdown sensitized cells to TRAIL-induced cell death

To further investigate the role of PKCη and Mcl-1 in TRAIL-induced cell death, we depleted PKCη or Mcl-1 in MCF-7 cells and monitored apoptotic cell death in response to TRAIL by YO-PRO-1 Assay. YO-PRO-1 is a green fluorescent nucleic acid dye that can permeate apoptotic cells. As shown in Figure 3 a, b, PKCη depletion modestly sensitized cells to TRAIL-induced cell death compared to the control non-targeting siRNA transfected cells. In contrast, Mcl-1 depletion by itself increased cell death compared to the control and further enhanced TRAIL-induced cell death (Figure 3 a, b). This data suggests that Mcl-1 depletion is sufficient in sensitizing MCF-7 cells to TRAIL-induced cell death.

PKCη protein level increases with breast tumor progression

Since PKCη overexpression conferred resistance to TRAIL (Figure 1) and depletion of PKCη modestly sensitizes MCF-7 cells to TRAIL-induced apoptosis (Figure 3), we next wanted to compare the protein levels of PKCη in normal breast epithelial cells and breast cancer cells. We utilized a series of isogenic human breast cancer cells [27] representing a tumor progression

model to determine if increased PKCη expression is associated with breast tumor progression. The MCF-10A series consists of cells that have the same genotype, but different phenotype. The MCF-10A cells are immortalized benign breast epithelial cells. The MCF-10AT1 cells are pre-malignant and were obtained by xenograft from cells that were transformed by HRAS. The MCF10CA1a and MCF10CA1d cells are metastatic clones. Since these cells lines are isogenic, they represent an advantageous model system for studying specific protein expression in breast tumor progression by eliminating genetic variations. Figure 4 shows a comparison of PKC isozyme protein levels in the MCF10A series. Compared to the normal MCF10A breast epithelial cells, PKCα levels remained similar in the cell lines, whereas PKCε and PKCδ levels increased in MCF10CA1d cells, but decreased in the metastatic cell line MCF10CA1a. PKCη increased ~2-fold in the pre-malignant MCF10AT1 cells and ~5-fold in both metastatic cell lines (Figure 4b). This data shows that increased protein level of the PKCη isozyme is associated with breast cancer progression.

DISCUSSION

The role of PKCη in tumor progression is cell type specific. PKCη was reported to be associated with renal carcinoma [28] whereas PKCη null mice were more susceptible to skin tumor formation [29]. In breast [24], lung [30], prostate [18] and Hodgkins lymphoma [31], PKCη was associated with resistance to chemotherapeutic agents. We have previously shown that overexpression of PKCη confers resistance to TNF-induced cell death; however, the mechanism by which PKCη exerts its anti-apoptotic effect is not well-established. This study substantiates the anti-apoptotic role of PKCη in breast cancer cells by the following observations. First, overexpression of PKCη was associated with resistance to TRAIL-induced cell death (Figure 1). Second, depletion of PKCη increased pro-apoptotic Bax dimerization and decreased anti-apoptotic Mcl-1 induction by TRAIL (Figure 2). Third, PKCη depletion modestly increased TRAIL induced cell death (Figure 3). Finally, using a series of cell lines representing a breast tumorigenesis model, PKCη protein level increased in the metastatic cells versus the normal/benign cells (Figure 4)

The Bcl-2 family of proteins consists of at least 20 members with opposing activities to regulate cell death [32]. The ratio of anti-apoptotic members (*e.g.,* Bcl-2, Bcl-xl, Mcl-1 and Bcl-w) to pro-apoptotic members (*e.g.,* Bax, Bad, and Bid) can decide the fate of the cell. There have been several reports linking PKC activation with alterations in expression of Bcl-2 family members leading to protection or resistance against apoptotic stimuli [22, 23, 33]. PKCε has

been shown to regulate several Bcl-2 family members to exert its anti-apoptotic effect [15]. Overexpression of PKCε increased anti-apoptotic Bcl-2 mRNA and protein level while decreasing pro-apoptotic Bid resulting in a net anti-apoptotic response contributing to TRAIL resistance [17]. PKCε also prevented Bax translocation to the mitochondrial membrane to protect MCF-7 cells from TNF-induced apoptosis [16]. Bax exists as a monomer in the cytosol and during induction of apoptosis, Bax translocates to the mitochondria, integrates into the outer mitochondrial membrane and undergoes dimerization [34] resulting in cytochrome c release and apoptotic cell death [34]. In the present study, PKCη depletion also regulated two opposing members of the Bcl-2 family, Bax and Mcl-1 (Figure 2). Depletion of PKCη by itself increased Bax dimer formation which was further increased in the presence of TRAIL. We also detected a concominant decrease in Mcl-1 induction by TRAIL. TRAIL induces NF-κB-mediated transcriptional induction of Mcl-1 in TRAIL-resistant cells [35]. Expression of c-myc reduced the induction of Mcl-1 by TRAIL and sensitized cells to TRAIL-induced cell death [35] suggesting Mcl-1 plays an important role in TRAIL resistance [36].

Tumor resistance to apoptotic cell death is often a hallmark of cancer and contributes to resistance to chemotherapeutic agents [5]. Although TRAIL has been used in clinical trials as a promising anti-cancer agent for its ability to target cancer cells while sparing normal cells [37, 38]; recombinant TRAIL therapy or anti-TRAIL-R therapies are limited to patients with TRAIL-sensitive tumors resulting in low overall efficacy [39-41]. Therefore, for patients demonstrating a

partial response to TRAIL therapy, combination therapy with TRAIL and agents targeting specific apoptosis signaling pathways can be beneficial.

In this study, we have shown that PKCη can potentially target two Bcl-2 family members, pro-apoptotic Bax and anti-apoptotic Mcl-1 in response to TRAIL (Figure 2). Whether or not PKCη can regulate Bax or Mcl-1 directly is subject to further investigation. It has been reported that Mcl-1 can be phosphorylated and inactivated by a pro-apoptotic member of the mitogen-activated protein kinase (MAPK) family, c-Jun NH(2)-terminal kinase (JNK) in response to oxidative stress [42]. Moreover, a recent report indicated that overexpression of PKCη inhibited JNK activity and its pro-apoptotic function in MCF-7 cells to confer protection against DNA-damage induced apoptosis [43]. Therefore, it is conceivable that the link between the anti-apoptotic function of PKCη and Mcl-1 may be via JNK inhibition.

We have shown that Mcl-1 depletion had more of an effect in enhancing TRAIL-induced cell death that PKCη (Figure 3). This could be explained in part by the role of Mcl-1 in regulating cell death. Depending on the cellular conditions, Mcl-1 plays two major roles in regulating apoptosis [44]. Under survival conditions, Mcl-1 binds and sequesters pro-apoptotic Bak on the mitochondrial membrane. Upon receiving apoptotic stimuli, Mcl-1 is displaced from Bak by BH3-only proteins leading to Bak oligomerization and release of cytochrome c. Mcl-1 can also heterodimerize with BH3-only proteins such as tBid, PUMA and Bim under survival conditions. During apoptosis, NOXA displaces Mcl-1 which releases BH3-only proteins, allowing them to interact with Bax which inserts into the mitochondrial membrane and causes the release of

cytochrome c. Therefore, Mcl-1 when present in sufficient amounts in the cell, acts as an anti-apoptotic protein by sequestering pro-apoptotic proteins. However, apoptotic stimuli can lead to the rapid downregulation and inactivation of Mcl-1. Thus, in cells that are resistant to apoptosis due to overexpression of Mcl-1, depletion of Mcl-1 can cause a robust apoptotic response in response to apoptotic stimuli.

