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A closer look

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Shikha Gupta

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Kathleen Meek
Major Professor's Signature

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DNA-PK

A closer look

By

Shikha Gupta

A DISSERTATION

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ABSTRACT

DNA-PK: A closer look

By

Shikha Gupta

Double strand breaks (DSBs) arise endogenously during normal cellular processes and exogenously by genotoxic agents such as ionizing radiation (IR). In higher eukaryotes these breaks are repaired by non homologous end joining (NHEJ) and to a lesser extent by homologous recombination. DNA-PK is a repair enzyme composed of a catalytic subunit, DNA-PKcs and a DNA targeting subunit, Ku70/80 heterodimer. The catalytic subunit is a DNA activated serine threonine protein kinase and has been shown to phosphorylate Ku70/80 and numerous other substrates. Studies suggest once bound to a DNA double strand break, Ku recruits the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs). Five DNA-PK phosphorylation sites have been identified in Ku70/80. This suggested that there is a phosphorylation-mediated regulation of Ku in NHEJ. Here I show that Ku containing serine/threonine to alanine mutations at these five sites is fully able to complement the radiation sensitivity of Ku negative mammalian cells. This led us to conclude that DNA-PK mediated phosphorylation of Ku is not required for DNA double strand break repair.

DNA-PKcs possesses an intrinsic, albeit weak DNA-end binding activity that is stabilized by Ku70/80. DNA-PKcs is a kinase made up of 4128 residues and has at least four identified domains: LRR, FAT, FATC and PI3kinase. However, besides the kinase

domain the contribution of other domains in DNA-PKcs's function in DSB repair is not known. In the second part of my study I show that disrupting the LRR motif in DNA-PKcs by point mutations affects its ability to function in NHEJ. I further show that the mutant protein does not bind DNA-cellulose as well as the wild type even though it interacts with Ku70/80 as well as the wild type. These results led me to conclude that the LRR domain in DNA-PKcs contributes to the intrinsic DNA-end binding property of DNA-PKcs, which is required for its role in NHEJ.

DEDICATION

To my loving family, who have supported me through my foolish endeavors

To Vipra, who found me somewhere between my whims and fancies

AND

To the pursuit of skepticism, empiricism and synicism

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FOREWORD

The work discussed in Chapter 3 of this thesis is published as referenced below:

DNA-PK dependent phosphorylation of Ku70/80 is not required for non homologous end joining. **Shikha Gupta,** Pauline Douglas, Nick Morrice, Katheryn Meek and Susan P.Lees-Miller.

DNA repair 2005 Aug 15;4(9):1006-18

Images in this dissertation are presented in color.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF ABBREVIATIONS

aa	amino acid
ALA	alanine
ASP	aspartic acid
ATM	ataxia talengiectasia mutated
CDK	cyclin dependent kinase
CHO	chinese hamster ovary
DNA-PK	DNA protein kinase
DNA-PKcs	DNA protein kinase catalytic subunit
ds	double stranded
DSB	double stranded break
EDTA	ethylene diamine tetra acetic acid
EGTA	ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetic Acid
EM	electron microscopic
EMSA	electromobility shift assay
GST	glutathione S transferase
HR	homologous recombination
IR	ionizing radiation
LRR	leucine rich region
LZ	leucine zipper
MALDI	Matrix assisted laser desorption/ionization
MAPK	mitogen activated protein kinase

MS	mass spectrometry
NHEJ	non homologous end joining
OA	okadaic acid
PAGE	polyacrylamide gel electrophoresis
PIKK	phosphatidylinositol 3-kinase-like kinase
PKA	protein kinase A
PKC	protein kinase C
PP2A	protein phosphatase 2A
RAG	recombination activating gene
RNAase	ribonuclease A
RSS	recombination signal sequence
SDS	sodium dodecyl sulphate
SSA	single strand annealing
SSB	single strand break
TdT	terminal deoxynucleotidyl transferase
VWA	vonWillebrand factor A
WRN	Werner syndrome protein
XRCC	X-Ray cross complementing

CHAPTER 1

INTRODUCTION

All through a cell's life cycle, its DNA is constantly broken and repaired. Double strand breaks (DSBs) are regarded as the most lethal of all DNA lesions. In a cell, DSBs can arise endogenously by cellular processes like V(D)J recombination, meiotic recombination and also during replication when the replication machinery encounters single strand breaks (SSBs) causing a replication fork to collapse, generating DSB (Haber, 2000; Karran, 2000; Norbury and Hickson, 2001; Bassing *et al.*, 2002). When left unrepaired, DSBs lead to the activation of cell cycle checkpoints and can also signal cell death (Jackson, 2001). Misrepaired DSBs can lead to genetic translocations and deletions, destabilize the genome and potentially cause cancer (van Gent *et al.*, 2001; Thompson and Schild, 2002; Vilenchik and Knudson, 2003). There are three main pathways that repair DSBs: homologous recombination, single strand annealing and non-homologous end joining. However, each of these pathways contributes differently in lower and higher eukaryotes (Haber, 2000; Lees-Miller and Meek, 2003; Vilenchik and Knudson, 2003; Shiloh and Lehmann, 2004).

Homologous recombination or gene conversion is favored in lower eukaryotes like yeast. This process relies on utilizing genetic information on the sister chromatid or the homologous chromosome to restore the DSB. Some of the proteins involved in this process are RAD51, RAD52, RAD54, RAD57, RAD59 (RAD 52 epistasis group) and a protein complex comprising of three proteins, RAD50/ MRE11 /XRS2. Homologous

recombination is the predominant repair pathway during the G2 and S phases of the cell cycle (Featherstone and Jackson, 1999; Thompson and Schild, 2001; van den Bosch *et al.*, 2002). Single strand annealing (SSA) is another pathway that requires regions of homology to repair the damaged DNA. It requires tandem repeats of homologous sequences on both strands and the strands anneal to each other after resection of DNA from 5' ends specifically. Hence, SSA results in a tremendous loss of genetic information. This process also requires RAD 52 (Featherstone and Jackson, 1999; Thompson and Schild, 2001; van den Bosch *et al.*, 2002).

Non-homologous end joining (NHEJ) is the primary pathway that repairs the majority of DSBs in all cell cycle phases, while HR contributes modestly in G1 and progressively more as cells move through the cycle in G2 (Rothkamm *et al.*, 2003). There are six known proteins that unequivocally constitute the core of NHEJ machinery; namely, Ku subunits (Ku70 and Ku80), DNA-PKcs, DNA ligase IV and its partner XRCC4, and the nuclease Artemis (Critchlow and Jackson, 1998; Featherstone and Jackson, 1999; Pierce *et al.*, 2001; Jackson, 2002; Lees-Miller and Meek, 2003). In addition, C1D, a nuclear matrix protein with high affinity for DNA (Yavuzer *et al.*, 1998; Erdemir *et al.*, 2002), polymerase μ and polymerase λ have also been implicated to play a role in NHEJ (Mahajan *et al.*, 2002; Havener *et al.*, 2003; Nick McElhinny and Ramsden, 2003, 2004).

The main focus of my study is the protein complex, DNA-PK (DNA dependent protein kinase). Of the six factors, DNA-PK is central to the process of NHEJ. It is a protein complex made up of three proteins- DNA-PKcs (serine/threonine kinase) and Ku (a heterodimeric DNA binding protein) (Gottlieb and Jackson, 1993). DNA-PKcs acts as a protein scaffold and binds to all five factors bringing them in close vicinity of each other. Cells that lack DNA-PKcs are radiosensitive and defective in DSB repair (Jackson and Jeggo, 1995; Jeggo *et al.*, 1995; Peterson *et al.*, 1995). Additionally, studies have established that DNA-PK's kinase activity is indispensable for NHEJ (Peterson *et al.*, 1997; Kurimasa *et al.*, 1999; Kienker *et al.*, 2000)

My studies have addressed two issues in NHEJ:

Role of Ku phosphorylation in NHEJ: DNA-PKcs has been shown to phosphorylate numerous proteins *in vitro* and/or *in vivo* including Ku, XRCC4, Artemis and DNA-PKcs itself (Chan *et al.*, 1999; Douglas *et al.*, 2002; Ma *et al.*, 2002; Lee *et al.*, 2004). Recently our lab and others have shown that auotophosphorylation of DNA-PKcs is important for DNA end processing in NHEJ (Ding *et al.*, 2003; Meek *et al.*, 2004; Reddy *et al.*, 2004). However, the physiological relevance of other phosphorylation events is yet to be established. In this study, I will test the hypothesis that phosphorylation of Ku is required for NHEJ.

Role of Leucine rich region in DNA-PKcs: The kinase activity of DNA-PKcs resides in amino acids 3745 to 4013, a PI3 kinase domain. Another recognized domain within

this large protein is a leucine zipper (LZ) motif, perhaps more appropriately designated a leucine rich region (LRR), that spans residues 1503-1602 (20% of the residues are leucines) (Hartley *et al.*, 1995). Whereas DNA-PK's kinase activity has been shown to be absolutely indispensable for its function in NHEJ, little is known about the functional relevance of the LRR. In 1998, Jackson and colleagues identified the nuclear matrix protein C1D as a factor that interacts specifically both *in vitro* and *in vivo* with the leucine rich region of DNA-PKcs (Yavuzer *et al*, 1998). In this study, using a mutagenic approach, I will determine the functional relevance of LRR in DNA-PKcs.

CHAPTER 2:

REVIEW OF LITERATURE

2.1 Model for NHEJ

Studies have established that binding of Ku to damaged DNA ends is the first step in NHEJ (Dynan and Yoo, 1998; Yoo and Dynan, 1999; Doherty and Jackson, 2001; Dudasova *et al.*, 2004). Ku forms a ring around DNA, leaving the DNA ends accessible to other factors such as DNA-PKcs (Walker *et al.*, 2001). By itself, DNA-PKcs possesses a very weak DNA binding activity (Hammarsten and Chu, 1998). The C terminal end of Ku80 binds to DNA-PKcs; this allows for direct contact of DNA-PKcs with the DNA terminus (Singleton *et al.*, 1999). In this conformation, DNA-PKcs mediates synapsis of two DNA ends and this DNA synapsis is also required to activate the kinase (DeFazio *et al.*, 2002). The kinase phosphorylates both Ku70 and Ku80 (Chan *et al.*, 1999) followed by translocation of Ku internally along the DNA (Dynan and Yoo, 1998; Calsou *et al.*, 1999; Yoo and Dynan, 1999; Doherty and Jackson, 2001).

DNA-PKcs also phosphorylates itself at two identified phosphorylation clusters (ABCDE and PQR) and these autophosphorylation events have been shown to reciprocally regulate the processing of DNA ends (Ding *et al.*, 2003) (Cui *et al*, 2005 unpublished data). Phosphorylation at ABCDE opens the DNA ends for access by end-processing factors namely, Artemis, polymerase μ and λ and TdT (in V(D)J recombination), and phosphorylation at the PQR cluster blocks this access.

Artemis has been shown to possess endonuclease and exonuclease activity *in vitro* and has been proposed to play a role in DNA end processing in NHEJ (Ma *et al.*, 2002; Schlissel, 2002). Ku and L/X have also been shown to interact with DNA polymerase μ (pol μ) (Mahajan *et al.*, 2002; Nick McElhinny and Ramsden, 2003). *In vitro* studies have shown that pol μ stabilizes the interaction between L/X, Ku and DNA and is also required for gap filling activity in NHEJ (Lee *et al.*, 2003). Polymerase λ is yet another polymerase implicated in NHEJ. Its yeast counterpart Pol4 has been linked both genetically and biochemically to NHEJ (Wilson and Lieber, 1999; Tseng and Tomkinson, 2002). Pol λ has also been shown to associate with the core NHEJ factors Ku and XRCC4-ligaseIV (Fan and Wu, 2004; Ma *et al.*, 2004; Nick McElhinny *et al.*, 2005). Recently, it has been shown that in NHEJ, mammalian cells employ pol μ, poly λ and TdT for distinctive end processing roles in DSB repair and V(D)J recombination (Nick McElhinny *et al.*, 2005).

Ku and DNA-PKcs finally recruit the ligase IV/XRCC4 (L/X) complex (Nick McElhinny *et al.*, 2000) and DNA-PKcs phosphorylates the XRCC4 component (Leber *et al.*, 1998; Chen *et al.*, 2000; Hsu *et al.*, 2002; Calsou *et al.*, 2003). Ligase IV and XRCC4 form a tight complex (Lee *et al.*, 2000) and this interaction stimulates ligase IV activity (Modesti *et al.*, 1999).

Besides the six factors, Dai *et al* have suggested the requirement of an unidentified factor for NHEJ (Dai *et al.*, 2003). Additionally, C1D, a 16 Kda DNA

Fig. 1. **Model for NHEJ**

1. **DNA damage:** Double strand breaks in DNA can be induced by a variety of agents and certain normal cellular processes.

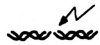
2. **Complex assembly:** Ku binds DNA ends and then recruits catalytic subunit. The catalytic subunit has been shown to possess three structural domains: two large globular domains ("head" and "palm") separated by the arm domain (Boskovic *et al*, 2003). The head is composed of the kinase, FAT and FATC domains (Rivera-Calzada *et al*, 2005)

3. **Synapsis and activation:** DNA binding induces a conformational change in the head and palm region. DNA-PKcs aligns two broken DNA ends to form a synaptic complex. This synapsis of DNA ends is required to activate the kinase (DeFazio *et al*, 2002).

4. **Autophosphorylation at ABCDE:** Phosphorylation at one or more sites in the ABCDE cluster (aa 2609-2647, shown in yellow) induces a conformational change that allows access of DNA ends to DNA-end modifying factors (Ding *et al*, 2003)

Fig. 1 Model For NHEJ

1.



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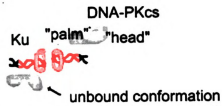
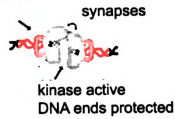


3. DNA-PK-bound conformation



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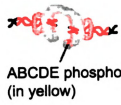


Model for NHEJ (cont.)

5. **End processing:** DNA-PK's kinase activity is required to activate the nuclease activity of Artemis (Ma *et al*, 2002). Pol μ and λ have been shown to be required for gap filling (Nick McElhinny *et al*, 2005). Other factors like polynucleotide kinase and TdT would presumably work at this point as well.

6. **Autophosphorylation of PQR:** Further phosphorylation at a second cluster named PQR (aa 2023-2056, shown in pink) in DNA-PKcs induces another conformational change which blocks the DNA-end processing factors to access DNA ends, thus preventing excessive nucleotide loss (Cui *et al*, unpublished data).

7. **Ligation:** At some point, XRCC4/Ligase IV complex is targeted to the processed ends for the ligation of the broken DNA ends.

8. **Complex Disassembly:** The dissociation of DNA-PKcs from Ku-bound DNA is phosphorylation dependent. Phosphorylation at two known clusters (ABCDE and PQR) is not required for complex disassembly. It is unclear what sites in DNA-PKcs induce its dissociation. It is also not known what and how Ku is released from DNA.

9. **Recycling:** A cellular phosphatase has been shown to interact with the phosphorylation site cluster (ABCDE) in DNA-PKcs. This likely facilitates recycling of the protein for future repair use.

5.

protein-protein
interaction
target
end-processing

6.

PQR phosphorylation
(in pink)

ABCDE phosphorylation
(in yellow)

7.

X4/L4

8.

further autophosphorylation
induces dissociation

9.

Modified from Meek et al, Immunol. Rev, 2004, 200 (1) 132-141

binding protein has been shown to interact with DNA-PK (Yavuzer *et al.*, 1998). C1D has also been shown to play a role in repairing the DSBs via NHEJ and homologous recombination in yeast (Erdemir *et al.*, 2002). Its role in DNA repair in higher eukaryotes is still under investigation.

Figure 1 is a simplified depiction of how the NHEJ pathway functions.

2.2 Role of Ku in NHEJ

Ku was identified as an autoantigen that cross-reacted with the immune sera from patients with autoimmune disorders like systemic lupus erythromatosus and polymyosytis-scleroderma (Mimori *et al.*, 1981; Mimori *et al.*, 1986). Its role in DSB repair was elucidated using a radiosensitive CHO cell line, xrs-6. Xrs-6 belongs to a group of rodent cell lines that have impaired DSB repair and V(D)J recombination (Lee *et al.*, 1996). Complementation studies helped identify the defective gene in these mutants to be a human gene XRCC5. (XRCC denotes X ray cross complementing) (Jeggo *et al.*, 1992). Ku80 was identified as the product of XRCC5 gene (Taccioli *et al.*, 1994). Human Ku80 cDNA was shown to substantially complement the DSB rejoining defect in xrs-6 cells (Ross *et al.*, 1995).

Ku is a heterodimeric protein comprised of Ku70 and Ku80. The heterodimer functions as the regulatory subunit of the protein complex DNA-PK (DNA-dependent protein kinase). Ku binds to the broken DNA ends, recruits and activates DNA-PKcs (DNA-PK catalytic subunit), a ~450 kDA serine/threonine kinase (Doherty and Jackson, 2001).

Ku70 (613 aa) and Ku80 (735 aa) tightly associate with each other and this association plays a role in stabilization of the two subunits in the cell (Singleton *et al.*, 1997). The central domain in Ku80 is comprised of amino acids 449 to 477 and has been shown to be essential for its interaction with Ku70 (Osipovich *et al.*, 1997; Cary *et al.*, 1998; Osipovich *et al.*, 1999). Also, the C terminal (12 aa) of Ku80 has been shown to be involved in its interaction with DNA-PKcs and this interaction is essential for DNA–PK activity. It is interesting to note that this C-terminal 12 aa is conserved in human, mouse and hamster Ku80 but is absent in the *C. elegans* and *S. cerevisae* Ku homologues. This correlates with the observation that orthologues of DNA-PKcs are present in all vertebrates, but not in yeast (Gell and Jackson, 1999; Singleton *et al.*, 1999).

Additionally, both Ku70 and Ku80 have an N-terminal domain that belongs to the vonWillebrand factor A (VWA) family of domains. These domains are known to mediate protein-protein interactions. Thus, the VWA domain in Ku heterodimer might be involved in recruiting proteins to the site of DNA damage (Wu and Lieber, 1996; Doherty and Jackson, 2001) For example, WRN (Werner syndrome protein), a RecQ family of DNA helicases with a 3'-5' exonuclease activity, has been shown to interact with this domain of Ku80 (Karmakar *et al.*, 2002). This binding has no effect on helicase activity of WRN but broadens the exonuclease specificity and enhances its processivity (Li and Comai, 2000). It is suggetsed that Ku might play a role in activation of WRN to remove certain replication blocks (Orren *et al.*, 2001).

Also, Doherty and Jackson speculated that this domain might play an important role in aligning the broken DNA ends via interaction between two Ku heterodimers in trans (Doherty and Jackson, 2001).

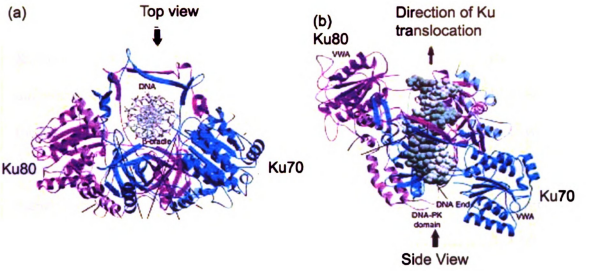
Goldberg and colleagues recently elucidated the crystal structure of the Ku heterodimer bound to DNA (Walker *et al.*, 2001). The structure showed Ku in an open ring shape forming a narrow bridge through which two turns of DNA helix are threaded. Majority of the contacts in the Ku-DNA complex are with sugar-phosphate backbone and not with bases directly. This provided an explanation for why Ku lacks significant sequence preference for DNA. This unusual mode of Dna binding by Ku also expains as to how Ku can translocate internally along DNA leaving the DNA ends accessible to other proteins (Fig. 2). Interestingly, the structure for free Ku heterodimer is similar to the DNA-bound form. This raises a major question as to how the threaded Ku complex becomes dissociated from the DNA once the DNA has religated?

