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Protein Kinase C and Apoptosis

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Summary

The protein kinase C (PKC) family consists of ten structurally related serine/threonine protein kinases. PKC isoforms are critical regulators of cell proliferation and survival and their expression or activity is altered in some human diseases, particularly cancer. The development and utilization of PKC isoform specific tools, including dominant inhibitory kinases, mouse models in which specific PKC isoforms have been disrupted, and PKC isoform specific antisense/siRNA, has allowed studies to define isoform-specific functions of PKC in the apoptotic pathway. From these approaches a pattern is emerging in which the conventional isoforms, particularly PKCα and PKCβ, and the atypical PKCs, PKCι/λ and PKCζ, appear to be anti-apoptotic/pro-survival. The novel isoform, PKCδ, is primarily pro-apoptotic, whereas PKCε in most studies appears to suppress apoptosis. The identification of both pro- and anti-apoptotic isoforms suggests that PKC isoforms may function as molecular sensors, promoting cell survival under favorable conditions, and executing the death of abnormal or damaged cells when needed.This chapter discusses what is currently known about the contribution of specific isoforms to apoptosis, and how signal transduction by PKC integrates with other molecular regulators to promote or inhibit apoptosis.

1. Introduction

Apoptosis was originally described by Kerr, Wyllie, and Currie as a series of morphologic changes to the cell which include membrane blebbing, nuclear condensation and DNA digestion *(1)*. It is now appreciated that this program of cell death is initiated by physiological stimuli, during development, and by a wide range of cellular toxins. Apoptosis is essential for the maintenance of tissue homeostasis in complex organisms, and alterations in this pathway underlie a variety of disease processes. During development, apoptosis mediates cell turnover and tissue remodeling and is important for the elimination of self-reactive cells in the immune system. Apoptosis is also important for the clearance of altered or damaged cells, and notably does so without eliciting an inflammatory response. However, inappropriate activation or inhibition of apoptosis is associated with a wide range of human diseases including cancer, autoimmune disease, and neurodegenerative disorders. In cancer and autoimmune disease, failure to eliminate defective or unwanted cells may contribute to disease *(2–8)*, whereas in neurodegenerative disorders there is an inappropriate loss of cells. Included in this later group are diseases such as heart failure and other types of acute and chronic tissue injury where apoptosis may contribute to excessive cell loss.

Genetic disruption of the apoptotic pathway is an extremely common feature of tumor cells and the ability to evade apoptosis is considered an essential "hallmark of cancer" *(9,10)*. Correlations between the expression of specific apoptotic markers and

From: *Apoptosis, Cell Signaling, and Human Diseases: Molecular Mechanisms, Volume 2*
Edited by R. Srivastava © Humana Press Inc., Totowa, NJ

clinical outcome underscore the relevance of this pathway to cancer biology *(11,12)*. For instance, increased expression of the anti-apoptotic protein, Bcl-2, or reduced expression of the pro-apoptotic protein, Bax, correlates with poor prognosis and increased metastasis in breast and other tumor types *(11,12)*. Bax expression is lost in a subset of human colon cancers and its loss is associated with increased cancer cell growth in vivo and in vitro *(13)*. Likewise, inhibition of apoptosis may underlie the resistance of many tumors to chemotherapeutic drugs. Loss of Bax in glioblastoma multiforme tumors results in resistance to apoptotic stimuli in vitro *(14)*. Mouse models also suggest that targeted suppression of the apoptotic pathway promotes tumor progression in mice expressing activated oncogenes. For instance, overexpression of Bcl-2 increases c-myc induced tumorigenesis in the mammary gland *(15)*, whereas loss of the pro-apoptotic protein, Bax, accelerates mammary tumor development in C3(1)/SV40-Tag transgenic mice *(16)*. Taken together, these studies suggest that normal apoptosis is critical for tumor suppression and that inactivation, or aberrant regulation of this pathway, may have important consequences for tumorigenesis or tumor progression.

PKC family members have been implicated in a wide range of cellular responses including cell permeability, contraction, migration, hypertrophy, proliferation, apoptosis, and secretion. In many cases these functions appear to be cell or tissue specific, implying that the specification of these responses relies on the interaction of PKC isoforms with other regulatory pathways in the cell. In particular, protein kinase cascades are emerging as important modulators of the apoptotic response *(17)*. These include the phosphoinositide 3-kinase/AKT (PI3-kinase/AKT) pathway, the c-Jun-N-terminal-Kinase (JNK) and p38 pathways, the Janus-Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway *(18–20)*, and many isoforms of PKC. Activation of these cascades can result in direct phosphorylation of apoptotic proteins, or regulate apoptosis by activating or inhibiting the transcription of pro- or anti-apoptotic genes. Recently, tools to decipher the function of specific PKC isoforms have been developed, including "knock-out" mouse models, enabling investigators to probe the roles played by specific members of this family. This chapter will focus on the evidence that specific members of this family play distinct roles in regulating apoptosis.

2. Apoptosis

Many laboratories have been involved in deciphering the molecular players that execute apoptosis as well as molecular regulators of the pathway. From these studies it has become clear that despite the disparity in signals that induce apoptosis, execution of the pathway relies on a common set of biochemical mediators. Critical genes in the apoptotic pathway were identified first in *Caenorhabditis elegans,* and homologs of these genes have since been cloned in mammalian cells, revealing a highly conserved pathway from nematodes to mammals *(21,22)*. Essential players in this pathway include the Bcl-2 family of pro- and anti-apoptotic proteins, and the cysteine-dependent aspartate-directed (caspase) proteases *(23,24)*.

