ELUCIDATING THE ROLE OF REDOX EFFECTS AND THE KU80 C-TERMINAL REGION IN THE REGULATION OF THE HUMAN DNA REPAIR PROTEIN KU

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My career goals have changed over the years, but the one thing that has remained the same is a strong interest in science. From my first chemistry class at Pioneer Jr./Sr. High School with Mrs. McClain, I fell in love with performing experiments and interpreting the data that was generated. During my time at the University of Saint Francis my interested grew as the experiments became more complex and the data more challenging. However, it wasn't until three years were spent working in quality control that I realized research would be the most interesting and challenging use of my knowledge of science. With this realization, I enrolled in graduate school at Indiana University School of Medicine and with the experience, knowledge and guidance gained my career goals have never been more certain.

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ABSTRACT

Sara M. McNeil

ELUCIDATING THE ROLE OF REDOX EFFECTS AND THE KU80 C-TERMINAL REGION IN THE REGULATION OF THE HUMAN DNA REPAIR PROTEIN KU

DNA double strand breaks (DSB) are among the most lethal forms of DNA damage and can occur as a result of ionizing radiation (IR), radiomimetic agents, endogenous DNA-damaging agents, etc. If left unrepaired DSB's can cause cell death, chromosome translocation and carcinogenesis. In humans, DSB are repaired predominantly by the non-homologous end joining (NHEJ) pathway. Ku, a heterodimer consisting of Ku70 and Ku80, functions in the recognition step of this pathway through binding DNA termini. Ku recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to create the full DNA-PK heterotrimer. Formation of DNA-PK results in autophosphorylation as well as phosphorylation of downstream proteins of the NHEJ pathway. Previous work showns that the extreme C-terminus of Ku80 stimulates the kinase activity of DNA-PKcs, and Ku DNA binding is regulated as a function of redox via stimulation of a conformational change when oxidized resulting in a decrease in DNA binding activity. To further understand these methods of regulation of Ku and DNA-PK, a pair of mutants has been constructed; one consisting of full length Ku70 and truncated Ku80 (Ku70/80ΔC) lacking 182 C-terminal amino acids. The removal of these amino

acids was shown to have little to no effect on the proteins expression, stability or DNA binding, as determined by SDS-PAGE, western blot analysis and electrophoretic mobility shift assay (EMSA). When oxidized Ku70/80ΔC showed a decrease in DNA binding similar to that seen in wild type, however when re-reduced the mutant did not recover to the same extent as wild type. A second mutant was constructed, containging amino acids 590-732 of Ku80 (Ku80CTR), to further understand the mechanism by which Ku80 C-terminus interacts with the rest of the Ku heterodimer. Possible protein-protein interactions were evaluated by Ni-NTA affinity, gel filtration chromatography, fluorescence polarization and two forms of protein-protein cross-linking. Ni-NTA agarose affinity, and gel filtration chromatography failed to reveal an interaction in the presence or absence of DNA. However, photo-induced cross-linking of unmodified proteins (PICUP) as well as EDC cross-linking demonstrated an interaction which was not affected by DNA. The work presented here demonstrates that the interaction between Ku80CTR and Ku is rather weak, but it does exist and plays a relatively large role in the NHEJ pathway.

John J. Turchi, Ph.D., Committee Chair

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Introduction

DNA double strand breaks (DSBs) can be caused by ionizing radiation (IR), reactive oxygen species (ROS), radiomimetic drugs and other endogenous and exogenous events. If these breaks are not repaired, they ultimately can result in cell death. Inaccurate repair or rejoining of these breaks can generate chromosomal translocations, deletions and mutations, which can lead to genetic instability and contribute to the development and progression of cancer. An increasing amount of research is drawing a close correlation between DNA repair and how it affects the development and treatment of cancer (1). The pathways that are showing the most promise in cancer therapy are those that are very well characterized and the mechanisms are well understood. Unfortunately, the pathways to repair a double strand break are not as well characterized, but have been found to be connected with radiosensitivity (2), a treatment for certain types of cancer.

Many drugs, such as those that enhance chemotherapy, function by manipulation of a certain pathway. To target a known pathway, it is critical to understand the mechanism of operation of each step of the pathway in order to optimize the method of manipulation. The mechanisms of each step of the pathway have very specific functions and tend to be the more minute details. The non-homologous end joining (NHEJ) pathway, for example, is one that could have great potential in being a target for small molecules that enhance radiation therapy treatment, but unfortunately the mechanisms of this pathway are not well understood.

The research presented herein is designed to bring new knowledge regarding the regulation mechanism of Ku and its role in DNA-PKcs activation.

There are two main pathways to repair DSBs, homologous recombination (HR) and NHEJ (3). HR is the more accurate pathway with minimal loss of genetic material and only occurs when a homologous chromosome is present to provide extensive regions of sequence homology. Because NHEJ does not require a homologous chromosome or significant regions of homology it is the predominant pathway to repair DNA DSBs in humans, however, it is error-prone. DSBs initiate signaling via ataxia-telangiectasia mutant protein (ATM), which results in downstream signaling beginning the NHEJ pathway (4). Once the early signaling events have begun and the NHEJ pathway is initiated by Ku, a heterodimeric protein comprised of Ku70 and Ku80 subunits, binds DNA termini generated from DSB with a strong affinity (Figure 1). The DNA dependent protein kinase catalytic subunit (DNA-PKcs) is then recruited to the site of a DSB through an interaction with both Ku and the DNA termini, thus generating the active DNA-PK holoenzyme (5). Active DNA-PK, a serine/threonine protein kinase, then undergoes autophosphorylation and phosphorylates other downstream NHEJ proteins. DNA-PK, specifically, has been implicated in the phosphorylation and activation of proteins such as the nuclease Artemis (6). As a result of a double strand break, the DNA termini often contain structural damage such as thymine glycols, ring fragmentation, 3' phosphoglycolates, 5' hydroxyl groups and abasic sites. These modifications require processing of the DNA termini to remove the damage before ligation by the XRCC4/Ligase IV/XLF complex can occur (7;8). Many enzymes have been implicated, but

