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Protein Kinase C δ Is Critical for Nucleotide Excision Repair of Cyclobutane Pyrimidine Dimers

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LOYOLA UNIVERSITY CHICAGO

PROTEIN KINASE C δ IS CRITICAL FOR NUCLEOTIDE EXCISION REPAIR OF CYCLOBUTANE PYRIDIMINE DIMERS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN CELL BIOLOGY, NEUROBIOLOGY, & ANATOMY

BY
CHRISTOPHER M. NEGRO
CHICAGO, ILLINOIS
AUGUST 2011
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<td>ATR</td>
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<td>BCC</td>
<td>Basal Cell Carcinoma</td>
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<td>BER</td>
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<td>COX2</td>
<td>Cyclooxygenase 2</td>
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<td>CPD</td>
<td>Cyclobutane Pyrimidine Dimer</td>
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<td>CS</td>
<td>Cockayne Syndrome</td>
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<td>DAG</td>
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<td>Murine Embryonic Fibroblast</td>
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<td>NER</td>
<td>Nucleotide Excision Repair</td>
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<td>TGFβ</td>
<td>Transforming Growth Factor β</td>
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<td>TTD</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<td>XP</td>
<td>Xeroderma Pigmentosum</td>
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<td>6-4PP</td>
<td>6-4 Photoproduct</td>
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ABSTRACT

Nucleotide excision repair (NER) is the process by which cells identify and repair bulky, helix-distorting DNA lesions such as ultraviolet (UV) radiation-induced cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP). NER in human cells is a complex biochemical process requiring several complementation groups. Defects in complementation group genes result in rare, autosomal recessive cancer syndromes such as xeroderma pigmentosum (XP), cockayne syndrome (CS), and trichothiodystrophy, supporting the tumor suppressive effects of NER. Protein Kinase C delta (PKCδ) expression is lost in squamous cell carcinomas where it functions as a tumor suppressor gene. Additionally, in response to UV radiation, PKCδ is known to function in the maintenance of the G2/M cell cycle checkpoint and apoptosis. Because cell cycle checkpoints are coupled to DNA damage repair pathways, this thesis investigates the role PKCδ plays in DNA damage repair of UV-induced adducts.

To examine the role of PKCδ in the repair of DNA damage CPD’s, wild type mouse embryonic fibroblasts (MEFs) were exposed to a non-apoptotic UV dose (5 mJ/cm²), allowed time for repair, and then fluorescently stained for CPDs. Through quantitative immunofluorescence microscopy, we found wild type MEFs were competent at repairing CPD’s whereas PKCδ null MEFs had significantly (p<0.001) reduced capacity for repair of UV-induced CPD adducts.
These results were also supported using southwestern blotting for CPDs in DNA isolated from wild type and PKCδ null cells exposed to high dose UV radiation.

We also found that re-expressing PKCδ in PKCδ-/- cells restores DNA damage repair of CPDs. Cells infected with PKCδ expressing vector obtained levels of CPD repair consistent with WT cells.

Immunofluorescence staining was also used to show decreased levels of nuclear xeroderma pigmentosum complementation groups A and C (XPA; XPC) protein in PKCδ-/- MEFs. This data was also supported by western blot data which showed low endogenous levels and delayed induction of XPA and XPC in PKCδ-/- cells after UV irradiation. Thus, our results indicate a role for PKCδ in DNA damage repair of CPDs through NER signaling.
CHAPTER ONE
INTRODUCTION

The Human Skin

Anatomy of the Skin

The human skin comprises the largest multifunctional organ of the body and accounts for roughly 15% of total body weight in adults. The epidermal and dermal layers constitute the two primary layers of the skin with a subcutaneous layer of loose connective and adipose tissue below the inner dermal layer\textsuperscript{1,2}. The major action of the skin is to provide protective, thermoregulatory, metabolic, and immunologic functions.

The fine structure of the skin shows considerable variations in thickness, vascularization, innervation, and cellular distribution. The epidermis is the outermost layer of the skin and continuously self-renews. The major cell type found in the epidermal layer is the keratinocyte which undergoes a differentiation process resulting in production of flattened, anucleate cells. Additionally, the epidermis also contains non-keratinocytes including immune cells called Langerhans cells, melanin (pigment) producing melanocytes, and pressure sensitive Merkel cells\textsuperscript{3}. The epidermis can be further broken down into several layers, the innermost basal layer, the stratum spinosum, the granular layer, and the most superficial cornified layer. Keratinocytes originate from mitotic divisions
of undifferentiated stem cells in the basal layer\textsuperscript{4,5}. These cell migrate upward toward the skin surface undergoing keratinization before shedding\textsuperscript{1,6}.

The dermis is a compressible, supportive connective tissue located below the epidermal layer. This thick, relatively acellular layer is comprised of several cells (fibroblasts and mast cells), blood vessels, nerve endings, hair follicles, and sebaceous glands\textsuperscript{7}. Separating the dermis and epidermal layer is a complex basement membrane synthesized by keratinocytes and dermal fibroblasts. This membrane plays a fundamental role in mechanical support and keratinocyte migration during wound healing. Additionally, it is also traversed by lymphocytes during immunologic and inflammatory processes\textsuperscript{8}. The basement membrane is composed mainly of laminins and type IV and VII collagens\textsuperscript{9}. Advanced, neoplastic cancers grow through this barrier by releasing matrix-metalloproteases which allow transformed epidermal cells to invade the dermis and enter blood vessels and metastasize to other areas of the body\textsuperscript{10}.

**Skin Cancer**

Cancers of the skin (including melanoma and non-melanoma skin cancer) are the most common form of cancer with greater than one million cases diagnosed each year\textsuperscript{11}. The primary factor contributing to the induction of skin cancer is unprotected skin exposure to ultraviolet (UV) radiation from either environmental (sun) or industrial, man-made (tanning beds) sources\textsuperscript{12-14}.

As previously noted, skin cancers are classified into melanoma and non-melanoma types. Non-melanoma skin cancers are further subdivided into
squamous (SCC) and basal cell carcinoma (BCC) subtypes. BCCs are the most common subtype of skin cancer. These cancers originate from transformation of epidermal keratinocytes and rarely metastasize\textsuperscript{15}. The second most common subtype, SCCs, are derived from keratinocytes that express squamous differentiation markers and also rarely metastasize. However SCCs are more metastatic than BCCs. The non-melanoma skin cancers occur most frequently on UV exposed areas of the body including the face, back, shoulders, and scalp\textsuperscript{16}. Despite the large incidence of non-melanoma skin cancers, in most cases they can be cured with minor surgery or local treatments such as topical chemotherapies, cryosurgery, and photodynamic therapy.

Melanoma is the least common type of skin cancer accounting for only ~5%, but is the most metastatic and causes the majority of skin cancer deaths. Melanoma begins in the melanin producing cells of the skin, the melanocytes. Development of melanoma can occur almost anywhere on the body but occurs most frequently on sun exposed regions such as the chest and back of males and on the legs of females\textsuperscript{17}. Therefore, UV is regarded as a major contributor to the development of both melanoma and non-melanomas skin cancers alike.

**Ultraviolet Radiation**

Exposure to UV light can, at times, lead to DNA lesions that, if not repaired correctly, threaten both cell and organism viability. In skin cancers, the main etiological factor responsible for DNA lesions is environmental UV light. The most damaging wavelengths of UV radiation, UVC, are absorbed by the
ozone layer and are therefore physiologically irrelevant. Therefore, cells need only to contend with UVA and UVB rays (315-400nm and 280-315nm respectively)\textsuperscript{14}.