2.3 Physiological relevance of DNA-PK's kinase activity in NHEJ

Cells lacking functional DNA-PKcs are radiosensitive and lack the ability to repair DSBs via NHEJ (Jackson and Jeggo, 1995; Peterson *et al.*, 1995). The repair defects can be reversed by ectopic expression of DNA-PKcs. However, deletion and site directed mutants in the kinase domain of DNA- PKcs cannot reverse the repair deficits of DNA-PKcs deficient cells demonstrating that the protein kinase activity of DNA-PKcs is requisite for its role in NHEJ (Kurimasa *et al.*, 1999; Kienker *et al.*, 2000).

Fig. 2. Crystal Structure of Ku heterodimer-DNA complex: Ku70 is depicted in blue strands and Ku80 in pink. The structure lacks the extreme carboxy terminus for both Ku70 and Ku80, however their relative positions are indicated. The side view highlights the orientation of Ku heterodimer with respect to the broken or free DNA end. Ku70 is located proximal and Ku80 distal, to the broken DNA end.

Fig. 2



Modified from Doherty and Jackson, Current Biology 2001,11; R920-923 and Walker et al, Nature 2001, 412; 607-614

2.4 Autophosphorylation of DNA-PKcs:

In vitro studies have shown that autophosphorylation of DNA-PK occurs on Ku70, Ku80 and DNA-PKcs (Chan *et al.*, 1999; Douglas *et al.*, 2002). When autophosphorylated, DNA-PKcs dissociates from the Ku-DNA complex which results in the loss of kinase activity (Chan and Lees-Miller, 1996; Merkle *et al.*, 2002). Cryo-electron microscopic studies have shown that the number of DNA-protein complexes is reduced when DNA-PK is phosphorylated and are restored in the presence of a protein phosphatase-1 (PP1). The loss of kinase activity was further corroborated by EMSA and kinase assays (Douglas *et al.*, 2001; Merkle *et al.*, 2002). Further, seven phosphorylation sites were identified in DNA-PKcs using Mass spectrometery (MS). Of these seven sites, four sites have been shown to be phosphorylated *in vivo* using phosphospecific antibodies (Douglas *et al.*, 2002).

Recently our lab has shown that mutating six phosphorylation sites in the above mentioned cluster in DNA-PKcs abolishes its capacity to restore radioresistance in DNA-PKcs deficient cell line, V3. The phosphorylation site mutant DNA-PKcs (ABCDE) was able to phosphorylate other substrates like XRCC4 and Artemis and thus was shown to have normal kinase activity (Ding *et al.*, 2003). Additionally, the mutant protein was shown to have reduced activity in V(D)J recombination. Sequence analysis of the joints that did recombine in the presence of mutant protein revealed a lack of DNA end processing.

Since autophosphorylation has been shown to cause disassembly of DNA-PKcs with concomitant loss in the kinase activity (Chan and Lees-Miller, 1996; Merkle *et al.*, 2002), the non-phosphorylatable DNA-PKcs should have a constitutive kinase activity and should be resistant to the loss of kinase induced by pre-autophosphorylation. Surprisingly, biochemical analysis using purified wild type and mutant DNA-PKcs (ABCDE) revealed that the mutant protein is only slightly more resistant to the loss of kinase activity compared to the wild type DNA-PKcs (Ding *et al.*, 2003) suggesting additional phosphorylation sites might be involved in the disassembly of DNA-PKcs from Ku/DNA complex. Moreover, this mutant protein still underwent substantial autophosphorylation *in vitro* (Ding *et al.*, 2003; Reddy *et al.*, 2004). Five potential phosphorylation sites outside of ABCDE were identified and these sites were mutated to alanine. The construct generated was designated as PQR (Cui *et al*, unpublished data). When expressed in V3 cells and compared to wild type and ABCDE mutant, phosphorylation at PQR was found to have a reciprocal function to ABCDE phosphorylation in regulating end access to processing factors. Ding *et al* proposed a model suggesting that the conformation of ABCDE (unphosphorylated DNA-PKcs) does not allow end processing factors to access the DNA termini and thus the breaks are left unrepaired, leading to increased cell death. This was further corroborated by studies from Reddy *et al* and Block *et al* in *in vitro* end-joining assays (Block *et al.*, 2004; Reddy *et al.*, 2004). The recent data with the PQR mutant (partially unphosphorylated DNA-PKcs) suggests an extension of the above model where phosphorylation at ABCDE would allow the DNA-ends to be accessible for end processing and subsequent phosphorylation at PQR would block this access and promote interaction with XRCC4/ligaseIV to ligate the

processed ends (Cui et al, unpublished data). There is further evidence that autophosphorylation state of DNA-PKcs may also guide DSBs to be either repaired by HR or NHEJ. Whereas initial phosphorylation at ABCDE promotes HR, subsequent phosphorylation at PQR would block HR.

Finally, neither ABCDE nor PQR phosphorylation events cause DNA-PKcs to dissociate from DNA. The autophosphorylation events that cause the dissociation and kinase inactivation are being currently investigated.

2.5 Phosphorylation of Ku heterodimer

The above studies have established autophosphorylation of DNA-PKcs to be a physiologically important step in NHEJ, making DNA-PKcs to be the first unequivocally physiologically relevant substrate. Ku is the DNA binding partner of DNA-PKcs and has been shown to be phosphorylated by DNA-PKcs (Yaneva and Busch, 1986; Boubnov and Weaver, 1995; Chan *et al.*, 1999). MALDI based studies on phosphorylation of Ku in Susan Lees-Miller's laboratory resulted in identification of DNA-PKcs target sites; serine 577, serine 580 and threonine 715 in Ku80, and serine 6 in Ku70 (Chan *et al.*, 1999). Jin and Weaver identified serine 51 as another phosphorylation site in Ku70 (Jin and Weaver, 1997).

It has been suggested previously that the preferred substrate consensus of DNA-PK is a serine/threonine followed by a glutamine (SQ/TQ motif) (Lees-Miller *et al.*, 1992; O'Neill *et al.*, 2000). However, *in vitro*, purified DNA-PK can phosphorylate

serine and threonine residues that are followed by hydrophobic amino acids such as tyrosine, leucine or alanine (Chan *et al.*, 1999; Douglas *et al.*, 2002; Yu *et al.*, 2003). Four out of five sites in Ku70/80 are S/T followed by a hydrophobic residue and serine 51 in Ku70 is a SQ site. When compared across vertebrates, serine 6 was conserved in humans, rat, mouse, hamster but not in frog. Serine 51 in Ku70 was conserved in all vertebrates. Similarly in Ku80, where serine 577 was conserved across the vertebrates, serine 580 was not conserved in frog and threonine 715 was not conserved in hamster and frog (Table 2).

In vitro studies have shown that Ku's translocation along DNA stimulates DNA ligation by XRCC4/Ligase IV (Kysela *et al.*, 2003). Eventually Ku must dissociate from the DNA to allow replication of the repaired DNA. Ku binds with high affinity to DNA in *in vitro* assays and how Ku dissociates from DNA remains to be elucidated (Doherty and Jackson, 2001). Although *in vitro* studies have shown that phosphorylation of Ku is not involved in dissociation of Ku from DNA (Chan *et al.*, 1999), there is some evidence suggesting that it might have a role in translocation of Ku along DNA (Calsou *et al.*, 1999; Frit *et al.*, 2000). Also, it is possible that phosphorylation of Ku might work in concert with DNA-PKcs phosphorylation to facilitate dissociation of DNA-PKcs from Ku bound DNA. In the face of several unanswered questions concerning phosphorylation events in NHEJ, Ku makes an interesting phosphorylation substrate to study.

TABLE 1. Phosphorylation sites in Ku70 and Ku80 compared across vertebrates:

Ku70 aa	6	51	% identity	%similarity
Human	---MSGWE **S** YYKT----- SLI FLV DASK AMFE	**S**	100%	100%
Rat	---MSEWE **S** YYKT------SLI FLV DASR AMFE	**S**	76%	83%
Mouse	---MSEWE **S** YYKT------SLI FLV DASR AMFE	**S**	76%	83%
Hamster	---MSGWE **S** YYKT------SLI FLV DASR AMFD	**S**	77%	83%
X. laevis	---MTEWG **D** HFVN------SLI FLV DASK PMFE	**S**	62%	76%

Ku80 aa	577 580	% identity	%similarity
Human	AKKLKTEQGGAHF **S** V **S** **S** L AE	100%	100%
Rat	AKKYKTEKEEGHI **S** I **S** **S** L AE	76%	86%
Mouse	AKKYKTEKEEDHI **S** I **S** **S** L AE	76%	86%
Hamster	AKKCKTEKEEGHI **S** I **S** **S** V AE	78%	86%
X. laevis	AKKL KEDD—EGF **S** L L **R** L AD	61%	78%

	715
Human	APKD KPSGD **T** A A V FE E GGDVDDLLDM I
Rat	TPKD KAKED **T** T G L EE - GGDVDDLLDM I
Mouse	APKD KAKED **T** T G P EE - AGDVDDLLDM I
Hamster	APKD KAKED **A** A G L EE - GGDVDDLLDM I
X. laevis	AQKE E- KVE **E** A A M ME D EGDVDDLLDMM

% Identity and % similarity represents homology across whole Ku70 or Ku80

2.6 Structure of DNA-PKcs

Electron microscopic structure of DNA-PKcs at 30 Å resolution is shown to contain three distinct regions which the authors have labeled as "head", "palm" and "arm" (Boskovic *et al.*, 2003). The arm region connects the head to the palm. The structure further showed that when bound to DNA, DNA-PKcs undergoes substantial conformational change compared to the unbound protein. When bound to DNA, the palm region of the protein bends and locks around DNA and comes in contact with the head region. (Fig. 3) The authors further speculated that this conformational change could be responsible for activation of the kinase. Previously, DeFazio *et al* had shown that

DNA-PKcs plays a role in bringing the broken ends together in a ligatable configuration, which they referred to as "synapsis". Using EM studies and pull down experiments, they further showed that DNA-PKcs dimerizes during this synaptic event and that addition of Ku enhances the ability of DNA-PKcs to mediate synapsis. The synapsis was also shown to be a required for the activation of kinase. These studies together suggest a model where in the two DNA-PKcs molecules bound to different DNA ends would form a bridge across the break. The conformational change accompanying the DNA end binding of DNA-PKcs and the "synapsis" of DNA ends would result in the activation of kinase, triggering the phosphorylation events (autophosphorylation of DNA-PKcs) required for NHEJ.

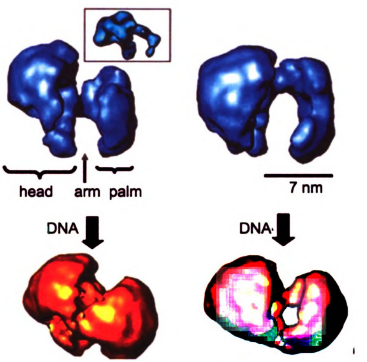
Recently, a cryo EM three dimensional structure of 13Å resolution was generated (Rivera-Calzada *et al.*, 2005) (Fig. 4). This higher resolution allowed more detailed

definition of structural domains and revealed several new features. Using threading and docking techniques, the authors placed the kinase domain in the head region of DNA-PKcs. At this higher resolution the palm domain was further resolved into two distinct segments or claws designated as proximal and distal claw. The two claws together maintain a palm structure observed in the low resolution, 30Å structure.

Sequence-based predictions led the authors to propose that the arm region is being formed by a succession of HEAT repeat segments. HEAT repeat is a member of a family of repeat motifs that form superhelices or solenoids. A HEAT repeat comprises two helices A and B, which form helical hairpin. The A helices from the convex surface of the superhelix and B helices form the concave surface. Brewerton *et al* had first identified the presence of HEAT repeats in the region N-terminal to the kinase domain in DNA-PKcs and had further hypothesized that the HEAT repeats are present in the more curved part of the DNA-PKcs (Brewerton *et al.*, 2004). Further, by placing major domains of the kinase in the structure, Rivera -Calzada *et al* could approximately predict the physical locations of "landmark" residues. The flow of the polypeptide placed the first ~800 residues to constitute the distal claw and associated arm region and residues 800-1900 forming the major segment of the arm and the proximal claw. The autophosphorylation sites (T2609, S2612, T2620, S2624, T2638, T2647) were mapped to the shoulder, which is the region connecting the arm to the head. This placement would make these sites inaccessible to the active site of the same molecule, hence suggesting autophosphorylation of DNA-PKcs occurs in trans via interaction with a second molecule. The LRR region maps to the proximal claw of the arm in the structure.

Fig. 3. Two views of DNA-PKcs 3D structure (30Å): The structures in blue are free DNA-PKcs molecules and orange are DNA-bound complexes. The insert shows a comparable view of ATM.

Fig. 3



Modified from Boskovic et al, EMBO J. 2003, 22(21); 5875-5882

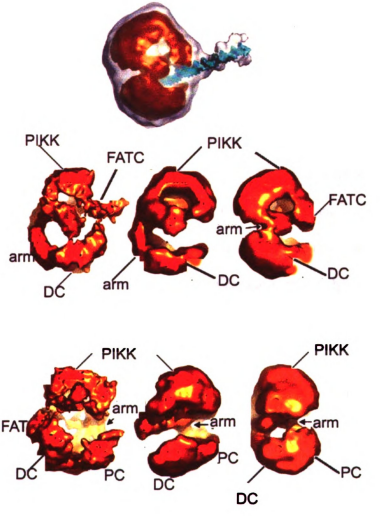
Fig. 4. Conformational changes of DNA-PKcs upon DNA binding:

(A) Negative-stain EM structure (gold) of DNA-PKcs bound to one end of a 54 bp DNA duplex. The DNA (blue) which is clearly visible at a lower contour level (white) passes between and is in contact with the edges of head and palm along its backbone, with the blunt end in contact with the inner face of the palm

(B) Montage of the 13Å cryo-EM structure of DNA-PKcs and the 30Å negative-stain structures for DNA-PKcs in the absence and presence of DNA. Functional domains identified in the cryo-EM structure and their counterparts in the negative-stain structures are labeled as follows: PIKK- catalytic domain; arm-man array of HEAT repeats; DC-distal calw of the palm; PC-proximal claw, FAT-proetruding end of the FAT domain, FATC-FATC domain.

(C) as **(B)** but rotated ~90° around the vertical. The change in position of FATC domain, FAT protrusion and claws on DNA binding is evident.

Fig. 4



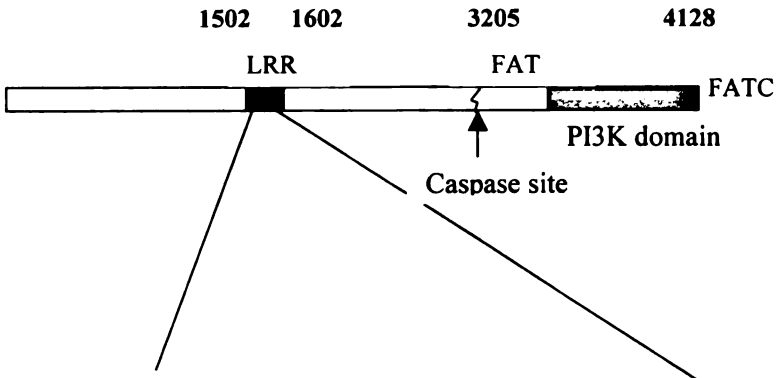
Modified from Rivera-Calzada et al, Structure 2005, 13; 243-255

2.7 Lecuine Rich Region in DNA-PKcs

A potential leucine zipper motif within the DNA-PKcs coding sequence spanning residues 1503-1538 was noted by Hartley *et al.* in the original sequence analysis of the molecule (Hartley *et al.*, 1995). It consists of 6 leucine residues, each separated by six residues. However, as observed by Fujimori *et al*, the first and fourth leucine residues (aa1502 and aa1524 respectively) are substituted in chicken and *Xenopus*, however the second and third leucine residues (aa 1510 and aa 1517) are conserved among all vertebrates (Fujimori *et al.*, 2000). More recently, other investigators consider this region (1503-1539) to be more appropriately designated as a leucine rich region (33% leucine). A more extensive overlapping region (1503-1602) is also relatively leucine rich (20%). DNA-PKcs is highly homologous (63-85%) in the six species for which sequences are available, (human, horse, dog, mouse, chicken and frog). The leucine rich region (LRR) is similarly well conserved (Fig. 5). The LRR has also been shown to interact with a nuclear matrix, DNA binding protein C1D (Yavuzer *et al.*, 1998). The authors further showed that C1D could activate DNA-PKcs in absence of DNA ends.

Fig. 5

A.



B.

```
                1502                                                1539
                   SLDPSCKRLASGLLELAFAFGGLCEHLVSLLLNTAVLS
human              ---L---Q----------------R-------P----
Equine             ----N--------------------------D-T---
Canine             ---------------------------D---D-----
Murine             -------S--N-------G-----D--------S-M--
Gallus             AV-I-S----DR--Q----IDDQ--E--------V---
Xenopus            ---I-S----E-------M---Q--E--------VI--
                                  **
WT DNA-PKcs        SLDLSCKQLASGLLELAFAFGGLCERLVSLLLNPAVLS...
LRR DNA-PKcs       SLDLSCKQLASGLLDPAFAFGGLCERLVSLLLNPAVLS...
```

C. % Homology with huDNA-PKcs

	complete	LRR
Equine	84.5	82.1
Canine	82.9	84.6
Murine	78.8	78.9
Gallus	68.4	61.5
Xenopus	63.3	74.4

Fig. 5. Digrammatic representation of DNA-PKcs: A. A line diagram depicting various domains in DNA-PKcs **B.** Mutations introduced in LRR. **C.** LRR compared across vertebrates

2.8 C1D

C1D was identified as a 16kDa polypeptide found to be covalently associated with genomic DNA covalently. The cDNA was first cloned by expression cloning using a monoclonal antibody to polypeptides released from rigorously extracted and nuclease-digested DNA (Nehls *et al.*, 1998). The authors proposed it to be an epigenetic factor involved in gene regulation. Later, C1D was found to be associated with the transcriptional repressor RevErb and nuclear corepressors NcoR and SMRT, which led to the hypothesis that C1D is a component of the complex involved in transcriptional repression and was also in agreement with the role previously proposed for C1D (Zamir *et al.*, 1997).

2.8.1 C1D's role in apoptosis:

Using human C1D cDNA as a probe with poly(A)$^+$ RNAs from 50 human tissues, C1D was shown to be expressed in all cells (Rothbarth *et al.*, 1999). Rothbarth *et al* also concluded that the expression level of the endogenous gene is cell type-specifically regulated and that different cell types require and /or tolerate different levels of C1D protein. When over-expressed in cells, C1D triggers apoptosis. Rothbarth *et al* speculated that over-expression of C1D mimics or substitutes for DNA strand breaks, resulting in an initial DNA-end independent activation of DNA-PK, which further results in p53 activation and finally leads to apoptosis. However, they did not provide any biochemical evidence for this pathway being activated by over-expression of C1D. Another study by the same authors showed that inhibition of proteasome-dependent proteolysis results in increase in the expression of C1D and similar increase in expression

on proteasome inhibition is also true for other apoptosis-related gene products like p53, p73, p21Cip1, p27Kip and caspases (Rothbarth *et al.*, 2002).

2.8.2 C1D in NHEJ:

Yavuzer *et al* in their study had shown that the protein and mRNA levels of C1D increase upon γ-irradiation. Also, they had identified C1D to be the first substrate that was capable of activating DNA-PK in the absence of free-ends (Yavuzer *et al.*, 1998). With C1D being also identified as a player in apoptosis, it is highly likely that C1D plays a role in DSB repair by either contributing to the activation of DNA-PK to help repair the breaks or triggering apoptosis if the damage is beyond repair. Further, C1D was identified as a polypeptide that might be involved in higher order chromatin folding. The polypeptides that are covalently bound to DNA, like C1D, form a part of nuclear matrix and some of them have been reported to be involved in targeting a subset of genomic DNA to the nuclear matrix (Neuer *et al.*, 1983; Neuer and Werner, 1985). Given the role of nuclear matrix in the regulation of important cellular events such as DNA replication, transcription, RNA splicing and DSB repair (Mullenders *et al.*, 1984; Nelson *et al.*, 1986; Nelson and Coffey, 1987; Getzenberg *et al.*, 1991; Koehler and Hanawalt, 1996), it seems possible that C1D plays an important role in DSB repair.