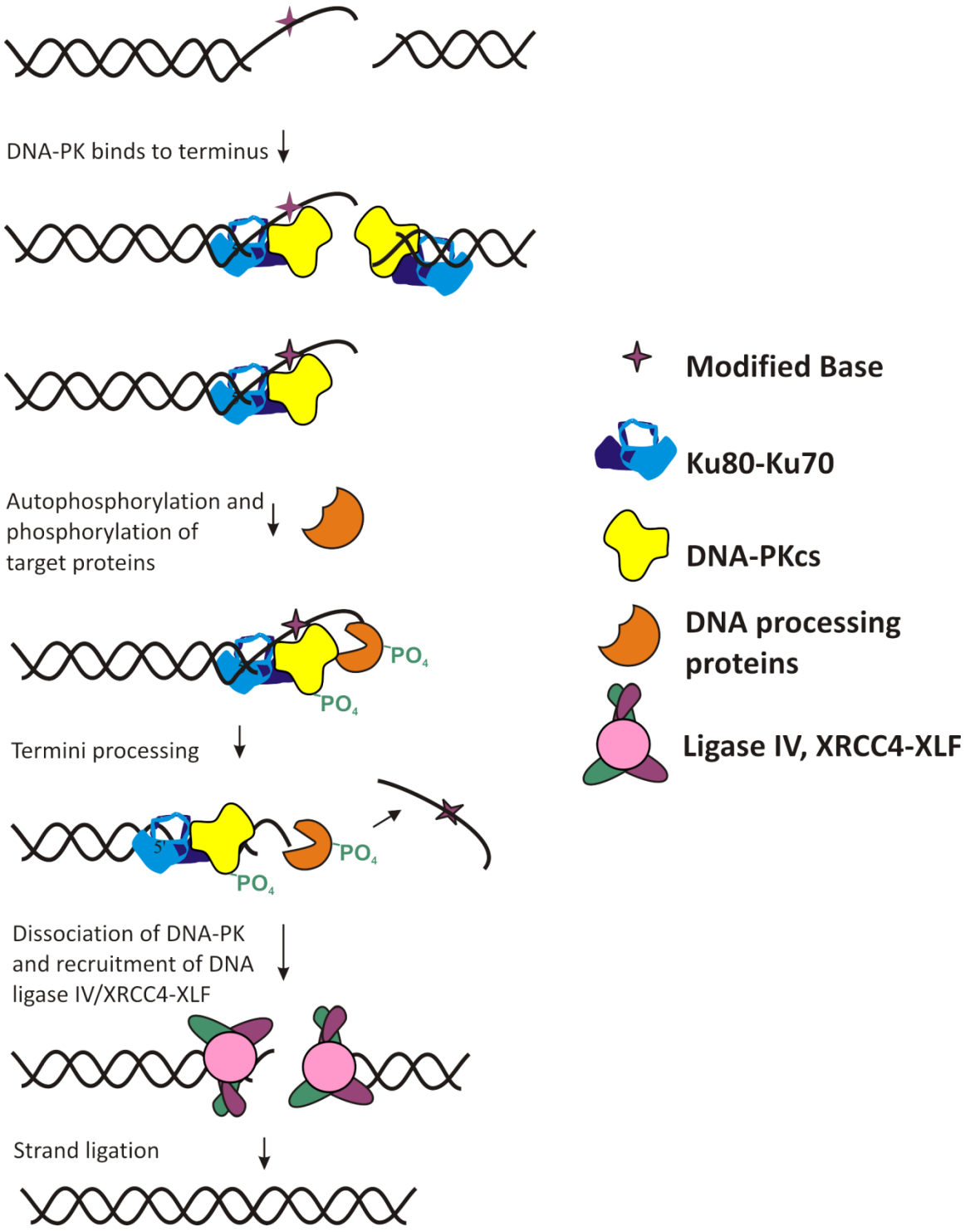

DNA-PK binds to terminus
Autophosphorylation and phosphorylation of target proteins
PO4
PO4
Termini processing
5'
PO4
PO4
Dissociation of DNA-PK and recruitment of DNA ligase IV/XRCC4-XLF
Strand ligation
Modified Base
Ku80-Ku70
DNA-PKcs
DNA processing proteins
Ligase IV, XRCC4-XLF

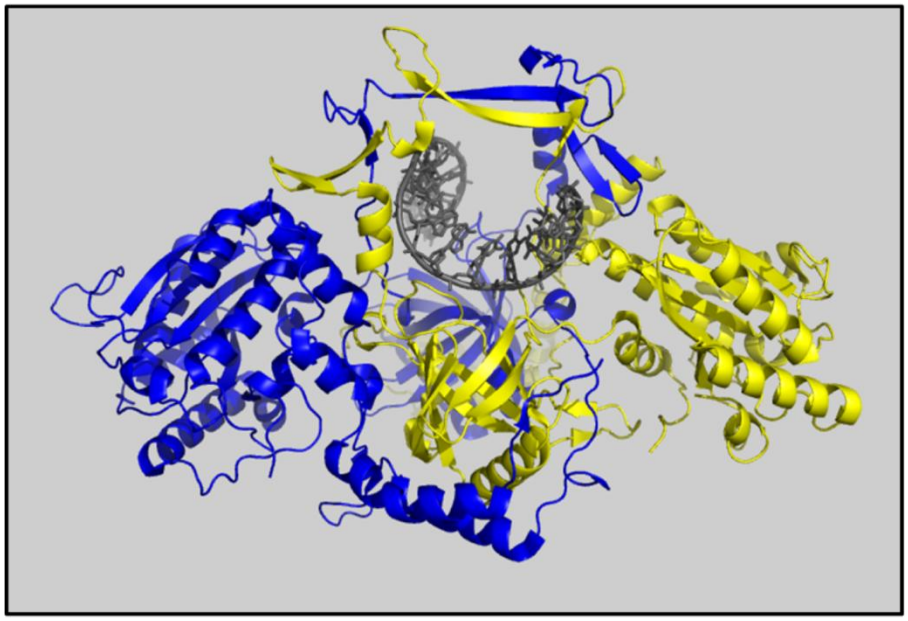
Figure 1. Model of Human Non-Homologous End Joining (NHEJ) DNA Repair Pathway. A DSB occurs and the pathway is initiated by the Ku protein binding damaged DNA termini, translocates inward, and recruits DNA-PKcs to form the DNA-PK heterotrimer. DNA-PK is activated as a serine-threonine kinase and undergoes autophosphorylation as well as phosphorylation of downstream molecules. DNA termini are processed to remove modified or damaged bases. By an unknown mechanism, DNA-PK dissociates from the DNA and DNA ligase IV/XRCC4-XLF complex facilitates DNA relegation. This model is based off of published data from multiple sources and prepared by KSP.

not fully defined, in DNA termini processing such as FEN-1 (9), polynucleotide kinase (PNK) (10), Werner protein (11;12), and Artemis (13) with more being discovered to have a connection with the NHEJ pathway.

The crystal structure of Ku revealed a bridge and pillar region comprised of both Ku70 and Ku80 subunits that form a ring around DNA (Figure 2a) (14). These studies revealed the ring shape exists in the presence and absence of DNA as well as a great deal of structural homology between the Ku70 and 80 subunits, despite the fact that they share minimal sequence homology (15). The three-dimensional structure of Ku enables the protein to slide or translocate along the length of a DNA molecule (16). However, it is unclear how Ku dissociates from the DNA upon completion of the NHEJ pathway when the termini are eventually ligated. Additional studies have demonstrated that upon DNA-PKcs binding, Ku translocates inward along the DNA in an ATP independent manner (17) consistent with the sliding model. Studies have shown that Ku binds DNA in a sequence independent fashion by way of several hydrophobic residues that make contact with the major groove of DNA and several basic residues that interact with the phosphate back bone (18;19). Photocrosslinking and crystal structure studies have shown that the Ku70 subunit is proximal to the DSB and Ku80 is distal to the DSB (20).