The Bcl-2 family consists of pro- and anti-apoptotic proteins that regulate the release of pro-apoptogenic factors from the mitochondria, such as cytochrome *c*, which is essential for caspase activation *(23,25)*. Apoptosis is suppressed through heterodimerization of anti-apoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-xL, with pro-apoptotic proteins such as Bak and Bax, thus the ratio of pro- to anti-apoptotic Bcl-2 proteins is an important

determinant of cell fate. In nonapoptotic cells, anti-apoptotic proteins bind to and neutralize pro-apoptotic proteins. Apoptotic stimuli alleviate the Bcl-2 mediated suppression of pro-apoptotic Bax and Bak, allowing these proteins to oligomerize into transmembrane pores in the mitochondria, induce cytochrome *c* release and activate caspases. Key to this pathway are the "BH3" only proteins, pro-apoptotic members of the Bcl2 family such as Bim, Bid, Bik, PUMA, Noxa, and Bad, which act as apical damage sensors *(26)*. BH3-only proteins are thought to function by antagonizing the action of pro-survival Bcl-2 proteins *(26)*. The diversity of this subfamily of Bcl-2 proteins suggests that they may have evolved in to response to diverse types of cell stress.

Caspases are expressed as inactive zymogens and are processed to an active form in response to apoptotic stimuli *(24,27,28)*. Activated initiator caspases cleave and activate other caspases, resulting in a cascade of caspase activation. The job of activated caspases is to dismantle the cell through cleavage of cell proteins, thus, caspase activation is central to the process of apoptosis and activation of caspases is generally viewed as an irreversible commitment to cell death. Whereas activation of the apoptotic pathway is critical for removal of unwanted cells, studies from mice lacking specific components of the apoptotic cascade suggest that this pathway is also absolutely required for development, because loss of caspase-3, -7, -8, or -9, or Bcl-2 results in either embryonic or perinatal death *(29–31)*.

Two pathways for the activation of caspases have been described (*see* Fig. 1) *(32)*. These pathways differ in the mechanism by which initiator caspases are activated, whereas the activation of downstream, or effector caspases, such as caspase 3, 6, and 7 is common to both pathways. The receptor-mediated, or extrinsic, pathway is initiated by ligand binding to death receptors such as tumor necrosis factor (TNF), Fas, and TNF-related apoptosis-inducing ligand (TRAIL) receptors *(33)*. Ligand binding leads to the formation of signaling complexes which activate caspases and lead to cell death. Key to this pathway is formation of the death inducing signaling complex (DISC). Death receptors contain a cytoplasmic domain, known as the "death domain" which, upon ligand binding, interacts with the death domain of the adaptor protein, Fas-associated death domain protein (FADD) or TRAIL-associated death domain protein (TRADD). Procaspase-8 is then recruited to the complex to form the DISC, resulting in auto-cleavage and activation of caspase-8 *(34)*. Activated caspase 8 in turn cleaves and activates downstream "effector" caspases, such as caspase-3, leading to cleavage of cellular proteins and cell death. Although this pathway was originally described as mitochondrial independent, it is now clear that active caspase-8 can cleave Bid, a member of the Bcl-2 family, and that cleaved Bid can amplify the death signal by promoting the release of apoptogenic proteins from the mitochondria *(35)*.

Drugs, chemicals, irradiation, and cell stress activate caspases via the intrinsic or mitochondria-dependent pathway. Although the specific cell signals delivered by these agents differ, all appear to converge at the mitochondria resulting in the release of cytochrome *c* and loss of mitochondrial membrane potential. Cytochrome *c*, together with Apaf1, ATP and pro-caspase-9, forms the "apoptosome" and leads to activation of caspase-9, and the subsequent activation of effector caspases. In addition to the Bcl-2 proteins discuss above, caspase activation is also regulated by the release of mitochondrial proteins such as the inhibitor of apoptosis proteins (IAP) that inhibit activated caspases, and SMAC/DIABLO which binds and inhibits IAPs *(36,37)*. Finally, a group of

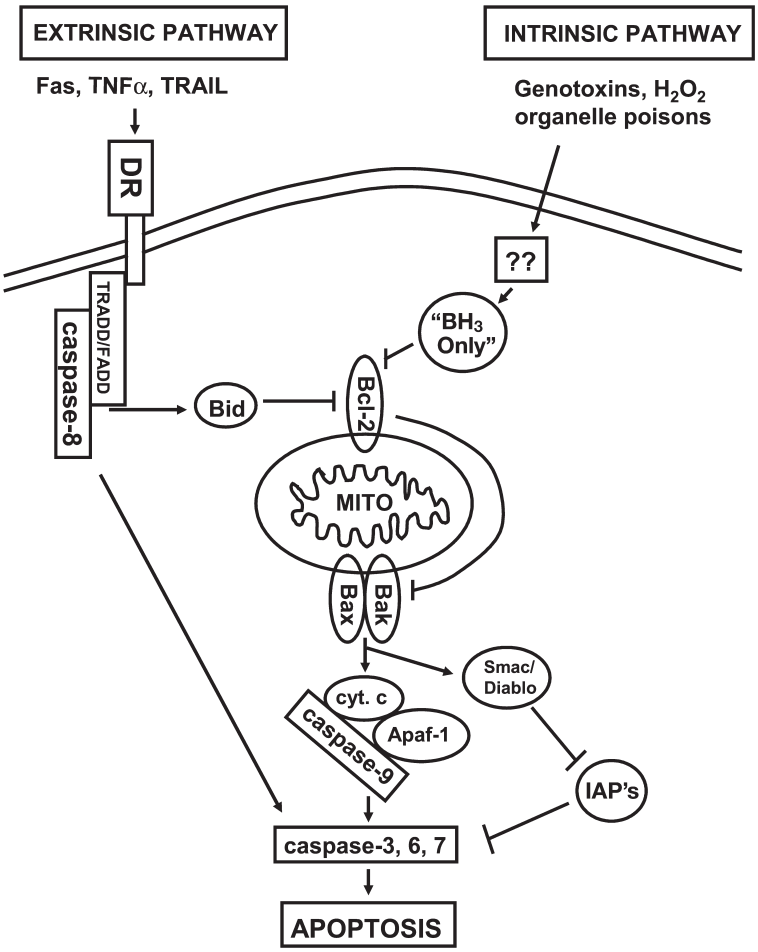

Fig. 1. Intrinsic and extrinsic apoptotic pathways. Apoptosis can be activated through the extrinsic/death receptor dependent pathways, or the intrinsic/mitochondrial dependent pathway. Both pathways converge to activate a common set of effector caspases. See text for details.