**UVA Radiation**

Roughly 95% of the UV radiation reaching the Earth’s surface is comprised of the wavelengths corresponding to UVA rays. UVA rays are linked to long term damage to skin such as wrinkles, photoaging, and skin cancer\textsuperscript{18,19}. This is largely attributed to chronic exposure of UVA rays that penetrate deeply into the dermis. Chronic exposure to UVA has been shown to generate reactive oxygen species that promote carcinogenesis by damaging DNA\textsuperscript{20-22}. However, UVA does not exist in nature without UVB and, environmentally, UVA has been shown to be inhibitory to UVB-induced apoptosis\textsuperscript{23}. With regard to photocarcinogenesis, apoptosis is a protective mechanism because of its function as an antimutagenic and anticarcinogenic cellular response. Therefore, the presence of UVA along with DNA damaging UVB will increase mutation burden and, similarly, skin cancer induction.

**UVB Radiation**

Despite UVA’s ability to augment UVB-mediated mutation and skin cancer formation, UVB is still the more directly carcinogenic of the two rays. UVB primarily causes damage to the cells of the epidermis with prolonged exposure often resulting in sunburn and reddening. The damage induced by UVB is universal affecting various biological macromolecules such as lipids, proteins,
and nucleic acids. However, the UV-induced mutations that give rise to skin cancers are derived from the modifications to DNA. Of particular concern for genome stability are bulky, helix-distorting dimeric pyrimidine photoproducts including cis-syn cyclobutane pyrimidine dimers (CPDs), 6-4 photoproducts (6-4PPs), and Dewar’s valence isomers. Sites of adjacent pyrimidine bases within a DNA strand are often the preferred sites of UV-induced photolesion formation. While most damage will be CPDs, UV radiation can induce a wide range of damage including protein-DNA crosslinks, oxidative base damage (e.g. 8-oxo-7,8-dihydroxyguanine), and single strand breaks. However, of these DNA adducts, CPDs are more difficult for cells to repair and are therefore the most carcinogenic lesion.

**UV-Induced Photolesions**

CPDs constitute the predominate form of UV-induced photodamage. They are formed from a [2+2] cycloaddition between the C5-C6 double bonds of adjacent pyrimidine nucleotides. The majority of CPDs occur between adjacent thymines but can theoretically be derived from any combination of pyrimidines as a direct result of the wavelengths, dose of irradiation, and adjacent nucleotide sequences.

6-4PPs are the second most prevalent form of induced photolesion. Like CPDs, 6-4PPs preferentially form between TT, CT, and CC nucleotides with an induction yield depending on wavelengths and adjacent nucleotide sequences. Upon further irradiation with UVB, 6-4PPs can further rearrange
to form Dewar’s valence isomers however the biological significance of this conformation change is unknown\textsuperscript{33}.

Despite being formed in a similar fashion, structural and biological differences exist between CPDs and 6-4PPs. Structurally, CPDs result in little strain in Watson-Crick base pairing whereas 6-4PPs result in a pronounced alteration that results in a loss of pairing. The amount of induced DNA burden accounts for differences in rate and efficiency of cellular processing of the photolesions. Because 6-4PPs cause a greater amount of strain DNA repair proteins can identify and excise these lesions more rapidly and with greater accuracy than CPDs\textsuperscript{34}.

CPDs are produced both by UVB and UVA radiation\textsuperscript{35}. Because UVA is not directly absorbed by DNA, the formation of CPDs occurs through a mechanism differing from the direct excitation pathway observed in UVB induced CPDs. This theory was derived from studies performed in bacteria in which bacteria exposed to UVA preferentially induced CPDs at adjacent thymine sites without any detectable formation of 6-4PPs\textsuperscript{36-38}. Additionally, the rate of removal of CPDs generated by UVA rays is lower than those produced by UVB irradiation in skin\textsuperscript{35}. However, whether these bulky photolesions are derived from UVA or UVB, CPDs and 6-4PPs are repaired through the nucleotide excision repair mechanism.

**DNA Damage Response**
Proper regulation of cellular propagation is dependent on the cell’s ability to maintain stable genetic material over many cell generations. However, various endogenous and exogenous agents cause DNA damage that constantly challenges genomic integrity. In order to combat both irradiation and chemically induced aberrations, the cell employs molecular mechanisms that repair damaged DNA and thus prevent tumorogenesis. These mechanisms include base-excision repair (BER), which excises damaged or inappropriately matched bases as single nucleotides, and nucleotide excision repair (NER), which is primarily concerned with the removal of bulky, helix distorting adducts such as CPDs and 6-4PPs\textsuperscript{39,40}.

**Nucleotide Excision Repair**

NER recognizes and repairs helix-distorting base lesions. This includes UV photolesions such as CPD, 6-4PPs, as well as polycyclic aromatic hydrocarbon adducts. This highly sophisticated process can be separated into two sub-pathways, discernable by the location and recognition of the lesion. Global-Genomic NER (GG-NER) repairs lesions over the entire non-transcribed regions of the genome, while repair of lesions that block transcription on template DNA strands is considered Transcription-Coupled NER (TC-NER). NER in human cells is a complex biochemical process requiring several proteins. The ability of NER machinery to discriminate damaged from undamaged DNA is contingent on several subunits of the NER multi-protein machine.
At sites of base damage, a multi-protein complex assembles in a step-wise fashion to excise the damaged oligonucleotide sequence. Studies of this complex have identified XPC, XPA, and RPA as the critical proteins for recognition of sites of damage. However, several additional proteins have proved vital for recruitment of other NER proteins and excision of damaged DNA regions\textsuperscript{33,41,42}. 
Figure 1: Essential Features of Nucleotide Excision Repair
Nucleotide excision repair (NER) operates on base damage caused by exogenous agents that cause alterations in Watson-Crick base pairing leading to bulky lesions in the DNA duplex. A. In Global Genomic NER (GG-NER) the lesion is recognized by heterodimer complex XPC-hHR23B. Initial recognition leads to recruitment of a pre-incision complex including TFIH, XPA, XPD, XPB, XPG, and RPA. This complex binds the lesion, unwinds the DNA, and recruits endonuclease XPF-ERCC1. After assembly, the complex cleaves and excises the damaged region of DNA. The excised region is replaced by a post-excision proteins Pol δ/ε, PCNA, RPA, RFC, and DNA ligase. B. Transcription coupled NER (TC-NER) differs in lesion identification. Instead of XPC-hHR23B, the lesion is identified by Pol III, CSA, and CSB. Despite this difference, subsequent proteins and processes are identical to GG-NER.
XPC, a 125-kD protein, is exclusive to GG-NER and is essential for initial recognition and binding of the site of DNA damage\textsuperscript{42}. Two observations characterized XPC as the first protein to bind DNA damage in the process of NER. First, cells deficient in XPC are only deficient in the process of GG-NER and not TC-NER which indicates an early role in NER signaling. Additionally, irradiating cells through a porous polycarbonate filter caused localized UV-induced damage which allowed colocalization studies of NER proteins with CPD damage\textsuperscript{43-45}. These studies observed that XPC colocalized with sites of damage in XPA deficient cells, while XPA could not be recruited in XPC deficient cells\textsuperscript{44}. Therefore these findings also established the presence of XPC as a necessity for the recruitment of XPA to the site of damage\textsuperscript{44}. In vitro studies of purified XPC have shown that it alone is sufficient to bind damaged DNA regions. However, XPC typically exists as a heterodimer that is tightly complexed to hHR23B. Binding to hHR23B improves XPC stability and efficiency of binding to DNA damage\textsuperscript{46}. A second protein, hHR23A, with similar homology to hHR23B, can also bind with XPC, but does not increase XPC efficiency for damage identification as well as hHR23B\textsuperscript{39}.