Using *S. cerevisiae* as a model organism, Erdimir *et al*, analysed the consequence of loss of functional C1D. In agreement with the proposed function of C1D, *yc1d* mutant was sensitive for growth at 39°C, which is a common phenotype of mutants defective in preservation of genomic integrity (Boulton and Jackson, 1996a, b; Hryciw *et al.*, 2002).

Like *yku70, yc1d* was slightly sensitive to γ-irradiation. Using plasmid based assays, the authors further showed that *yc1d* mutant was defective in DNA-end joining, specifically 3'-end processing. Also, in the absence off Yc1dp, 50% of the 3'-overhanging breaks were repaired inaccurately, suggesting that Yc1dp might play a role to suppress error prone DSB repair pathway and help in maintaing genomic integrity. When tested for HR, *yc1d* showed two fold reduction in HR efficiency compared to the wild type. Further, the double knock out *yc1d/yku70* were more deficient in 3'-end joining when compared to the single mutants, suggesting the two proteins co-operate in this process (Erdemir *et al.*, 2002) in yeast.

In spite of all the above evidence, the role of C1D in DSB repair in higher eukaryotes is still a subject of speculation. Yavuzer *et al* had shown that C1D interacts with DNA-PKcs directly through the leucine rich region (LRR) of DNA-PKcs and also activates the kinase. Additionally, since the LRR is approximately mapped to the proximal claw of the arm region, the LRR might be a putative DNA binding domain in DNA-PKcs.

Thus, in mystudy I disrupted the LRR motif in DNA-PKcs, using site directed mutagensis, to better understand the role of C1D in DSB repair and its interaction with DNA-PKcs.

2.9 DNA binding of DNA-PKcs in the absence of Ku

Studies with purified DNA-PKcs have shown that DNA-PKcs is capable of binding to DNA by itself. This DNA binding activity was strongest in low salt (Hammarsten and Chu, 1998) and decreased sharply when the salt concentration increased. Further, this complex was biochemically functional and this DNA binding can activate the kinase, however the activation was specific for dsDNA ends. This study was corroborated by another study from Yoo and Dynan who concluded that DNA-PKcs makes extensive contacts with both strands of the DNA in the vicinity of DNA termini (Yoo and Dynan, 1999). Using iodopyridinated probes and cross linking experiments the authors further concluded that DNA-PKcs caps the free DNA ends and displaces Ku toward interior positions on DNA. These studies suggest that DNA-PKcs has DNA binding domains, which might play a role in DNA-PKcs function in DNA repair. The 13Å structure of DNA-PKcs bound to DNA does provide some insight into the regions making contacts with DNA (Rivera-Calzada *et al.*, 2005). The EM images suggest the stabilization of DNA termini by the arm, palm and FATC domain of DNA-PKcs (Fig 4).

CHAPTER 3

DNA-DEPENDENT PHOSPHORYLATION OF KU70/80 IS NOT REQUIRED FOR NON HOMOLOGOUS END JOINING

ABSTRACT:

The Ku70/80 heterodimer is a major player in the non-homologous end joining pathway for the repair of IR-induced DNA double-strand breaks (DSBs). Once bound to a DSB, Ku recruits the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) to form the DNA-dependent protein kinase holoenzyme complex (DNA-PK). Our collaborator Susan Lees-Miller had previously identified four DNA-PK phosphorylation sites on the Ku70/80 heterodimer: serine 6 of Ku70, serine 577 and 580 and threonine 715 of Ku80. Jin and colleagues had identified serine 51 as yet another phosphorylation site in Ku70. This raised the interesting possibility that DNA-PK dependent phosphorylation of Ku provide a mechanism for regulation of non-homologous end joining. Here, using site directed mutagenesis of each of these sites we show that phosphorylation at any or all of these sites is not required for non-homologous end joining. Ku containing serine/threonine to alanine mutations at these sites was fully able to complement the radiation sensitivity of Ku negative mammalian cells, indicating that phosphorylation at these sites is not required for Ku's function in non-homologous end joining. Surprisingly, data from our collaborator's laboratory showed that okadaic acid-induced phosphorylation of Ku did not require the presence of either DNA-PKcs or the

related protein kinase, ATM, but was sensitive to the protein kinase inhibitor, staurosporine. This suggested that in addition to DNA-PKcs, Ku is phosphorylated by a staurosporine sensitive kinase. In sum, we conclude that DNA-PKcs is not the only kinase that phosphorylates Ku in cells and that DNA-PK mediated phosphorylation of Ku is not required for DNA double strand break repair.

INTRODUCTION:

The Ku70/80 heterodimer is required in the non-homologous end joining (NHEJ) pathway for the repair of (IR) induced DNA double-strand breaks (DSBs) (Ross *et al.*, 1995; Lee *et al.*, 1996). *In vitro*, Ku binds to ends of double stranded (ds) DNA with high affinity (Blier *et al.*, 1993; Dynan and Yoo, 1998). Once bound to a DNA end, Ku recruits the catalytic subunit of the DNA-dependent protein kinase, DNA-PKcs, to the DSB, which stimulates the activity of DNA-PKcs approximately ten-fold (Gottlieb and Jackson, 1993; Smith and Jackson, 1999). The protein kinase activity of DNA-PK is essential for NHEJ (Kurimasa *et al.*, 1999; Kienker *et al.*, 2000) and inhibitors of DNA-PK activity inhibit NHEJ *in vitro* and *in vivo* (Baumann and West, 1998; Chernikova *et al.*, 1999). Since DNA-PK assembles and becomes active at a DSB, it is reasonable to assume that its physiological targets could also be present and be phosphorylated at the DSB. Indeed, all factors known to be required for NHEJ can be phosphorylated by DNA-PK *in vitro*, and, in some cases, *in vivo*. Chan *et al* had shown previously that DNA-PK phosphorylates serine 6 of Ku70, and serines 577, 580 and possibly 579, as well as threonine 715 of Ku80 *in vitro* (Chan *et al.*, 1999). Additionally, serine 51 in Ku70 was also identified as a DNA-PK target site (Jin and Weaver, 1997). Other components of the NHEJ pathway that are phosphorylated by DNA-PK *in vitro* include XRCC4 (serines 260 and 318) (Yu *et al.*, 2003; Lee *et al.*, 2004), DNA ligase IV (threonine 650 and either serine 668 or 672) (Wang *et al.*, 2004), and Artemis (Ma *et al.*, 2002). Phosphorylation of DNA ligase IV is not required for DNA end joining *in vitro*, but may affect the stability of DNA ligase IV *in vivo* (Wang *et al.*, 2004). Similarly, phosphorylation of XRCC4 is not required for NHEJ (Yu *et al.*, 2003). DNA-PKcs has been shown to

autophosphorylate itself both *in vitro* and *in vivo* and these autophosphorylation events have been shown to be required for NHEJ (reviewed in section 2.4).

Since Ku is the DNA targeting subunit of DNA-PK, we considered it an excellent candidate for a physiological substrate of DNA-PK. Ku70 and Ku80 each contain a central DNA binding region that is flanked by unique amino terminal and carboxy-terminal regions (Gell and Jackson, 1999). The central DNA binding region of the Ku70/80 heterodimer forms a basket like structure that cradles a strand of dsDNA (Walker *et al.*, 2001). In addition, Ku70 contains a highly acidic amino terminal region of unknown function, and carboxy terminal SAP (SAF-A/B, Acinus and PIAS) domain that may be important for DNA binding (Walker *et al.*, 2001). Ku80 contains a C terminal domain comprised of a helix-turn helix-motif (Harris *et al.*, 2004) and, at the extreme C terminus, a small region of approximately 12 amino acids that represents the minimal sequence required for interaction with DNA-PKcs (Gell and Jackson, 1999; Singleton *et al.*, 1999; Falck *et al.*, 2005). Interestingly the identified DNA-PK phosphorylation sites all lie in the unique N and C terminal domains of Ku70/80 (Chan *et al.*, 1999). These regions are close to the DNA-binding canal and/or are required for the interaction of Ku with DNA-PKcs.

The protein kinase activity of DNA-PK is required for NHEJ and Ku70 and Ku80 are phosphorylated by DNA-PKcs *in vitro*, therefore Ku70 and Ku80 were candidate physiological substrates of DNA-PK. Given the proximity of the *in vitro* phosphorylation sites to the DNA binding region of the Ku heterodimer, one attractive

hypothesis was that DNA-PK-mediated phosphorylation of Ku could displace Ku from DNA ends prior to religation. Thus, we hypothesized that phosphorylation of Ku by DNA-PK could be important for NHEJ. However, when tested *in vivo* by expressing alanine or aspartic acid mutants of the phosphorylation sites, none of the sites seem to play a role in NHEJ. To further investigate the physiological importance of Ku phosphorylation, our collaborator Dr. Lees Miller's laboratory raised phosphospecific antibodies to serine 6 of Ku70, and serines 578, 580 and threonine 715 of Ku80. Using these antibodies, they show that Ku is phosphorylated at these sites *in vivo* in okadaic acid treated cells under conditions previously shown to inhibit protein phosphatase 2A (PP2A). Surprisingly, OA-induced phosphorylation of Ku was ablated by preincubation with staurosporine but not with wortmannin. Further, using phosphospecific antibodies in DNA-deficient and ATM-deficient cells, they show that Ku gets phosphorylated at these sites *in vivo* even in the absence of DNA-PKcs or ATM.

Thus, we conclude that a staurosporine sensitive kinase phosphorylated Ku in addition to DNA-PKcs and that DNA-PK dependent phosphorylation of Ku is not required for DSB repair.

MATERIALS AND METHODS:

Immunoblotting:

To assess expression of transfected Ku70 and Ku80, the indicated amounts of whole cell extracts from clonal transfectants were analyzed after electrophoresis on a 7.5% SDS polyacrylamide gel and transfer to PVDF membrane. Polyclonal rabbit antisera raised against Ku80 or Ku70 (Serotech, Raleigh, NC) was used as the primary antibody (1:5000 dilution). In some experiments, V5 tagged Ku70 was detected with an anti-V5 conjugated horseradish peroxidase (1:5000 dilution). Anti-rabbit IgG conjugated to horseradish peroxidase was used as the secondary antibody (1:5000 dilution). Membranes were incubated with a chemiluminescent substrate (ECL, DuPont) according to the manufacturer's recommendations.

Phosphorylation of Ku by DNA-PKcs:

Purified DNA-PKcs (600 ng) and Ku 70/80 (200 ng) were incubated in the presence of 50 mM Hepes/NaOH (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.25 mM unlabelled (non radioactive) ATP, plus sonicated calf thymus DNA at (10 mg/ml). Samples were analyzed by SDS/PAGE on 10% acrylamide gels followed by coomassie blue staining and autoradiography.

Cell culture and cell extracts and dsDNA cellulose pulldown assays:

The *xrs*-6, Ku80 deficient cell line was a generous gift from Dr. David Roth (New York University). Cells were maintained in F12 nutrient medium with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD). Stable transfectants were maintained with 2.5 μg/ml Blasticidin. Transfectants co-expressing human Ku80 and human Ku70 were maintained with 2.5 μg/ml blasticidin and 100 μg/ml G418. *xrs*-6 transfectants expressing human Ku70 and/or human Ku80 were derived by stably transfecting cells with 40μg PvuI I linearized plasmid. Transfections were performed in 60 mm dishes using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were placed under selection conditions (2.5μg/ml blasticidin or 100μg/ml G418) and isolated clones derived. Individual colonies were screened for Ku80 or Ku70 expression by immunoblot analysis and further cultured.

$Ku70^{-/-}$ ES cells were a generous gift from Dr. Fred Alt (Harvard University Medical School). Cells were maintained in DMEM supplemented with 20% fetal calf serum and ESGRO (Chemicon, Temecula, CA) in gelatinized 60mm dishes. Stable transfectants were maintained with 5μg/ml blasticidin. $Ku70^{-/-}$ ES cell transfectants expressing Ku70 were derived by stably transfecting cells with 25 μg PvuI I linearized plasmid. Transfections were performed as described above.

Oligonucleotides:

The following oligonucleotides (and complements) were used generate Ku 70 and 80 mutants.

70S6A : 5' ATT TGC GGC CGC AGA TGT CAG GGT GGG AGG CCT ATT

70S51A : 5' GGC TAT GTT TGA AGC TCA GAG TGA AGA TGA G

70S6D : 5' ATT TGC GGC CGC AGA TGT CAG GGT GGG AGG ACT ATT

70S51D: 5' ATG TTT GAA GAC CAG AGT GAA G

80S577-80A : 5' GGA GCC CAC TTC GCT GTC TCC GCT CTG GCT GAA GGC

80S715A: 5' AGT GGA GAC GCT GCA GCT GTA T

80S577-80D : 5' GGA GCC CAC TTC GAC GTC TCC GAC CTG GCT GAA GGC

80S715D: 5' AGT GGA GAC GAC GCA GCT GTA T

Construction and transfection of expression plasmid:

Human Ku80 and Ku70 cDNAs were cloned from human mRNA into bacterial expression plasmids. For expression in mammalian cells, the Ku80 cDNA was subcloned into pcDNA6A (Invitrogen, Carlsbad, CA) and the Ku70 cDNA was subcloned into plncx2 and pEF6-V5/His C (Invitrogen, Carlsbad, CA).

Assessment of Radiation Sensitivity:

Cells (1×10^3) in serum free media were exposed to various amounts of ionizing radiation using a ^{60}Co source and immediately seeded in complete medium. After 7 days, cell colonies were fixed with methanol, stained with crystal violet and colony numbers are assessed.

V(D)J Recombination assays:

To assess V(D)J recombination in xrs-6 cells, RAG-1 and RAG-2 expression constructs (3 μg each), expression plasmids encoding Ku80, or the pcDNA6A vector control (6 μg) and a recombination substrate (pJH201 or pJH290; 1 μg) were transfected by liposome using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals). In some experiments, 6 μg of a terminal deoxynucleotidyl transferase (Tdt) expression plasmid was included in the transfections. Forty-eight hours later, plasmid substrates were rescued from the cells by alkaline lysis. Alkaline lysates were restricted with *Dpn*I and then used to transform chemically competent *Escherichia coli*. Transformed bacteria were spread onto two Luria-Bertani agar plates, one containing 100 μg of ampicillin/ml and the other containing 100 μg of ampicillin/ml plus 22 μg of chloramphenicol/ml. For sequence analyses, plasmid DNA was prepared from individual chloramphenicol-resistant colonies.

MTT Assays:

Cells (15×10^3) in serum free media were exposed to various amounts of ionizing radiation using a ^{60}Co source and immediately seeded into complete medium in gelatinized 24 well plates. After 5 days, the cells were incubated with MTT (1mg/ml) for 2-3 hours at 37°C. The cells were then solubilised in 200μl of acidic iso-propanol. Cell survival was assessed by plotting absorbance (590nm-650nm).

Generation and purification of Baculovirus expressed Ku:

Human Ku70 and Ku80 cDNA cloned into pFastBac Dual was a generous gift from Dr. Dale Ramsden's lab. A 300bp PCR product (using the Ku70 (S6,51A) in plncx2 as the template) was subcloned into pFastBac Dual at BamH1/ EcoR1 sites to generate alanine substitutions at serine 6 and 51 in Ku70. A 1Kb PCR product (using Ku80 (S577,580A, T715A) in pcDNA6A as the template) was subcloned into the pFastBac Dual at Sma1/Nhe1 sites to generate alanine substitutions at serine 577,580 and threonine 715 in Ku80. In each of these constructs Ku70 and Ku80 were co-transcribed and Ku70 was synthesized with a C-terminal His tag.

Pellets from Sf9 cells infected with baculovirus expressing Ku70 and Ku80 were lysed in Buffer C (20mM HEPES pH7.9, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, Complete Mini- Protease Inhibitor Cocktail tablets EDTA free (Roche), 5 mM imidazole) and incubated with Ni^{+}-agarose beads (Qiagen) (preequilibrated with Buffer C) for 1 hour at 4°C. The beads were washed three times in buffer C containing 50mM imidazole. The protein was then eluted in buffer C containing 500mM imidazole and dialyzed overnight against Buffer D (20mM HEPES pH 7.9, 20% glycerol, 100mM KCl, 0.2mM EDTA). For phosphorylation assays, either wild type Ku or Ku containing 5 serine/threonine to alanine mutations (Ku ALA) was incubated with purified human DNA-PKcs under standard autophosphorylation conditions as described above.

RESULTS:

Single site phosphorylation at S577 or S580 or T715 in Ku80 does not alter its role in NHEJ:

We first addressed if phosphorylation of any single site in Ku80 was important for its role in NHEJ. To address this, we used site directed mutagenesis to mutate the phosphorylation sites (serine or threonine) to alanine. Alanine, unlike serine or threonine, lacks a hydroxyl group and thus cannot be phosphorylated. Hence, mutating serine/threonine to alanine makes a protein non-phosphorylatable. Dr. Lees-Miller's laboratory supplied us with bacterial expression constructs for fusion proteins of the human Ku80 phosphorylation site mutants S577A, S580A and T715A. These were sub-cloned into eukaryotic expression vectors before I joined the laboratory. I started by stably transfecting these constructs in *xrs-6*, a cell line deficient in Ku80. The stable clones were screened by western blotting for protein expression (Fig. 6A) and evaluated for radioresistance.

Using single site mutants we wanted to first address if phosphorylation at any one site by itself was important for Ku's role in NHEJ. Since threonine 715 is located towards the C-terminus of Ku80 and is extremely close to the 12aa tail that interacts with DNA-PKcs, it seemed plausible that phosphorylation at threonine 715 might be involved in regulation of dissociation of Ku from DNA-PKcs and DNA. In that case, blocking phosphorylation at threonine 715 would block this disassembly and the downstream factors from accessing DNA ends. This would lead to a block in the repair of DSBs making cells radiosensitive.

However, as can be seen in Fig. 6B, cells expressing T715A mutant for Ku80 were as radioresistant as the cells expressing wild type Ku80. The same was also true for S577A and S580A mutants. Thus, I concluded that blocking phosphorylation at either S577, 580 or threonine 715 in Ku80 does not block Ku's role in NHEJ.

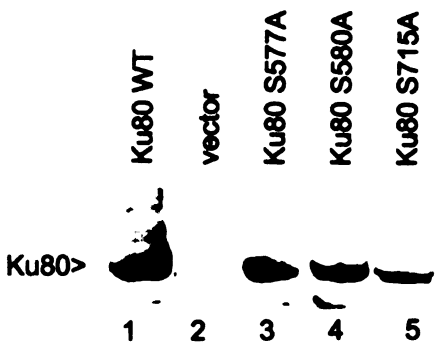
Multi-site phosphorylation in Ku80 does not alter its role in NHEJ:

Next, I blocked phosphorylation at all three sites in Ku80 by constructing a combined mutant cDNA (Ku80 ALA) to test whether multi-site phosphorylation (as opposed to single site phosphorylation) is required for Ku80's role in NHEJ. To address whether or not phosphorylation makes Ku non-functional, I also generated a cDNA expression vector for aspartic acid mutant of Ku80 where the three phosphorylation sites were mutated to aspartic acid (Ku80 ASP) (aspartic acid sometimes can mimic phosphoserine and phosphothreonine). Similar to above, stable transfectants were generated and screened by western blotting for protein expression (Fig. 7A, 8A). These clones were then assessed for radiosensitivity and compared to the stable clones expressing wild type Ku80 (Fig. 7B, 8B). Both, Ku80 ALA and ASP mutants reversed the NHEJ defect in *xrs-6* to the wild type levels. Further, transient V(D)J recombination assays revealed no functional deficits compared to wild type (Table 1). From this data, I conclude that Ku80 phosphorylation is not required for Ku's function in NHEJ.