mitochondrial proteins have been identified that induce apoptosis independently of caspase activation *(38,39)*. These include Apoptosis Inducing Factor *(38,39)* and endonuclease G *(40)*, which are released from the mitochondria in response to an apoptotic signal and translocate to the nucleus to trigger nuclear condensation and DNA fragmentation *(39)*.

3. PKC Structure/Activation

The PKC family contains 10 structurally related serine/threonine protein kinases that were originally characterized by their dependency upon lipids for activity (*see* Fig. 2) *(41,42)*. The lipid dependence of these enzymes has facilitated the identification of upstream activators. Physiologic regulators of PKC, including growth factors and hormones, activate PKC via receptor stimulated activation of phosphatidylinositol-specific phospholipase C (PI-PLC). Activation of PI-PLC results in the generation of diacylglycerol (DAG), an increase in intracellular Ca++ via generation of Ins(1,4,5)P3, and the

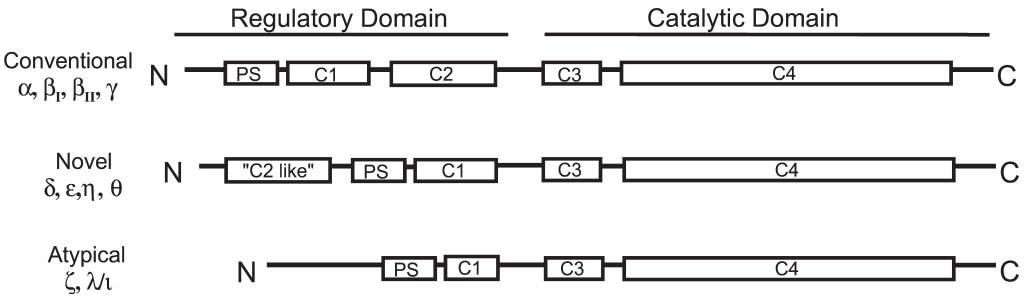

Fig. 2. Structural representation of PKC isozymes and their subfamilies. PS, pseudosubstrate; C1, binds DAG/phorbol esters; C2, binds phosphatidyl serine and Ca2+; C3 and C4, kinase domain.

subsequent activation of PKC. Sub-families of PKC are defined by their requirement for these activators, with the classical isoforms (PKCα, β, and γ) requiring DAG and calcium, the novel isoforms (PKCδ, ε, η, and θ) requiring DAG, but not calcium, and the atypical isoforms (PKCζ and λ/ι) requiring neither. The domains critical for binding these activators have been defined and reside in the N-terminal regulatory portion of the protein. These include the C1 domain which binds DAG and the C2 domain which binds Ca++. These domains also function to target PKC to membranes via DAG and anionic phospholipids *(43)*. The N-terminal regulatory domain also contains binding sites for anchoring proteins which are thought to target the activated kinase to specific subcellular sites. These include the Receptors for Activated C Kinase (RACK's) as well as other PKC-interacting proteins *(44)*.

The C-terminal kinase domain of PKC is highly conserved between isoforms and phosphorylation at three sites in this domain is required to generate a mature form of the kinase that can be recruited to membranes *(45–51)*. The first of these phosphorylation events occurs at a conserved threonine in the activation loop; phosphorylation at this site appears to be essential for activity of most isoforms *(48,51)*. Several laboratories have identified the PIP3 regulated kinase, PDK-1, as the kinase responsible for PKC activation loop phosphorylation *(46,52)*. Phosphorylation at two additional C-terminal sites contributes to the stability of the kinase. These include an autophosphorylation site and a C-terminal hydrophobic site *(48)*. Phosphorylation at these sites renders PKC protease and phosphatase resistant, and catalytically competent. However PKC is still in an inactive conformation in which the substrate binding pocket is occupied by the pseudosubstrate domain. Generation of the second messengers, DAG and Ca^{++}, increases the affinity of "primed" PKC for the membrane resulting in release of the pseudosubstrate from the substrate binding pocket and activation of the kinase. Tyrosine phosphorylation of some PKC isoforms, particularly PKCδ, is seen in response to many stimuli including apoptotic stimuli such as UV, H_2O_2 and etoposide *(53–63)*. Tyrosine residues important for apoptosis have been identified by mutagenesis and will be addressed below.

4. PKC and Apoptosis

PKC plays a fundamental role in the regulation of cell proliferation and differentiation and recent studies suggest that it is also involved in the regulation of cell survival. Early approaches to defining the role of PKC in apoptosis relied upon activation of PKC

by phorbol-12-myristate-13-acetate (PMA), which targets the conventional and novel isoforms, and inhibition by pharmacological agents. These agents are problematic both because of their broad specificity within the PKC family, and in the case of inhibitors, their potential for inhibition of other enzymes. Nonetheless, using these types of approaches, investigators have clearly demonstrated a role for PKC in regulating apoptosis induced by both death receptors (extrinsic pathway), and by DNA damaging agents and cell toxins (intrinsic pathway). Whether alterations in PKC activity enhance or suppress apoptosis appears to depend on the initiating signal as well as the specific cell type. Most studies indicate that activation of PKC with PMA blocks Fas, TRAIL, and TNF-α induced apoptosis *(64–69)*. In some cases this appears to result from disruption of DISC formation *(66,67)*. The protective effect of PMA on Fas-induced apoptosis has also been attributed to activation of the extracellular regulated kinases (ERK) and NF-κB pathways *(70)*. However, in some studies PMA induces apoptosis, or sensitizes cells to death receptor induced apoptosis *(71–73)*. This supports the notion that the functional outcome of PKC activation reflects the specific PKC isoform expression profile of a given cell type.