In addition to inducing apoptosis, TRAIL can also induce survival pathways through the activation of transcription factor, nuclear factor-κβ (NF-κβ) [45]. The activity of NF-κB is sequestered by binding to the inhibitor of κB (IκB) [46]. Upon phosphorylation of IκB by IκB kinase β (IKKβ), NF-κβ is released, translocates to the nucleus and induced transcription of survival proteins [46]. Therefore, small molecule inhibitors of IKKβ can have toxic properties [47, 48]. It was reported in B-cell lymphoma cells resistance to IKKβ inhibitors was due to a compensatory effect of IκB kinase α which phosphorylated IκB in the absence of IKKβ [49]. Depletion of IKKα in combination with IKKβ inhibitors successfully sensitized resistant B-cell lymphoma cells to cell death [49]. Therefore, cancer cells may engage in several compensatory signaling pathways to promote survival or sustain proliferation. We have shown that depletion of PKCη caused only a modest sensitization to TRAIL-induced cell death, which may be due to compensatory effects from other anti-apoptotic proteins.

Using the MCF-10A series representing a breast tumor progression model, we have observed that PKCη protein levels increase in the metastatic cell lines versus the normal cell line (Figure 4). It is possible that these cells utilize PKCη to advance to the metastatic state and further

studies should elucidate the mechanism by which PKCη can promote tumorigenesis in these cells. The expression and activation of PKCε has been reported to be decreased in malignant breast [50] and pancreatic [51] compared to the adjacent normal tissue. It is possible that PKCη and PKCε can play differential roles in normal versus breast cancer cells where PKCε has a predominant role in the normal cells and PKCη a predominant role in metastatic cells.

PKCη and Mcl-1 have been implicated as anti-apoptotic proteins whose overexpressions confer resistance to TRAIL-induced cell death. The results from this study provide a potential mechanism by which PKCη exerts its anti-apoptotic effect by regulating the Bcl-2 family members Bax and Mcl-1. This study provides valuable insight to target PKCη via the mitochondrial cell death pathway to sensitize breast cancer cells to TRAIL-induced cell death.

Figure 1. Effect of PKCη overexpression on TRAIL-induced apoptosis

MCF-7 cells were transfected with pcDNA3 empty vector and PKCη-pcDNA3 constructs. G418 resistant pooled cells were treated with the indicated concentrations of TRAIL for 12 h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. GAPDH was used as a loading control. Arrow indicates cleaved PARP band. Results were representative of at least 2 independent experiments.

Figure 1.

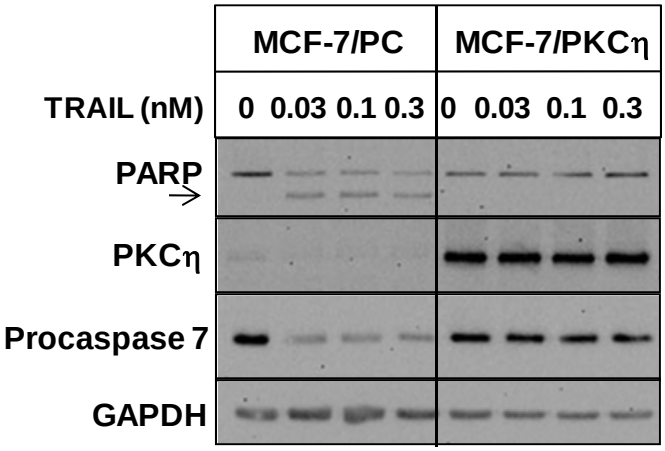

MCF-7/PC
MCF-7/PKCη
TRAIL (nM)
0 0.03 0.1 0.3
0 0.03 0.1 0.3
PARP
PKCη
Procaspase 7
GAPDH

Figure 2. Effect of PKCη depletion on TRAIL-induced cell death.

MCF-7 cells were transfected with non-targeting siRNA (Con) or siRNA against PKCη. 48 h after transfection, cells were treated with 0.1 nM TRAIL for 12 h. (a) Western blot analysis was performed with total cellular extracts using PKC antibodies. (b) Western blot analysis was performed using Bax, Mcl-1, Bid and Bcl-2, Dotted arrow indicates Bax dimer. Actin was used as a loading control. Results were representative of 2 independent experiments.

Figure 2.

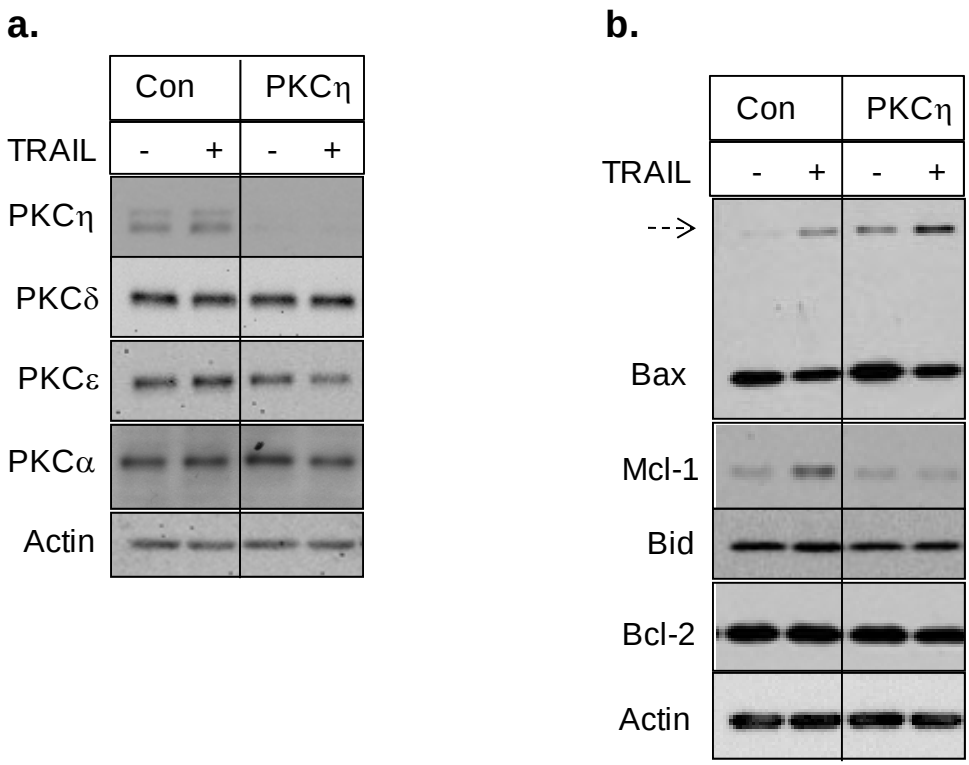

a.
Con
PKCη
TRAIL
- + - +
PKCη
PKCδ
PKCε
PKCα
Actin
b.
Con
PKCη
TRAIL
- + - +
-->
Bax
Mcl-1
Bid
Bcl-2
Actin