Transcription factor IIH (TFIIH) is a ten subunit subcomplex, essential for GG-NER and TC-NER, and is typically associated with initiation of RNA polymerase II transcription. However this subcomplex is also recruited to DNA adduct sites during UV-induced DNA damage\textsuperscript{47,48}. Central to the overall function of this subcomplex are proteins XPB and XPD which exhibit DNA-dependent
ATPase and helicase functions\textsuperscript{49}. The unwinding associated with TFIIH generates junctions between double-stranded DNA that are fundamental for correct excision of damaged DNA\textsuperscript{39,50,51}. This critical process occurs in two, well defined, steps. Firstly, TFIIH is recruited through protein-protein interaction with XPC-hHR23B to the site of damage. This step is ATP-independent. In an second ATP-dependent step, XPB and XPD unwinds DNA providing space for subsequent NER proteins\textsuperscript{52,53}.

XPA, a metalloprotein containing a DNA binding Zn\textsuperscript{2+} finger, is another NER protein essential for recognition of DNA damage. XPA is crucial for the early stages of both GG-NER and TC-NER. It’s ability to recognize damaged regions is contingent on two fundamental occurrences: disruption of normal Watson-Crick base pairing and altered structural chemistry of bases of the damaged strand\textsuperscript{54,55}. XPA is recruited to the site of damage through a physical interaction with checkpoint ataxia-telangiectasia mutated and RAD3-related (ATR) kinase which translocates the protein from the cytosol into the nucleus\textsuperscript{56}. Additionally, XPA maintains crucial contacts with other core repair factors including RPA. It is suggested that the specificity of RPA is contingent on interactions with XPA that provide great specificity in regards to strand position\textsuperscript{57}.

RPA is a single-stranded DNA binding protein composed of three subunits of 70, 32, and 14 kD each with DNA binding domains\textsuperscript{58,59}. RPA displays two inherent binding patterns, one spanning 8-10 nucleotides and another roughly \~30. The latter of which occludes the undamaged single-strand DNA formed by the TFIIH subcomplex\textsuperscript{60-62}. 
Two additional NER proteins recruited to the site of damage are the endonucleases XPG and XPF-ERCC1. XPG is an endonuclease specific for 3' end incision\textsuperscript{63}. Beyond its role as an endonuclease, XPG has also been shown to stabilize the preincision protein complex through binding to RPA. XPG is also required for the 5’ incision by XPF-ERCC1 heterodimer by stabilizing the double strand bubble created by the helicases of TFIIH\textsuperscript{64,65}. XPF-ERCC1 are the last proteins to join the complex before excision of the damaged region. This heterodimer is recruited to the site of damage by XPA and is the major protein responsible for the transition between the incision making protein complex and the repair synthesis\textsuperscript{66-69}.

The repair and ligation steps in NER take place in the same fashion as that of replication. This process requires polymerase processivity factor PCNA, PCNA-dependent DNA polymerase δ or ε, RPA, pentameric clamp loader RFC, and DNA ligase I\textsuperscript{58,70-73}. This mechanism is well defined and serves to reestablish intact double-stranded DNA.

**Gene Regulation of NER Proteins**

The effectiveness of NER is contingent on the proteins that comprise the process. Therefore, repair of damage is dependent on regulation of the genes encoding the NER proteins. The mechanism that governs NER protein regulation is not completely understood, however p53 and low-dose UVB have been shown to act in this photoprotective response. NER genes are induced in keratinocytes exposed to low levels of UVB, whereas exposure at high doses of
UVB downregulates NER genes and is associated with preparation for cell death\textsuperscript{74}. These results were found to be true of nearly all NER genes. This observation has led to the belief that there exists a common regulatory pathway for all genes in the NER pathway.

**NER Deficiency Syndromes**

The phenotypic consequences of a defect in one of the NER proteins are illustrated by the occurrence of rare autosomal recessive disorders: xeroderma pigmentosum (XP), cockayne syndrome (CS), and trichothiodystrophy (TTD). Several complementation groups have been identified that contribute to the NER-defective class of XP patients (XPA through XPG), two in CS patients (CSA and CSB), and three associated with TTD (XPB, XPD, and TTDA)\textsuperscript{75}. Cells from these individuals share an impaired capacity to repair UV induced DNA photolesions located either in the transcribed regions of DNA or within inactive regions of the genome.

XP patients carry cutaneous hallmarks including red, blotchy parchment skin and freckles and are characterized by a severe predisposition for the development of skin cancers on UV exposed regions of the body such as the face, head, neck, and arms. Due to the defective nature of their NER machinery, UV radiation from sun exposure generally leads to degenerative alterations of the skin and eyes. XP patients develop melanoma at normal rates but frequently develop squamous cell and basal cell carcinomas\textsuperscript{39}. Due to their molecular phenotype, the UV exposed cells experience increased mutational burden and
readily undergo neoplastic transformation. Severity of repair deficiency and symptoms experienced vary depending on which of the several XP proteins are truncated by mutation or deleted. XPA, XPB, XPD, and XPG patients suffer a severe deficiency in NER repair of genomic damage. However, XPD and XPG maintain low residual activity that can be >50% repair synthesis levels in some cases. Additionally, patients with an absence of GG-NER specific protein, XPC, experience wild-type sensitivity to sunburn because transcription-coupled photolesions are removed normally. Typically, XPC deficient cells have residual repair levels of roughly 15-30% and are less sensitive to UV than patients deficient in XPA.

CS is caused by a mutational inactivation of genes encoding TC-NER proteins CSA and CSB. This inactivation results in increased sensitivity to UV and other DNA damaging agents. CS is a very pleotropic disease causing not only photosensitivity, but also physical and mental retardation. However, despite increased photosensitivity these patients shockingly do not exhibit a predisposition for developing skin cancer. Physical anomalies including skeletal deformation (curving of the spine), microcephaly, and osteoporosis have been observed. Furthermore, neurological irregularities such as progressive neurological degeneration, myelination abnormalities, retinopathy, and sensorineural hearing loss are frequently observed.

The development of CS has unveiled many difficulties with regard to explaining the observed phenotypes through NER insufficiency alone. It has been shown that CSB knockout mice experience reduced symptoms of CS while
CSB/XPA double mutant mice fail to grow and die before weaning \(^{81}\). Additionally, CS cells have increased difficulty repairing ionizing radiation inducing oxidative damage to the transcribed DNA \(^{80,82}\). XPG deficient patients experience some symptoms similar to CS patients, yet impaired transcription-coupled repair of oxidative damage is unique to cells from CS patients \(^{83}\). Nevertheless, the additional neurological and developmental symptoms observed in CS suggest that transcription coupled repair proteins CSA and CSB may have functions beyond those observed in NER.

Individuals with TTD suffer from several mental and physical conditions including mental retardation, failure to thrive, ichthyotic skin, and brittle hair and nails \(^{39,84}\). Several syndromes fall into the category of TTD including Pollitt syndrome, Tay’s syndrome, Amish brittle hair syndrome, and Sabinas syndrome \(^{75}\). Most cases of TTD present with photosensitivity, but this is not true of all reported TTD patients. NER deficiency in TTD afflicted individuals was described through discovery of a mutation in XPD that revealed severe physical symptoms atypical of XP alone \(^{85}\).