While much is known about the biochemical activities of Ku, its physiological regulation is less well understood. A common effect of IR-induced DNA damage is the generation of free radical species accompanied by a local change in the cellular

A



B

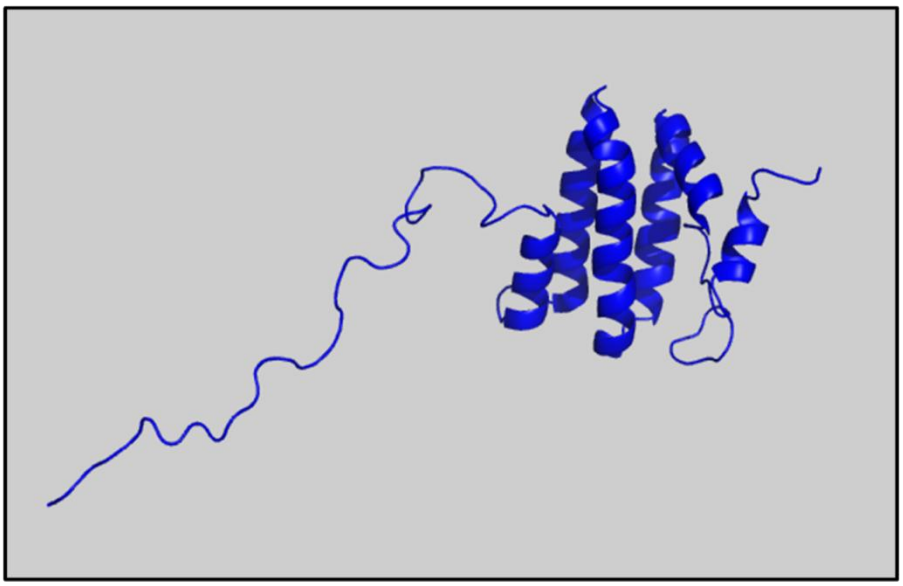


Figure 2. A) The crystal structure of Ku is depicted as a ribbon diagram in solid 3-dimensional rendering. Ku70 is presented in yellow (amino acids 34-534), Ku80 in blue (6-545), and the DNA molecule in dark gray with the simulated damaged DNA termini coming out of the page. Imaged adapted from PDB file 1JEY(21). B) The nuclear magnetic resonance (NMR) image of the C-terminus of Ku80 amino acids 590-732. Image adapted from PDB file 1RW2 (22).

oxidation/reduction status of proteins including those found in the NHEJ pathway. It has been shown that oxidative stress has a significant effect on the NHEJ pathway (23-25). Previous studies have shown that under oxidative conditions there is a marked decrease in DNA-PK activity (26-30). More specifically, oxidative stress has been shown to impair Ku's ability to bind DNA, and a conformational change in Ku under oxidized conditions leads to a significantly higher K_{off}. The effect oxidative stress has on Ku is a curious issue when thinking in terms of the crystal structure of Ku. The crystal structure does not reveal any disulfide bonds; however, it is lacking several amino acids, containing amino acids 6-545 of the 732 in the Ku80 subunit alone, and in particular a cysteine in the C-terminal region of Ku80.

Previous studies have also shown that the C-terminus of Ku80 is essential for efficient activation of DNA-PKcs, and only the final 12 amino acids are sufficient to bind DNA-PK (31-33). Upon removal of the C-terminus of Ku80 the kinase activity of DNA-PK is drastically decreased. Due to the highly flexible nature of the C-terminus of Ku80 (34-36), it is not present in the crystal structure of Ku (Figure 2) (37) and little is known about its interaction with the rest of the Ku molecule or DNA-PKcs. Previous work was capable of showing the full Ku molecule using small angle X-ray scattering (SAXS) (38). These studies have revealed that the C-terminus of Ku80 is capable of extending from the Ku molecule to a distance suitable for an interaction with a DNA-PKcs molecule that is bound to the same DSB end, as well as a DNA-PKcs molecule on an adjacent double strand break creating a synaptic complex (Figure 3).

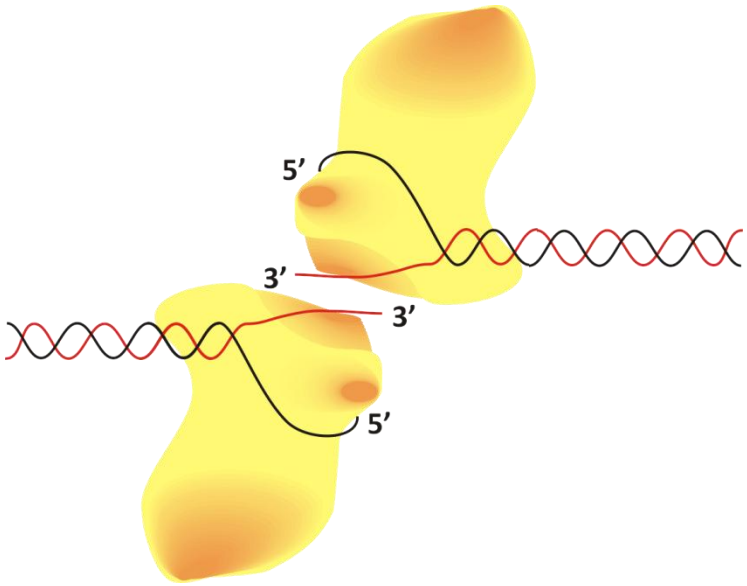


Figure 3. Synaptic complex model. DNA threads through the kinase and the ends separate. The 5’ end threads itself through the periphery of the kinase while the 3’ is possibly searching for regions of microhomology. Figure is adapted from ref (39).

To further understand the regulatory effect of the C-terminus of Ku80 we constructed, purified and analyzed two mutants of Ku. The first mutant contains a truncated form of Ku80 in conjunction with full length Ku70 (Ku70/80ΔC). This mutant was employed to confirm that no DNA binding activity is lost when the Ku80 C-terminus is removed and that DNA-PKcs kinase activity is drastically decreased. The truncation mutant was also analyzed in redox conditions to further understand the role of the Ku80 C-terminus and more specifically cysteine 638. Our studies show that there is a slight change in conformation upon oxidation and re-reduction that is not attributable to the removal of the C-terminus of Ku80, thus revealing a small possible role for the C-terminus of Ku80 in the recovery process of oxidative stress. The second mutant contained the final 142 amino acids, 590-732, of Ku80. This mutant was utilized in conjunction with the Ku70/80ΔC truncation mutant to reveal a protein-protein interaction. This interaction was detected by two methods of zero-distance crosslinking. Zero distance crosslinking is characterized by covalently binding of two molecules directly together without the aid of a linker arm (40). A catalyst is used to activate the side chain of a specific amino acid that then forms an intermediate. This intermediate then acts as a nucleophile and attacks the side chain of an adjacent amino acid forming a covalent bond between two amino acids that were not endogenously bound by the peptide bond found in polypeptides. Zero distance crosslinking schemes allow amino acids to be bound together that are in close proximity, such as that found in a protein-protein interaction. These forms of crosslinking are more specific for an interaction due

to the lack of the linker region tethering together two molecules that are simply within range of the linker arm and possibly not involved in a genuine interaction (41).