Figure 3. Effect of PKCη or Mcl-1 depletion on TRAIL-induced cell death

(a) MCF-7 cells were transfected with non-targeting siRNA (Con) or siRNA against PKCη or Mcl-1. Two days after transfection, cells were treated with the indicated concentrations of TRAIL for 12 h. Cells were stained with YO-PRO-1 dye for 15 mins and visualized under 20X magnification using the fluorescent Zeiss Axiovert 40 inverted microscope. YO-PRO positive cells were detected with green fluorescence. (b) Densitometric quantification of apoptotic cells as the ratio of YO-PRO+ cells to total cell number. Results represent at least 2 independent experiments.

Figure 3.

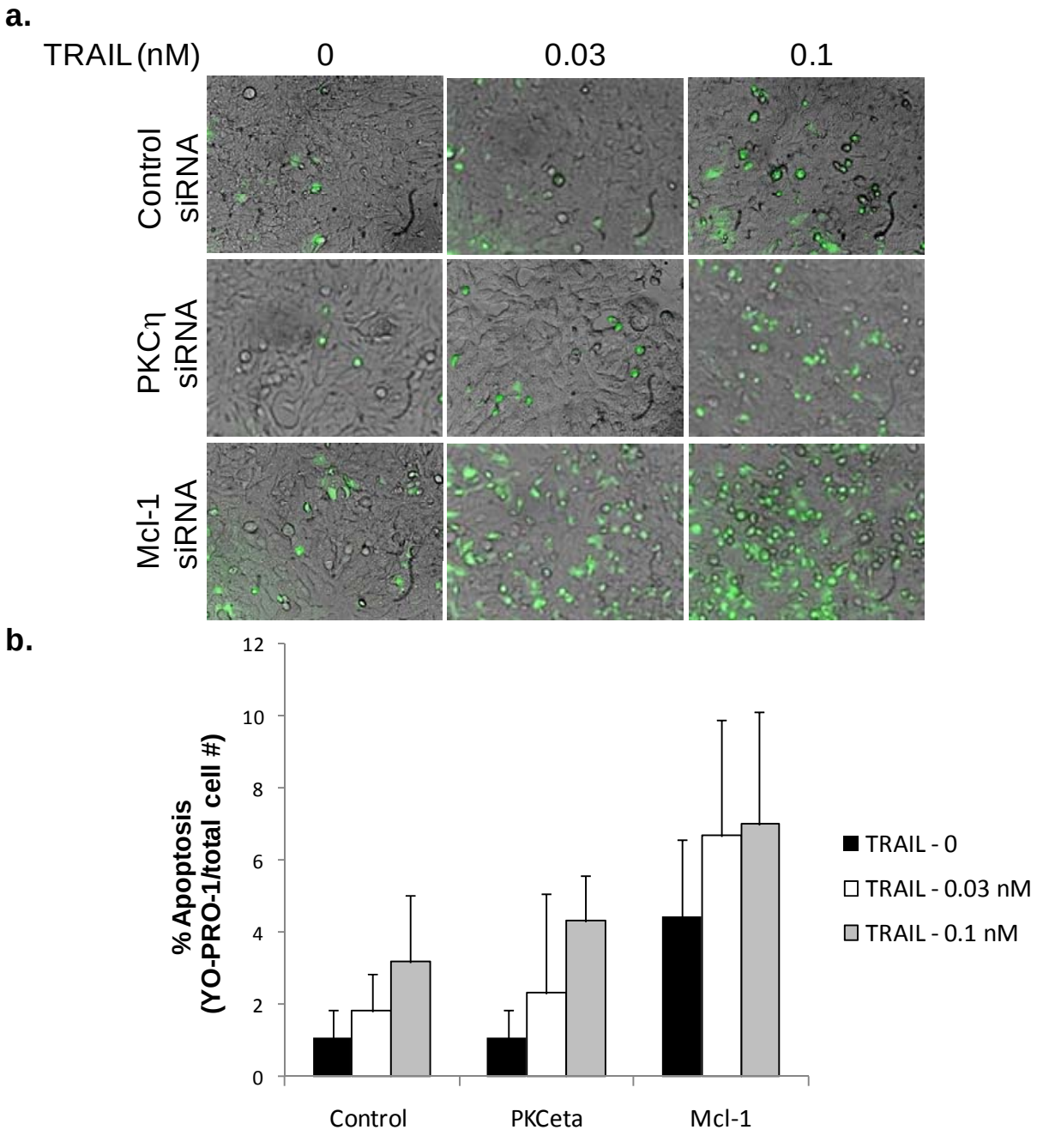
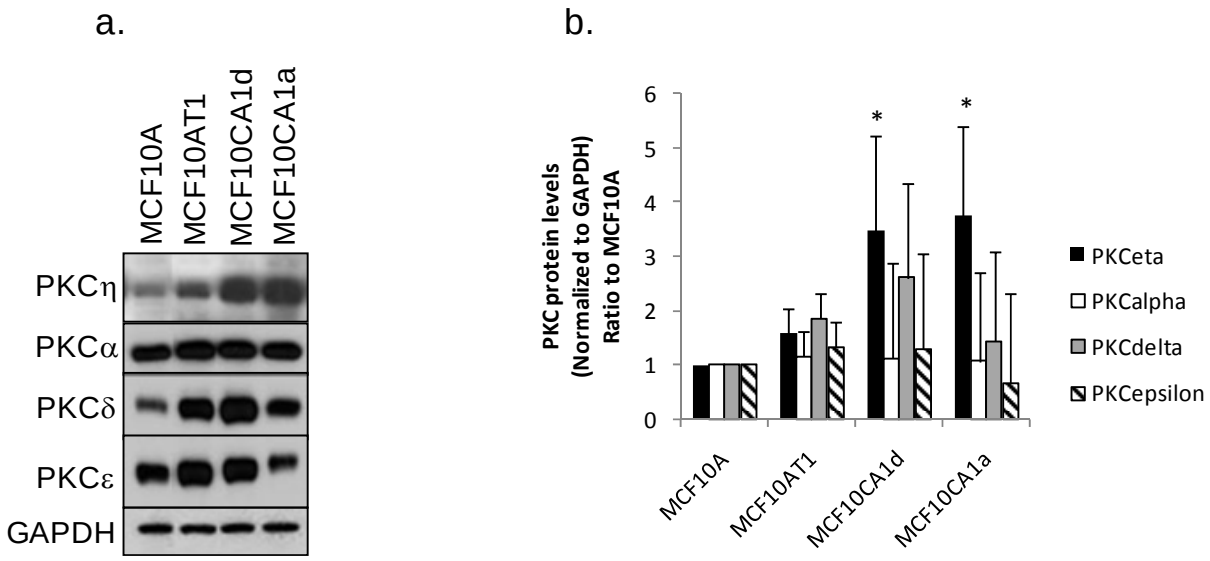

a.
TRAIL (nM)
0
0.03
0.1
Control siRNA
PKCη siRNA
Mcl-1 siRNA
b.
% Apoptosis (YO-PRO-1/total cell #)
12
10
8
6
4
2
0
Control
PKCeta
Mcl-1
TRAIL - 0
TRAIL - 0.03 nM
TRAIL - 0.1 nM

Figure 4. Comparison of PKC isozyme protein levels in MCF10A series.