Fig. 6. Alanine mutants at single site in Ku80 exhibit wild type complementation of NHEJ deficit in *xrs-6*: A. 100 μg of whole cell extracts from *xrs-6* cells stably transfected with vector alone (vector, lane 2) or wild type Ku80 (Ku80 WT, lane 1) or alanine mutant at single site in Ku80 (Ku80 S577A, lane 3; Ku80 S580A, lane 4; Ku80 T715A, lane 5) were analyzed by western blot for Ku80 expression. **B.** Radioresistance of *xrs-6* cells stably transfected with vector alone (crosses, solid line) or wild type Ku80 (solid circles, dotted line) or alanine mutant at serine 577 (Ku80 S577A, open circles/ solid line) or alanine mutant at serine 580 (Ku80 S580A, open squares/solid line) or alanine mutant at threonine 715 (Ku80 T715A, closed triangles/solid line) was determined by clonal survival assays as described in section 3.1.

Fig. 6

A.


Ku80 WT
vector
Ku80 S577A
Ku80 S580A
Ku80 S715A
Ku80>
1
2
3
4
5

B.

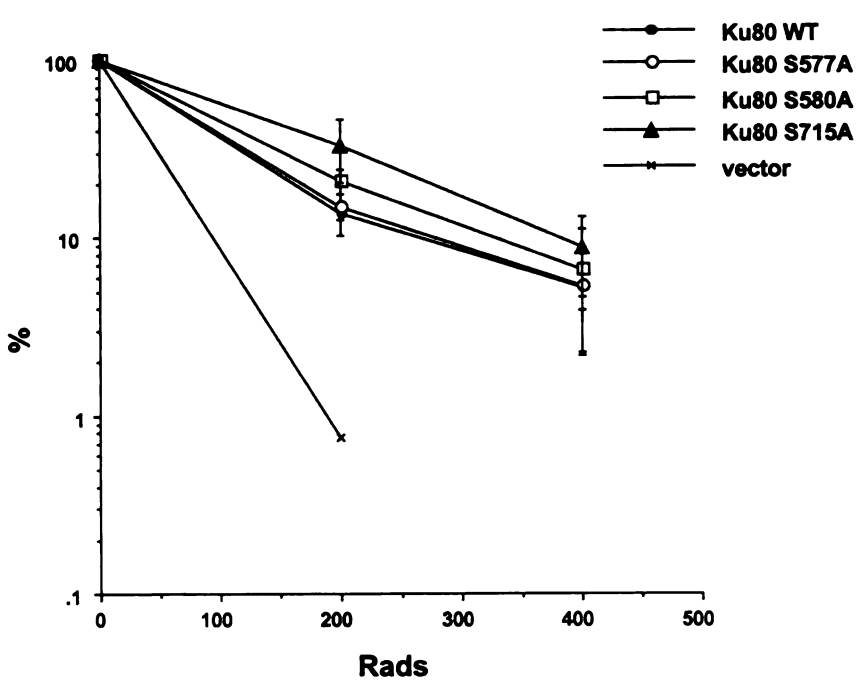

Ku80 WT
Ku80 S577A
Ku80 S580A
Ku80 S715A
vector
100
10
1
.1
%
0
100
200
300
400
500
Rads

Fig. 7. Multi site alanine mutant in Ku80 exhibit wild type complementation of NHEJ deficit in *xrs-6*: A. 100 μg of whole cell extracts from Chinese hamster ovary cells (CHO 9, lane 1) or *xrs-6* cells stably transfected with vector alone (vector, lanes 2-3) or wild type Ku80 (WT C, lane 4; WT D, lane 7) or Ku80 with serines 577, 580 and threonine 715 mutated to alanine (TM ALA 6, lane 5; TM ALA 8, lane 8) were analyzed by western blot for Ku80 expression. **B.** Radioresistance of *xrs-6* cells stably transfected with vector alone (open circles/solid line) or Ku80 WT (WT C, WT D, solid squares/solid line) or TM ALA (TM ALA 6, TM ALA 8, solid triangles/solid line) or CHO 9 (closed circles/solid line) was determined by clonal survival assays as described in section 3.1.

Fig. 7

A.


CHO 9
vector
vector
WT C
TM ALA 6
WT D
TM ALA 8
Ku80>
1 2 3 4 5 6 7 8

B.

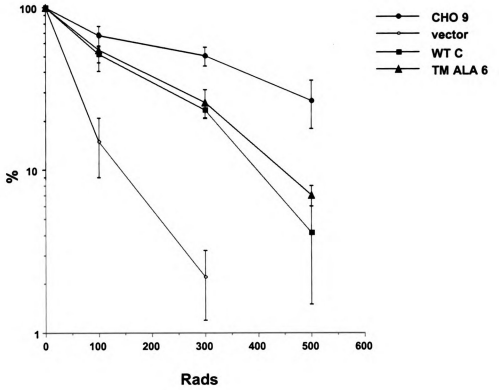

100
10
1
%
0
100
200
300
400
500
600
Rads
CHO 9
vector
WT C
TM ALA 6

Fig. 7B (cont.)

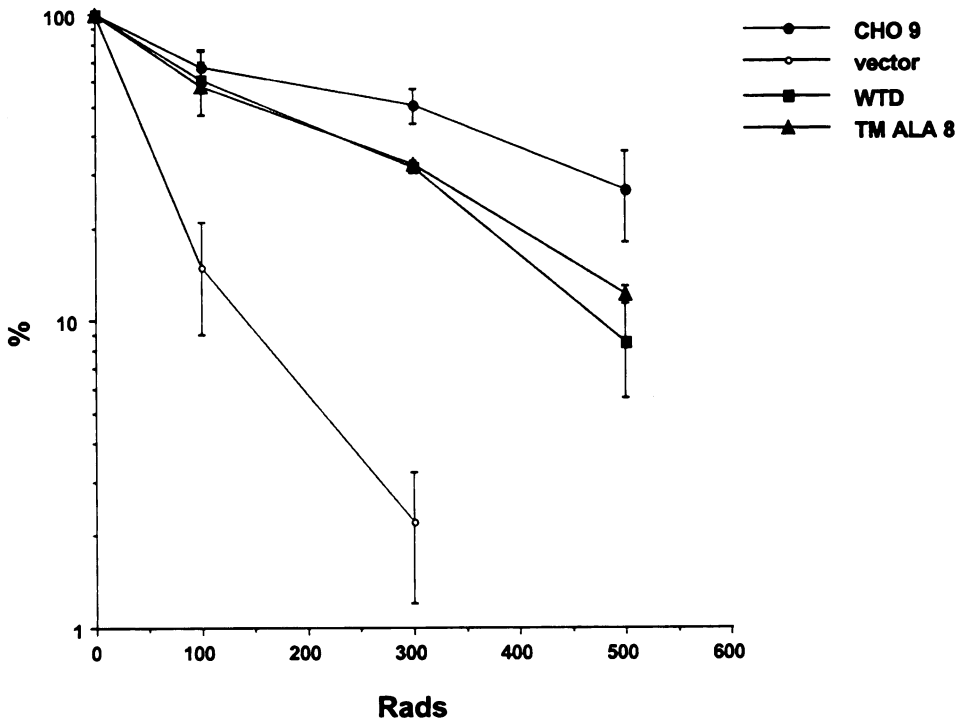
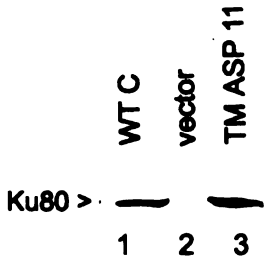


Fig. 8. Multi site aspartic acid mutant in Ku80 exhibit wild type complementation of NHEJ deficit in *xrs-6*: A. 100 μg of whole cell extracts from *xrs-6* cells stably transfected with vector alone (vector, lane 2) or wild type Ku80 (WT C, lane1) or Ku80 with serines 577, 580 and threonine 715 mutated to aspartic acid (TM ASP 11, lane 3) were analyzed by western blot for Ku80 expression. **B.** Radioresistance of *xrs-6* cells stably transfected with vector alone (open circles/solid line) or Ku80 WT (WT C, solid circles/solid line) or TM ASP (TM ASP 11, solid squares/solid line) was determined by clonal survival assays as described in section 3.1.

Fig. 8

A.


WT C
vector
TM ASP 11
Ku80 >
1
2
3

B.

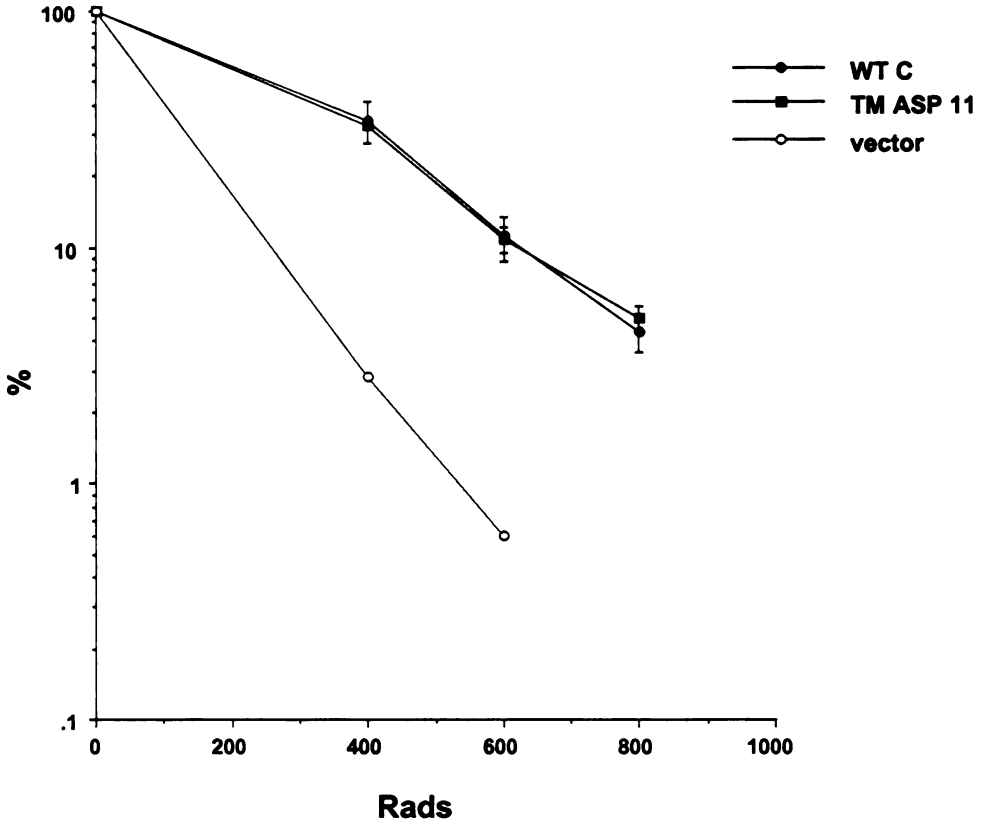

100
10
1
.1
%
0
200
400
600
800
1000
Rads
WT C
TM ASP 11
vector

Table 2.

coding joints (pJH290)

Transfected plasmids	#Amp Cam/ #Amp	Recombination (%)
RAGS only	2/47,040	0.004
RAGS + Ku80WT	39/68,600 60/140,532 35/53,312	0.05 0.042 0.065
RAGS + Ku80ALA	65/35,280 14/117,600 47/123,480	0.13 0.012 0.038

Table 2. Ku80ALA supports wild type levels of V(D)J recombination: Transient V(D)J recombination assays were performed as described in Experimental Procedures. RAG expression from plasmid vectors initiates recombination in the V3 cells, as assessed by the plasmid substrate pJH290 that detects coding joints or the pJH201 substrate that detects signal joints as indicated. Numbers of ampicillin (amp) and ampicillin/chloramphenicol (amp+cam) resistant colonies from at least three separate experiments are presented. Recombination rate (%R) is calculated as the number of chloramphenicol resistant colonies divided by ampicillin resistant colonies X 100.

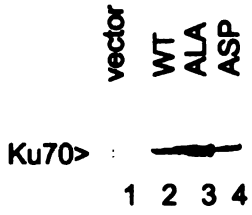
Ku70 phosphorylation is not required for NHEJ:

Since Ku is a heterodimer made up of two proteins Ku70 and Ku80, it was also important to address if phosphorylation of Ku70 is required during NHEJ. As mentioned previously, there are two phosphorylation sites identified in Ku70, serine 6 and serine 51. To address the relevance of these sites, I constructed cDNA(s) where serine 6 and 51 were mutated to either alanine (Ku70 ALA) to block phosphorylation or aspartic acid (Ku70 ASP) to mimic constitutive phosphorylation. Wild type Ku70, Ku70 ALA or Ku70 ASP were expressed in Ku70 $^{-/-}$ ES cells (Fig. 9A) and clones with similar Ku70 expression were evaluated for radioresistance (Fig. 9B). Both the alanine and aspartic acid Ku70 mutants reversed the radiosensitive phenotype of Ku70$^{-/-}$ ES cells similarly to wild type Ku70. Hence, I conclude that phosphorylation of Ku70 at either serine 6 or serine 51 is not required for NHEJ.

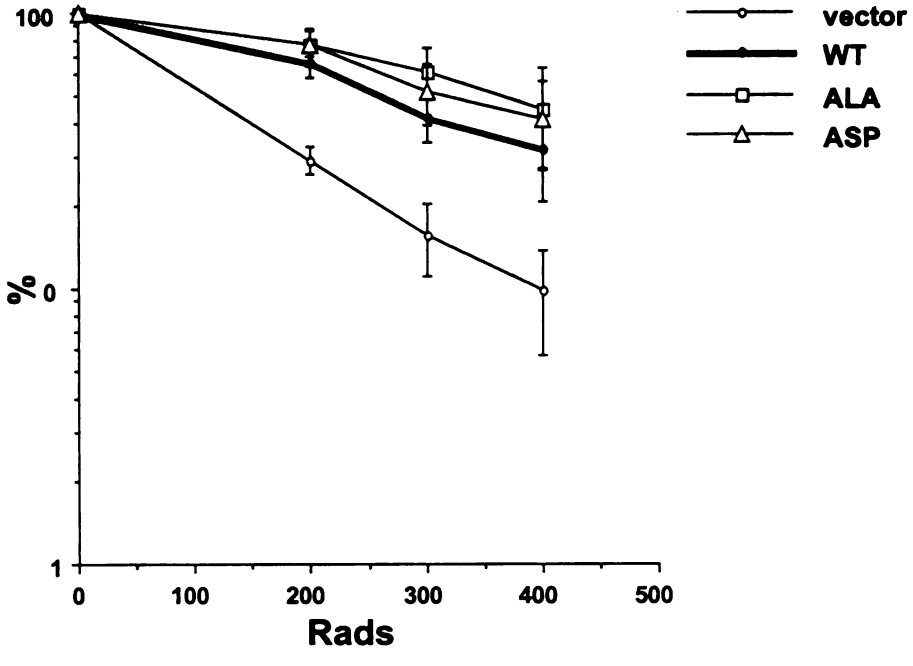
Fig. 9. Alanine and aspartic acid mutants of Ku70 exhibit wild type complementation of NHEJ deficit in Ku70$^{-/-}$ ES cells. A. 100 μg of whole cell extracts from Ku70$^{-/-}$ cells stably transfected with vector alone (vector, lane 1) or wild type V5-Ku70 (WT, lane2) or V5-Ku70 with serines 6 and 51 mutated to either alanine (ALA, lane 3) or aspartic acid (ASP, lane4) were analyzed by western blot for Ku70 expression using anti-V5 antibody. **B.** Radioresistance of Ku70$^{-/-}$ cells stably transfected with vector alone (open circles/solid line) or wild type V5-Ku70 (Ku70 WT, solid circles/ bold line) or V5-Ku70 ALA (open squares/solid line) or V5-Ku70 ASP (open triangles/solid line) was determined using MTT assays as described in section 3.1.

Fig. 9

A.


vector
WT
ALA
ASP
Ku70>
1 2 3 4

B.


100
%
0
1
0
100
200
300
400
500
Rads
vector
WT
ALA
ASP

DNA-PK phosphorylation sites in Ku70/80 are not required for NHEJ:

As noted previously, DNA-PKcs autophosphorylates on multiple sites within two major clusters spanning amino acids 2609-2647 and 2023-2056 and this autophosphorylation is required for NHEJ (Ding *et al.*, 2003). Moreover, our lab has shown that phosphorylation at only one or two sites within a cluster of five or six sites can suffice functionally in DNA-PKcs. Thus, it was possible that in the above experiments phosphorylation of one protein (Ku70) can suffice for the block in phosphorylation of the other (Ku80) in *xrs-6* cells and vice versa for $Ku70^{-/-}$ ES cells.

Additionally, even though the phosphorylation sites were not present in the crystal structure for Ku, the arrangement of the polypeptide chain in the structure suggested that the two sites in Ku70 and three sites in Ku80 are in close proximity to with each other. Thus, we next considered that phosphorylation at all five sites in Ku70 and Ku80 might act in concert and play a role in NHEJ. To address if phosphorylation of Ku70 and Ku80 together is required for NHEJ, we needed a cell strain that was genetically deficient in both Ku70 and Ku80. *xrs-6* is only genetically defective in Ku80. For Ku70, whereas mRNA levels are similar to wild type CHO cells, the amount of protein detected in *xrs-6* is minimal. The reason for this difference between the mRNA and protein levels of Ku70 has been attributed to the instability of Ku70 in the absence of Ku80. It has been shown previously that endogenous hamster Ku70 is stabilized by ectopic expression of Ku80. However, expression of human Ku80 only partially reverses the NHEJ deficit in the *xrs*-6 cell line (Smider *et al.*, 1994). This partial complementation has been attributed to the structural differences between hamster and

human Ku80 (76% identity). Considering these structural differences between hamster and human Ku80, I hypothesized that human Ku70, when co-expressed with human Ku80 in *xrs*-6 cells, should act as the dominant partner and should out compete endogenous hamster Ku70 for heterodimerization. This would result in instability of endogenous hamster Ku70 and would enable me to test the functionality of mutant Ku (with all five sites mutated to alanine and aspartic acid).

To test this hypothesis, I generated an expression vector encoding a functionally inactive (for NHEJ) N-terminal Ku70 deletion mutant that has previously been shown to efficiently heterodimerize with Ku80 (Jin and Weaver, 1997). This N-terminal deletion mutant was expressed in *xrs*-6 cells expressing WT Ku80 (Fig. 10A). If human Ku70 preferentially heterodimerizes with human Ku80, expression of the deletion mutant should act as a dominant negative in the *xrs*-6 transfectants expressing wild type Ku80. Stable clones were assessed for Ku70 expression (Fig. 10A) and as can be seen, the amount of the deletion mutant protein is less than the endogenous hamster Ku70. However, co-expressing the deletion mutant does not affect the levels of endogenous hamster Ku70. This we believe is due to the reason that the deletion mutant is not expressed at high levels (deletion mutants are often unstable) to out compete the endogenous protein completely or partially. Also, it is possible that I was unable to detect the slight decrease in the levels of endogenous hamster Ku70 in a western blot. However, when tested in irradiation assays, the cells co-expressing the human Ku70 deletion mutant and wild type hamster Ku70 have a radiosensitive phenotype (Fig. 10B) demonstrating that a non-functional Ku70 mutant (even in low amounts) can out compete

endogenous hamster Ku70 and can act as a dominant negative partner for Ku80 (albeit incomplete).

Using this approach, we next addressed whether expression of human Ku heterodimers with all five phosphorylation sites substituted with either alanine or aspartic acid functionally complement the radiosensitive phenotype of *xrs*-6 cells. However, co-expression of either alanine or aspartic acid Ku70 mutants with the corresponding Ku80 mutants did not induce radiosensitivity (Fig. 11B and 12B). This is true even in transfectants that express substantial levels of the mutant Ku70 proteins (Fig. 11A and 12A). Thus, we conclude that phosphorylation at these five sites in the Ku heterodimer is not required for IR induced double strand break repair.