Materials and Methods

Mutant Construction – Ku80ΔC was prepared by PCR sub-cloning using an anti-sense primer inserting a stop codon after amino acid 548 (Table 1). Ku80ΔC was purified with or without a $[His]_6$ tag. To acquire the $[His]_6$ tag, PCR product was subcloned into pRSET B. The tagged construct was then subcloned into pBacPAK 8 and used to generate a recombinant baculovirus via co-transfection with bacpak6 viral DNA (Clonetech; Mountain View, CA). For the construct that did not contain a $[His]_6$ tag, the PCR product was subcloned directly into pBacPAK 8 and used to generate a recombinant baculovirus as described by the manufacturer (Clonetech). Briefly, SF9 cells grown in Grace's complete media were seeded at $1X10^6$ total cells in a 35-mm dish and allowed to adhere to the plate for 1 hour. Media was removed and replaced with Grace's Basic Media and incubated for 15 min. The Bacfectin mixture, containing 500 ng plasmid DNA and BacPAK6 viral DNA, was prepared in a final volume of 96 μl. 4 μl of bacfectin was added to the Bacfectin mixture and incubated at room temperature for 15 min. After the media was removed from cells, the Bacfectin mixture was added dropwise and incubated at 27^oC for 72 hours. Following incubation the cells were removed and the viral supernatant collected via centrifugation. The supernatant is now considered the primary transfectant. Recombinant baculovirus was then purified via plaque assay and amplified as described in the Clonetech BacPAK Baculovirus Expression System user manual. Briefly, cells were plated at $1X10^6$ total cells in a 35-mm dish; a serial dilution of virus was prepared from the primary transfectant and introduced to cells for 1 hour.

Table 1. DNA Oligonucleotides

Primer name	Sequence (5'→3')
Sense	ATACCGTCCCACCATCGGGC
Antisense	GAATTCCTAAGCAGTCACTTGATCCTTTT
30A	CCCCTATCCTTTCCGCGTCCTTACTTCCCC
30C	GGGGAAGTAAGGACGCGGAAAGGATAGGGG

Following incubation, virus inoculum was removed and cells were covered with a 1% agarose and complete media solution, allowed to solidify and another layer of complete media was added followed by an incubation of 4-5 days at 27^{o}C. Plaques were then visualized with neutral red staining and picked from the plate. The selected plaque picks were then incubated overnight in complete media and added to 5X10^{5} total Sf9 cells and incubated for 3-4 days. Following incubation media was transferred to a sterile tube and the cells were kept to analyze for protein production via western blot analysis. This virus was designated as a passage one virus and was further amplified as described briefly. A 150-cm^{2} flask was seeded with 1X10^{7} total cells with virus to achieve a multiplicity of infection (M.O.I.) of 0.1, assuming a viral titer of 5X10^{5}-1X10^{7}. Following an incubation of 4-6 days at 27^{o}C, cells were removed from the media and the media was then considered a passage 2 viral stock. This was amplified further by infecting 100 ml 5X10^{5} cells/ml with virus to achieve an M.O.I. of 0.1-0.5, assuming a viral titer of 1X10^{8}, and incubated in suspension for 4-6 days. Cells were removed from the media via centrifugation and supernatant was then considered a passage 3 virus. This was used to infect Sf9 cells for protein expression upon viral titer calculation. Viral titer was determined by plaque assay as described above. Protein production of the Ku70/80ΔC and wt Ku was achieved by co-infection with wild type $[His]_6$ Ku70 virus as previously described (42).

The Ku80 C-terminal region (Ku80CTR) mutant construct contained genetic sequence encoding amino acids 599-732. The final 432 bases of the Ku80 gene were synthesized by GenScript Corporation into pUC57 with an Nde1 restriction enzyme cut

site at the 5' end and BamH1 restriction enzyme cut site at the 3' end. This fragment was then subcloned into pET15b to achieve a $[His]_6$ tag on the N-terminus of the protein that can be removed via thrombin cleavage.

Protein Purification – Human Ku was purified from Sf9 cells infected with recombinant baculovirus. Briefly, 200 ml of $1X10^6$ Sf9 cells were co-infected with baculovirus containing Ku70 and either wild type Ku80 or Ku80ΔC with an M.O.I. of 5 and 10 respectively. Cells were incubated for 48 hours and lysed in buffer containing 50 mM sodium phosphate pH 8.0, 1 M potassium chloride, 10% glycerol, 0.25% triton X-100, and 7 mM 2-mercaptoethanol. Wild type and Ku70/80ΔC mutants were purified by sequential Ni-NTA and Q-Sepharose column chromatography as previously described (43;44). Fractions containing Ku were identified based on SDS-PAGE and visualized by Coomassie blue staining. Peak fractions were pooled and dialyzed overnight in either Buffer A or HEPES buffer (buffer A: 25 mM Tris pH 8.0, 75 mM potassium chloride, 10% glycerol, 0.0025% triton X-100 and 2 mM DTT; HEPES buffer: 20 mM HEPES pH 6.0, 75 mM potassium chloride, 10% glycerol, 0.005% triton X-100, 2 mM DTT) and stored at -80^{o}C.

Ku80CTR was purified from Bl21 *E.coli* cells. Briefly, pET15b-Ku80CTR was transformed into BL21 *E.coli* cells and allowed to grow on LB agar plates with ampicillin overnight at 37^{o}C. From these plates a colony was picked and allowed to grow in LB broth with ampicillin till log phase of growth was achieved. The cells were then induced with 0.4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for one hour. Cells were

then harvested via centrifugation and lysed with a buffer containing 50 mM sodium phosphate pH 8.0, 1 M potassium chloride, 10% glycerol, 0.25% triton X-100, and 7 mM 2-mercaptoethanol. Cell free extract was then supplemented with 20 mM imidazole and applied to a 2 ml Ni-NTA chromatography column at a flow rate of 1 ml/min. Protein was eluted with lysis buffer containing 350 mM imidazole. Fractions containing Ku80CTR were identified based on SDS-PAGE and visualized by Coomassie blue staining. Peak fractions were pooled and dialyzed overnight in either Buffer A or HEPES buffer and stored at -80^{o}C.

Thrombin Cleavage – Ku80CTR $[His]_6$ tag was removed via thrombin cleavage. Cleavage reactions were carried out in cleavage buffer containing 20 mM Tris-HCl, 150 mM NaCl, and 2.5 mM $CaCl_2$, pH 8.4. 400 μg Ku80CTR and 0.05 units of thrombin (Novagen) diluted in 50 mM sodium citrate, 200 mM NaCl, 0.1% PEG-8000, and 50% glycerol pH 6.5 in a final reaction volume of 500 μl. Reactions were incubated at room temperature for 2 hours. Imidazole was then added to a final concentration of 20 mM and reactions were applied to a Ni-NTA spin column that had been equilibrated with cleavage buffer supplemented with 20 mM imidazole. Columns were centrifuged at 270 x g for 5 minutes and flow through, containing cleaved Ku80CTR, was collected and dialyzed overnight against either Buffer A or HEPES buffer.