(a) Western blot analysis was performed with total cellular extracts using the indicated antibodies. GAPDH was used as a loading control. (b) Densitometric quantification of PKCη, –α, –δ and -ε protein levels from 3 separate experiments corrected for loading and compared as a ratio to MCF10A cells. The asterisk (*) indicates significant difference from MCF10A ($P<0.01$) using Student's *t*-test.

Figure 4.


a.
MCF10A
MCF10AT1
MCF10CA1d
MCF10CA1a
PKCη
PKCα
PKCδ
PKCε
GAPDH
b.
PKC protein levels
(Normalized to GAPDH)
Ratio to MCF10A
0
1
2
3
4
5
6
*
*
MCF10A
MCF10AT1
MCF10CA1d
MCF10CA1a
PKCeta
PKCalpha
PKCdelta
PKCepsilon

REFERENCES

1. Bellamy, C.O., et al., *Cell death in health and disease: the biology and regulation of apoptosis.* Semin Cancer Biol, 1995. **6**(1): p. 3-16.
2. Lockshin, R.A. and Z. Zakeri, *Programmed cell death and apoptosis: origins of the theory.* Nat Rev Mol Cell Biol, 2001. **2**(7): p. 545-50.
3. Yuan, J. and B.A. Yankner, *Apoptosis in the nervous system.* Nature, 2000. **407**(6805): p. 802-9.
4. Green, D.R. and G.I. Evan, *A matter of life and death.* Cancer Cell, 2002. **1**(1): p. 19-30.
5. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
6. Vaux, D.L. and R.A. Flavell, *Apoptosis genes and autoimmunity.* Curr Opin Immunol, 2000. **12**(6): p. 719-24.
7. Bold, R.J., P.M. Termuhlen, and D.J. McConkey, *Apoptosis, cancer and cancer therapy.* Surg Oncol, 1997. **6**(3): p. 133-42.
8. Hengartner, M.O., *The biochemistry of apoptosis.* Nature, 2000. **407**(6805): p. 770-6.
9. Gross, A., J.M. McDonnell, and S.J. Korsmeyer, *BCL-2 family members and the mitochondria in apoptosis.* Genes Dev, 1999. **13**(15): p. 1899-911.
10. Schulze-Osthoff, K., et al., *Apoptosis signaling by death receptors.* Eur J Biochem, 1998. **254**(3): p. 439-59.
11. Rajalingam, K., et al., *Smac/DIABLO is required for effector caspase activation during apoptosis in human cells.* Apoptosis, 2007. **12**(8): p. 1503-10.
12. Vogelstein, B. and K.W. Kinzler, *Cancer genes and the pathways they control.* Nat Med, 2004. **10**(8): p. 789-99.
13. Reyland, M.E., *Protein Kinase C and Apoptosis*, in *Apoptosis, Cell Signaling, and Human Diseases Molecular Mechanisms, Volume 2* R. Srivastava, Editor. 2007, Humana Press. p. 31-55.

14. Basu, A., *The involvement of novel protein kinase C isozymes in influencing sensitivity of breast cancer MCF-7 cells to tumor necrosis factor-alpha.* Mol Pharmacol, 1998. **53**(1): p. 105-11.
15. Basu, A. and U. Sivaprasad, *Protein kinase Cepsilon makes the life and death decision.* Cell Signal, 2007. **19**(8): p. 1633-42.
16. Lu, D., et al., *Protein kinase C-epsilon protects MCF-7 cells from TNF-mediated cell death by inhibiting Bax translocation.* Apoptosis, 2007. **12**(10): p. 1893-900.
17. Sivaprasad, U., E. Shankar, and A. Basu, *Downregulation of Bid is associated with PKCepsilon-mediated TRAIL resistance.* Cell Death Differ, 2007. **14**(4): p. 851-60.
18. Sonnemann, J., et al., *Down-regulation of protein kinase Ceta potentiates the cytotoxic effects of exogenous tumor necrosis factor-related apoptosis-inducing ligand in PC-3 prostate cancer cells.* Mol Cancer Ther, 2004. **3**(7): p. 773-81.
19. Lessene, G., P.E. Czabotar, and P.M. Colman, *BCL-2 family antagonists for cancer therapy.* Nat Rev Drug Discov, 2008. **7**(12): p. 989-1000.
20. Ali, A.S., et al., *Exploitation of protein kinase C: a useful target for cancer therapy.* Cancer Treat Rev, 2009. **35**(1): p. 1-8.
21. Basu, A., *The potential of protein kinase C as a target for anticancer treatment.* Pharmacol Ther, 1993. **59**(3): p. 257-80.
22. Guo, B.C. and Y.H. Xu, *Bcl-2 over-expression and activation of protein kinase C suppress the trail-induced apoptosis in Jurkat T cells.* Cell Res, 2001. **11**(2): p. 101-6.
23. Farrow, B., et al., *Activation of conventional PKC isoforms increases expression of the pro-apoptotic protein Bad and TRAIL receptors.* Int J Gastrointest Cancer, 2002. **32**(2-3): p. 63-72.
24. Akkaraju, G.R. and A. Basu, *Overexpression of protein kinase C-eta attenuates caspase activation and tumor necrosis factor-alpha-induced cell death.* Biochem Biophys Res Commun, 2000. **279**(1): p. 103-7.