DNA polymerases synthesize DNA. However, they also play a role in DNA damage tolerance and carcinogenesis. Tolerance occurs when specialized polymerases, such as DNA polymerase \(\zeta\) and \(\eta\), place a base-pair opposite DNA damage adducts and bypass the damage. This process is known as translesion DNA synthesis (TLS) and is responsible for many point mutations in the cell \(^{86}\). However, in the absence of these error-generating DNA polymerases, no translesion bypass will occur which results in replication fork collapse, leading to
double strand breaks and genome instability, which increases the incidence of cancer.\textsuperscript{87,88}

**Structure and Regulation of Protein Kinase C**

Protein kinase C (PKC) comprises a family of 9 serine/threonine kinases with distinct and sometimes opposing roles in transducing signals associated with cell proliferation, differentiation, apoptosis, and angiogenesis.\textsuperscript{89,90}

The structure of the PKC molecule consists of two major domains, a regulatory and catalytic at the amino and carboxy terminal ends respectively. Both domains consist of highly conserved regions (C1-C4) and regions of great variability (V0-V5) (Figure 2). The C1 and C2 regions are important in PKC activation and are found in the regulatory domain.\textsuperscript{91} The C3 and C4 are located in the catalytic fragment and are the ATP-binding and kinase regions respectively.\textsuperscript{92}

PKCs exist in an inactive conformation due to binding of the regulatory domain to the catalytic domain through an inhibitory pseudosubstrate sequence.\textsuperscript{93} The PKC family is divided into three groups differing in their cofactor requirements for activation. These groups include the conventional PKC isoforms (α, β, and γ) that are activated by Ca\textsuperscript{2+} and diacylglycerol (DAG); novel PKC isozymes (δ, ε, η, and θ) that require only DAG for activation; and atypical (ζ, and λ/ι) that require neither Ca\textsuperscript{2+} nor DAG but can be stimulated by agents such as phosphatidylserine.\textsuperscript{94} DAG is produced by the cleavage of phosphoinositol bisphosphate cleavage by phospholipase C.\textsuperscript{95} Cofactor binding causes release of inhibition by pseudosubstrate and an increase in PKCs affinity.
for cell membranes. Additionally, PKCs are activated by proteolytic cleavage in the hinge region, which, in turn, releases the active catalytic fragment from the pseudosubstrate domain.

Figure 2: Protein Kinase C Isozyme Structure and Classification
The general structure of PKC has 4 conserved domains (C1-C4): C1 contains motifs that form the DAG and phorbol ester binding sites. C2 contains calcium and acidic lipid recognition sites. C3 and C4 form the ATP and substrate binding lobes of the catalytic site. Deviations from general structure include the C2 domain of novel PKCS which lacks amino acids to bind calcium and atypical PKCs which lack structure to bind DAG and calcium.

Along with the variations in structure and activation, PKC isozymes also differ in biological function, tissue distribution, subcellular localization, and substrate specificity. Generally, PKC isozymes reside in the cytosol of the cell and, upon activation, translocate to the cell membrane or a cellular organelle.
Cell specific isozyme functions are relative to their subcellular localization. Furthermore, compartmentalization contributes to discrepancies in activation\textsuperscript{99,100}. These discrepancies in activation, subcellular localization, and compartmentalization raise the possibility of developing therapeutics that target specific PKC isozymes in tumor cells.

**PKCs and Cancer**

The function of PKCs in cancer is complex. Increased and decreased PKC levels have been associated with malignant transformation in cell lines associated with breast, lung, skin, gastric carcinomas, and many other cancers\textsuperscript{101,102}. However, deviations from normal levels that give rise to cancers are dependent on the primary functions of specific PKC isozymes. For instance, PKCβ has been implicated in cellular proliferation and differentiation. In the early stages of colon cancer, the expression of the PKCβ\textsubscript{II} splice variant increases significantly via the cyclooxygenase 2 (COX2) and transforming growth factor β (TGFβ) pathways\textsuperscript{103,104}. Likewise, PKC activation and inhibition has been correlated with induction of apoptosis. Several PKCs including α and η are phosphorylated by the PI3K pathway and, upon activation, phosphorolate AKT\textsuperscript{105-107}. Furthermore, PKCα phosphorylates anti-apoptotic protein BCL2 signaling cell survival\textsuperscript{108}. However, the PKCδ isozyme, is a the major isozyme responsible for pro-apoptotic tumor suppression\textsuperscript{109,110}.

**Protein Kinase C δ Structure and Function**
Protein kinase C Delta (PKCδ) is a calcium independent (novel) PKC isozyme activated by DAG and phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA)\textsuperscript{111,112}. In humans, PKCδ is ubiquitously expressed and has several known functions in cell cycle regulation and apoptosis. The structure of PKCδ is analogous to all novel PKCs with the exception of the C4 region of the catalytic domain. In PKCδ, the C4 domain contains a nuclear localization sequence that is essential for PKCδ's role in apoptosis\textsuperscript{113,114,115}. Typically, PKCδ is regulated by tyrosine phosphorylation within the hinge region or regulatory domain at Tyr 52, 187, 311, 512 and 523 residues\textsuperscript{116-118}. Tyrosine phosphorylation is usually associated with inhibition of PKCδ, but has been shown in some cases (Tyr 311) to promote functional activation when cells are stimulated with substances such as phorbol esters, hormones, and growth factors\textsuperscript{119-122}. In response to several pro-apoptotic stimuli such as ionizing radiation, reactive oxygen species (ROS), ultraviolet radiation, growth factors, and cytokines, PKCδ is activated through proteolytic cleavage by caspase-3 to a 40-kDa catalytic fragment (Figure 3). Once activated PKCδ translocates from the cytoplasm to the nucleus or to the mitochondria\textsuperscript{93}. Activated PKCδ phosphorylates serine/threonine residues of specific substrates impeding cell cycle progression and promoting apoptosis, while its downregulation or inhibition is involved in tumorigenesis. Thus, PKCδ functions as a tumor suppressor.
**Protein Kinase C δ Signaling**

Upon activation, PKCδ phosphorylates serine/threonine residues in specific substrates. Synthetic peptides have shown a general phosphorylation motif $RXX(S/T)XRX$, where $X$ can be any amino acid$^{123}$. Specifically, phosphorylation by PKCδ requires a hydrophobic amino acid at position +1 at the C-terminal of the phosphorylation site (Ser), glycine at position -1 and basic amino acids at positions -6, -5, -4, and -2$^{123}$. Ultimately, the optimal amino acid sequence for PKCδ phosphorylation is $A(A/R)R(K/A)RKGSFF(Y/F)GG$ where the

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**Figure 3: PKCδ structure**
The structure of PKCδ consists of two domains, a regulatory and a catalytic domain separated by a hinge region with the caspase-3 cleavage site.
serine adjacent to the phenylalanine is particularly important for PKCδ recognition. PKCδ has several protein targets include p53, lamin B, and Rad9, that regulate several important cellular functions once phosphorylated ⁹³,¹²⁴.

**PKCδ Apoptosis Signaling**

As previously stated, PKCδ is activated by several stimuli (reactive oxygen species, ionizing radiation, anti-cancer agents, UV radiation, growth factors, cytokines) which initiate molecular mechanisms such as tyrosine phosphorylation and caspase-3 cleavage that initiate its pro-apoptotic activity ¹²⁵-¹²⁸. Apoptotic signaling is contingent on a predominance of pro-apoptotic signal. Regulation occurs through positive feedback in which PKCδ regulates caspase-3 increasing its own cleavage ¹²⁹-¹³³.