Ku70ALA expressed in baculovirus is phosphorylated on additional sites:

Since mutating five known phosphorylation sites did not affect Ku's role in NHEJ, there was a possibility of additional phosphorylation site(s) present in the heterodimer, which might be functionally relevant for Ku's role in NHEJ. To address this, I expressed wild type and mutant (all five sites mutated to alanine) Ku70 and Ku80 in Sf9 cells using baculovirus expression vectors (see Materials and Methods). The proteins were partially purified and phosphorylated in the presence of purified DNA-PKcs and γ-^{32}P [ATP]. As shown in Fig.13, the ability of purified DNA-PKcs to phosphorylate the alanine mutant Ku proteins *in vitro* was reduced to approximately 30% (for Ku70) and less than 10% (for Ku80) as compared to wild type Ku expressed in baculovirus. This suggested that the identified sites in Ku70 and Ku80 represent the

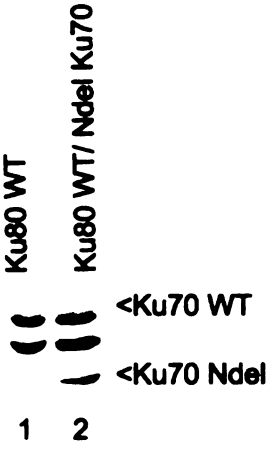
major *in vitro* DNA-PK phosphorylation sites. Our collaborators also corroborated this by mass spectrometric analyses of *in vitro* phosphorylated Ku.

Also, during the course of my studies our collaborator generated a panel of phosphospecific antibodies to the identified DNA-PK phosphorylation sites in Ku. Using these phosphospecific antibodies, it was confirmed that in the presence of okadaic acid, serine 6 of Ku70 and serines 577, 580 and threonine 715 are phosphorylated *in vitro* and *in vivo*. However, this phosphorylation was abrogated in the presence of protein kinase inhibitor staurosporine and was not affected by wortmannin (DNA-PKcs inhibitor). This suggested that *in vivo,* in addition to DNA-PKcs, Ku is also phosphorylated by a putative staurosporine sensitive kinase whose identity is currently being investigated.

Fig. 10. Co-expressing N-terminal deletion of Ku70 (NdelKu70) with wild type Ku80 confers radiosensitivity in *xrs-6* cells. A. 100μg of whole cell extracts from *xrs-6* cells stably transfected with wild type Ku80 (WT C, lane 1) or both wild type Ku80 and NdelKu70 (Ku70 Ndel/Ku80 WT, lane 2) was analyzed by western blot for Ku70 expression. **B.** *xrs-6* cells stably transfected with wild type Ku80 (solid circles/solid line) or both wild type Ku80 and NΔKu70 (open squares/solid line) or vector alone (crosses/solid line) were irradiated at the indicated doses and cell death was assessed by colony formation as described in section 3.1.

Fig.10

A.


Ku80 WT
Ku80 WT/ Ndel Ku70
<Ku70 WT
<Ku70 Ndel
1
2

B.

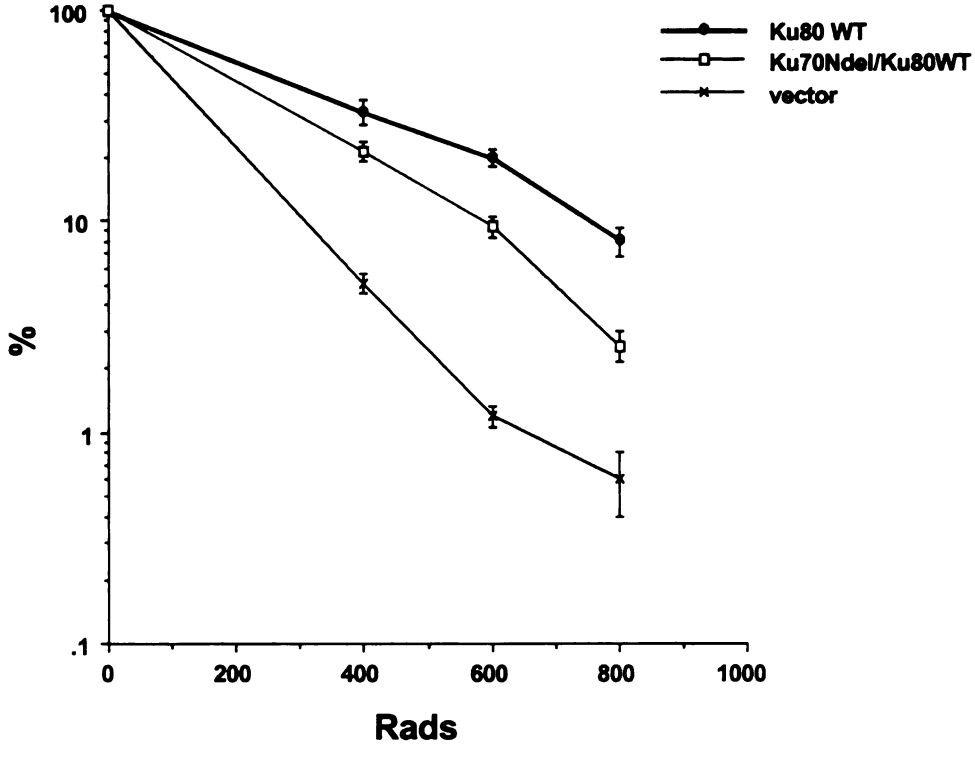
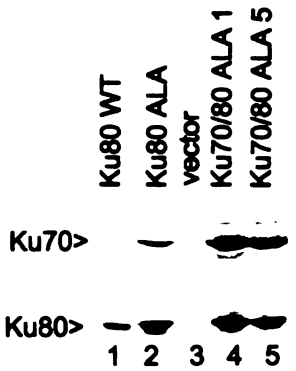

100
10
1
.1
%
0
200
400
600
800
1000
Rads
Ku80 WT
Ku70Ndel/Ku80WT
vector

Fig. 11. Co-expressing alanine mutants of Ku70 and Ku80 do not confer radiosensitivity. A. 100 μg of whole cell extracts from *xrs-6* cells stably transfected with vector alone (vector, lane 3) or wild type Ku80 (WT C, lane 1) or alanine mutant Ku80 (TM6, lane 2) or both alanine mutant Ku70 and alanine mutant Ku80 (Ku70/80 ALA 1 and 5, lanes 4 and 5) were analysed by western blot for Ku70 and Ku80 expression. **B.** *xrs-6* cells stably transfected with wild type Ku80 (solid circles/bold line) or alanine mutant Ku80 (solid circles/solid line) or both alanine mutant Ku70 and alanine mutant Ku80 (open squares/solid line and open triangles/solid line) or vector alone (crosses/solid line) were irradiated at the indicated doses and cell death was assessed by colony formation as described in section 3.1.

Fig. 11

A.


Ku80 WT
Ku80 ALA
vector
Ku70/80 ALA 1
Ku70/80 ALA 5
Ku70>
Ku80>
1 2 3 4 5

B.

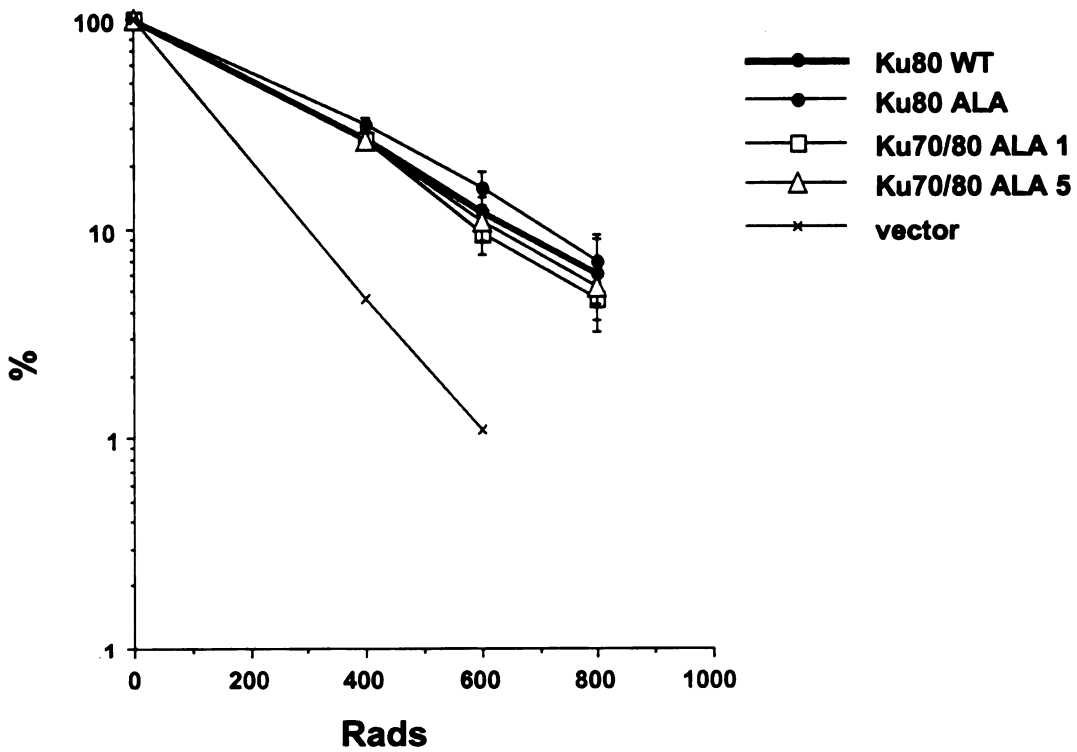
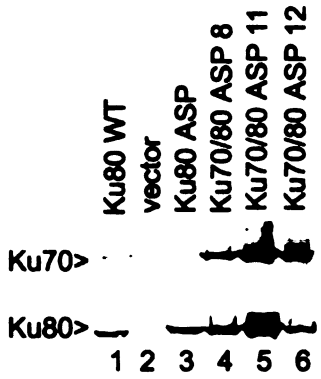

100
10
1
.1
%
0
200
400
600
800
1000
Rads
Ku80 WT
Ku80 ALA
Ku70/80 ALA 1
Ku70/80 ALA 5
vector

Fig.12. Co-expressing aspartic acid mutants of Ku70 and Ku80 do not confer radiosensitivity. A. 100μg of whole cell extracts from *xrs-6* cells stably transfected with vector alone (vector, lane 3) or wild type Ku80 (WT C, lane 1) or aspartic acid mutant Ku80 (TM ASP, lane 2) or both aspartic acid mutant Ku70 and aspartic acid mutant Ku80 (Ku70/80 ASP 8,11 and 12, lanes 4, 5 and 6) were analysed by western blot for Ku70 and Ku80 expression. **B.** *xrs-6* cells stably transfected with wild type Ku80 (filled circles/bold line) or aspartic acid mutant Ku80 (filled squares/solid line) or both aspartic acid mutant Ku70 and aspartic acid mutant Ku80 (open circles/solid line, open squares/solid line and open triangles/solid line) or vector alone (crosses/solid line) were irradiated at the indicated doses and cell death was assessed by colony formation as described in section 3.1.

Fig. 12

A.


Ku80 WT
vector
Ku80 ASP
Ku70/80 ASP 8
Ku70/80 ASP 11
Ku70/80 ASP 12
Ku70>
Ku80>
1 2 3 4 5 6

B.

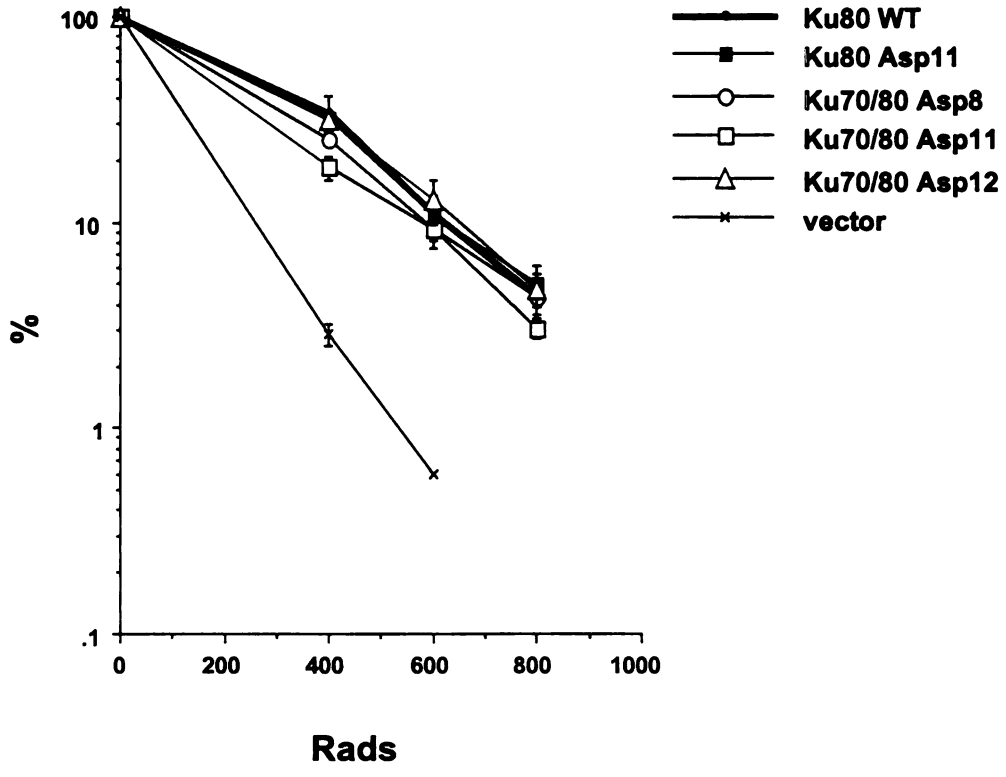
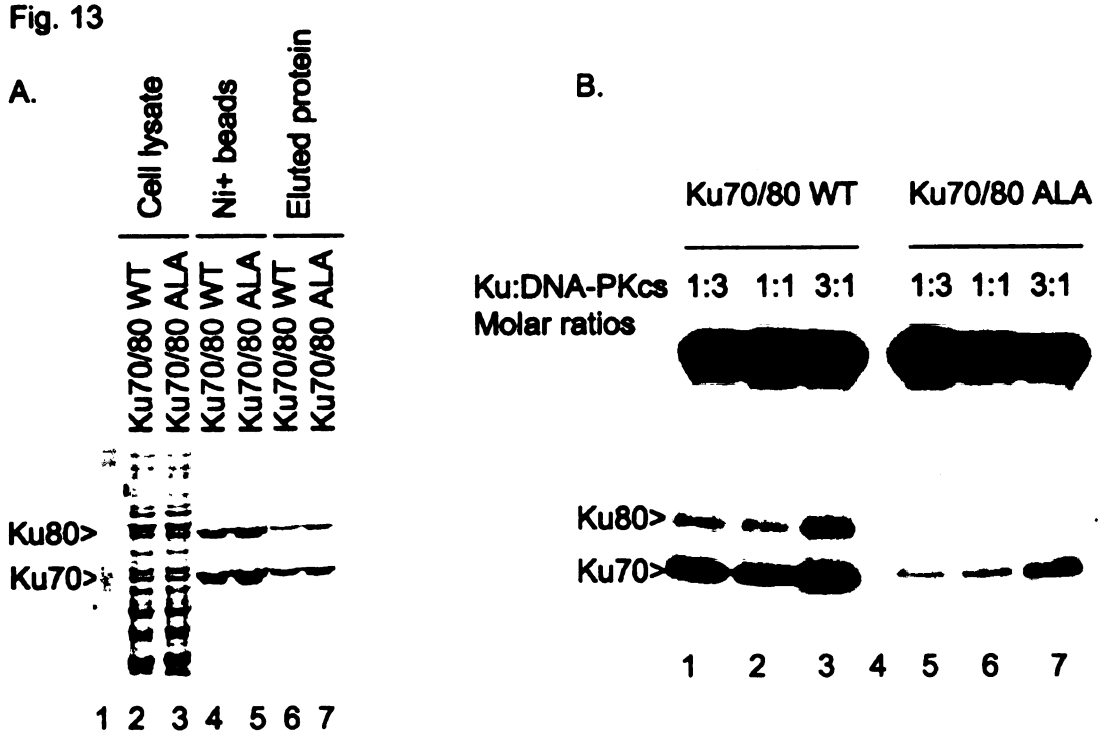

100
10
1
.1
%
0
200
400
600
800
1000
Rads
Ku80 WT
Ku80 Asp11
Ku70/80 Asp8
Ku70/80 Asp11
Ku70/80 Asp12
vector

Fig. 13. Mutating three phosphorylation sites in Ku80 and two phosphorylation sites in Ku70 reduces *in vitro* phosphorylation of Ku70/80 by DNA-PKcs:

A. Ku70/80 WT (lane 2, 4 and 6) or Ku70/80 ALA (lane 3, 5 and 7) were expressed in Sf9 cells and 15μl of cell lysate (lanes 23), 15μl Ni^{+}-agaorse beads (lanes 4,5), or 10μl of eluted protein (lanes 6,7) was analyzed on a SDS-PAGE gel followed by coomassie blue staining.

B. Partially purified Ku70/80 WT (lanes1-3) or ALA (lanes 5-7) was phosphorylated in the presence of purified DNA-PKcs and γ32-[ATP] using three molar ratios of Ku:DNA-PKcs (1:3, 1:1 and 3:1) and phosphorylation was analyzed by SDS-PAGE followed by autoradiography.

Fig. 13


A.
Cell lysate
Ni+ beads
Eluted protein
Ku70/80 WT
Ku70/80 ALA
Ku70/80 WT
Ku70/80 ALA
Ku70/80 WT
Ku70/80 ALA
Ku80>
Ku70>
1 2 3 4 5 6 7
B.
Ku70/80 WT
Ku70/80 ALA
Ku:DNA-PKcs Molar ratios
1:3 1:1 3:1
1:3 1:1 3:1
Ku80>
Ku70>
1 2 3 4 5 6 7

DISCUSSION:

Our collaborator Susan Lees-Miller's laboratory had shown that both Ku70 and Ku80 are phosphorylated *in vitro* by purified DNA-PK (Chan *et al.*, 1999). Further, using phosphospecific antibodies, they showed that in the presence of protein phosphatase inhibitor, okadaic acid, Ku is phosphorylated at these sites *in vivo.*

Thus, DNA-PK-dependent phosphorylation of the Ku heterodimer presented an attractive model for the regulation of NHEJ. To test this hypothesis, I generated a panel of cell lines expressing either the alanine or aspartic acid mutant of Ku80 (serines 577,580 and threonine 715 to either alanine, Ku80 ALA, or aspartic acid, Ku80 ASP) and Ku70 (serines 6 and 51 mutated to either alanine Ku70 ALA or aspartic acid, Ku70 ASP). Expressing either Ku80 ALA or Ku80 ASP in *xrs-6* or Ku70 ALA or ASP in $Ku70^{-/-}$ ES cells complemented the IR sensitivity of these cells like the wild type Ku80 or Ku70. Co-expressing the Ku70 ALA or ASP and Ku80 ALA or ASP did not confer a radiosensitive phenotype to *xrs-6* cells.

As mentioned above, *in vivo* phosphorylation of Ku was observed only in cells treated with okadaic acid. Surprisingly, this OA-induced phosphorylation was abrogated by nanomolar concentrations of the protein kinase inhibitor, staurosporine. Although staurosporine was originally identified as an inhibitor of PKC, subsequent studies showed that it also inhibits CDK2/cyclinA, PKA, phosphorylase kinase and MAP kinase with IC_{50} of 20 nM or below (Johnson *et al.*, 2002). Staurosporine does not inhibit protein kinase activity of either ATM or DNA-PKcs (Feng *et al.*, 2004; Goodarzi *et al.*, 2004;

Goodarzi and Lees-Miller, 2004) and *in vitro* phosphorylation of of Ku by DNA-PKcs was unaffected by staurosporine.