The particular cellular mix of PKC's maybe even more critical in the context of the intrinsic apoptosis pathway. Activation of PKC with PMA blocks irradiation induced apoptosis in Jurkat cells *(74)* and singlet oxygen induced apoptosis in HL-60 cells *(75)*. However, in some cell types, including salivary epithelial cells *(71)* and prostate cancer cells *(72,76)*, PMA induces apoptosis. Likewise, the PKCδ inhibitor, rottlerin, suppresses genotoxin induced apoptosis in most cells, however in some cell types pretreatment with rottlerin enhances apoptosis *(77,78)*. The complexity and potential redundancy of the PKC signaling network has prompted the development of PKC isoform specific tools including dominant inhibitory kinases, mouse models in which specific PKC isoforms have been disrupted, and PKC isoform-specific antisense/siRNA to define isoform-specific functions of PKC in the apoptotic pathway. From these approaches a pattern is emerging in which the conventional isoforms, particularly PKCα and PKCβ, and the atypical PKC's, PKCι/λ, and PKCζ, appear to be anti-apoptotic/pro-survival. The novel isoform, PKCδ, is primarily pro-apoptotic, whereas PKCε in most studies appears to suppress apoptosis. What is currently known about the contribution of specific isoforms to apoptosis, and how signal transduction by specific PKC isoforms integrates with other molecular regulators to promote or inhibit apoptosis is discussed in the next section.

5. Pro-survival PKC Isoforms

Pro-survival PKC isoforms have been defined chiefly based on the ability of these protein kinases to suppress apoptotic signaling and/or promote cell survival.

5.1. PKCα

Most evidence suggests that PKCα promotes cell survival and that loss of PKCα activity either induces death outright, or sensitizes cells to death signals. A notable exception is LNCaP prostate cancer cells *(79,80)*. In these cells PKCα activation by a synthetic DAG analog that activated PKCα, but not PKCδ, was shown to induce apoptosis, and this could be blocked by expression of a dominant-negative PKCα mutant *(79)*. However, in melanoma cell lines *(81)*, COS1 cells, bladder carcinoma cell lines

(82), glioma cells *(83)* and salivary gland epithelial cells *(84)*, depletion of PKCα activity by expression of dominant negative of PKCα, or by PKCα depletion with antisense or siRNA, induces apoptosis. In the case of salivary epithelial cells and glioma cells, PKCα and PKCδ have been shown to be reciprocal regulators of apoptosis, with PKCα promoting cell survival and PKCδ promoting cell death *(83,84)*. Finally, ceramide induced apoptosis is thought to function at least in part by inhibiting phosphorylation, and thereby activation, of PKCα *(85)*.

An important question remaining is whether apoptosis induced by loss of PKCα is secondary to loss of a proliferative signal, or occurs through a direct effect on the apoptotic machinery. Proteins involved in the execution of apoptosis have been identified as potential targets of PKCα. Overexpression of PKCα increases Bcl-2 phosphorylation at serine 70 and suppresses apoptosis in human pre-B REH cells *(86)*. Interestingly, phosphorylation at serine 70 has been shown to stabilize and increase the anti-apoptotic function of Bcl-2 *(87)*. In a similar vein, depletion of PKCα in COS cells induces apoptosis and this correlates with down regulation of Bcl-2 expression *(82)*. PKCα has also been implicated in transduction of the Akt/PKB survival signal via direct phosphorylation of Akt at serine 473, and by of activation of the serine/threonine protein kinase Raf-1 *(88,89)*. Overexpression of PKCα in 32D myeloid progenitor cells activates endogenous Akt, consistent with its pro-survival function *(90)*.

Overexpression of PKCα is seen in a variety of human tumors, arguing that it may be a pro-proliferative signal as well as an anti-apoptotic signal in these cells *(91,92)*. Overexpression of PKCα increases the proliferative capacity of thymocytes, MCF-7 breast cancer cells and glioma cells *(91,93,94)*. PKCα has been shown to regulate a number of pathways involved in cell proliferation including the MAPK, AP-1 and NF-kB pathways, and promotes cell-cycle progression in some cells. The increased expression of PKCα in tumor cells has prompted the development of therapies directed at reducing its expression or activity *(91,93,94)*. Studies in vitro using a PKCα antisense oligonucleotide to decrease PKCα expression in tumor cell lines showed decreased proliferation and increased expression of p53, suggesting that PKCα depletion therapy may sensitize tumors cells to apoptosis *(95–97)*. Likewise, PKCα antisense oligonucleotides significantly reduced tumor growth in a xenograph model *(97)*. However, clinical trials to assess the efficacy of this strategy for the treatment of human tumors have been disappointing.

5.2. PKCβ

Two forms of PKCβ, which differ in their C-terminus, are generated by alternative splicing, PKCβI and PKCβII. Studies from mice deficient for both isoforms indicate that PKCβ is essential for signaling via the B-cell antigen receptor and that loss of PKCβ results in decreased NF-κB activation and B cell survival *(100)*. Most in vitro data indicates a pro-survival function for PKCβII. In vivo studies show that overexpression of PKC β_{II} protects small cell lung cancer cells against c-myc induced apoptosis *(101)* and Whitman et al. have shown that activation of PKCβII suppresses Ara-C induced apoptosis in HL-60 cells and increases the level of the anti-apoptotic protein, Bcl-2 *(102)*. Nuclear translocation and activation of PKCβII is also associated with v-Abl mediated suppression of apoptosis in IL-3 dependent hematopoietic cells *(103)*. Reported substrates for PKCβII include the pro-survival kinase, Akt, and lamin B1. In antigen stimulated mast cells PKCβII phosphorylates Akt at serine 473, consistent with a pro-survival function *(104)*.