PKCδ apoptotic signaling begins with caspase-3 activation resulting in translocation of PKCδ catalytic fragment to the mitochondria where it facilitates cytochrome c release from the outer membrane of the mitochondria ¹³⁴. This occurs through a pathway in which the catalytic fragment of PKCδ induces turnover of Mcl-1, which is an inhibitor of Bax and Bak. This releases inhibition on Bax causes release of cytochrome c and induction of apoptosis ¹³²,¹³⁵.

**PKCδ and Cell Cycle Regulation**

In addition to PKCδs function in apoptosis, it also plays a role in cell cycle regulation in response to DNA damage. UV-induced cell cycle arrest is influenced by the active catalytic fragment of PKCδ, which induces a pronounced G₂/M cell cycle arrest in murine embryonic fibroblasts (MEFs) ¹³⁶. This cell cycle
A regulatory role occurs downstream of ATM/ATR kinases which are activated in response to different forms of DNA damage such as UV, ionizing radiation, and X-rays\textsuperscript{137}. These findings have established PKC\(\delta\) involvement in the DNA damage response because, if PKC\(\delta\) is absent, a cell with damaged DNA would carry mutations through the G\textsubscript{2}/M cell cycle checkpoint while at the same time evading apoptosis.

**Loss of PKC\(\delta\) in Skin Tumors**

The loss of tumor suppressors at either the genomic or protein level is necessary for development of human cancers. In chemically and UV-induced mouse skin tumors and in human SCCs, PKC\(\delta\) protein levels are significantly reduced\textsuperscript{138-140}. PKC\(\delta\) is lost at the transcriptional level in SCC\textsuperscript{101}. However, overexpression of PKC\(\delta\) in SCC cell lines and mouse epidermis induces apoptosis and suppresses tumorigenicity\textsuperscript{138,139}. Therefore, re-expressing PKC\(\delta\) restores apoptotic functionality again representative of PKC\(\delta\)s tumor suppressing function\textsuperscript{101}. The mechanism of PKC\(\delta\) downregulation in SCCs is unknown however it is likely that PKC\(\delta\) is negatively regulated by active Ras which has been previously shown to down-regulated PKC\(\delta\) protein and RNA in human keratinocytes\textsuperscript{101,138,140}. This is supported by findings that ras is active in \(\sim\)60\% of human SCCs and >90\% of chemically induced mouse skin tumors\textsuperscript{141,142}. These mouse tumors with ras mutations were also deficient in PKC\(\delta\) protein levels and activity suggesting an association between ras mutation and PKC\(\delta\) loss\textsuperscript{139}. 
Summary

Rationale and Hypothesis

Bulky, helix-distorting DNA adducts, such as CPDs induced by UV radiation, are repaired by NER. Defects in NER complementation group genes give rise to familial cancer syndromes such as xeroderma pigmentosum, supporting the important tumor suppressive effects of NER. PKCδ expression is lost in squamous cell carcinomas where it functions as a tumor suppressor gene. PKCδ is activated in response to DNA damage downstream of ATM/ATR, and participates in inducing apoptosis and maintain the G2/M cell cycle checkpoint in response to UV radiation. Since cell cycle checkpoints are coupled to DNA damage repair pathways we hypothesis that cells deficient in PKCδ will have impaired ability to repair UV induced photolesions. Furthermore, PKCδ loss will inhibit some aspects of the NER machinery.
CHAPTER TWO
MATERIALS AND METHODS

Cell Culture and UV Treatment

Spontaneously immortalized MEFs from wild-type or PKCδ-/ mice (provided by Dr. Anning Lin University of Chicago) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen-11965-092) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). UV irradiation was performed using a UV Panelite unit emitting UV wavelengths of approximately 65% UVB, 34% UVA, and <1% UVC spectrums.

Southwestern Blotting

Cell lysates were collected by scraping cells in 500µL DNA lysis buffer (10 mM Tris-HCl pH=7.8, 5mM EDTA, 0.3 M NaOAC, 1%SDS) and 5 µL proteinase K. Samples were incubated overnight at 55°C. Lysate DNA was purified from samples using the DNeasy Blood & Tissue Kit from Qiagen (69504). DNA concentration was determined using an Ultrospec 3000 UV/visible spectrophotometer from Pharmacia Biotech. DNA samples were heat denatured at 100°C for 10 minutes. A slot blot apparatus was used to vacuum transfer DNA samples onto Hybond-N+ membrane. DNA was crosslinked to the membrane using a UV stratalinker 1800 (Stratagene) at 70,000 Joules. Overnight membrane blocking was carried out using denatured herring testes DNA
(Clontech-S0277) diluted in 1x TBS with 10% BSA. Primary antibody used for immunostaining was Anti-CPD antibody (1:50; Kamiya Biomedical Company-TD-M-2). Goat anti-mouse IgG conjugated to Alexafluor-680 was used as a secondary antibody for detection with a LI-COR infrared scanning system (LI-COR Biosciences).

**UV Absorbance/Transmittance**

UVA, UVB, and UVC absorbance of Dulbecco’s modified Eagle’s medium (Invitrogen-11965-092), Medium 154CF (Invitrogen-M154CF), Dulbecco’s Phosphate Buffered Saline 1X without calcium chloride and magnesium chloride (Invitrogen-14190-144), and Milli-Q water was determined using an Ultrospec 3000 UV/Visible spectrophotometer (Pharmacia Biotech). Percent transmittance was determined using the following calculation: Absorbance = -log(percent transmittance/100).

**Immunofluorescence**

Cells, plated on sterilized coverslips in 6 well culture dishes, were fixed in a 1:1 acetone/methanol solution at -20°C for 10 minutes. A solution of 70 mM NaOH in 70% ethanol was added to fixed cells and incubated at room temperature for 2 minutes. Cells were permeabilized in 0.1% Triton X-100. Primary antibodies against Thymine Dimers (CPD) (1:150, 1:200; Kamiya Biomedical Company-TD-M-2), XPA (1:50; Santa Cruz-sc-853), and XPC (1:50; Santa Cruz-sc-30156) were used along with secondary antibodies goat anti-
rabbit IgG-Alexa Fluor-568, goat anti-rabbit IgG-Alexa Fluor-488, goat anti-
mouse IgG conjugated to Alexa Fluor-488. Secondary antibody was followed by
staining with 4',6-diamidino-2-phenylindole (DAPI). Coverslips were mounted
onto slides using Gelvatol. Images were generated using an Olympus Provis
Microscope with Retiga 4000R 4.2 megapixel monochrome camera at 20x and
40x magnification.

**Retroviral Constructs, Packaging, and Infection**

PKCδ-green fluorescent protein (GFP) constructs were provided by Dr.
Mary E. Reyland (University of Colorado Health Sciences Center). Viral
constructs and retroviral supernatant were prepared by Edward LaGory and
incubated at -80 °C\textsuperscript{136}. For infection, polybrene (hexadimethrine bromide, sigma
H-9268) was added to viral supernatant to a final concentration of 4 µg/ml.
Retroviral Infection was performed in 6 well culture dishes by adding 2 mL of
virus with polybrene to each well and incubated at 37° C for 2 hours. Virus was
redistributed over the plate every 15 minutes to ensure even distribution of virus
over cultured cells.