Thus, taken together the above results led us to conclude that phosphorylation on serine 577, 580 and threonine 715 in Ku80 or serine 6 and 51 in Ku70 is not required for DNA DSB repair by NHEJ and that *in vivo*, in addition to DNA-PKcs, Ku is phosphorylated by a staurosporine sensitive kinase. This suggests that phosphorylation of Ku might play a role in some processes other than NHEJ. However, to narrow down the hunt, the logical step would be to identify the staurosporine sensitive kinase that phosphorylates Ku.

CHAPTER 4

THE LEUCINE RICH REGION OF DNA-PKCS FACILITATES ITS TARGETING TO DNA

ABSTRACT:

DNA-PK, a serine-threonine protein kinase is a central player in non-homologous end joining (NHEJ), the favored pathway to repair double strand breaks (DSBs) in vertebrates. The kinase consists of the DNA binding, regulatory subunit [Ku] and the larger ~465 kDa catalytic subunit [DNA-PKcs]. The kinase activity of DNA-PKcs resides in amino acid 3745 to 4013, a PI3 kinase domain. Another recognized domain within this large protein is a leucine zipper (LZ) motif, or perhaps more appropriately designated a leucine rich region (LRR), that spans residues 1503-1602 (20% residues are leucines). Whereas DNA-PK's kinase activity has been shown to be absolutely indispensable for its function in NHEJ, little is known about the functional relevance of the LRR. Here we show that DNA-PKcs with point mutations in the LRR can only partially reverse the radiosensitive phenotype and V(D)J recombination deficits of V3 cells, a DNA-PKcs deficient cell line.

Disruption of the LRR motif affects the ability to purify DNA-PKcs by virtue of its binding to DNA-cellulose. Thus, extracts from V3 cells expressing the mutant protein display reduced kinase activity compared to wild type controls using a standard pull down assay. These data suggest that the LRR region of DNA-PKcs in living cells may contribute to its intrinsic DNA affinity.

INTRODUCTION:

The DNA-dependent protein kinase (DNA-PK) plays an essential role in non-homologous DNA end joining [NHEJ] by initially localizing to DNA breaks. Its immense size [~465kD] suggests that the catalytic subunit of DNA-PKcs (DNA-PKcs) may function (at least in part) as a scaffold to organize a DNA repair complex. Indeed, DNA-PKcs has been shown to interact with several other polypeptides including the Ku heterodimer, the XRCC4/DNA ligase IV complex, the Artemis endonuclease, and the nuclear matrix protein C1D. An emerging consensus of DNA-PK's function is that after initial binding (one DNA-PK complex per DNA end), the synapsis of the two complexes activates DNA-PK's kinase activity (known to be requisite for NHEJ), and the active complex targets other repair factors to the site of DNA damage (reviewed in Lees-Miller *et al.*, 2003). To date, the only DNA-PK phosphorylation event shown conclusively to be required in NHEJ is phosphorylation of DNA-PKcs itself (Chan *et al.*, 2002; Ding *et al.*, 2003; Soubeyrand *et al.*, 2003). Consistent with a model whereby DNA-PKcs serves a scaffolding role is data from our laboratory and several others suggesting that DNA-PKcs regulates access of broken DNA ends to repair factors via autophosphorylation (Ding *et al.*, 2003, Block *et al.*, 2004; Reddy *et al.*, 2004). Thus, autophosphorylation induced conformational changes of DNA-PK may orchestrate an as yet undefined sequence of repair events.

Recent structural studies suggest that DNA-PKcs has three major domains, and that it shares this organization with the related kinase, ATM. The three domains have been termed palm, arm, and head (Boskovic *et al.*, 2003; Rivera-Calzada *et al.*, 2005).

The head contains both the enzymatic (PI3 kinase) motifs as well as regions previously implicated in DNA-PKcs's interaction with Ku. The arm is a connecting domain and likely contains important regulatory autophosphorylation sites. The palm consists of two protruding regions termed claws. The proximal claw is possibly comprised of the extreme N-terminus of DNA-PKcs; the distal claw likely contains the LRR. In the DNA bound confirmation, a significant conformational change occurs such that the head and palm clamp together, possibly stabilizing the protein/DNA interaction.

In 1998, Jackson and colleagues identified the nuclear matrix protein C1D as a factor that interacts specifically both *in vitro* and *in vivo* with the leucine rich region of DNA-PKcs (Yavuzer *et al.*, 1998). Here, using a mutagenic approach, we address the functional relevance of DNA-PK's LRR. We demonstrate that DNA-PKcs deficient cells expressing the LRR mutant (LRRm1) protein are considerably more radiosensitive and less proficient at V(D)J recombination than are the cells that express wild type DNA-PKcs. We further show that DNA-PKcs harboring a mutation within the LRR (LRRm1) fractionates poorly with DNA cellulose as compared to the wild type protein. However, pull down experiments demonstrate that the LRR mutant (LRRm1) has wild type interaction with the DNA binding protein, C1D. Also, *in vitro* the LRRm1 is recruited by Ku to DNA ends similar to the wild type DNA-PKcs. The poor fractionation on DNA cellulose is therefore attributed to the disruption of a putative motif in the LRR region of DNA-PKcs that may contribute to the innate DNA binding capabilities of DNA-PKcs. Thus, we conclude that in addition to being recruited to DNA breaks by its DNA end

binding partner Ku, the intrinsic interaction of DNA-PKcs with DNA is functionally important.

MATERIALS AND METHODS:

Cell lines and culture conditions:

The V3, DNA-PKcs deficient double strand break repair [DSBR] mutant CHO cell line (Whitmore *et al.*, 1989) was the generous gift of Dr. Martin Gellert. Cells were maintained in αMEM with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD). Stable transfectants were maintained with 400 μg/ml G418 (Invitrogen). Sf9 cells were maintained in Sf-900 II SFM medium (Invitrogen) containing 1% antibiotic-antimycotic (Invitrogen). The cells were grown at 27°C without CO_2.

Oligonucleotides:

Oligonucleotides used in this study are as follows (5' > 3'). The two oligonucleotides flanking the LRR were KAM 110 (GGAACAAGAGAATGGAGATGA) and KAM 111 (GCTTCTGTGCATGTGCTTGAC) The two oligonucleotides with the desired mutations spanning across the LRR were KAM 112 (GACTTCTGG**ACCCAG**GCTTTGC) and KAM 113 (GCAAAGG**CTGGGT**CCCAGAAGTC). Positions at which glutamic acid and leucine were changed to aspartic acid and proline are shown in bold face. The mutations introduce identical substitutions as on construct studied in yeast two hybrid experiments by Jackson and colleagues (Yavuzer *et al*, 1998).

Oligonucleotides used to clone human C1D cDNA were KAM 281 (TTGCGGCCGCGCACTTTTACTTTTTCCTTTATTGGC) and KAM 282 (TTAAGCTTCAGCCATAATGGCAGGTGAA).

Oligonucleotides used to clone human C1D in Baculovirus were KAM 390 (CGCTCTAGATCTGCCATAATGCATCACCATCACCATCACGCAGGTGAAGAAA TTAATGAAGAC) and KAM 391 (TCTAGACTCGAGCGGCCGCGCTTAACTTTTACTTTTTCC)

Oligonucleotides used to subclone LRR in pGEX 5X-3 were KAM 439 (CGTGGGATCCCCAGGATGTCCACAGATTTG) and KAM 440 (GCGGCTCGAGTCCATTTTGGTATTATCCAC)

Oligonucleotides used to substitute proline for a leucine at position 1510 in GST-LRR were KAM 482 (CTCAGTTGTAAGCAGCCGGCCAGCGGACTTCTG) and KAM 483 (CAGAAGTCCGCTGGCCGGCTGCTTACAACTGAG).

Construction and transfection of expression plasmids:

Construction of the wild-type human DNA-PKcs expression vector was described previously (Shin *et al.*, 2000). To generate the expression plasmids encoding the LRR mutant, duplex oligonucleotides KAM 112 and KAM 113 flanking the LRR were used to amplify a 5Kb fragment using human DNA-PKcs cDNA as the template DNA. The subsequent PCR fragment was cloned using the Topo-TA cloning kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The resulting plasmid was utilized to generate the LRR mutant by Quik change mutagenesis according to the manufacturer's protocol (Stratagene, La Jolla, Calif.). This PCR fragment spans two Ehe I sites at

positions 1770 and 6280 in the open reading frame of the human DNA-PKcs cDNA. This fragment was subsequently subcloned into the wild type DNA-PKcs expression vector after sequencing analyses confirmed that no additional substitutions were introduced during PCR amplifications.

V3 transfectants expressing human DNA-PKcs were derived as described previously (Ding *et al.*, 2003).

DNA cellulose pulldown of DNA-PK, measurement of protein kinase activity, and nuclear matrix preparations:

Whole cell extracts were prepared and kinase activity was measured as described previously (Ding *et al.*, 2003). To assess fractionation of DNA-PKcs onto DNA-cellulose, whole cell extracts were absorbed onto DNA-cellulose in 500 ul buffer A [25mM Hepes, pH 7.9; 50mM KCl, 10mM $MgCl_2$, 10% (v/v) glycerol; 1mM EDTA; 1mM EGTA; 1mM DTT and the indicated concentration of NaCl] for 1 hour at 4°C. Beads were washed three times in the same buffer and washed beads were suspended in 2X SDS-PAGE buffer (25mM Tris-Cl pH 6.8, 10% (v/v) glycerol, 1% SDS, 0.04 % Bromophenol Blue and 25µl 2-mercaptoethanol added fresh to 475 µl of the buffer). The beads were then heated for 5 minutes at 70°C, centrifuged at 1.5 rpm for 2 minutes. The supernatant was then analysed by immunoblotting as described previously (Ding *et al.*, 2003). When pulled down with C1D, the kinase reaction was supplemented with activation buffer and partially purified Ku (expressed in Baculovirus).

To visualize mobilization of DNA-PKcs in responsed to DSBs, V3 transfectants were treated for 1 hour with bleomycin (Sigma). Membrane insoluble fractions were prepared as described previously (Drouet *et al*, 2005).

V(D)J recombination assays:

Extrachromosomal recombination assays were performed as described previously (Chapter 3, Materials and Methods).

Assessment of Radiation Sensitivity:

Cells (3×10^3) were exposed to various amounts of ionizing radiation using a ^{60}Co source and immediately seeded in complete medium containing 10% fetal bovine serum. After 7 days, cell colonies were fixed with ethanol, stained with crystal violet, and colony numbers were assessed.

Expression of fusion proteins and pulldown experiments:

A cDNA encoding for full-length human C1D was ampliflied from human brain mRNA by RT-PCR using oligonucleotides KAM 281 and KAM 282. The termination codon was not included, and the amplified fragment was cloned into pcDNA-6V5/His expression vector (Invitrogen) that encodes a C-terminal V5-HIS tag. The above cDNA was then used as a template to amplify and subclone C1D into BamH1 and XhoI sites in pFastBac I (generous gift from Dale Ramsden's lab), a baculovirus expression vector using oligonucleotides KAM 390 and KAM 391. The oligos were designed to encode a

N-terminal His tag. Human Ku70 and Ku80 cDNA cloned into pFastBac Dual was a generous gift from Dr. Dale Ramsden's lab.

Whole cell extracts from V3 transfectants (2mg) and whole cell extracts from either C1D-infected or Ku-infected or control virus-infected Sf9 cells were co-incubated for 30 minutes in buffer A at 4°C. Subsequently, 30 μl of Ni^+ agarose was added, and the extracts were absorbed in buffer A with 25mM imidazole. After 1h, the Ni^+ agarose was washed three times with buffer A containing 50mM imidazole. Proteins were eluted with SDS-PAGE buffer and analyzed by immunoblotting.

To express, LRR fusion proteins in bacteria, the LRR (aa1471- aa1592) was amplified using DNA-PKcs plasmids (either wild type or LRR mutant) as the template using oligoucleotides KAM 439 and KAM 440. The PCR product was digested and subcloned into BamH1 and Xho1 sites in pGEX-5X-3 (Amersham) to generate GST-LRR or GST-LRRm1 expression plasmids. The GST-LRRm1 was then utilized as a template to generate GST-LRRm2 (L1510P, E1516D, L1517P) by Quik change mutagenesis (Stratagene) using oligonucleotides KAM 482 and KAM 483. The GST-LRRdel was generated by deleting the Xba1-Nco1 fragment (aa 1504-1551) from GST-LRR. All plasmids were sequenced to confirm that no extra substitutions were introduced during the PCR.

The expression plasmids for GST-fusion proteins were transformed into *E.coli* BL-21 (DE3) (Stratagene, LaJolla, Calif.). To express GST-fusion proteins, a 5ml

overnight culture was used as a seed culture for 100ml LB-broth. The bacteria were grown at 37°C to reach the Abs_{600nm} of 0.6 and then induced with 1mM IPTG at 27°C for 4 hours. The cells were harvested and suspended in 5ml Lysis buffer (50mM Hepes pH 7.5, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 10% Glycerol, 1% Triton X-100 and Protease inhibitor cocktail tablet (Complete Mini, Roche)) and sonicated for 15 minutes on ice. The lysate was then centrifuged at 14,000 rpm for 15 minutes at 4°C. The induction and solubility was assessed by SDS-PAGE followed by coomassie staining.

Whole cell lysate from bacteria expressing either GST alone or equal amounts of GST-LRR, GST-LRRm1, GST-LRRm2 or GST-LRRdel and whole cell extracts from His tagged C1D-infected Sf9 cells were co-incubated in buffer A for 30 minutes at 4°C. Subsequently, 30 µl of pre-swollen Glutathione-agarose (pre-equilibrated in buffer A) was added and incubated for 1h at 4°C. After an hour, the Glutathione-agarose was washed three times with buffer A containing 2mM Glutathione (reduced form). Proteins were eluted with SDS-PAGE buffer and analyzed for C1D by immunoblotting using anti-His (Qiagen) as the primary antibody (1:5000 dilution). A Goat anti-mouse conjugated to horseradish peroxidase (Signal Transduction labs) was used as the secondary antibody (1:5000 dilution). The membrane was then incubated with a chemiluminescent substrate (ECL, DuPont) according to manufacturer's recommendations.

RESULTS:

LRR DNA-PKcs mutant can only partially reverse radiosensitive phenotype in V3 cells:

A potential leucine zipper motif within the DNA-PKcs coding sequence spanning residues 1503-1538 was noted by Hartley *et al.* in the original sequence analysis of the molecule (Hartley *et al.*, 1995). It consists of 6 leucine residues, each separated by six residues. However, the fourth leucine in the putative zipper is not conserved in chicken and frog. Thus, more recently, other investigators consider this region (1503-1539) to be more appropriately designated as a leucine rich region (33% leucine). A more extensive overlapping region (1503-1602) is also relatively leucine rich (20%). DNA-PKcs is highly conserved (63-85%) in the six species for which sequences are available, (human, horse, dog, mouse, chicken and frog). The LRR is similarly well conserved (Fig. 3). In the report from Yavuzer *et al,* demonstrating that C1D interacts with the LRR of DNA-PKcs, two site specific mutants of a DNA-PKcs polypeptide that ablated its interaction with C1D were studied. One of the mutants contains two point mutations substituting aspartic acid and proline for the glutamic acid and leucine residues at amino acids 1516 and 1517 as indicated in Fig. 5. This mutant peptide was not able to interact with CID as assessed by yeast two hybrid assays (Yavuzer *et al.*, 1998). Also, as observed by Fujimori *et al*, the first and fourth leucine residues (aa1502 and aa1524 respectively) are substituted in chicken and *Xenopus*, however the second and third leucine residues (aa 1510 and aa 1517) are conserved among all vertebrates (Fujimori *et al*, 2000) (Fig. 5), consistent with the previous results that these two leucines are essential for binding of the C1D protein

(Yavuzer *et al.*, 1998), further suggesting that the physical association of C1D and DNA-PKcs is biologically significant.

To begin to assess the functional relevance of the LRR of DNA-PKcs in living cells, one of these mutations was introduced into the complete DNA-PKcs cDNA. Wild type and mutant constructs encoding DNA-PKcs [designated WT and LRRm1] were stably transfected into DSBR mutant V3 cells that lack DNA-PKcs and are thus defective in NHEJ. Clones with similar levels of DNA-PKcs expression were selected for further study (Fig. 14A). To assess the ability of the wild type and mutant constructs to reverse the known radiosensitive phenotype of V3 cells, cell irradiation assays were performed. As can be seen (Fig. 14B), although expression of the LRR mutant protein renders cells significantly more radioresistant than cells transfected with vector alone, cells expressing the mutant protein are still substantially more radiosensitive than cells expressing equivalent levels of wild type DNA-PKcs. Thus, we conclude that the LRR mutant protein can only partially complement the radiosensitivity of V3 cells.

DNA-PKcs mutant, LRRm1 only supports reduced levels of either coding or signal end joining in V3 cells:

The ability of the mutant DNA-PKcs to support RAG induced V(D)J recombination in V3 cells was tested. The wild type DNA-PKcs substantially complements the coding end joining deficit of V3 cells (assessed with substrate pJH290) (Table 3). In contrast, LRRm1 supports only reduced level of coding end joining (1.4 to 14 fold reduced) as compared to wild type DNA-PKcs. We and others have reported previously that V3 cells

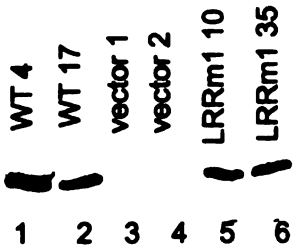
have a significant signal end joining deficit that is substantially reversed by transfecting wild type DNA-PKcs (Ding *et al.*, 2003; Kurimasa *et al.*, 1999; Woods *et al.*, 2002). Consistent with those results, we find here that wild type DNA-PKcs substantially increases the recovery of signal joints from V3 cells (assessed with substrate pJH201). Although co-transfection of LRRm1 also increases the recovery of signal joints from V3 cells, the numbers are consistently reduced (1.2 to 8 fold) as compared to transfections with wild type DNA-PKcs. As reported previously, co-transfection of an ATP binding site mutant (K>M) does not alter the levels of either coding or signal joints in transient assays as compared to transfections with no DNA-PKcs at all (Kienker *et al.*, 2000). Thus, we conclude that LRRm1 can only partially support either signal or coding end joining during V(D)J recombination.

Recently, work from our lab has demonstrated that mutations in DNA-PKcs's numerous autophosphorylation sites dramatically affect end processing of coding joints (Ding *et al.*, 2003; Block *et al.*, 2004; Reddy *et al.*, 2004). However, sequence analysis of coding joints mediated by the LRR mutant as compared to those mediated by the wild type protein were comparable with regards to nucleotide loss, percent complete coding ends, and the number of joints with short sequence homologies apparent at the joint (Table 4). In sum, we conclude that coding joints mediated by the LRR mutant protein are structurally normal.

Fig. 14. DNA-PKcs with point mutations in LRR motif only partially complements the radiosensitivity in V3 cells. (A) Immunoblot analysis of whole cell extracts from V3 transfectants expressing either full length DNA-PKcs (lanes1 and 2), vector alone (lanes 3 and 4) and LRRm1 (lanes 5 and 6). (B) The radiation resistance of V3 transfectants expressing wild type DNA-PKcs, vector alone or LRRm1 was assessed as described in Materials and Methods. Data are presented as percent survival to that of non -irradiated controls (set at 100%). Error bars, standard error of the mean of three separate experiments.

Fig. 14

A.


WT 4
WT 17
vector 1
vector 2
LRRm1 10
LRRm1 35
1
2
3
4
5
6

B.