PKCβII phosphorylation on lamin B1 in etoposide treated rat fibroblasts appears to precede caspase-6 cleavage of lamin B1 and dissolution of the nuclear membrane.

In line with a pro-survival/anti-apoptotic function, animal cancer models as well as human tumor studies suggest that PKCβII expression may contribute to tumor promotion or progression. For instance, PKCβII expression is specifically increased in patients with diffuse large B-cell lymphomas and inhibition of PKCβ in cultured cells from these patients induces apoptosis *(106)*. In mice, expression of a PKCβII transgene results in hyperplasia of intestinal epithelial cells and increased sensitivity to chemical carcinogens *(107,108)*. In a mouse xenograph model, growth of hepatocellular carcinomas induced by overexpression of vascular endothelial growth factor (VEGF) could be blocked, and apoptosis induced, by oral administration of an inhibitor of PKCβ *(109)*. Recently, the PKC-β selective inhibitor, Enzastaurin, has been shown to suppress growth and induce apoptosis in xenographs of human colon cancer and glioblastoma *(110)*.

Whereas PKCβII functions primarily to promote cell survival, the role of PKCβI in survival/apoptosis is less clear. Inhibition of PKCβI in W10 B cells increases apoptosis, presumably by suppressing activation of the ERK pathway *(111)*. Likewise, overexpression of PKCβI suppresses the apoptotic response of gastric cancer cells to the COX-2 inhibitor, SC-236, whereas antisense depletion of PKCβI sensitizes these cells to chemotherapeutic drugs *(95,112)*. In contrast, a pro-apoptotic function for PKCβI has been demonstrated using a HL-60 variant, HL-525 cells, which are deficient in PKCβ *(113)*. HL-525 cells are suppressed in death receptor induced apoptosis, but this response can be recovered by transfection of PKCβI *(113,114)*. Likewise, in U-937 myeloid leukemia cells PMA treatment induces apoptosis in a PKCβ dependent manner *(73)*.

5.3. PKCε

PKCε expression/activation is often associated with cell transformation and tumorigenesis and the ability of PKCε to promote tumorigenesis is in many cases related to the suppression of apoptosis *(115–120)*. Early in vitro studies showed that PKCε is required for PMA mediated protection of U937 cells from TNF-α or calphostin C induced apoptosis *(121)*. Caspase cleavage of PKCε occurs in some cells undergoing apoptosis, and Basu et al. have shown that in MCF-7 cells treated with TNF-α, caspase cleavage generates an active, anti-apoptotic form of PKCε *(122,123)*. In glioma cells, expression of PKCε suppresses TRAIL induced apoptosis. This protection can be enhanced by expression of a caspase-resistant form of PKCε, suggesting the caspase cleavage of PKCε contributes to its pro-survival function *(124)*. Likewise, expression of PKCε promotes survival of lung cancer cells and increases their resistance to chemotherapeutic drugs *(120)*. In contrast, PKCε has been shown to be required for UV induced apoptosis through regulation of the JNK and ERK signaling pathways via activation of Ras/Raf *(125)*. This may be a common effector of PKCε because studies from other labs suggest that the oncogenic function of PKCε is through activation of the Ras/Raf pathway *(126–128)*. PKCε has also been shown to enhance survival through activation of the NF-κB pathway *(129)*.

Other studies have suggested that changes in PKCε expression may be associated with tumor progression in humans. The PKCε gene is amplified in 28% of thyroid cancers and a chimeric/truncated version of PKCε has been cloned from human thyroid cancer cells *(116)*. Expression of this chimeric/truncated PKCε protein in PCCL3 cells

made them resistant to apoptosis, suggesting that this may contribute to tumor proliferation or progression *(116)*. In contrast, a later study from the same group showed no mutations in PKCε in a study of 31 thyroid cancers; however some tumors had decreased expression of PKCε *(130)*. Analysis of a panel of melanoma cell lines showed that PMA sensitizes cells to TRAIL induced apoptosis and that sensitization correlates with low expression of PKCε*(131)*.

Overexpression of PKCε is a common feature of human prostate tumors *(132)*. Studies in human prostate carcinoma cells show that overexpression of PKCε is associated with conversion from an androgen dependent to androgen independent state, and that in a CWR22 xenograft model, PKCε is upregulated in recurrent prostate tumors *(117)*. This study further shows that endogenous PKCε is required for resistance to apoptosis in CWR-R1 cells, a cell line selected from the recurrent CWR22 tumors *(117)*. In other studies from the same lab, the resistance of prostate cancer cells to apoptosis was shown to result from the interaction of PKCε with the pro-apoptotic protein, Bax *(133)*. Moreover, the association of PKCε with Bax correlated with the progression of prostate cancer cells to an apoptosis resistant state *(133)*.

5.4. PKCλ and PKCζ

The atypical PKC isoforms, PKCλ/ι, and PKCζ are associated with survival in many cells. These isoforms are downstream effectors of PI-3 kinase, are required for mitogenic activation in oocytes and fibroblasts, and for NF-κB activation in NIH3T3 cells and U937 cells *(134,135)*. Most studies indicate a correlation between the expression or activation of PKCλ/ι and/or PKCζ and sensitivity to apoptosis. PKCζ suppresses Fas-FasL induced apoptosis in Jurkat cells by inhibiting DISC formation *(136)*. Murray and Fields have shown that PKCλ/ι protects human K562 leukemia cells from apoptosis induced by taxol or okadaic acid *(137)*. Protection against these agents appears to be specific for PKCλ/ι, as expression of PKCζ had no effect *(137)*. This same lab has also shown that Bcr-Abl mediated resistance to apoptosis requires PKCι*(138)*. Plo et al. have reported that overexpression of PKCζ protects U937 cells from etoposide and mitoxantrone-induced apoptosis via inhibition of topoisomersase II activity *(139)*. In line with these studies, inhibition of PKCζ sensitizes U937 cells to etoposide and TNF-α induced apoptosis *(140)*. Berra et al. have shown that exposure of cells to apoptotic stimuli such as UV radiation, leads to a dramatic decrease in the activity of the atypical PKC isoforms, PKCζ and/or PKCλ *(141)*. PKCζ is cleaved by caspases and down regulation of cleaved PKCζ has been shown to occur through the ubiquitin-proteasome pathway *(142,143)*.