Bradford Assay – Final protein concentrations were determined via Bradford assay. A standard curve was established containing a titration of BSA ranging in concentration

from 0 to 12.5 μg/ml. Reactions were performed in a final volume of 200 μl containing BioRAD protein assay reagent (BioRAD) with a dilution factor of 5 and either 5 or 10 μl or the protein final dialysis buffer. Unknown samples were diluted to a factor of 40, 28.6, and 20 or 200, 100, and 40. Samples were prepared in a 96 well plate and readings were taken at 595 nm. The standard curve was calculated and the final protein concentrations were determined.

SDS-PAGE and Western Blot – Proteins were separated via SDS-PAGE. Samples were denatured with 6X loading dye, heated to 95^{o}C for 5 minutes and loaded onto an SDS-PAGE electrophoresis gel (Invitrogen), Gels were ran according to manufacturers specifications. Gels were either stained with Coomassie blue or transferred to PVDF membrane for Western blot analysis according to manufacturer's specifications. Membranes were blocked with 2% non-fat dry milk in TBS-Tween and probed with the primary antibodies indicated in the figure legends (Table 2). Bound antibodies were detected with a horse radish peroxidase (HRP) conjugated goat anti-mouse IgG and visualized via chemiluminescence detection and images captured on a Fujifilm LAS-3000 CCD system.

EMSA – Electrophoretic Mobility Shift Assays (EMSAs) were performed as previously described (45;46). Briefly, reactions were performed in a volume of 20 μl containing 50 mM Tris-Cl pH7.8, 10 mM $MgCl_2$ and 50 mM NaCl. The protein preparations were assessed for DNA binding activity in an EMSA containing 500fmol of ^{32}P-labeled 30-bp

Table 2. Antibodies

Antibody Name	Subunit Specificity
Ku (p80) Ab-2	Ku80CTR or C-terminus of Ku80
Ku (p80) Ab-7	Ku80ΔC or N-terminus of Ku80
Ku (p70) Ab-4	Ku70

double strand DNA as previously described using oligonucleotides 30A and 30C (Table 1) (47;48). Oxidized conditions were achieved by incubating Ku for 15 min on ice in 2 mM diamide. Re-reduced conditions were achieved by incubating oxidized Ku with 5 mM DTT for 15 min on ice. Reaction products were then separated by electrophoresis on a 6% native polyacrylamide gel. The gels were then dried and exposed to a PhosphorImager screen (Amersham Biosciences; Piscataway, NJ) and quantified using ImageQuant software. Quantification of the data is presented as the averages and standard deviations of at least three independent measurements.

Ni-NTA Pull-Down Assay – Ni-NTA pull down assays were performed via Qiagen spin columns as well as batch columns. Reactions were performed in a volume of 400 μl containing 700 nM Ku, 1.4 μM Ku80CTR, and Buffer A supplemented with 20 mM imidazole (25 mM Tris pH 8.0, 150 mM KCl, 10% glycerol, 0.005% Triton X-100, and 2 mM DTT). Reactions were incubated on ice for 1-2 hours. Qiagen spin columns were equilibrated with Buffer A supplemented with 20 mM imidazole and reactions were loaded onto the column by centrifugation at 270Xg for 5 minutes. Columns were then washed with Buffer A supplemented with 20 mM imidazole, and proteins eluted with Buffer A supplemented with 350 mM imidazole by centrifugation at 890Xg for 2 minutes. For Ni-NTA batch columns, columns were equilibrated with Buffer A supplemented with 20 mM imidazole. Reactions were incubated with column matrix for 1 hour with gentle agitation at 4^{o}C. Columns were then washed with Buffer A supplemented with 20 mM imidazole, and eluted with Buffer A supplemented with 350

mM imidazole, and matrix was separated via centrifugation for 1 min at 5,000Xg. Load, flow through and elution fractions were analyzed by SDS-PAGE and western blot procedures.

Gel Filtration Chromatography – Gel filtration chromatography was performed on a Bio-Silect SEC250-5 column (BioRAD); 300 mm in length, 7.8 mm in width, 14.5 ml bed volume, with a molecular weight capacity ranging between 10-300 kDa. The SEC250 column was equilibrated in running buffer (50 mM Tris pH 6.8, 200 mM NaCl and 1 mM DTT). Ku70/80ΔC and Ku80CTR were applied to the column sequentially and in a mixture. Reactions were prepared with a final concentration of 6.2 μM of Ku80CTR and/or 1.6 μM Ku70/80ΔC, incubated on ice for 5 minutes, applied to SEC250 gel filtration chromatography column and separate at 0.5 ml/min. Fractions were collected and analyzed by SDS-PAGE and western blot.

PICUP – Photo-induced crosslinking of unmodified proteins (PICUP) was performed. Reactions were carried out in buffer containing 15 mM NaPi pH 7.5, 150 mM NaCl, 2.5 mM APS and 0.125 mM Ruthenium as indicated. Through time course studies and titration of Ku70/80ΔC and Ku80CTR we established that 900 nM Ku70/80ΔC with varied concentrations of Ku80CTR would be exposed to intense white light for 20 seconds. Reactions were performed in the presence and absence of 22 pmol 30-bp double strand DNA 30A and 30C (Table 1) and 3 μg BSA as indicated. Reactions were then placed 6 inches from an intense white light source shining through a 1% Copper Sulfate solution

to dissipate heat and exposed for 20 seconds. Reactions were stopped with either the addition of DTT or 6X SDS loading dye. Reactions were then separated on SDS-PAGE gels and stained with Coomassie blue or transferred to PVDF membrane for western blot analysis.

EDC Coupling – Additional protein crosslinking experiments were performed with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) in the presence of reaction stabilizing reagent *N*-hydroxysulfosuccinimide (NHS). Proteins involved in EDC coupling were dialyzed in HEPES buffer (20 mM HEPES pH 6.0, 75 mM KCl, 10% glycerol, 0.005% triton X-100 and 2 mM DTT). Reaction buffer contained 100 mM MES pH 6.0 and 500 mM NaCl. Optimal one step EDC coupling conditions were determined by titration of EDC, titration of Ku80CTR, and time course studies. From these studies it was determined that the optimal conditions were 1 mM EDC, 1.4 mM NHS, 900 nM Ku70/80ΔC and varied concentrations of Ku80CTR. Reactions were formed in the presence and absence of 22 pmol 30-bp double strand DNA 30A and 30C (Table 1) and 3 μg BSA as indicated. Reactions were incubated for 30 minutes at room temperature and stopped by the addition of 6X SDS loading dye. Samples were then loaded onto SDS-PAGE gels followed by transfer to PVDF membrane for western blot analysis.