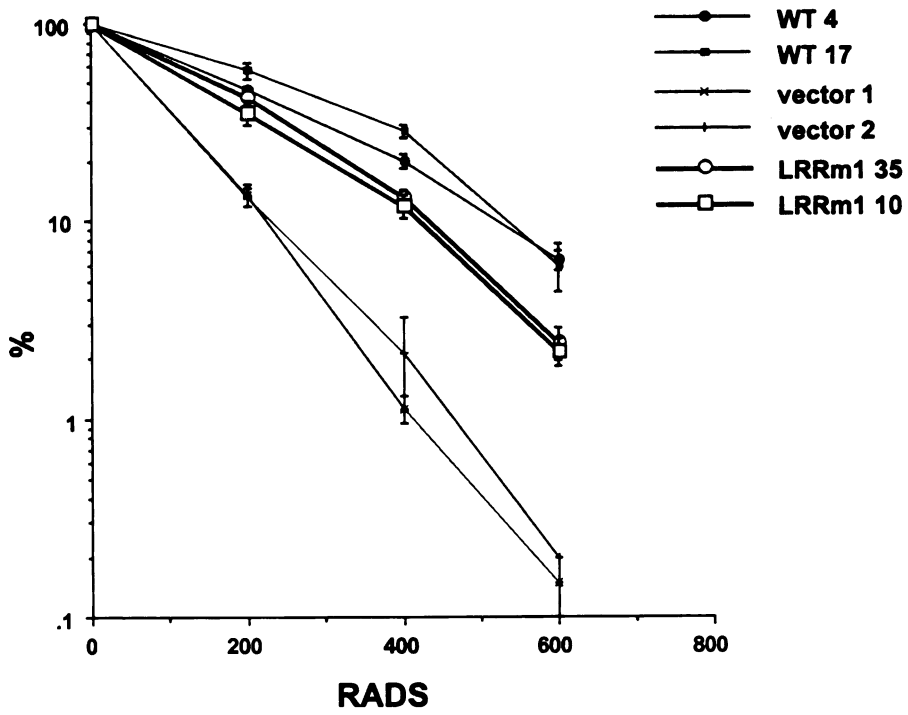

100
10
1
.1
%
0
200
400
600
800
RADS
WT 4
WT 17
vector 1
vector 2
LRRm1 35
LRRm1 10

Table 3. LRRm1 supports reduced levels of V(D)J recombination. Transient V(D)J recombination assays were performed as described in Experimental Procedures. RAG expression from plasmid vectors initiates recombination in the V3 cells, as assessed by the plasmid substrate pJH290 that detects coding joints or the pJH201 substrate that detects signal joints as indicated. Numbers of ampicillin (amp) and ampicillin/chloramphenicol (amp+cam) resistant colonies from four separate experiments are presented. Recombination rate (%R) is calculated as the number of chloramphenicol resistant colonies divided by ampicillin resistant colonies X 100.

Table 3.

	coding joints (pJH290)		signal joints (pJH201)	
Transfected plasmids	#Amp Cam/ #Amp	Recombination (%)	#Amp Cam/ #Amp	Recombination (%)
RAGS only	1/2,500	0.04	2/6750	0.030
	1/33,250	0.003	0/18,750	0
	0/22,500	0	2/23,500	0.0085
	0/33,750	0	2/11,750	0.017
	0/6,500	0	3/3,000	0.1
RAGS + wild type	53/5,000	1.06	184/25,750	0.715
	108/103,000	0.105	106/17,000	0.624
	99/41,750	0.237	113/27,500	0.411
	96/51,250	0.18	157/13,750	1.14
	67/8750	0.76	95/4,500	2.1
RAGS + LRRm1	8/11,000	0.073	38/12,250	0.310
	28/106,250	0.026	4/5250	0.076
	36/76,000	0.047	45/24,250	0.186
	30/23,250	0.13	440/54,000	0.8
	11/2250	0.48	77/4500	1.7
RAGS + K>M	5/44,250	0.011	6/17,750	0.034
	0/7500	0	0/10,750	0
	1/38,500	0.003	0/15,750	0
	0/6,000	0	8/41,500	0.019
	0/5,000	0	3/3750	0.08

Table 4. Sequence analysis of coding joints recombined by LRRm1

	# Sequences	Base loss/ joint	% complete ends	% SSH	P segments/ complete end
Wild Type	72	4.8	29 (42/144)	42 (30/72)	31 (13/42)
LRRm1	26	3.65	31 (16/52)	27 (7/26)	50 (8/16)
RAGS only	16	14.69	41 (13/32)	31 (5/16)	92 (12/13)

LRRm1 has wild type interaction with C1D:

To test if the functional deficits of LRRm1 could be attributed to disruption of its interaction with C1D, a baculovirus vector expressing His-tagged C1D was constructed and expressed in Sf9 cells, for pull down experiments (Fig. 15A). Extracts from C1D-infected Sf9 or control virus-infected Sf9 cells were incubated with the cell extracts from V3 transfectants expressing either wild type or LRRm1 DNA-PKcs and Ni^{+} agarose. To our surprise (and in contrast with the yeast two hybrid studies of Yavuzer *et al.*), both wild type and mutant proteins fractionate similarly with immobilized C1D onto Ni^{+} agarose (Fig. 15B).

LRRm1is targeted to the nuclear matrix similar to the wild type DNA-PKcs:

There is a large body of evidence that DNA and RNA modifying enzymes are sequestered to particular nuclear compartment, in some cases by association with the nuclear matrix reviewed in (Berezney *et al*, 2002). Recently, Drouet *et al* have shown that in response to DNA double strand breaks, DNA-PKcs is mobilized to the triton non-extractable fraction of the nuclear compartment (the nuclear matrix). Yavuzer *et al* had previously hypothesized that C1D might be involved in targeting DNA-PKcs to the nuclear matrix in response to DNA damage. To corroborate that LRRm1 does indeed interact with C1D as well as the wild type DNA-PKcs both *in vitro* and *in vivo* in response to DNA damage, we next examined the mobilization of LRRm1 to the nuclear matrix in response to DNA damage using the protocol outlined by Drouet *et al.* V3 transfectants expressing either the WT or the LRR mutant DNA-PKcs were treated with Bleomycin for one hour to induce double strand breaks. The treated cells were washed

and extracted as outlined by Drouet *et al.* The extracted fraction was then analyzed by immunoblotting for DNA-PKcs levels.

As shown in Fig. 16, there is a significant mobilization and concentration of DNA-PKcs and γ-H2AX and not Lamin B in the nuclear matrix fraction in response to the induction of DNA DSBs. Furthermore, both wild type and LRRm1 DNA-PKcs associate with the nuclear matrix equally well. Thus, we conclude that the NHEJ defect in the LRR mutant is neither because of the disruption of interaction with C1D nor because of loss of association with nuclear matrix.

Additional regions outside the LRR contribute to DNA-PKcs's interaction with C1D:

To understand the discrepancy between our results and the results published by Yavuzer *et al*, we considered that perhaps the LRR motif is not completely disrupted in the full length LRR m1 DNA-PKcs. To address the possibility that second and third leucines (aa1510 and aa1517 respectively) together contribute to DNA-PKcs's interaction with C1D, we generated several GST fusion proteins spanning the LRR. These included wild type DNA-PKcs, GST-LRR (spanning residues1471-1592), the mutant GST-LRRm1 (with the same mutations already tested in the full length protein), an additional mutant GST-LRRm2 (changing the second leucine, L1510P as well as the previous mutations E1516D, and L1517P), and a deletion mutant GST-LRRdel (that completely deletes residues 1504-1551 from this region) (Fig. 17A). These GST fusion proteins were then co-incubated with extracts from C1D infected Sf9 cells and immobilized onto glutathione agarose beads and analyzed for C1D by immunoblotting. As can be seen

(Fig. 17B), GST-LRRm1 is substantially reduced in its ability to interact with C1D in agreement with the yeast two-hybrid results published by Yavuzer *et al.* However, mutating the second leucine (GST-LRRm2) or deleting the region spanning the conserved leucines (aa1510 and aa1517 implicated in DNA-PKcs's interaction with C1D) disrupts the LRR interaction with C1D to the same level as the GST-LRRm1 suggesting mutating glutamic acid to aspartic acid and leucine to proline (E1516D, L1517P) does indeed disrupt the LRR motif in a 100 aa peptide (GST-LRRm1) as well as in full length DNA-PKcs (LRRm1). Further, since the mutations in GST-LRRm1 are identical to the full-length LRRm1, and the fact that C1D interaction is disrupted with GST-LRRm1 but not with full length LRRm1, we conclude that regions outside the LRR contribute to DNA-PKcs's interaction with C1D, explaining the full-length LRRm1 interacting with C1D similar to the wild type DNA-PKcs.

Fig. 15. LRRm1 has wild type interaction with C1D: A. Whole cell extract from Sf9 cells infected with baculovirus expressing His-tagged C1D was incubated with Ni^+ agarose beads. Beads were washed and analyzed for C1D expression by SDS-PAGE followed by coomassie blue staining (lane 3). Lane 1 represents 5 μl of Pre stained protein marker. **B.** Whole cell extracts (2mg) from V3 transfectants expressing either WT (lanes 1 and 4) or LRRm1 (lanes 2 and 5) was incubated with whole cell lysate from either control virus infected-Sf9 (lanes 1-2) cells or C1D-infected (lanes 4-5) Sf9 cells. The C1D-DNA-PKcs complex was then absorbed onto Ni^+ agarose beads. Beads were washed and immunoblotted for DNA-PKcs.

Fig. 15

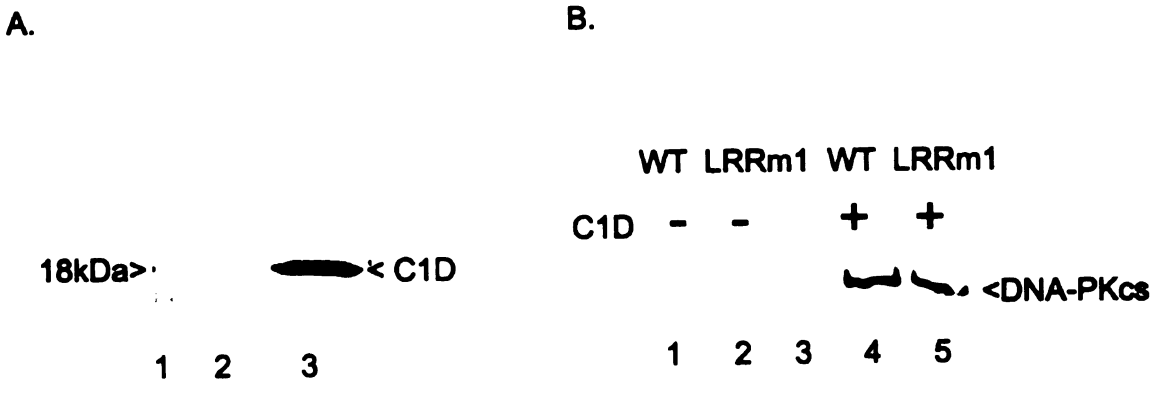


Fig. 16. LRRm1 is targeted to the nuclear matrix similar to the wild type DNA-PKcs: V3 transfectants expressing either wild type (lane1-2) or LRRm1 (lanes 3-4) were left untreated or were treated with Bleomycin (140μM). Cells were then extracted twice with Triton X-100 containing buffer and treated with RNase and DNase (as outlined by Drouet *et al*). The pellet fraction (nuclear matrix) after DNase digestion was solubilized in SDS-containing gel loading buffer. The samples were then immunoblotted for DNA-PKcs, γH2AX and Lamin B.

Fig. 16

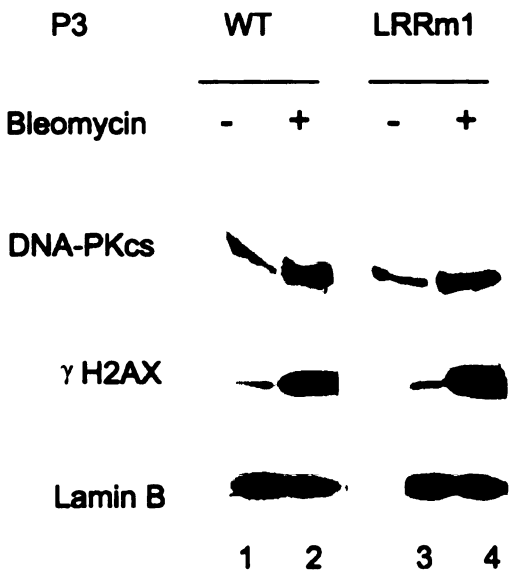
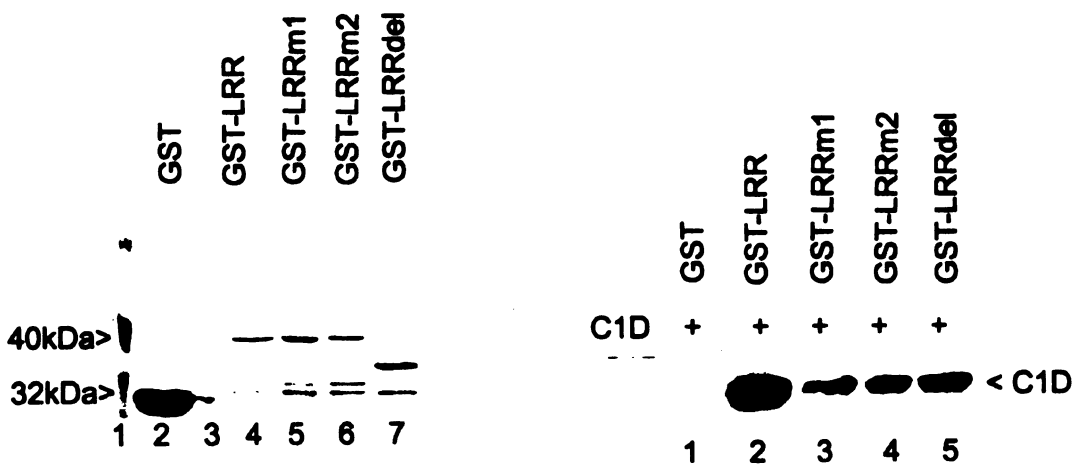


Fig. 17. GST-LRRm1 does not interact with C1D as well as the wild type GST-LRR: A. Whole cell lysates from bacteria expressing GST (lane 2) or GST-LRR (lane 4), GST-LRRm1 (lane 5), GST-LRRm2 (lane 6) and GST-LRRdel (lane 7) were incubated with Glutathione-agarose beads. Beads were washed and analyzed for protein expression by SDS-PAGE followed by coomassie-blue staining. **B.** Whole cell lysates from bacteria (same amount as in A.) expressing GST (lane 1) or GST-LRR (lane 2), GST-LRRm1 (lane 3), GST-LRRm2 (lane 4) and GST-LRRdel (lane 5) was co-incubated with whole cell lysates from Sf9 cells infected with baculovirus expressing His-C1D in 500μl of buffer A. The extracts were then absorbed onto Glutathione-agarose. Glutathione beads were washed and immunoblotted for His-C1D.

Fig. 17


GST
GST-LRR
GST-LRRm1
GST-LRRm2
GST-LRRdel
40kDa>
32kDa>
1 2 3 4 5 6 7
C1D
+ + + + +
< C1D
1 2 3 4 5

LRRm1 binds poorly to DNA-cellulose:

To explain the NHEJ defects in V3 transfectants expressing LRR mutant, we tested the mutant for its enzymatic activity. LRRm1 consistently displayed approximately two-fold lower kinase activity as compared to the wild type (Fig. 18A) in standard pulldown assays. Further, we analyzed the DNA-cellulose fraction used for the kinase assays, using western blots. As shown in Fig. 18B, the mutant DNA-PKcs fractionates poorly with DNA cellulose. To ascertain whether the mutant is compromised in only DNA-binding and/or kinase activity, I quantitated three different DNA-cellulose pull down assays. The densitometric analysis of these blots correlated well with the two-fold reduction in kinase activity of the mutant (Fig. 18C) suggesting that the mutant protein's reduced ability to bind DNA explains its reduced kinase activity as opposed to an intrinsic enzymatic defect.

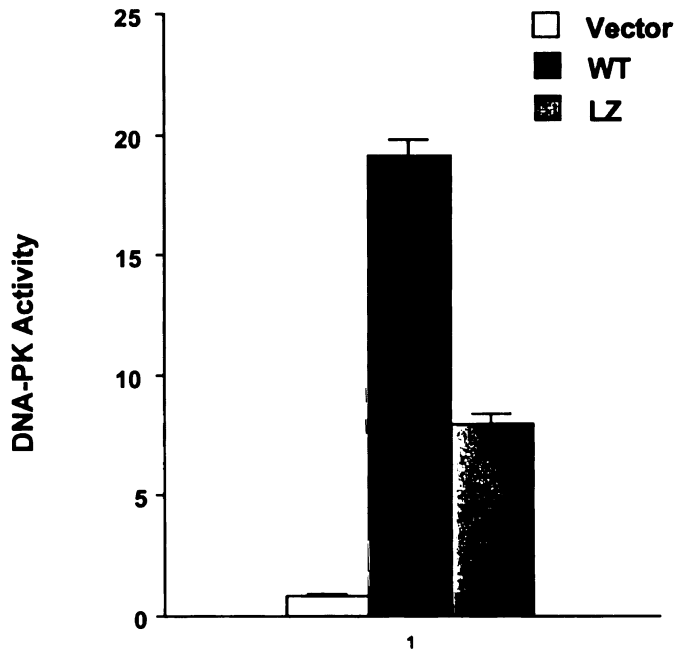
The LRR mutant has wild type levels of kinase activity:

To conclusively prove that the mutant displays low kinase activity only due to a defect in binding to DNA-cellulose, we used C1D to pull down DNA-PKcs (as described in section 4.2). As discussed in section 4.2.3, both wild type and mutant DNA-PKcs interact equally well with Ni^{+}- agarose immobilized C1D (Fig. 19A). These Ni^{+}-agarose beads were then used to phosphorylate p53, as in standard DNA-PK pull down assays. As shown in Fig. 19B, LRRm1 displayed kinase activity similar to the wild type DNA-PKcs. Thus, we conclude that disrupting LRR does not alter the kinase activity of DNA-PKcs.

Fig. 18. LRR mutant of DNA-PKcs has reduced kinase activity by virtue of reduced DNA-binding. A. Whole-cell extracts (250 μg) prepared from V3 cells transfected with either vector alone, WT DNA-PKcs, LRR mutant were assayed for enzymatic activity. Phosphorylation of the p53 substrate was assessed after 60 min. Each cell extract was tested in duplicate and three independent extracts were tested for each cell line. **B.** Immunoblot analysis of extracts (500μg) from V3 transfectants expressing wild-type (WT) or mutant (LRRm1) DNA-PKcs (lane 1-2) and DNA-cellulose fractions of extracts (500μg) from V3 transfectants expressing WT and LRRm1 (lane3-4) DNA-PKcs. **C.** Densitometric analysis of three independent DNA-cellulose fractions of extracts (500μg) from V3 transfectants expressing WT and LRRm1 DNA-PKcs. Error bars, standard error of the mean.

Fig. 18

A.


25
20
15
10
5
0
DNA-PK Activity
Vector
WT
LZ
1

B.

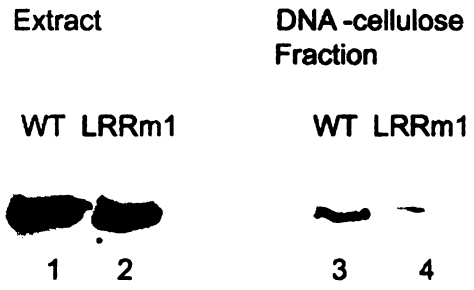
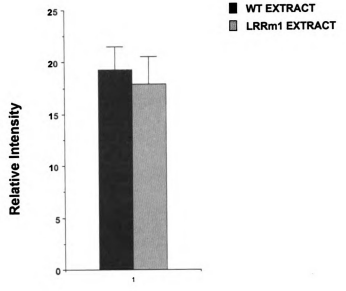

Extract
DNA-cellulose Fraction
WT LRRm1
WT LRRm1
1 2
3 4

Fig. 18 C (cont.)