Studies investigating the molecular mechanisms by which atypical PKC's inhibit apoptosis have revealed two potential pathways. PKCζ activity in apoptotic cells is negatively regulated by it's interaction with the pro-apoptotic protein, Prostate Apoptosis Response-4 (PAR-4). Expression of PAR-4 induces apoptosis by coordinately activating the Fas-FasL death receptor pathway and suppressing the pro-survival NF-κB pathway *(144)*. Data from several laboratories suggests that PAR-4 functions in part by regulating pro-survival signaling mediated by the atypical PKCs. PAR-4 has been shown to interact with, and suppress, the enzymatic activity of PKCζ and PKCλ in apoptotic cells *(145)*. Further studies revealed that the atypical PKC's are critical for activation of the NF-κB pathway, and that sequestration of PKCζ and PKCλ by PAR-4 is one mechanism by which PAR-4 suppresses NF-κB activation *(146–149)*. PKCζ has also been

shown to interact with p38 in apoptotic chondrocytes, resulting in inhibition of PKCζ*(150)*. Recently it has been shown that p38 interacts with the regulatory domain of PKCζ and suppresses PKCζ activity by blocking autophosphorylation of the kinase *(151)*. Interestingly, PKCζ and PKCλ have also been shown to bind to, and inactivate, the pro-survival kinase, Akt, in response to ceramide and growth factors *(152–154)*. This finding suggests that through regulation of pro-survival pathways, the atypical PKC's may act as a switch between cell death and cell proliferation.

6. Pro-apoptotic PKC Isoforms

The novel isoforms, PKCδ and PKCθ, are often grouped together as pro-apoptotic. This is based primarily on the finding that both isoforms are cleaved by caspase-3 to generate a constitutively activated form of the kinase, which, when introduced into cells can induce apoptosis *(155,156)*. However, the contribution of PKCθ to apoptosis has been investigated to only a limited extent, with some studies indicating that it is required for T-cell survival *(157,158)*, whereas others indicate a pro-apoptotic function *(155,156,159,160)*. In contrast, the function of PKCδ in apoptotic cells has been studied extensively and it is clear that in most cellular contexts PKCδ promotes, and in many instances is required for, execution of the apoptotic program.

6.1. PKCδ

PKCδ is a ubiquitously expressed isoform that has been implicated in both regulation of cell proliferation and cell death. In addition, recent studies on PKCδ$^{-/-}$ mice have identified diverse roles for this signaling molecule in control of immunity *(161,162)*, apoptosis *(163)*, and cell migration *(164)*. In most cells overexpression of PKCδ results in inhibition of proliferation, and/or apoptosis, and loss of PKCδ is associated with cell transformation *(165–169)*. However, some studies have attributed an anti-apoptotic or pro-survival function to PKCδ, particularly in transformed or tumor cells *(170–176)*. PKCδ is required for activation of ERK downstream of the epidermal growth factor receptor (EGF) *(177–179)* and for signal transduction downstream of the insulin growth factor-1 (IGF-1) receptor in some tumor cells *(180–182)*. These studies suggest that PKCδ has the potential to both positively and negatively regulate cell proliferation and cell death, and hence may act as a "switch" to direct a cell into an appropriate pathway depending on the cellular milieu *(183)*.

PKCδ is activated by numerous apoptotic stimuli and PKCδ activity is required for apoptosis induced by ultraviolet (UV) irradiation, genotoxins, taxol and brefeldin A *(184,185)*, phorbol ester *(186)*, oxidative stress *(187)*, and death receptors *(188)*. Suppression of PKCδ function through treatment with the chemical inhibitor rottlerin *(185)*, expression of kinase dead PKCδ (PKCδKD) *(184)* or the PKCδ regulatory domain *(186)*, or by introduction of a competitive PKCδ specific RACK RBS peptide *(189)*, all inhibit apoptosis in different cell types.

Our laboratory and other groups have utilized inhibitors of PKCδ to probe where in the apoptotic pathway PKCδ functions. In parotid C5 cells exposed to etoposide, expression of PKCδKD inhibits distal apoptotic events such as DNA fragmentation and the morphological features of apoptosis, as well as proximal apoptotic events including loss of mitochondrial membrane potential *(184)*. Indeed, other reports have shown inhibition of mitochondrial apoptotic features upon suppression of PKCδ function

(186,187). This indicates that PKCδs role in apoptosis is at, or prior to, signaling events at the mitochondria. Consistent with these findings, mice in which the PKCδ gene has been disrupted are defective in mitochondria-dependent apoptosis *(163)*.

6.2. Caspase-Cleavage of PKCδ

Caspase cleavage of PKCδ is emerging as an important mechanism for amplification of the apoptotic pathway. Early studies in U937 cells showed that irradiation activated a 40-kD myelin basic protein kinase that was subsequently identified as a stable, proteolytically cleaved, yet catalytically competent fragment of PKCδ *(190)*. Cleavage of PKCδ was shown to occur in the hinge domain of the protein at a consensus caspase-3 cleavage sequence, thus causing the release of the N-terminal regulatory region of the enzyme and generating a C-terminal, constitutively active kinase fragment (*see* Fig. 3). Production of this PKCδ fragment could be inhibited by overexpression of Bcl-2 or treatment of cells with a pharmacological caspase inhibitor. In an accompanying study, expression of the PKCδ catalytic fragment (PKCδCF) was shown to be sufficient to rapidly induce apoptotic cell death independent of a killing stimulus, thus revealing a role in apoptosis for PKCδ *(191)*.