Limited Proteolysis – Limited tryptic proteolysis was performed according to established procedures (49) with the following modification. The Ku preparations were analyzed for potential structural changes under control, conditions as well as oxidized and re-

reduced conditions. Oxidized conditions were achieved by incubating Ku for 15 min on ice in 2 mM diamide. Re-reduced conditions were achieved by incubating oxidized Ku with 5 mM DTT for 15 min on ice. Ku protein preparations (4 μg) were subjected to limited proteolysis by the addition of 200 ng of sequencing grade bovine trypsin (Roche Diagnostics). Reactions were performed in buffer A and incubated at 37^{o}C for 10 minutes. Reactions were terminated by the addition of SDS loading dye and samples were separated by 15% SDS-PAGE. Products were visualized via Coomassie Blue staining and images were captured using Image Reader LAS-3000 (FujiFilm). Images were visualized and quantified using MultiGuage V3.0.

Limited Proteolysis With Crosslinking – Limited tryptic proteolysis with crosslinking was performed similar to limited proteolysis described above with slight modifications. wtKu and DNA-PK were analyzed for Ku80CTR interaction under native and PICUP conditions with or without the addition of DNA or ATP. 8 μg wtKu were subjected to PICUP crosslinking conditions in the presence or absence of 30-bp double strand DNA 30A and 30C (Table 1) as indicated followed by limited proteolysis in Buffer A with 20ng trypsin at 37^{o}C for 1 hour. PICUP reactions were stopped with the addition of 5 mM DTT, and proteolysis reactions were stopped with the addition of protease inhibitors or 6X SDS loading dye. Products were then separated by 8-20% gradient SDS-PAGE and transferred to PVDF membrane for western blot analysis. DNA-PK (1.5 μg) was allowed to undergo phosphorylation for 15-30 minutes at 37^{o}C in a reaction containing 27 pmol 30-bp double strand DNA 30A and 30C (Table 1), 250 μM ATP, 1 μCi ^{32}P ATP, 20 mM

HEPES pH 7.5, 8 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 100 mM KCl. PICUP reagents were added to each reaction as indicated and allowed to undergo crosslinking. Crosslinking was stopped with the addition of DTT and allowed to digest at 37°C overnight in the dark with 5 μg trypsin. Digestion reactions were stopped with the addition of 6X SDS loading dye and heated to 95°C. Products were then separated by 4-20% gradient SDS-PAGE, transferred to PVDF membrane and visualized by western blot analysis.

DNA-PK Kinase Assay – Kinase assays were performed at 37°C in a final volume of 20 μl containing 20 mM HEPES, pH 7.5, 8 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 125 μM ATP, [γ-^{32}P] ATP (0.5 μCi), 2 pmol 30-bp double strand DNA 30A and 30C (Table 1), 500 μM p53 synthetic peptide, and 80 fmol DNA-PKcs was incubated with 1 pmol wtKu, 1 pmol Ku70/80ΔC, or 10 pmol Ku80CTR as indicated. Reactions were incubated at 37°C for 15 minutes and stopped with 30% acetic acid. Reaction products were spotted on P81 phosphocellulose filter paper that was then washed 5 times for 5 minutes each in 15% acetic acid, once in 100% methanol and allowed to dry. Samples were exposed to PhosphorImager and analyzed using ImageQuant software (Molecular Dynamics). Kinase assay was performed by KSP.

Results

Identification and Mutation of Potential Amino Acids Involved in Ku Regulation – Previous analysis identified the extreme C-terminus of Ku80 as an effecter of DNA-PKcs activation and potentially involved in redox regulation of Ku DNA binding activity. These analyses showed that the removal of the extreme C-terminus of Ku80 dramatically decreases the kinase activity of DNA-PK (50). Chemical reactivity probes also identified the C-terminal domain of Ku, which contains C638, as an area of interest in redox regulation that has the potential to influence the DNA binding activity of Ku (51). To further understand the role of the C-terminus of Ku80, we created two mutants. The first mutant contains a truncated form of Ku80 PCR was used to introduce a stop codon after amino acid 548. This C-terminal truncation mutant is designated Ku80ΔC. The composition of this construct was verified by restriction enzyme mapping. This mutant was then used to generate a recombinant baculovirus that was verified to produce a single subunit protein at the correct molecular weight that reacted with a Ku80 N-terminus specific antibody (Table 2, Figure 4b and c). The recombinant baculovirus was then co-infected with a $[His]_6$-wtKu70 into Sf9 cells to express the heterodimer Ku70/80ΔC. Ku70/80ΔC and wtKu recombinant proteins were purified via Ni-agarose column chromatography followed by fractionation on a Macro-prep Q anion exchange matrix. SDS-PAGE analysis of the purified proteins is presented in Figure 4. A 1:1 stoichiometry is of the utmost importance for the Ku molecules due to the requirement that both subunits are present for the heterodimer to actively bind DNA and recruit

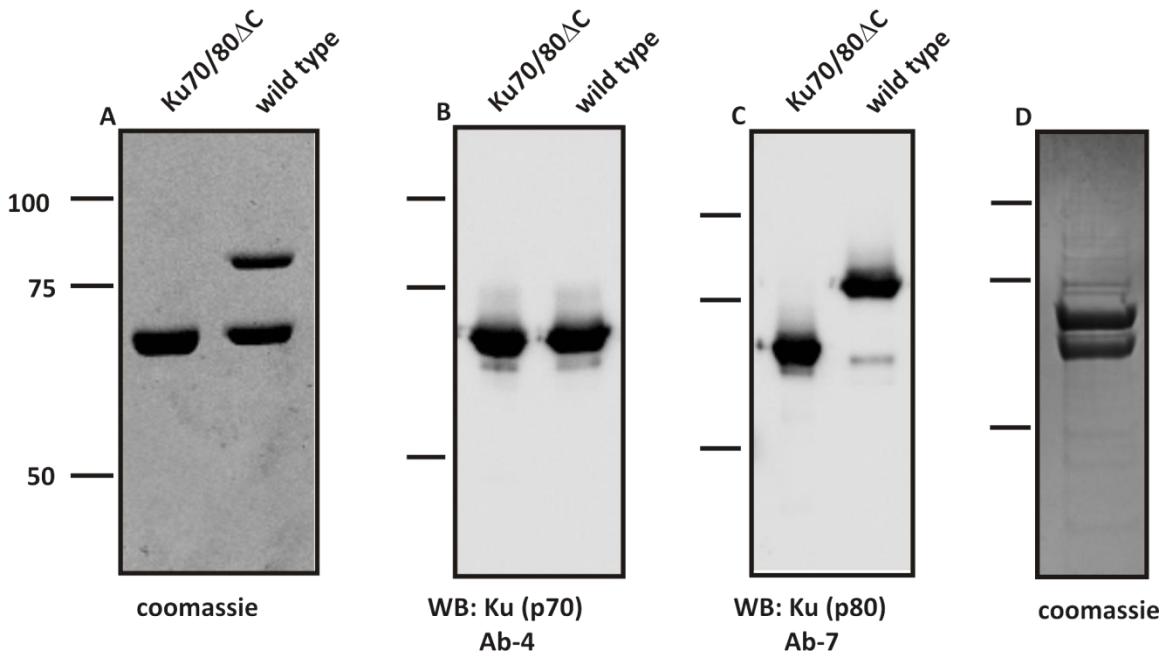


Figure 4. Ku heterodimer complexes purity and stoichiometry. The wtKu and Ku70/80ΔC with a $[His]_6$-tag on both Ku70 and Ku80ΔC were subjected to SDS-PAGE and visualized with Coomassie blue staining (A) western blot analysis and detected with Ku (p70) Ab-4 (B) or Ku (p80) Ab-7 (C). (D) ku70/80ΔC with $[His]_6$-tag on Ku70 only was separated by SDS-PAGE and stained with Coomassie. Panels A, B and C were adapted from ref (52).