25. Lu, D., J. Huang, and A. Basu, *Protein kinase Cepsilon activates protein kinase B/Akt via DNA-PK to protect against tumor necrosis factor-alpha-induced cell death.* J Biol Chem, 2006. **281**(32): p. 22799-807.
26. Clarke, D.M., et al., *Targeted induction of apoptosis via TRAIL and cryoablation: a novel strategy for the treatment of prostate cancer.* Prostate Cancer Prostatic Dis, 2007. **10**(2): p. 175-84.
27. Miller, F.R., et al., *Xenograft model of progressive human proliferative breast disease.* J Natl Cancer Inst, 1993. **85**(21): p. 1725-32.
28. Brenner, W., et al., *Protein kinase C eta is associated with progression of renal cell carcinoma (RCC).* Anticancer Res, 2003. **23**(5A): p. 4001-6.
29. Chida, K., et al., *Disruption of protein kinase Ceta results in impairment of wound healing and enhancement of tumor formation in mouse skin carcinogenesis.* Cancer Res, 2003. **63**(10): p. 2404-8.
30. Sonnemann, J., et al., *Down-regulation of protein kinase Ceta by antisense oligonucleotides sensitises A549 lung cancer cells to vincristine and paclitaxel.* Cancer Lett, 2004. **209**(2): p. 177-85.
31. Abu-Ghanem, S., et al., *PKCeta expression contributes to the resistance of Hodgkin's lymphoma cell lines to apoptosis.* Cancer Biol Ther, 2007. **6**(9): p. 1375-80.
32. Youle, R.J. and A. Strasser, *The BCL-2 protein family: opposing activities that mediate cell death.* Nat Rev Mol Cell Biol, 2008. **9**(1): p. 47-59.
33. Zong, Z.P., et al., *Phorbol myristate induces apoptosis of taxol-resistant sarcoma cells in vitro.* Eur J Pharmacol, 2004. **489**(1-2): p. 3-11.
34. D'Alessio, M., et al., *Oxidative Bax dimerization promotes its translocation to mitochondria independently of apoptosis.* Faseb J, 2005. **19**(11): p. 1504-6.
35. Ricci, M.S., et al., *Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death.* Cancer Cell, 2007. **12**(1): p. 66-80.

36. Kim, S.H., M.S. Ricci, and W.S. El-Deiry, *Mcl-1: a gateway to TRAIL sensitization.* Cancer Res, 2008. **68**(7): p. 2062-4.
37. Ashkenazi, A., et al., *Safety and antitumor activity of recombinant soluble Apo2 ligand.* J Clin Invest, 1999. **104**(2): p. 155-62.
38. Walczak, H., et al., *Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo.* Nat Med, 1999. **5**(2): p. 157-63.
39. Johnstone, R.W., A.J. Frew, and M.J. Smyth, *The TRAIL apoptotic pathway in cancer onset, progression and therapy.* Nat Rev Cancer, 2008. **8**(10): p. 782-98.
40. Greco, F.A., et al., *Phase 2 study of mapatumumab, a fully human agonistic monoclonal antibody which targets and activates the TRAIL receptor-1, in patients with advanced non-small cell lung cancer.* Lung Cancer, 2008. **61**(1): p. 82-90.
41. Hotte, S.J., et al., *A phase 1 study of mapatumumab (fully human monoclonal antibody to TRAIL-R1) in patients with advanced solid malignancies.* Clin Cancer Res, 2008. **14**(11): p. 3450-5.
42. Inoshita, S., et al., *Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress.* J Biol Chem, 2002. **277**(46): p. 43730-4.
43. Rotem-Dai, N., et al., *PKCeta confers protection against apoptosis by inhibiting the pro-apoptotic JNK activity in MCF-7 cells.* Exp Cell Res, 2009. **315**(15): p. 2616-23.
44. Akgul, C., *Mcl-1 is a potential therapeutic target in multiple types of cancer.* Cell Mol Life Sci, 2009. **66**(8): p. 1326-36.
45. Gaur, U. and B.B. Aggarwal, *Regulation of proliferation, survival and apoptosis by members of the TNF superfamily.* Biochem Pharmacol, 2003. **66**(8): p. 1403-8.
46. Li, Z.W., et al., *The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis.* J Exp Med, 1999. **189**(11): p. 1839-45.

47. Lam, L.T., et al., *Small molecule inhibitors of IkappaB kinase are selectively toxic for subgroups of diffuse large B-cell lymphoma defined by gene expression profiling.* Clin Cancer Res, 2005. **11**(1): p. 28-40.
48. Karin, M., Y. Yamamoto, and Q.M. Wang, *The IKK NF-kappa B system: a treasure trove for drug development.* Nat Rev Drug Discov, 2004. **3**(1): p. 17-26.
49. Lam, L.T., et al., *Compensatory IKKalpha activation of classical NF-kappaB signaling during IKKbeta inhibition identified by an RNA interference sensitization screen.* Proc Natl Acad Sci U S A, 2008. **105**(52): p. 20798-803.
50. Lavie, Y., et al., *Tamoxifen induces selective membrane association of protein kinase C epsilon in MCF-7 human breast cancer cells.* Int J Cancer, 1998. **77**(6): p. 928-32.
51. El-Rayes, B.F., et al., *Protein kinase C: a target for therapy in pancreatic cancer.* Pancreas, 2008. **36**(4): p. 346-52.

CHAPTER V

CONCLUSIONS

Protein kinase C (PKC) is the receptor for tumor-promoting phorbol esters and plays a major role in regulating signaling events involved in cell proliferation and cell death [1, 2]. Prolonged treatment with phorbol esters leads to activation and downregulation of PKCs [3]. Downregulation of PKCδ by phorbol esters was associated with tumor promotion [4]. We and others have reported that in contrast to other PKCs, PKCη is the only isozyme that is upregulated by prolonged treatment with phorbol esters [5-8] and downregulated by treatment with PKC inhibitors [6] indicating PKCη may play a role in tumor-promotion. An understanding of the cellular and molecular mechanisms of proteins involved in cancer progression is critical in developing effective cancer therapies. PKCη plays a role in cell proliferation, cell-cycle control and resistance to chemotherapeutic agents in various cancer types [9-12]. The purpose of this dissertation was to investigate the molecular mechanisms of PKCη upregulation and downregulation by PKC activators and inhibitors. The results described here demonstrated that (i) phosphorylation of PKCη protects it from downregulation (ii) downregulation of PKCη is mediated by two distinct mechanisms: proteasome-dependent and proteasome-independent and (iii) PKCη downregulation moderately sensitizes breast cancer cells to TRAIL-induced cell death via inhibition of Mcl-1 and increased dimerization of Bax.

Phosphorylation of PKCs can regulate their maturation, stability and function [13-17]. PKCs are generally targeted for downregulation after prolonged treatment with activators and the downregulation was usually associated with dephosphorylation of PKCs [18-22]. The first part of this dissertation investigated if the phosphorylation status of PKCη regulates its stability in response to activation by phorbol esters. We found that prolonged treatment with phorbol ester PDBu increased phosphorylation of PKCη at conserved serine/threonine sites in the activation loop, turn motif and hydrophobic motif. Furthermore, substitution of a single phosphorylation site from a serine/threonine to alanine which renders PKCη "non-phosphorylatable" resulted in destabilization of PKCη. Thus, phosphorylation plays an important role in protecting PKCη from downregulation. It has been shown that phosphorylation at the turn motif and hydrophobic motif sites stabilize PKC and other AGC kinases in a form that is resistant to degradation by proteases and phosphatases [13, 23]. Our results suggest that phosphorylation of all three sites is important for PKCη stability and mutation at just one site can destabilize the protein. It is not clear whether the phosphorylation at one site of PKCη can affect the phosphorylation of another site and this phenomenon should be tested in further experiments.