The current evidence suggests that full length PKCδ, as well as PKCδCF, contribute to apoptosis. It is known that expression of the caspase generated PKCδCF is sufficient to induce cell death, and Sitailo et al have shown that expression of PKCδCF is associated with activation of the pro-apoptotic protein, Bax, and cytochrome *c* release *(192,193)*. Furthermore, a caspase resistant mutant of PKCδ protects keratinocytes from UV-induced apoptosis *(194)*. However, evidence supports a role for full length PKCδ (PKCδFL) in apoptosis as well, because a caspase uncleavable form of PKCδ can induce apoptosis *(192)* and PKCδ is important for cell death in response to the toxins ceramide and phorbol esters which do not induce PKCδ cleavage *(76,195–197)*. Thus PKCδ may function at two or more points in the apoptotic pathway; a likely scenario being that PKCδFL contributes to activation of caspase-3 and generation of PKCδCF which then feeds back to amplify the apoptotic pathway. How PKCδ regulates specific apoptotic events is not clear, although insight can be gathered from the large number of studies that have analyzed PKCδ activation, subcellular localization and phosphorylation targets in apoptotic cells.

6.3. Tyrosine Phosphorylation of PKCδ

Phosphorylation of PKCδ on tyrosine residues is an early response to many stimuli including apoptotic stimuli such as UV, H_2O_2 and etoposide*(53–56)*. Functionally important tyrosine residues in PKCδ have been identified by mutagenesis and include Y64 and Y187 in glioma cells treated with etoposide *(53)*, Y311, Y332 and Y512 in response to H_2O_2 *(56)*, and Y52, Y64 and Y155 in response to Sindbis virus infection *(198)*. Recently, Okhrimenko et al. have identified PKCδ Y155 as being important for the anti-apoptotic effects of PKCδ in glioma cells treated with TRAIL *(176)*. These studies suggest that phosphorylation of PKCδ on specific tyrosine residues may regulate the cell or stimulus specific functions of PKCδ.

Although in many cases the tyrosine kinases upstream of PKCδ have not been defined, studies point to nonreceptor tyrosine kinases, specifically c-Abl and the Src-like kinase, Lyn, as being important in response to apoptotic agents *(54,56,58–60,199,200)*.

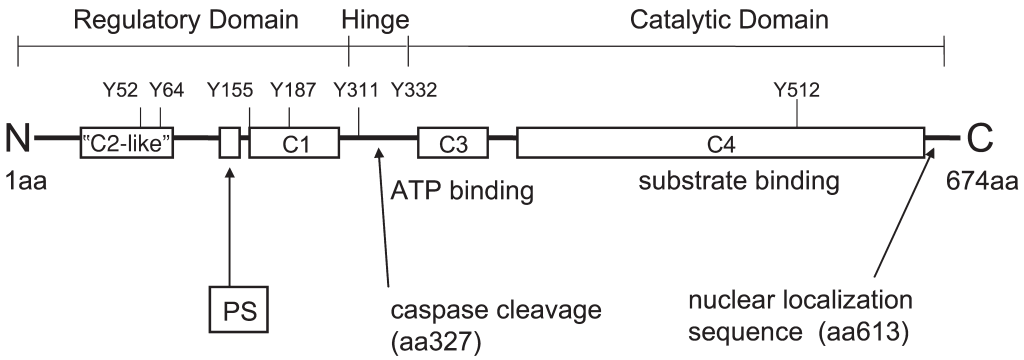

Fig. 3. PKCδ and apoptosis. Sites in the mouse PKCδ protein important for regulation and activation in apoptotic cells are shown. These include Y52, Y64, Y155, Y187, Y311, Y332, and Y512; the caspase cleavage site at aa327; and the C-terminal nuclear localization sequence. *See* text for more details.

Apoptotic agents activate c-Abl and Lyn and induce their association with PKCδ *(55,201,202)*. Yuan et al. report that in MCF-7 cells c-Abl constitutively associates with PKCδ, and that PKCδ becomes phosphorylated on tyrosine in cells induced to undergo apoptosis with ionizing radiation *(55)*. Studies from this same lab show that in H_2O_2 treated cells, c-Abl phosphorylates PKCδ and PKCδ can also phosphorylate and activate c-Abl *(201)*. Yoshida et al. have reported that expression of dominant negative Lyn can suppress tyrosine phosphorylation of PKCδ in Ara-C treated cells *(202)*. Whereas Lyn and c-Abl are found in both the nucleus and cytosol, it is the nuclear form of these kinases that is activated by DNA damaging agents *(203,204)*. Tyrosine phosphorylation may link PKCδ to downstream events in the apoptotic pathway by regulating its catalytic activity, subcellular localization, caspase cleavage or interaction with other proteins such as substrates.

6.4. Subcellular Localization of PKCδ

PKCδ localizes to a variety of subcellular compartments in apoptotic cells in a cell and stimulus dependent fashion. Translocation of PKCδ to the mitochondria has been demonstrated in response to PMA, whereas oxidative stress induces mitochondrial accumulation of PKCδ in U937 cells *(186,187,196,197)*. In these studies translocation of PKCδ to the mitochondria results in cytochrome *c* release and loss of mitochondrial membrane potential. Mitochondrial association of PKCδ in prostate cancer cells amplifies ceramide formation and promotes apoptosis *(205)*. However, treatment of HeLa cells with ceramide induces PKCδ localization to the Golgi compartment, and translocation to the Golgi was shown to be indispensable for apoptosis *(195)*. In contrast, treatment of glioma cells with TRAIL results in the accumulation of PKCδ in the endoplasmic reticulum and suppression of apoptosis *(176)*.