**Immunoblotting**

Western Blot analysis was performed using 12.5% and 8.5% SDS gel for
XPA and XPC staining respectively. Cell lysates were collected by scraping cells
in IP buffer (10 mM Tris-HCL, pH=7.5, 100 mM NaCl, 0.5% Triton X-100, and
protease and phosphates inhibitors). Lysates were briefly sonicated and
centrifuged at 14,000 rpm for 5 minutes to remove cellular debris. Protein concentrations were determined using standard Bradford Reagent methodology. Primary Antibodies used for western analysis included XPA (1:50; Santa Cruz-sc-853), XPC (1:50; Santa Cruz-sc-30156), and β-Actin (1:5000; ICN, 691001). Secondary antibodies used were goat anti-rabbit IgG-Alexa Fluor-680, donkey anti-rabbit IgG-IRDye-800 and goat anti-mouse IgG conjugated to AlexaFluor-680 for detection using a LI-COR infrared scanning system (LI-COR Biosciences).
CHAPTER THREE

RESULTS

Abstract

PKCδ is a tumor suppressor frequently lost in chemically and UV-induced mouse and human skin tumors. This decline is due to downregulation of pro-apoptotic PKCδ at the transcriptional level resulting in increased survival of these skin tumors. Additionally, in response to UV-induced DNA damage, the catalytic fragment of PKCδ functions downstream of the ATM/ATR pathway and is essential for G2/M cell cycle checkpoint maintenance. Because cell cycle checkpoints are coupled to DNA damage repair pathways, we hypothesize that PKCδ may play a role in UVB-induced DNA damage repair. Our data demonstrates that PKCδ is essential for repair of CPDs following UVB-induced damage.

PKCδ is Necessary for Efficient Repair of CPDs

Cells exposed to UV radiation accrue damage that halts cell cycle progression. Stalled cell cycle progression is essential for the repair of DNA damage and prevention of transmission of damage to daughter cells. Because PKCδ is essential for maintaining UV-induced G2/M cell cycle arrest, southwestern blotting was used to determine if Mouse embryo fibroblasts (MEFs) cells lacking PKCδ were capable of repairing UV-induced DNA adducts.
Southwestern blotting involves the transfer of pure DNA to a membrane which can then be visualized using immunostaining. Cultured MEF cells were exposed to a 75 mJ/cm² dose of UVB radiation. After exposure, cells were given set amounts of time to repair their DNA (0 minutes, 10 minutes, 24 hours, 48 hours, and 72 hours). After repair, cells were lysed, DNA was extracted and purified, and then DNA was transferred onto a membrane using a vacuum suction slot blot apparatus. The membrane then underwent immunostaining with an antibody specific for CPDs. From quantitation of band intensity, we then extrapolated degree of repair over time. At 10 minutes after UV, we observed comparable levels of CPDs between WT and PKCδ-/- cells. However, at 48 and 72 hours, we observed decreased amounts CPDs in WT MEFs when compared to PKCδ-/- cells (Figure 4A). Quantitation of this blot, supported these observations showing equivalent damage at 10 minutes and decreased CPD density after 48 hours in WT cells (Figure 4B).
Figure 4. Analysis of UV-induced DNA Damage Repair

Southwestern Blot of Wild-type and PKCδ-/- MEF DNA. A. 200 ng DNA sample with anti-thymine dimer antibody staining. The reduction in band intensity signifies cellular repair. B. Band intensity quantitation of 200 ng DNA quantity.
Media UV Absorption and Percent Transmission

MEFs are routinely grown in DMEM medium with 10% FBS added. However, because of the presence of serum proteins and phenol red, this medium is likely to absorb significant amounts of UV radiation. With this in mind, we explored the possibility of administering UV to cells in different media that vary in absorption of UV wavelengths in order to decrease UV levels into a non-apoptotic range. We measured UV absorbance of wavelengths (200nm-400nm) of H₂O, DPBS, DMEM with 10% FBS, and Media 154 (Figure 5A, 5B). From these data, we were able to identify DPBS as an appropriate medium for dispensing UVB radiation because of its low UV absorbance/high percent transmittance, and thus accurate dosimetry. This medium allowed us to use a UVB dose of 5 mJ/cm² in which no apoptosis was observed.
Figure 5. UV Absorption and Percent Transmission

A. UV absorbance spectrophotometer measurements (200nm-400nm) of \( \text{H}_2\text{O} \), PBS-, DMEM, and Media 154. B. Percent Transmittance measurements. Absorbance = -log(\%transmittance/100).
Maximum Nuclear CPD Fluorescence After UVB Exposure

UV radiation causes formation of CPDs immediately upon exposure. However, maximum antibody detection of photolesions in cells is not observed immediately after irradiation. This observation is largely attributed to chromatin unwinding which provides space for NER proteins and may increase CPD antibody access. In order to observe repair of CPDs, we established a time point at which we could observe the greatest number of CPDs indicated by a maxima in CPD fluorescence intensity. We then performed immunofluorescence staining to visualize the kinetics of CPD detection in WT MEF cells. A low, non-apoptotic UVB dose of 5 mJ/cm\(^2\) was administered to cells plated in 6-well culture dishes in DPBS. Cells were then returned to DMEM media and fixed using methanol/acetone at 10 min., 30 min., 1 hour, 2 hours, and 4 hours after UVB exposure. Using fluorescent imaging, we then observed DNA damage using levels of nuclear CPD fluorescence intensity (Figure 6A). We then quantitated the relative CPD fluorescence intensity per cell nuclei. After quantitation, it was found that, on average, maximum CPD fluorescence was observed at approximately 1-2 hours after UV irradiation in WT cells (Figure 6B). From these results, we chose a 2 hour fixation time point for subsequent experiments.
Figure 6. Maximum Nuclear CPD Fluorescence After UVB Exposure

Immunofluorescence staining for CPDs at 10 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours after UV irradiation with 5 mJ/cm² UVB in WT MEFs. Quantitation using Image J software indicates maximum detection of CPDs at 1 hour after UV radiation is administered.
PKCδ is Required for Repair of UVB-Induced CPDs

Using southwestern blotting, we showed that PKCδ−/− cells were defective in repairing UVB-induced damage. In order to confirm this observation, we performed quantitative immunofluorescence on WT and PKCδ−/− MEFs fixed 2 and 48 hours after UVB-induced damage using a low dose (5 mJ/cm²) of UVB. These data, in figure 7A, show that irradiated WT cells were capable of repair of most CPD damage after 48 hours while PKCδ−/− cells had not repaired CPDs at 48 hours. The maximum CPD damage at 2 hours was similar in both WT and PKCδ−/−. In addition to imaging, nuclear CPD fluorescence was quantified at 0, 2, and 48 hours after UVB treatment. Quantitation in figure 7B shows comparable levels of CPD fluorescence at 2 hours and supports the observation that CPD levels are higher in PKCδ−/− cells at 48 hours after UVB treatment. In figure 7C, the 48 hour time point is displayed as an intensity distribution histogram and clearly shows two populations of cells that differ in their CPD repair capabilities. These results have proven to be both reproducible and highly significant (p<0.001). PKCδ−/− cells, however, did show a slight reduction in CPD fluorescence levels indicating that these cells are most likely capable of some level of CPD repair.
A. Hours After 5mJ/cm² UVB

WT MEF

PKCδ-/- MEF

CPD Immunofluorescence

B. Average CPD Fluorescence Per Cell Nuclei

WT MEF
PKCδ-/- MEF

Control 2 48

Hours After UV Treatment

P<0.001
Figure 7. Repair of UVB-Induced CPDs

Immunofluorescence staining using anti-thymine dimer CPD antibody. A. Fluorescent intensities were similar 2 hours after receiving 5 mJ/cm² of UVB radiation. PKCδ−/− cells show an appreciably higher CPD fluorescence intensity at 48 hours indicating reduced CPD repair. B. Average fluorescence of WT (N=50) and PKCδ null (N=50) MEF at 0, 2, and 48 hours after UVB irradiation. C. Histogram of CPD fluorescence intensity distribution 48 hours after UV irradiation.
Re-expressing PKCδ in PKCδ-/- Cells Restores CPD Repair