PKCη has been shown to be upregulated in various cancers and promotes resistance to chemotherapeutic agents [9, 10, 24-26]. In the second part of the dissertation, we focused on elucidating the downregulation mechanisms of PKCη. Our results show that contrary to other PKC isozymes, PKCη is downregulated by two inhibitors: the general PKC inhibitor Gö 6983 and PI3K inhibitor Ly294002. Both inhibitors promote dephosphorylation of PKCη by a

calyculin A-sensitive phosphatase, most likely protein phosphatase 1 (PP1). The inhibitors are distinct in their ability to degrade PKCη through the ubiquitin/proteasome-mediated pathway. Downregulation by Gö 6983 is proteasome-independent, whereas downregulation by Ly294002 is proteasome-dependent. Based on these studies, PKCη can be targeted for downregulation by dephosphorylation via inhibition of two separate upstream pathways: PKC and PI3K. PKCα downregulation is also mediated by two distinct mechanisms involving a proteasome-dependent pathway that does not require dephosphorylation and a proteasome-independent pathway requiring dephosphorylation and caveolar trafficking to degradation compartments [27]. Activation of PKCα by two different activators can trigger both downregulation pathways at the same time [27]. In the case of PKCη, the two inhibitors have two distinct downregulation mechanisms indicating the inhibitors may affect different kinases or phosphatases to act on distinct sites on PKCη. One limitation of using pharmacological inhibitors is the toxic side effects caused by non-specific targeting. Since these the two inhibitors downregulate only PKCη and not other PKC isozymes, non-specific effects are reduced.

Downregulation of PKCη was associated with sensitization of lung [28] and prostate cancers to chemotherapeutic agents [29]. We have previously shown that overexpression of PKCη protected MCF-7 breast cancer cells from TNF-induced apoptosis [24]. The third part of the dissertation investigated the anti-apoptotic role of PKCη in the breast adenocarcinoma MCF-7 cells in response to TRAIL-induced cell death. The results demonstrate that overexpression of

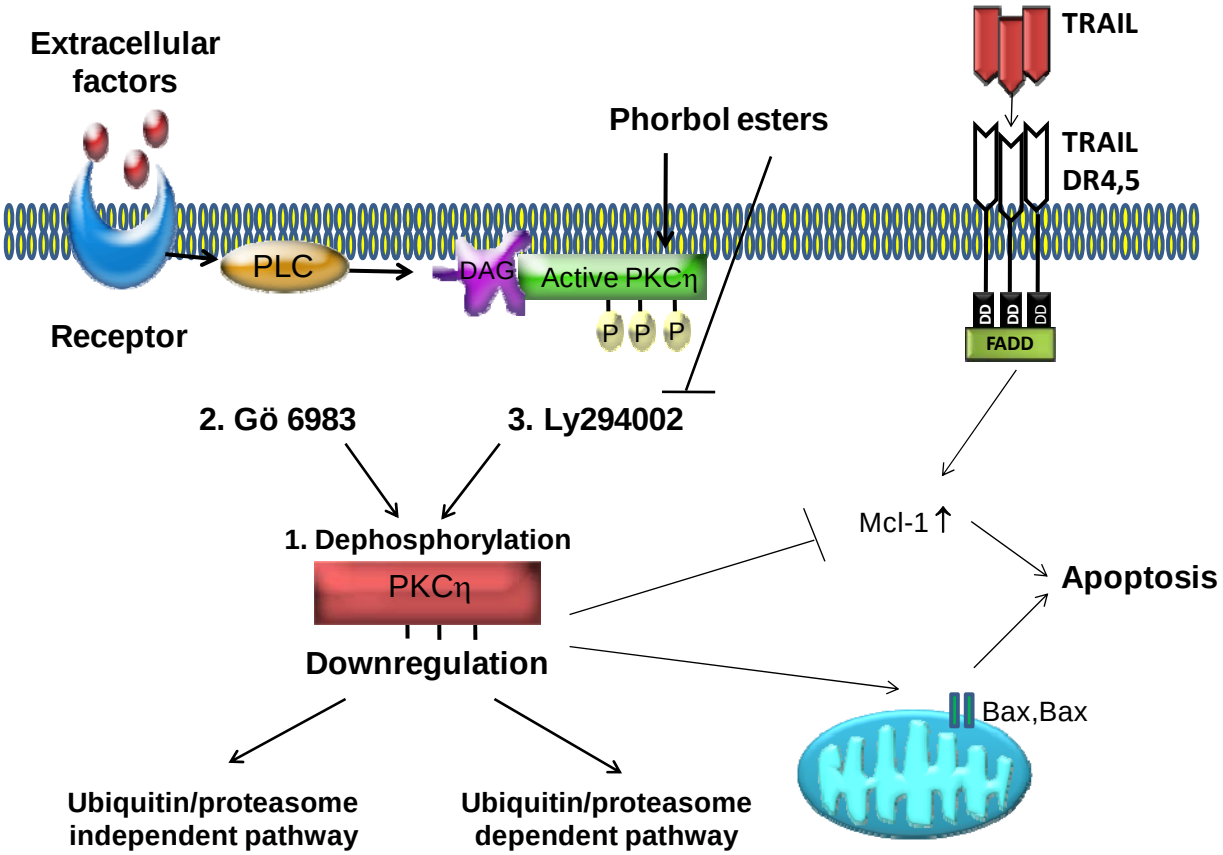
PKCη confers resistance to TRAIL-induced cell death in MCF-7 cells. Depletion of PKCη increased Bax dimer formation and decreased induction of Mcl-1 to sensitize MCF-7 cells to TRAIL-induced cell death. Therefore, Bax and Mcl-1 could be potential downstream targets of PKCη in TRAIL-induced cell death. Since PKCη depletion by itself did not enhance cell death in MCF-7 cells, this indicates that in MCF-7 cells, PKCη may not directly regulate cell death signaling and other anti-apoptotic proteins may compensate of the loss of PKCη. Similarly, although Akt plays a major role in survival, its involvement in cancer metastasis depends on the specific Akt isoform [30]. Thus, it is important to understand the mechanism of regulation for specific isozymes in order to develop more specific and specialized therapies.

In summary, this dissertation demonstrated that PKCη can be specifically targeted for downregulation by three pathways: 1) dephosphorylation at conserved sites 2) inhibition by Gö 6983 and 3) inhibition by Ly294002. We have shown that depletion of PKCη blocks the induction of Mcl-1 by TRAIL and increases Bax dimer formation indicating Mcl-1 and Bax may be potential targets of PKCη (Figure 1). This dissertation provides novel information about PKCη regulation by phosphorylation, downregulation and potential downstream targets. The information honed from this study makes PKCη an attractive target for cancer therapy in cancers that upregulate PKCη to promote cell proliferation and cell survival. We have identified downregulation and upregulation mechanisms that are specific to PKCη and not other PKCs which can be pivotal in developing inhibitors of PKCη for therapeutic intervention.