PKCδ translocates from the cytosol to the nucleus in response to etoposide, γ-irradiation, Fas ligand and IL-2 deprivation *(53,55,192,206)*. We have defined a nuclear localization sequence (NLS) in the carboxy-terminus of PKCδ that is required for nuclear import of both full-length PKCδ and the PKCδCF and have shown that nuclear localization of PKCδCF is required for its ability to induce apoptosis *(192)*. Strikingly, parotid C5 cells

transfected with GFP-tagged PKCδ catalytic fragments exhibit rapid nuclear accumulation and induction of apoptosis, independent of an apoptotic stimulus. This suggests that caspase cleavage facilitates nuclear translocation of PKCδ in parotid C5 cells, while in glioma cells, nuclear localization may precede caspase cleavage *(53,192)*. Taken together, these studies suggest a nuclear function for PKCδ in some apoptotic cells. This hypothesis is supported by the observation that the many of PKCδ substrates in apoptotic cells are nuclear proteins.

6.5. Targets of PKCδ in Apoptotic Cells

Substrates of PKCδ in apoptotic cells include transcription factors, protein kinases, structural proteins, DNA repair and checkpoint molecules, membrane lipid modification enzymes, and Bcl-2 family members. Intriguingly, these substrates are localized to mitochondria, plasma membrane, and nuclear compartments, suggesting that PKCδ can regulate apoptosis from various organelles within the cell. In UV exposed cells, PKCδ can phosphorylate and activate phospholipid scramblase 3 (PLS3) at the mitochondria *(207)*. In Fas treated cells, PKCδ phosphorylates and activates another member of this family, PSL1, at the plasma membrane *(208)*. In apoptotic monocytes PKCδ associates with, phosphorylates, and increases the activity of caspase-3 *(209)*.

The majority of PKCδ's substrates in apoptotic cells are nuclear proteins. For instance, lamin B, a nuclear structural protein, is phosphorylated by PKCδ in Ara-c exposed cells and contributes to this protein's degradation and the subsequent destruction of the nuclear infrastructure *(210)*. Also, within the nucleus, PKCδ interacts with and phosphorylates DNA-PK in cells exposed to genotoxins *(211,212)*. Phosphorylation of DNA-PK inhibits DNA binding, suggesting that PKCδ mediated phosphorylation inactivates the DNA double strand break repair function of this protein *(212)*. PKCδ also phosphorylates the multifunctional molecule hRad9, which regulates DNA repair and can act as a BH3 only death molecule by binding Bcl-2 *(211)*. PKCδ may also regulate the transcription of death genes through activation of the transcription factors p53, p73β, and STAT1. One study found that downregulation of PKCδ inhibits the basal transcription of p53 whereas other studies report PKCδ dependent accumulation of the p53 protein in apoptotic cells *(213–215)*. p73β, a transcription factor that mediates genotoxin induced cell killing, is phosphorylated by PKCδCF, inducing p73β dependent reporter transcription *(216)*. Our laboratory has shown that PKCδ and STAT1 interact in etoposide treated cells and that STAT1 is required for PKCδCF mediated DNA fragmentation and apoptosis *(217)*.

Although possibly not direct targets, activated PKCδ has been shown to interface with downstream signaling cascades to regulate the apoptotic machinery. Indeed, in apoptotic cells, the PI3-kinase/AKT pathway, the ERK, JNK and p38 pathways and the JAK-STAT pathway all appear to be regulated at least in part by PKCδ. PKCδ activates the JNK pathway in irradiation and Ara-c induced apoptosis, possibly through phosphorylation and activation of MEKK1 *(202)*. PKCδ also enhances radiation-induced apoptosis via ERK1/2 activation and suppression of radiation-induced G2-M arrest, and *(218)*. In keratinocytes p38δ is a downstream effector of PKCδ *(219)*. In cardiomyocytes exposed to ischemia, Akt is dephosphorylated and inactivated in a PKCδ dependent manner and this is accompanied by dephosphorylation and loss of Bad sequestration, allowing this potent apoptotic protein to induce cell death *(220)*. Finally, the binding of HSP25

to PKCδ has been shown to inhibit cell death, suggesting that the pro-apoptotic function of PKCδ may be negatively regulated through specific protein-protein interactions *(221)*.

7. Conclusions

The identification of both pro- and anti-apoptotic isoforms suggests that PKC may function as a molecular sensor, promoting cell survival under favorable conditions and executing the death of abnormal or damaged cells when needed. Although it is convenient for the purpose of discussion to classify PKC isoforms as pro- or anti-apoptotic, most PKC isoforms classified as primarily pro-survival/anti-apoptotic can also function to promote apoptosis and vis a versa. This observation underscores the importance of cellular context and begs the question of how the function of these isoforms is specified. Although this is not well understood, it is clear that specification of function is likely to involve post-translational modifications as well as function-specific changes in subcellular localization. Post-translational modifications, such as phosphorylation, may allow for protein–protein interactions with other molecular regulators of apoptosis, and/or target the active kinase to a specific subcellular location where these interactions may occur. Understanding the molecular basis for regulation of apoptosis by PKC isoforms may contribute to the development of therapeutic strategies to treat diseases such as cancer and neurodegenerative disorders.

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http://www.springer.com/978-1-58829-882-9

Apoptosis, Cell Signaling, and Human Diseases
Molecular Mechanisms, Volume 2
Srivastava, R. (Ed.)
2007, XVI, 402 p. 49 illus., 1 illus. in color., Hardcover
ISBN: 978-1-58829-882-9
A product of Humana Press