DNA-PKcs. Fortunately, we were able to demonstrate a 1:1 stoichiometry for each preparation, and the correct protein subunits were confirmed by western blot analysis (Figure 4b and c). Western blot analysis was particularly useful for the Ku70/80ΔC construct that contained a $[His]_6$-tag on both Ku70 and Ku80ΔC as the truncated $[His]_6$-tagged Ku80 migrates very closely to the $[His]_6$-tagged Ku70 as judged by Coomassie Blue stained gel. A second mutant construct was made to aid in our understanding of the role of the extreme C-terminus. This construct was designed and synthesized to contain amino acids 590-732 of Ku80, and is referred to as Ku80 C-terminal region (Ku80CTR). This synthesized mutant insert was subcloned into pET15b to create an N-terminal $[His]_6$-tag. The insert was then subjected to DNA sequence analysis and proper cloning was confirmed. The plasmid was then transformed into Bl21 *E.coli,* cells and over-expressed Ku80CTR was purified via Ni-agarose affinity chromatography. Upon purification of Ku80CTR, thrombin cleavage was utilized to remove the $[His]_6$-tag. SDS-PAGE analysis of the purified protein before and after thrombin cleavage is presented in Figure 5. The result of the one column purification scheme yielded a well expressed, highly pure recombinant protein with a $[His]_6$-tag that can be removed via thrombin cleavage.

DNA Binding of Ku is Independent of the Ku80CTR – DNA binding of the purified Ku variants was assessed in an EMSA using a 30-bp duplex DNA 30A and 30C (Table 1) under reduced conditions, which we have shown allow for maximal DNA binding activity of Ku (53;54). The results presented in Figure 6 demonstrate that the Ku70/80ΔC

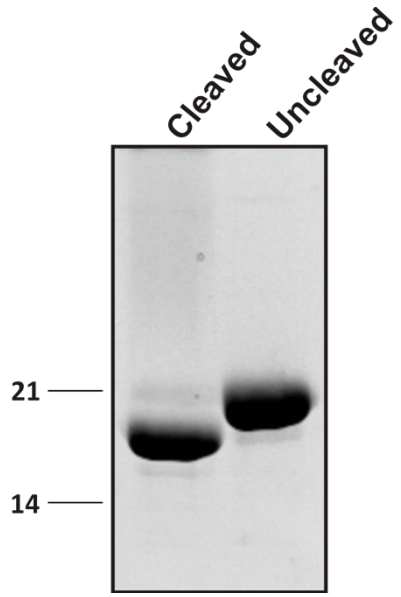


Figure 5. Purity of Ku80CTR. Cleaved and uncleaved forms of Ku80CTR were applied to SDS-PAGE and stained with Coomassie.

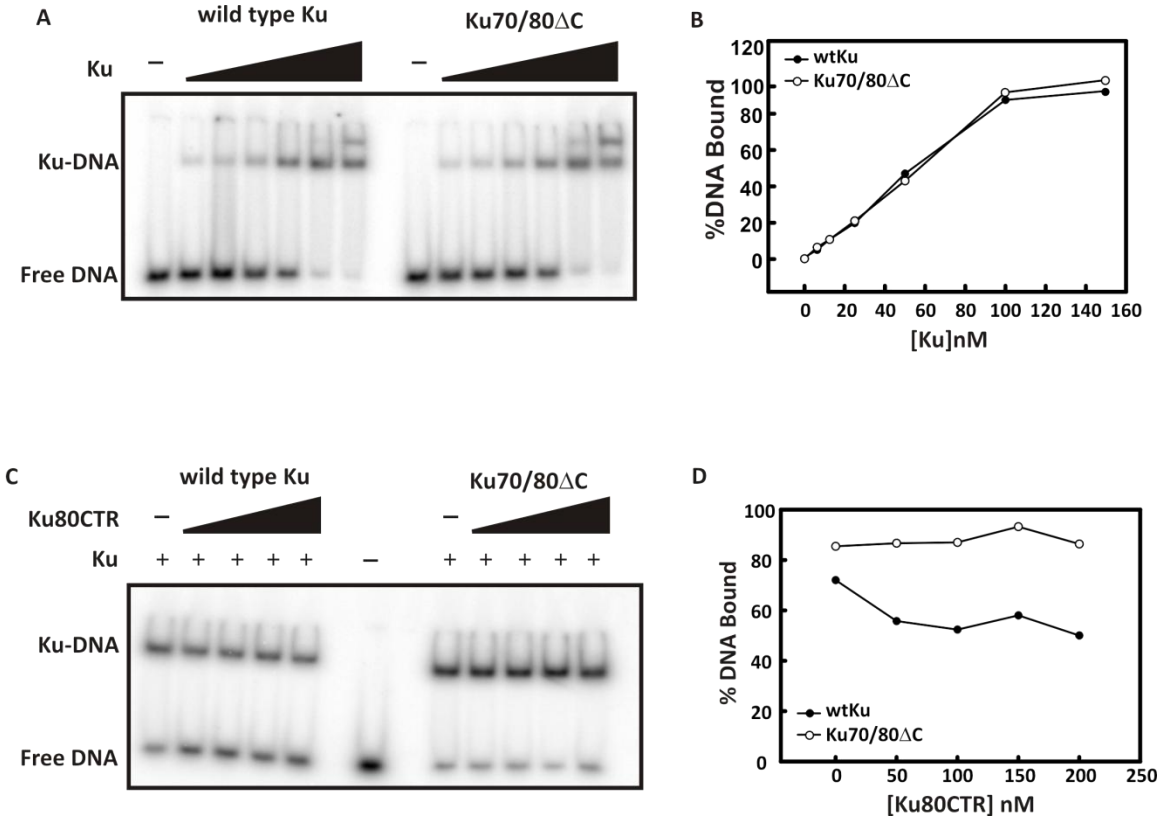


Figure 6. DNA binding activity is not affected by truncation or the addition of Ku80CTR. (A) wtKu and Ku70/80ΔC (0-150 nM) were assessed for binding to duplex 30-bp DNA substrate and visualized via PhosphorImager. (B) Quantification of the results in panel A was performed via PhosphoImager analysis. Filled circles represent wtKu and open circles represents Ku70/80ΔC. (C) wtKu (25 nM) and Ku70/80ΔC (50 nM) were supplemented with Ku80CTR (0-200 nM) and subjected to similar analysis as panel A. (D) Quantification of panel C. Filled circles represent wtKu and open circles represent Ku70/80ΔC.

variant lacking the C-terminal domain is capable of binding DNA with little to no reduction in binding affinity when compared to wtKu (Figure 6a). Nor was this binding affinity of Ku70/80ΔC affected by the addition of Ku80CTR (Figure 6c). Quantification of the data is presented in Figure 6b and d. While the C-terminal domain of Ku80 has been shown to be involved in activation of DNA-PKcs (55), these results demonstrate that DNA binding is only moderately affected, if at all, by removal of this domain. This result is consistent with the crystal structure of the Ku heterodimer bound to DNA (56). These data support the contention that no dramatic alteration in DNA binding activity is manifested by the introduced C-terminal truncation in the protein's primary structure.