Figure 1. Conclusion

We have reported that upon prolonged treatment with phorbol esters such as PDBu, PKCη is upregulated and phosphorylated at three conserved sites: activation loop, turn motif and hydrophobic motif. PKCη is downregulated by two inhibitors, the PKC inhibitor, Gö 6983 and PI3K inhibitor, Ly294002. Both inhibitors lead to dephosphorylation of PKCη by a calyculin A-sensitive phosphatase. Downregulation of PKCη by these two inhibitors occurs by two distinct mechanisms: proteasome-dependent and proteasome-independent. Gö 6983-mediated downregulation of PKCη occurs via a proteasome-independent pathway, whereas Ly294002-mediated downregulation of PKCη occurs by a proteasome-dependent pathway. Ly294002-mediated downregulation of PKCη is also inhibited by PDBu treatment. Depletion of PKCη by siRNA can block the induction of anti-apoptotic protein Mcl-1 by TRAIL and increase dimer formation of pro-apoptotic Bax which can lead to apoptosis.

Figure 1.


Extracellular factors
Receptor
PLC
DAG
Active PKCη
P P P
Phorbol esters
TRAIL
TRAIL DR4,5
DD DD DD
FADD
2. Gö 6983
3. Ly294002
1. Dephosphorylation
PKCη
Downregulation
Mcl-1
Apoptosis
Bax,Bax
Ubiquitin/proteasome independent pathway
Ubiquitin/proteasome dependent pathway

FUTURE DIRECTIONS

We have demonstrated that phosphorylation of PKCη by phorbol esters protects PKCη from downregulation. The kinases or phosphatases involved in the regulation of PKCη by phosphorylation remain to be identified. Since we have shown that both Gö 6983 and Ly294002 leads to dephosphorylation and downregulation of PKCη; it is likely that autophosphorylation or transphosphorylation events may be involved. Several PKC isozymes including PKCη have already been reported to cross-regulate and phosphorylate one another [31-35]. Thus, investigating the transphosphorylation of PKCη by other PKCs may be worthwhile approach to identify the kinase involved in PKCη phosphorylation in response to phorbol esters. PKCι/λ has been reported to phosphorylate PKCη at the hydrophobic motif site allowing PDK1 to dock and phosphorylate PKCη at the activation loop in mouse fibroblasts in response to parvovirus infection [31]. Whether or not PKCη can be phosphorylated by PKCι/λ in response to phorbol esters in human cancers is subject to future investigation. In addition to identifying the kinase involved in phosphorylating PKCη, identification of the phosphatase involved in dephosphorylating and downregulating PKCη can also have important implications in PKCη regulation. In the second chapter, we identified that PP1 may be the potential phosphatase regulating PKCη dephosphorylation since it is sensitive to calyculin A inhibition, but not okadaic acid. It would be worthwhile to investigate the role of PP1 in regulating PKCη dephosphorylation and downregulation in cancer cells that promote survival by upregulating

PKCη. Studies investigating the mechanism of PKCε dephosphorylation at the hydrophobic Ser729 site reported that dephosphorylation was not mediated by PP1, PP2A or PP2B, but was mediated by mTOR, MAPK and PKCδ [16, 36, 37]. Therefore the involvement of mTOR, MAPK and other PKCs should also be investigated in the downregulation of PKCη. In addition to identifying the kinase or phosphatase involved in regulating PKCη phosphorylation, identification of specific PKCη phosphorylation sites that are important for its stability could be beneficial for the development of PKCη-specific therapeutic agents.

We have identified two potential downstream targets of PKCη (Bax and Mcl-1) involved in sensitizing MCF-7 cells to TRAIL-induced apoptosis. Further experiments should confirm if PKCη can directly interact with Bax or Mcl-1 by coimmunoprecipitation or fluorescent resonance energy transfer (FRET) microscopy. PKCε was reported to directly interact with and phosphorylate components of the cardiac mitochondrial pore in mice to inhibit pore opening contributing to its cardioprotective function [38]. PKCε was also reported to interact with Bax and prevent its dimerization and translocation to the mitochondria to protect MCF-7 cells from TNF-induced apoptosis [39]. Since PKCη shares the most structural similarity to PKCε [40], further studies should identify the subcellular localization of PKCη in response to TRAIL-induced cell death to determine if PKCη can translocate to the mitochondria to exert its anti-apoptotic effect.

Since depletion of PKCη had a modest effect on cell death by apoptosis, further studies should determine if PKCη may play a role in other cell death pathways such as autophagy. PKCη was shown to mediate cell proliferation in glioblastoma cells by regulating the Akt and mTOR signaling pathways [41]. Both Akt and mTOR pathways have been reported to play a key role in regulating autophagy [42]; therefore it is conceivable that PKCη may mediate autophagy or cell proliferation in breast cancer cells via Akt or mTOR. We have shown in Chapter II that PKCη can be downregulated by the inhibitor of PI3K/Akt, Ly294002 in MCF-7 cells suggesting PKCη can act via the PI3K pathway. Given the role of PKCη in glioblastoma proliferation [5] and cell cycle control in MCF-7 cells and NIH-3T3 cells [43], depletion of PKCη could result in an anti-proliferative effect in MCF-7 breast cancer cells. Further studies should elucidate if PKCη depletion decreases cell proliferation in response to TRAIL in MCF-7 cells. Since MCF-7 cells contain a moderate level of PKCη protein, it may be worthwhile to test the effect of PKCη depletion in other cell lines expressing higher levels of PKCη such as MCF10CA1a or MCF10CA1d to determine its effect on cell death.

The mechanism of PKCη downregulation in response to inhibitors and increased phosphorylation in response to inhibitors has been limited to breast cancer cells and HEK 293 cells in this study. Future studies should investigate if the regulation of PKCη described in this dissertation can be applied to other cancer cell types such as lung[28], prostate [29], renal [25] and Hodgkins lymphomas [9] where PKCη plays a role in resistance or tumor progression.

The findings of this dissertation provide valuable insight on the mechanism of PKCη downregulation and its anti-apoptotic function in breast cancer. This information can lead to potential therapeutic agents targeting PKCη downregulation to overcome resistance to TRAIL in breast cancer cells.

REFERENCES

1. Blumberg, P.M., *Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture.* Cancer Res, 1988. **48**(1): p. 1-8.
2. Stabel, S. and P.J. Parker, *Protein kinase C.* Pharmacol Ther, 1991. **51**(1): p. 71-95.
3. Young, S., et al., *Down-regulation of protein kinase C is due to an increased rate of degradation.* Biochem J, 1987. **244**(3): p. 775-9.
4. Lu, Z., et al., *Tumor promotion by depleting cells of protein kinase C delta.* Mol Cell Biol, 1997. **17**(6): p. 3418-28.
5. Hussaini, I.M., et al., *Phorbol 12-myristate 13-acetate induces protein kinase ceta-specific proliferative response in astrocytic tumor cells.* J Biol Chem, 2000. **275**(29): p. 22348-54.
6. Basu, A., *The involvement of novel protein kinase C isozymes in influencing sensitivity of breast cancer MCF-7 cells to tumor necrosis factor-alpha.* Mol Pharmacol, 1998. **53**(1): p. 105-11.
7. Resnick, M.S., et al., *Selective up-regulation of protein kinase C eta in phorbol ester-sensitive versus -resistant EL4 mouse thymoma cells.* Cancer Res, 1997. **57**(11): p. 2209-15.
8. Kang, B.S., et al., *Activation-dependent degradation of protein kinase C eta.* Oncogene, 2000. **19**(37): p. 4263-72.
9. Abu-Ghanem, S., et al., *PKCeta expression contributes to the resistance of Hodgkin's lymphoma cell lines to apoptosis.* Cancer Biol Ther, 2007. **6**(9): p. 1375-80.
10. Martin, P.M. and I.M. Hussaini, *PKC eta as a therapeutic target in glioblastoma multiforme.* Expert Opin Ther Targets, 2005. **9**(2): p. 299-313.
11. Fima, E., et al., *PKCeta enhances cell cycle progression, the expression of G1 cyclins and p21 in MCF-7 cells.* Oncogene, 2001. **20**(46): p. 6794-804.