WT EXTRACT
LRRm1 EXTRACT
Relative Intensity
25
20
15
10
5
0
1

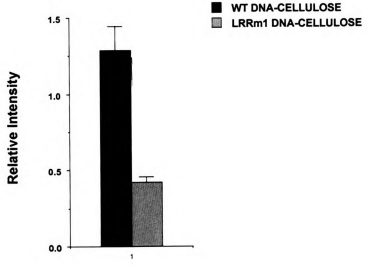
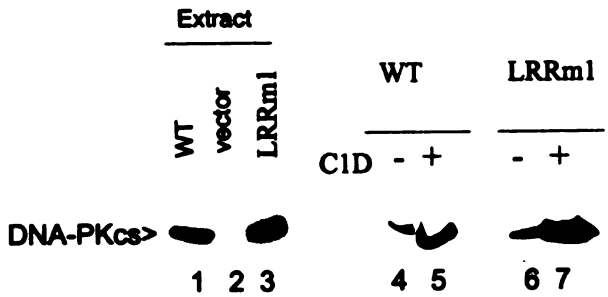

WT DNA-CELLULOSE
LRRm1 DNA-CELLULOSE
Relative Intensity
1.5
1.0
0.5
0.0
1

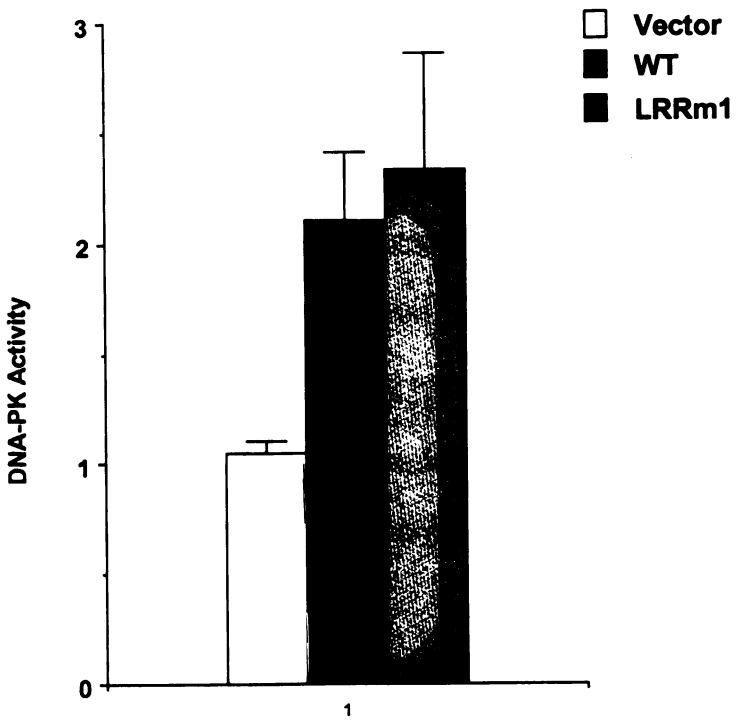
Fig. 19. LRRm1 has wild type levels of kinase activity A. Immunoblot analysis of whole cell extracts (100μg) from V3 transfectants expressing either WT (lane 1), vector (lane 2) or LRRm1 (lane 3). Whole cell extracts (2mg) from V3 transfectants expressing either WT (lanes 4-5) or LRRm1 (lanes 6-7) was incubated with whole cell lysate from either control virus infected-Sf9 cells (lanes 4-5) or C1D-infected Sf9 cells (lanes 6-7). The C1D-DNA-PKcs complex was then absorbed onto Ni^+ agarose beads. Beads were washed and immunoblotted for DNA-PKcs. **B.** DNA-PKcs absorbed onto Ni^+-agarose beads was assayed for enzymatic activity. Phosphorylation of the p53 substrate was assessed after 60 min. Three independent extracts were tested for each cell line. Error bars, standard error of the mean.

Fig. 19

A.


Extract
WT
vector
LRRm1
WT
LRRm1
CID - + - +
DNA-PKcs>
1 2 3 4 5 6 7

B.


Vector
WT
LRRm1
DNA-PK Activity
3
2
1
0
1

LRRm1 has wild type interaction with Ku:

Ku associates with DNA-PKcs near the C-terminus (3002-3850aa), partially overlapping its kinase domain (Jin *et al*, 1997). This region is separated by approximately 1500 aa from LRR. However, electron microscopic studies of DNA-PKcs suggest a head-arm-palm structure for DNA-PKcs (Boskovic *et al*, 2003; Rivera-Calzada *et al*, 2005) where it folds into a globular conformation to form a channel around dsDNA. Thus it is likely that intramolecular interactions within DNA-PKcs occur between regions far separated on the linear polypeptide chain. Since the mutant protein exhibits a defect in DNA-binding, we considered the possibility that the LRR facilitates the interaction of DNA-PKcs with Ku bound to DNA. To address this, His-tagged Ku70/80 was expressed in baculovirus (Fig. 20A). Whole cell extracts from either Ku-infected or control-infected Sf9 cells were incubated with extracts from transfectants expressing wild type or LRR mutant DNA-PKcs. The Ku-DNA-PKcs complex was then absorbed onto Ni^{+} agarose beads. As shown in Fig. 20B, wild type and LRRm1 DNA-PKcs are recruited to Ku bound DNA equally well. In sum, these data suggest that both DNA-PKcs's intrinsic DNA binding capacity as well as its interaction with the Ku affect its ability to associate with a DSB.

The LRR contributes to DNA-PKcs's intrinsic affinity to bind DNA:

To delineate the functional defect in the LRRm1 mutant, the following *in vitro* analyses were performed. Previous studies have established that DNA-PKcs can bind DNA in the absence of Ku (Chan *et al*, 1996; Dynan *et al*, 1998; Hammersten *et al*, 1998), however this binding is salt labile. Thus, we tested the ability of LRR mutant to bind DNA in various salt conditions by incubating whole cell extracts from stable

transfectants with DNA cellulose. As can be seen in Fig. 21, the LRR mutant protein fractionates poorly onto DNA cellulose in both salt concentrations tested, however, in the low salt conditions the difference between wild type and LRRm1 is far greater than in the high salt conditions. This can also be seen in the fractions analyzed after DNA-binding (the post fractions). The post fraction from wild type protein has less protein than the post fraction from LRRm1. This difference in the post fractions is more pronounced in low salt than in high salt. This result suggests that the LRR contributes to the innate binding properties of DNA-PKcs and thus disrupting the LRR alters the DNA binding properties of DNA-PKcs. An alternative explanation could also be that the LRRm1 is not assembling in a DNA-binding complex as well as the wild type because it is not interacting with a component, most likely a DNA-binding protein. The two identified DNA-binding proteins in the NHEJ complex are Ku and possibly C1D. As shown previously, LRRm1 has wild type interaction with both Ku and C1D. Thus, we conclude that LRRm1 binds poorly to DNA because the mutant DNA-PKcs has reduced intrinsic affinity for DNA.

Fig. 20. LRRm1 has wild type interaction with Ku: A. Lane 1 represents 5μl of pre-stained protein marker. Lane 2 represents 10μl of whole cell lysate from Sf9 cells infected with baculovirus expressing His-tagged Ku. Whole cell extract from Sf9 cells infected with baculovirus expressing His-tagged Ku was incubated with Ni^{+} agarose beads. Beads were washed and analyzed for Ku expression by SDS-PAGE followed by coomassie blue staining (lane 3). **B.** Whole cell extracts (2mg) from V3 transfectants expressing either WT (lanes1 and 4) or LRRm1 (lanes 2 and 5) was incubated with whole cell lysate from either control virus infected-Sf9 cells or Ku-infected Sf9 cells. The Ku-DNA-PKcs complex was then absorbed onto Ni^{+} agarose beads. Beads were washed and immunoblotted for DNA-PKcs.

Fig. 20

A.

B.

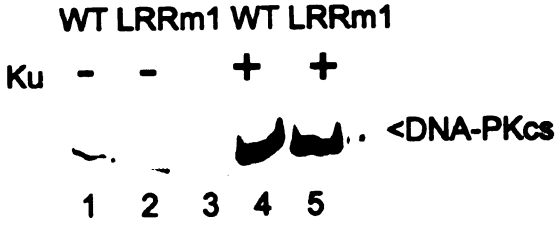
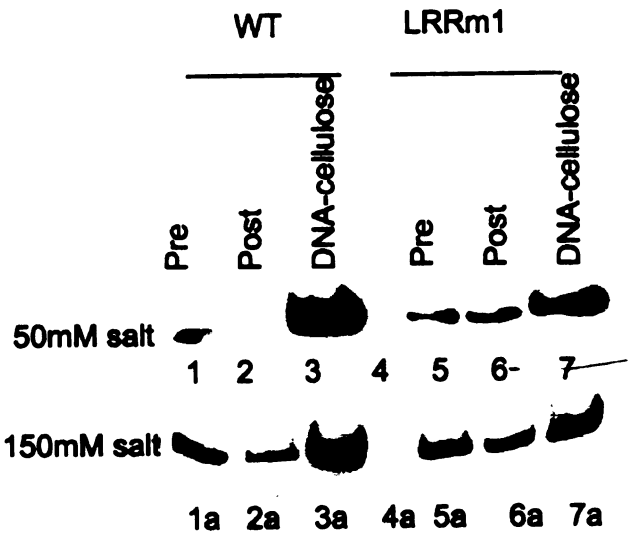


Fig. 21. The LRR contributes to DNA-PKcs's intrinsic affinity to bind DNA: Immunoblot analysis of DNA-cellulose fractions of extracts (500μg) from V3 transfectants expressing wild-type (WT) DNA-PKcs in either 50mM salt (lane 3) or 150 mM salt (lane 3a) and from DNA-cellulose fractions of extracts (500μg) from V3 transfectants expressing LRRm1 in either 50mM salt (lane 7) or 150mM salt (lane 7a). Pre (lanes 1,1a, 5, 5a) represents 2% of the total input and post (lanes 2, 2a, 6, 6a) represents 2% of the protein fraction left after incubation with DNA-cellulose.

Fig. 21


WT
LRRm1
Pre
Post
DNA-cellulose
Pre
Post
DNA-cellulose
50mM salt
1 2 3 4 5 6- 7
150mM salt
1a 2a 3a 4a 5a 6a 7a

DISCUSSION:

C1D is a nuclear matrix associated protein and binds DNA with very high affinity. Previous work from Jackson and colleagues had shown that DNA-PKcs interacts directly with C1D both *in vitro* and *in vivo* (Yavuzer *et al*, 1998). The authors inferred that this interaction requires an intact LRR region because in a yeast two-hybrid experiment utilizing a DNA-PKcs fragment with the LRR mutated failed to interact with C1D. Thus, at the onset of our studies, our working model was that DNA-PKcs is targeted to the nuclear matrix or chromatin network via its interaction with C1D and after DNA damage, Ku targets DNA-PKcs to DNA ends. To test this model, we introduced two mutations in the LRR region of DNA-PKcs (E1516D, L1517P); identical to the construct used in the yeast two hybrid studies to disrupt the LRR region by Yavuzer *et al.* The LRR mutant was expressed in V3 cells and compared to wild type DNA-PKcs for its function in IR repair and V(D)J recombination. The mutant DNA-PKcs was found to be compromised in both assays.

Having disrupted the LRR region in DNA-PKcs, we hypothesized that the mutant is not being targeted to DNA and /or the nuclear matrix via its association with C1D. However, when tested for its interaction with C1D, the LRR mutant was found to interact with C1D as well as wild type DNA-PKcs. In the previous study Yavuzer *et al* had shown that the interaction between C1D and DNA-PKcs is direct and is not mediated by Ku. Since C1D is also a DNA-binding protein, it was possible that C1D was pulling down DNA-PKcs through a DNA-bound complex and is thus more a reflection of Ku-DNA-PKcs interaction rather than C1D-DNA-PKcs interaction. To ascertain this, we

analyzed our pull down samples for Ku70 and Ku80. Unlike DNA-PKcs, Ku did not come down with Ni^{+}-agarose immobilized C1D (data not shown). These results established that in our *in vitro* system, interaction between DNA-PKcs and C1D was independent of Ku. However, there was still a possibility that *in vivo* the mutant DNA-PKcs is unable to interact with C1D in response to DNA-damage and this was not being detected in an *in vitro* pull down assay. Since C1D is not implicated in NHEJ directly, the only way to test whether or not LRRm1 was interacting with C1D *in vivo*, was to test for the proposed role of C1D in targeting DNA-PKcs to the nuclear matrix in response to DNA-damage. Thus, we followed the procedure outlined by Drouet *et al*, and the results established that the mutant DNA-PKcs was mobilized to the nuclear matrix similar to the wild type DNA-PKcs. This result also suggested that the mutant protein, although structurally and functionally capable of mobilizing to the right compartment to repair the breaks, it is somehow compromised at a downstream event leading to the repair of DSBs.

When tested for its ability to phosphorylate p53, the mutant displayed two fold less activity. However, the LRR mutant also fractionated poorly onto DNA-cellulose, which explains its reduced kinase activity in standard pull down assays. To confirm that the mutant DNA-PKcs had normal kinase activity, we replaced the DNA-cellulose fractionation in kinase assay with DNA-PKcs pulled down by Ni^{+}-agarose immobilized C1D. This result established that similar amounts of wildtype or mutant protein have similar protein kinase activity. Although the mutant fractionated poorly with DNA-cellulose, it was targeted to DNA ends by Ku similar to wild type DNA-PKcs. Therefore; we propose that the LRR of DNA-PKcs contributes to the intrinsic ability of DNA-PKcs

to bind DNA. The point mutations introduced in this region, although not sufficient to ablate its interaction with C1D, do alter the domain architecture to affect the intrinsic DNA-binding property of DNA-PKcs. This was further corroborated by the fact that the mutant DNA-PKcs was particularly reduced in its capability to bind DNA in low salt conditions, which earlier studies have established to be the conditions in which DNA-PKcs can bind DNA in the absence of its DNA-binding partner Ku (Chan *et al*, 1996; Dynan *et al*, 1998, Hammarsten *et al*, 1998). Our hypothesis is also in agreement with the recent Cryo-electron microscopic structure of DNA-PKcs (Rivera-Calzada *et al*, 2005) that suggests the palm region of DNA-PKcs undergoes a conformational change in the presence of a dsDNA and is utilized by DNA-PKcs to make contacts with DNA. The cryo-EM structure resolves the palm domain into two "claws" namely, proximal and distal. The authors further predict that the first ~800 residues form the distal claw and residues 800-1900 constitute the proximal claw of the palm domain. This would suggest that LRR (aa1492-1571) is a part of the proximal claw of DNA-PKcs and altering the structural conformation of this region can alter the intrinsic DNA binding property of DNA-PKcs. Taken together, these studies suggest that several distinct regions with in DNA-PKcs may contact DNA and our work demonstrates that the LRR contributes to the DNA binding property of DNA-PKcs, which is required for its complete function to repair DNA breaks.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

DNA-PK is composed of a 465 kDa serine/threonine protein kinase- DNA-PKcs and DNA binding heterodimer Ku. Genetic and biochemical studies have established that the protein kinase is activated when bound to a DSB and plays a central role in DSB repair. However, the precise role of the kinase in repairing the breaks is still a topic of speculation. One hypothesis is that the kinase modifies activity of other factors by phosphorylation. One substrate that unequivocally falls in this category is DNA-PKcs itself, which undergoes conformational changes post-phosphorylation and regulates end processing in NHEJ. Ku, being a part of DNA-PK complex, is a possible target for DNA-PKcs. However, from my studies I conclude that phosphorylation at known sites in Ku70 and Ku80 does not alter Ku's function in NHEJ as tested in irradiation assays. Also, additional work from our collaborator's laboratory shows that there is yet another kinase that phosphorylates Ku in cells. This kinase was found to be sensitive to the protein kinase inhibitor staurosporine. This raises an interesting possibility of Ku's role in processes other than NHEJ. However, to dissect that role it becomes important to identify the kinase. Ku has also been shown to be phosphorylated by cyclinA1/CDK2 *in vitro* and this phosphorylation has been hypothesized to have a role in DSB repair. However, the authors did not confirm if Ku was indeed an *in vivo* target for cyclinA1/CDK2 (Muller-Tidow *et al.*, 2004).

Some of the other known functions of Ku are in regulation of apoptosis and maintenance of telomeres. In addition to being phosphorylated, Ku70 can also get acetylated and this acetylation has been shown to regulate Bax-mediated apoptosis in cells (Cohen *et al.*, 2004). There are three lysines identified as potential acetylation sites in Ku70, which lie in the DNA binding ring. Whether or not acetylation of Ku is required for its function in NHEJ is not known. Its possible that acetylation of Ku helps it dissociate from DNA after or during DNA repair. This can be addressed by generating acetylation site mutants (K>A) using site directed mutagenesis. These mutants can then be tested for their function in DSB repair in irradiation assays.

As noted above, how DNA-PKcs repairs DSBs is still unclear. Recent work from our laboratory has shown that phosphorylation at two separate clusters in DNA-PKcs regulates end processing in NHEJ. However, the mechanics of the process are still not very well understood. There are several other steps in the process of DSB repair that require a detailed mechanistic study e.g., the signaling of DSB to the repair machinery, the choice of the pathway (HR and NHEJ) to repair the break, translocation of Ku along DNA, the synapsis, activation of the kinase, disassembly of the repair machinery. Cryo EM structures of DNA-PKcs have provided more insight into some of these aspects, but it is really important to be able to look at crystal structure of DNA-PKcs with or without DNA and in complex with some other factors. However, this might be a very difficult task owing to the huge size of the protein.

DNA-PKcs is a multi-domain protein, capable of acting as a scaffold, which is required to bring the various participating factors in close proximity to each other during the process of repair. The LRR region might have a role in this function. However, at this point LRR does not have a well-characterized role in the structural or functional aspect of DNA-PKcs. My work represents the first steps in identifying the importance of LRR and the results suggest that LRR is one of the DNA binding domains in DNA-PKcs and lack of a functional LRR renders DNA-PKcs only partially functional in NHEJ. An alternative explanation for reduced DNA binding of LRRm1 can be that disrupting LRR in DNA-PKcs has disrupted the interaction between DNA-PKcs and an unidentified DNA-binding protein, thus destabilizing the DNA binding of DNA-PKcs.

LRR may also be involved in other functions that were not measured by the techniques I have employed. It is possible that there are some critical residues in this region, which are important for either interaction with C1D or intramolecular interactions in DNA-PKcs and those residues might not have been affected by the mutations introduced (E1516D, L1517P). To address that, we are trying to generate a mutant that has the LRR deleted from the full length DNA-PKcs. It would be interesting to see how the mutant behaves in NHEJ and also how it interacts with other proteins in the pathway.

A previous study from Yavuzer *et al* had shown that a nuclear matrix protein C1D interacts with DNA-PKcs through the LRR motif. At the outset of this study we wanted to disrupt this interaction and address the role C1D in NHEJ. However, from my data I conclude that there are regions outside LRR in DNA-PKcs that contribute to the

interaction between C1D and DNA-PKcs. To determine if this is the case one could use the yeast two-hybrid assay using cDNA for different regions/domains of DNA-PKcs as the bait and cDNA for C1D as the prey, and vice versa. This information can then be used to disrupt interaction between C1D and DNA-PKcs and help unravel the role of C1D in NHEJ in mammalian cells.

The other way to address C1D's role in DNA repair is to knock out C1D. However, the lack of any commercial antibodies makes this is a formidable task. During the course of my study, I considered this approach and generated a peptide antibody. However, the antibody was unable to detect any endogenous or over-expressed C1D in mammalian cells. It did however detect C1D expressed in baculovirus. I think, this approach is worth a second try and instead of generating a peptide antibody, we can purify C1D expressed in baculovirus and use the whole protein to generate a second antibody. This antibody will, in all probability, be more sensitive than the peptide antibody. Once we have an antibody to detect endogenous levels of C1D, it would not be a difficult task to knock out or knock down the expression level of C1D using siRNA. In the absence of an antibody, quantitative RT-PCR can also employed to detect mRNA levels of C1D, however this would be a more labour intensive approach than the western blot analysis. Knocking down C1D will also help in understanding the role of a nuclear matrix protein in DNA repair.

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