If PKCδ is solely responsible for the observed repair deficiency in the PKCδ-/- cells, then re-expressing PKCδ in PKCδ-/- cells should result in improved repair. In order to re-express PKCδ in PKCδ-/- cells, we used a retroviral system to transduce full length, GFP-tagged PKCδ gene into cells lacking PKCδ. Once cells were transduced with PKCδ-GFP retrovirus, GFP immunofluorescence was performed to confirm transduction efficiency which was ~70%. After exposure to 5 mJ/cm² UVB, we observed that WT MEFs repaired nearly all damage and PKCδ-/- cells retained their CPDs (Figure 8). However, the PKCδ-/- cells transduced with PKCδ showed levels of nuclear CPD fluorescence intensity that were representative of both WT and PKCδ-/- repair capabilities (Figure 8A). Quantitation of nuclear CPD fluorescence indicated that PKCδ-/- cells transduced with PKCδ showed a high level of variability of the mean nuclear fluorescence intensity (Figure 8B). A CPD intensity distribution histogram at 48 hours after UV treatment shows that the large standard deviation observed in PKCδ transduced cells was due to two separate cell populations that were consistent with WT and PKCδ-/- cells (Figure 8C). Additionally, these results were consistent with the roughly 70% transduction efficiency observed in PKCδ transduced cells (determined by GFP fluorescence). These results indicate that lack of CPD repair in PKCδ-/- cells is not a secondary effect, but responsible for the observed defect in NER of CPDs in PKCδ-/- MEFs. Therefore, a cell that has lost PKCδ (which occurs in SCCs) can regain normal DNA damage repair capabilities if PKCδ expression is rescued.
A. Hours After 5ml/cm² UVB

WT MEF

PKCδ-/- MEF

PKCδ-/- MEF +PKCδ

B. CPD Fluorescence Intensity Per Cell Nuclei

WT MEF

PKCδ-/- MEF

PKCδ-/- MEF +PKCδ

Hours After UV Treatment
Figure 8. PKCδ Rescues CPD Repair Defect

PKCδ rescues defective NER of CPDs in PKCδ-/- cells. A. Immunofluorescence staining using anti-thymine dimer CPD antibody. Cells exposed to 5 mJ/cm² UVB have comparable CPD fluorescence intensity after 2 hours. However, 48 hours after UVB treatment, WT MEFs repair CPD damage, PKCδ-/- cells retain CPD a majority of CPD damage, and PKCδ transduced cells show cell populations that have both repaired and retained CPD damage. B. Average CPD fluorescence intensity per cell nuclei of WT (N=23), PKCδ-/- (N=17), and PKCδ-/- +PKCδ (N=19) MEFs at 0, 2, and 48 hours after UVB irradiation. C. Histogram of CPD fluorescence intensity 48 hours after UVB treatment.
**PKCδ-/- Cells Have Decreased XPA and XPC Protein Levels**

The mechanism used to repair CPDs involves several essential proteins for identification and excision of the bulky lesions. However, not all of these proteins are essential for both GG-NER and TC-NER processes. Additionally, nearly all NER genes are upregulated when cells are exposed to low dose of UVB\textsuperscript{74}. This upregulation leads to an increase in NER protein levels which can then be detected by western blot. In order to identify where in the NER process PKCδ is required, we performed western blots using whole cell lysates from cells that were exposed to low-dose UVB radiation. We then immunostained cells for DNA damage binding proteins XPC and XPA. XPC is critical for initial identification of DNA photolesions and is exclusive to GG-NER whereas XPA acts in both GG-NER and TC-NER and binds DNA damage in order to ensure excision of the lesion. For this experiment, we collected cell lysates at 0, 1, 3, and 6 hours after irradiation (Figure 9). We observed that NER proteins XPA and XPC were lower in non-irradiated PKCδ -/- cells and thus non-irradiated WT MEFs. Additionally, XPA increased after UVB treatment and was highest at 6 hours in WT cells. PKCδ-/- cells showed only a slight increase in XPA after 6 hours (Figure 9A). UVB treatment also caused an increase in XPC protein in WT cells 1 hour after treatment. This was in contrast to PKCδ-/- cells which showed an increase in XPC 6 hours after UVB treatment.
A.

<table>
<thead>
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<th>Hours post-UV (5mJ/cm²)</th>
<th>WT MEF</th>
<th>PKCδ/-/</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
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<td>3</td>
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<td>6</td>
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B.

![Graph showing XPA Density over Time After UV Irradiation]
Figure 9: UVB Increases NER Protein Concentration Only In WT Cells

Immunostaining for affect of UVB on NER Protein Levels. A. WT MEFs increase XPA and XPC protein levels in response to UVB treatment. B. Band Quantitation shows differences in both endogenous XPA and XPC and UV induced protein increases of these NER proteins.
**XPA is Not Recruited to the Nuclei of PKCδ−/− MEFs by UVB**

In response to UVB radiation, cytoplasmic XPA is recruited to the damaged nucleus requiring a physical interaction with ATR\(^{56,144}\). In figure 10, immunofluorescence staining of XPA was used to observe UVB-induced increases in nuclear XPA levels in WT and PKCδ−/− cells. Fixed cells were stained at 6 hours after UVB treatment. The XPA stained WT MEF cells showed an increase in nuclear XPA staining after 6 hours whereas the PKCδ null cells experienced only a slight, non-significant increase in XPA levels (Figure 10A). Additionally, this difference in XPA fluorescence intensity at 6 hours was statistically significant (P<0.001) (Figure 10B). These findings indicate that XPA nuclear localization is regulated by a pathway involving PKCδ.
**Figure 10: PKCδ is Crucial for UV-Induced Increase in Nuclear XPA**

Immunofluorescence staining for NER protein XPA after UV irradiation.  
A. At 6 hours after UV irradiation, WT cells show a higher nuclear XPA fluorescence intensity than PKCδ-/- cells.  
B. Average fluorescence intensity for XPA in WT and PKCδ-/- MEFs at 0 and 6 hours (P<0.001) after UV irradiation.
XPC is Not Recruited to the Nuclei of PKCδ-/MEFs by UVB

XPC is part of a NER heterodimer complex that is the first protein in the GG-NER pathway. Beyond its DNA damage binding role, it also recruits subsequent NER proteins of the preincision complex to the site of damage. Additionally, like XPA, the XPC gene is also shown to be induced by low levels of UVB. In figure 11, immunofluorescence staining for XPC revealed that nuclear XPC protein increases significantly after 6 hours in WT cells. This UVB-induced increase was completely absent in PKCδ-/cells (Figure 11A). Additionally, quantitation of nuclear XPC fluorescence revealed significantly lower nuclear XPC fluorescence in PKCδ-/MEFs compared to WT MEFs at 6 hours after UVB treatment (Figure 11B). Thus, one possible explanation for the observed low levels of nuclear XPA is the loss of recruitment signals from XPC. Reduced XPC could lead to impaired or complete loss of XPC mediated recruitment of succeeding NER proteins resulting in inhibition of normal functioning GG-NER.
Figure 11: PKCδ is Crucial for UV-Induced Increase in Nuclear XPC

Immunofluorescence staining for NER protein XPC after UV irradiation. A. At 6 hours after UV irradiation WT cells show a higher nuclear XPA fluorescence intensity than PKCδ−/− cells. B. Average fluorescence intensity for XPA in WT and PKCδ−/− MEFs at 0 and 6 hours (P<0.001) after UV irradiation.
**CHAPTER FOUR**

**DISCUSSION**

**PKCδ is a Proapoptotic Tumor Suppressor**

PKCδ has several well-characterized roles in cell growth, differentiation, cell cycle arrest, and apoptosis. However, PKCδ is often lost in skin tumors potentially resulting in progression of cells with damaged DNA through cell cycle checkpoints and evasion of apoptosis. Because cell cycle checkpoints ensure the fidelity of genetic material both by arresting cell cycle progression and facilitating DNA repair pathways, it is highly probable that proteins regulating cell cycle checkpoints such as PKCδ may also be involved in the DNA damage repair.