12. Rotem-Dai, N., et al., *PKCeta confers protection against apoptosis by inhibiting the pro-apoptotic JNK activity in MCF-7 cells.* Exp Cell Res, 2009. **315**(15): p. 2616-23.
13. Newton, A.C., *Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm.* Biochem J, 2003. **370**(Pt 2): p. 361-71.
14. Newton, A.C., *Protein kinase C: structure, function, and regulation.* J Biol Chem, 1995. **270**(48): p. 28495-8.
15. Parker, P.J. and S.J. Parkinson, *AGC protein kinase phosphorylation and protein kinase C.* Biochem Soc Trans, 2001. **29**(Pt 6): p. 860-3.
16. Parekh, D.B., W. Ziegler, and P.J. Parker, *Multiple pathways control protein kinase C phosphorylation.* Embo J, 2000. **19**(4): p. 496-503.
17. Ikenoue, T., et al., *Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling.* Embo J, 2008. **27**(14): p. 1919-31.
18. Nelsestuen, G.L. and M.D. Bazzi, *Activation and regulation of protein kinase C enzymes.* J Bioenerg Biomembr, 1991. **23**(1): p. 43-61.
19. Bornancin, F. and P.J. Parker, *Phosphorylation of protein kinase C-alpha on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state.* J Biol Chem, 1997. **272**(6): p. 3544-9.
20. Parker, P.J., et al., *Protein kinase C (PKC)-induced PKC degradation: a model for down-regulation.* Biochem Soc Trans, 1995. **23**(1): p. 153-5.
21. Lee, H.W., et al., *Dephosphorylation of activated protein kinase C contributes to downregulation by bryostatin.* Am J Physiol, 1996. **271**(1 Pt 1): p. C304-11.
22. Hansra, G., et al., *Multisite dephosphorylation and desensitization of conventional protein kinase C isotypes.* Biochem J, 1999. **342 (Pt 2)**: p. 337-44.
23. Cenni, V., et al., *Regulation of novel protein kinase C epsilon by phosphorylation.* Biochem J, 2002. **363**(Pt 3): p. 537-45.

24. Akkaraju, G.R. and A. Basu, *Overexpression of protein kinase C-eta attenuates caspase activation and tumor necrosis factor-alpha-induced cell death.* Biochem Biophys Res Commun, 2000. **279**(1): p. 103-7.
25. Brenner, W., et al., *Protein kinase C eta is associated with progression of renal cell carcinoma (RCC).* Anticancer Res, 2003. **23**(5A): p. 4001-6.
26. Karp, G., A. Maissel, and E. Livneh, *Hormonal regulation of PKC: estrogen up-regulates PKCeta expression in estrogen-responsive breast cancer cells.* Cancer Lett, 2007. **246**(1-2): p. 173-81.
27. Leontieva, O.V. and J.D. Black, *Identification of two distinct pathways of protein kinase Calpha down-regulation in intestinal epithelial cells.* J Biol Chem, 2004. **279**(7): p. 5788-801.
28. Sonnemann, J., et al., *Down-regulation of protein kinase Ceta by antisense oligonucleotides sensitises A549 lung cancer cells to vincristine and paclitaxel.* Cancer Lett, 2004. **209**(2): p. 177-85.
29. Sonnemann, J., et al., *Down-regulation of protein kinase Ceta potentiates the cytotoxic effects of exogenous tumor necrosis factor-related apoptosis-inducing ligand in PC-3 prostate cancer cells.* Mol Cancer Ther, 2004. **3**(7): p. 773-81.
30. Sheng, S., M. Qiao, and A.B. Pardee, *Metastasis and AKT activation.* J Cell Physiol, 2009. **218**(3): p. 451-4.
31. Lachmann, S., et al., *Parvovirus interference with intracellular signalling: mechanism of PKCeta activation in MVM-infected A9 fibroblasts.* Cell Microbiol, 2008. **10**(3): p. 755-69.
32. Rybin, V.O., et al., *Cross-regulation of novel protein kinase C (PKC) isoform function in cardiomyocytes. Role of PKC epsilon in activation loop phosphorylations and PKC delta in hydrophobic motif phosphorylations.* J Biol Chem, 2003. **278**(16): p. 14555-64.

33. Rybin, V.O., et al., *Protein kinase Cepsilon (PKCepsilon) and Src control PKCdelta activation loop phosphorylation in cardiomyocytes.* J Biol Chem, 2007. **282**(32): p. 23631-8.
34. Brandlin, I., et al., *Protein kinase C (PKC)eta-mediated PKC mu activation modulates ERK and JNK signal pathways.* J Biol Chem, 2002. **277**(8): p. 6490-6.
35. Durgan, J., et al., *The identification and characterization of novel PKCepsilon phosphorylation sites provide evidence for functional cross-talk within the PKC superfamily.* Biochem J, 2008. **411**(2): p. 319-31.
36. England, K., et al., *Signalling pathways regulating the dephosphorylation of Ser729 in the hydrophobic domain of protein kinase Cepsilon upon cell passage.* J Biol Chem, 2001. **276**(13): p. 10437-42.
37. Parekh, D., et al., *Mammalian TOR controls one of two kinase pathways acting upon nPKCdelta and nPKCepsilon.* J Biol Chem, 1999. **274**(49): p. 34758-64.
38. Baines, C.P., et al., *Protein kinase Cepsilon interacts with and inhibits the permeability transition pore in cardiac mitochondria.* Circ Res, 2003. **92**(8): p. 873-80.
39. Lu, D., et al., *Protein kinase C-epsilon protects MCF-7 cells from TNF-mediated cell death by inhibiting Bax translocation.* Apoptosis, 2007. **12**(10): p. 1893-900.
40. Bacher, N., et al., *Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin, and heart.* Mol Cell Biol, 1991. **11**(1): p. 126-33.
41. Aeder, S.E., et al., *PKC-eta mediates glioblastoma cell proliferation through the Akt and mTOR signaling pathways.* Oncogene, 2004. **23**(56): p. 9062-9.
42. Baehrecke, E.H., *Autophagy: dual roles in life and death?* Nat Rev Mol Cell Biol, 2005. **6**(6): p. 505-10.
43. Shtutman, M., et al., *PKCeta associates with cyclin E/Cdk2 complex in serum-starved MCF-7 and NIH-3T3 cells.* Exp Cell Res, 2003. **286**(1): p. 22-9.