PKCδ is known to associate with several DNA damage proteins associated with double-strand breaks including Rad9, DNA-dependent protein kinase (DNA-PKcs), and topoisomerase IIα. The association of PKCδ with Rad9 is necessary for formation of the Rad9-Hus1-Rad1 complex, which is essential for regulation of checkpoint activation and the apoptotic response to DNA damage. Additionally, PKCδ catalytic fragment phosphorylates DNA-PKcs inhibiting them from phosphorylating and activating downstream target p53.

PKCδ’s role in phosphorylating Rad9 and DNA-PKcs accentuate the importance of PKCδ in DNA damage-induced apoptosis. Introduction of a
caspase-resistant mutant of PKCδ in keratinocytes inhibits activation of PKCδ-cat and results in partial prevention of cytochrome c release in mitochondria and loss of antiapoptotic Mcl-1. Additionally, the addition of ATM/ATR inhibitor caffeine to MEFs exposed to UV, results in progression of cells through cell cycle checkpoints and results in apoptosis in WT cells. However, PKCδ-/- cells treated with caffeine and exposed to UV do not undergo apoptosis. Therefore, cells that either lack or inhibit proper function of PKCδ are predicted to be especially susceptible to mutagenesis due to failure of UVB DNA damage repair and resistance to apoptosis. This is analogous to the tumor suppressor p53, which also functions in DNA damage response as the guardian of the genome, and in apoptosis as a cellular proofreader.

**PKCδ is Essential For Effective Repair of CPD**

Induction of DNA damage by UV light activates several cellular responses including NER and ATR-CHK1 signaling. Great progress has been made towards understanding the signaling along these pathways, but the details of how these responses are coordinated are still very poorly understood. We addressed the question of whether PKCδ was involved in DNA damage repair by performing southwestern blotting and immunofluorescence studies on fibroblasts isolated from WT and PKCδ-/- mice in order to visualize and quantitate DNA damage and NER protein nuclear localization. Using these two techniques, we were able to determine that PKCδ is necessary for efficient DNA damage repair of CPDs in response to UV radiation. Additionally, because bulky, DNA distorting lesions,
such as CPDs, are repaired via the NER mechanism, PKCδ signaling must affect some aspect of this mechanism.

**PKCδ Transduction Rescues NER Defect**

In order to provide conclusive evidence that the lack of PKCδ, and not an off-target effect, is responsible for the observed NER deficient phenotype in PKCδ null MEFs, we performed a retroviral transduction of PKCδ gene into PKCδ-/- cells. Because transduction of PKCδ rescued cellular ability to repair CPD damage, we were able to determine that loss of PKCδ alone is accountable for the observed deficiency in CPD repair in PKCδ-/- MEFs. As reported in the discussion of figure 8, we achieved a transduction efficiency of roughly 70%. By determining how many cells fell within a particular range of CPD fluorescence intensity, we could ascertain that the number of cells that were capable of repairing CPD damage was consistent with our transduction efficiency. These results support our previous findings that PKCδ is essential for repair of CPDs.

**Several Possible Targets Exist for PKCδ**

DNA damage response affects a wide range of cellular processes. Central to DNA damage response are the PI3 kinase-like kinases ATM and ATR which are activated in response to double strand breaks and DNA bulky lesions respectively. ATM/ATR act by phosphorylating substrates that inhibit cell cycle progression and coordinate repair of lesions. Downstream proteins of ATM/ATR include Chk1, Chk2, p53, and PKCδ. The significance of
proper functioning ATM/ATR signaling is accentuated by the number of known debilitating disease associated with mutations in the kinases or mutations in other related protein components of DNA damage response. These diseases include xeroderma pigmentosum, ataxia telangiectasia, Seckel syndrome, and BRCA1 and BRCA2 in breast cancer\textsuperscript{154,155}. Recent discoveries concerning ATM/ATR signaling in skin cells have identified ATR as essential for translocation of NER protein XPA into the nucleus in response to UV induced adduct detection\textsuperscript{56,144}. Additionally, previous work from our lab has identified that the catalytic fragment of PKCδ also operates downstream of ATM/ATR in order to maintain G\textsubscript{2}/M arrest in response to UV radiation\textsuperscript{136}. Because XPA and PKCδ are both downstream proteins of ATR signaling, we were interested in looking at whether PKCδ could also be affecting NER protein recruitment. Using quantitative immunofluorescence, we were interested in determining where in the NER process PKCδ is involved. After observing a statistically significant difference in nuclear XPA levels at 6 hours post UV treatment, we were able to determine that PKCδ does affect localization of this protein as determined by immunofluorescence microscopy (Fig. 10). However, nuclear XPA recruitment is also contingent on DNA damage binding protein XPC. In the beginning stages of GG-NER XPC-hHR23B heterodimer identifies and binds the UV-induced adduct. XPC binding recruits proteins of the NER pre-incision complex to the site of the photolesion. This pre-incision complex includes XPA. We also performed quantitative immunofluorescence staining on nuclear XPC. Again, we saw nuclear accumulation of this XP protein in WT cells only. From these results, we
were able to determine that PKCδ is involved in nuclear localization/recruitment of the essential NER proteins XPA and XPC. However, it remains unclear as to whether the lack of nuclear XPA is due to PKCδ regulation of nuclear XPC levels. Western blot analysis of whole cell lysates also revealed differences in regulation of XPA and XPC between WT and PKCδ-/- MEFs. These differences were observed in both endogenous and UV-induced protein levels. Experiments using low-dose UVB have shown increase in NER gene transcription. This observation has given rise to the thought that all NER proteins are regulated by a common signaling cascade in which PKCδ may be a part of.

It remains unknown as to the exact location within NER PKCδ is functioning. However, this research has identified an additional role for PKCδ in repair of UV-induced photoproducts, and is the first work to identify a role for PKCδ in the repair of DNA damage. Characterizing this additional role for PKCδ in NER will both enhance our knowledge of DNA damage molecular signaling and strengthen our appreciation for the complexity of cellular response to DNA damage. Continued expansion of our knowledge of DNA damage repair signaling will undoubtedly present interesting opportunities for possible cancer prevention therapeutics in future research.


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While attending Loyola, Christopher was elected treasurer of the graduate student council and actively participated in several university organizations including the Loyola chapter of The American Physicians Scientists Association (APSA). Additionally, Christopher was selected for an oral presentation at the 2011 annual meeting of the Society for Investigative Dermatology and also received the Albert M. Kligman travel fellowship to attend the conference.

Christopher intends to enter medical school where he will continue his studies of medicine and biomedical science. He currently lives in Chicago, Illinois.