Redox Effects on DNA Binding – To assess the effect of redox on Ku binding we used the cysteine specific oxidant, diamide, to oxidize Ku and then analyzed binding in a reaction performed in the absence of added DTT. Previously, we have shown that under these conditions Ku exhibits a reversible oxidation event that impairs DNA binding (57;58). Based on previous results, 2 mM diamide was sufficient to oxidize wtKu (59). A similar line of experimentation was performed comparing the truncation mutant's DNA binding activity to that of wtKu (Figure 7), and again Ku DNA binding was reduced in both protein preparations as a function of diamide. We also reversed the conditions to re-reduce the protein by incubation with additional excess DTT. The result of this treatment was the restoration of DNA binding activity for the wild type protein and Ku70/80ΔC truncation variant. Interestingly, full DNA binding activity was not observed

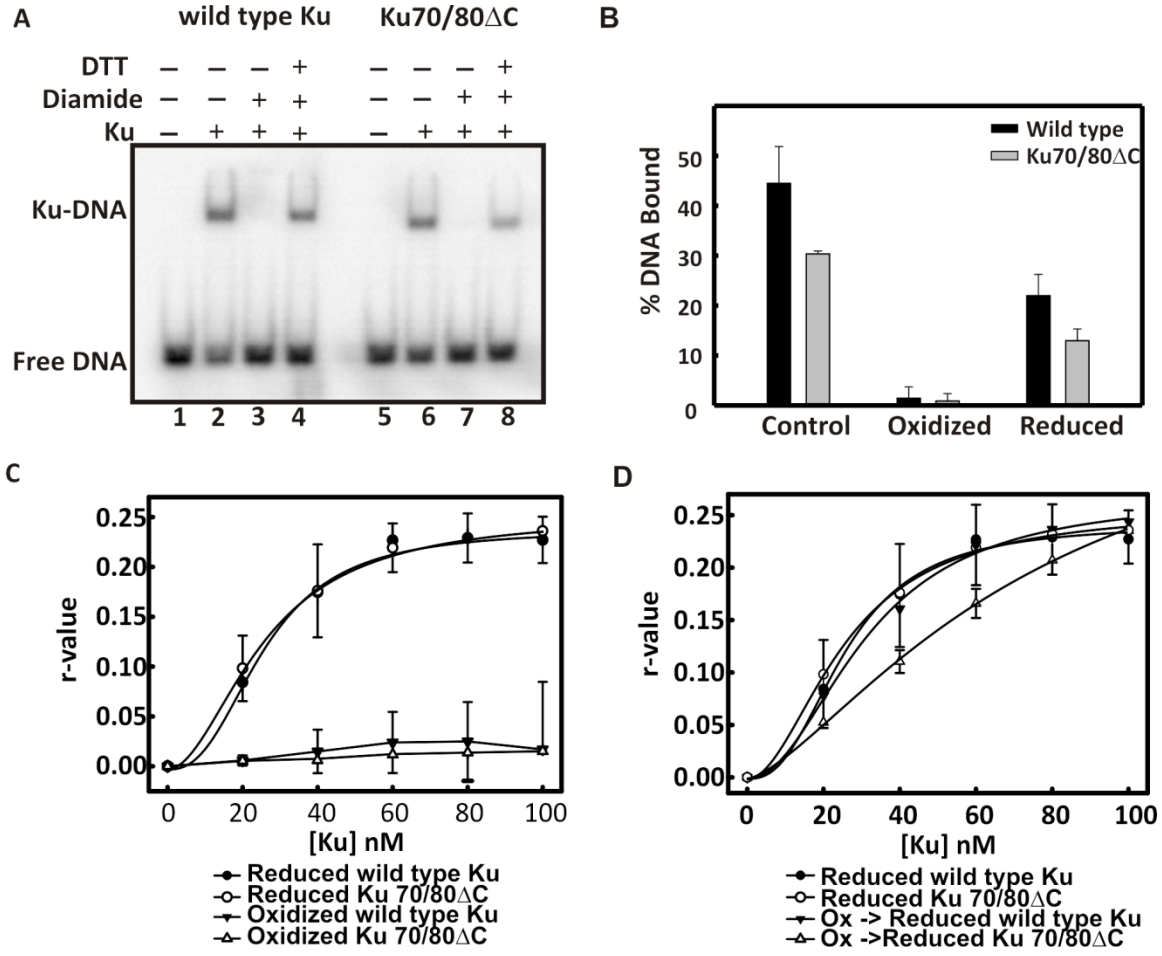


Figure 7. The effects of oxidation on DNA binding of wtKu and Ku70/80ΔC. (A) For oxidized conditions wtKu and Ku70/80ΔC were incubated with 2 mM diamide, for re-reduced conditions, diamide incubation was followed by incubation with 5 mM DTT. DNA binding was then assessed as described in Figure 6 legend. (B) Quantification of the results in panel A was performed via PhosphoImager analysis. Results are presented as the average and standard deviation of triplicate analysis. Anisotropy assays were performed in triplicate as described previously (52) under control verses oxidized conditions (C) and Control versus re-reduced conditions (D). Data presented represents average and standard deviation of three analyses. Ku preparations used in each series are designated in each figure. Figure adapted from ref (52).

for either protein preparation, with Ku70/80ΔC having a more decreased level of recovery when compared to wtKu, thus the potential for a persistent redox-dependent structural change exists in Ku (Figure 7). To determine if in fact a persistent structural alteration in Ku exists following diamide oxidation and re-reduction, we performed a limited tryptic digest of wtKu and Ku70/80ΔC that was treated with DTT before diamide addition at a ratio that does not result in Ku oxidization (control) and Ku that was first treated with diamide to oxidize the proteins, was then re-reduced with excess DTT. The final concentrations of all components were identical prior to the tryptic cleavage reactions. The results presented in Figure 8 demonstrate that there is somewhat more tryptic cleavage under the control conditions compared to the re-reduced Ku, and this is apparent in both the wild-type and Ku70/80ΔC mutant. This is also apparent in the prominence of the lower molecular mass peptides (<37 kDa) under control conditions and the prominence of the full length proteins in the re-reduced conditions. This is somewhat surprising in that the better DNA binding conditions (control) result in greater tryptic susceptibility. Quantification of the data, which is presented in Figure 8b and c, bears out the interpretation that re-reduced Ku is slightly less susceptible to tryptic cleavage. A difference in peptide product distribution was observed between the wild type and Ku70/80ΔC under both conditions that could not be attributed to the truncation. This suggests that the folding or conformation of the Ku70/80ΔC protein differs from that of wild type Ku. Interestingly, this minor difference may account for the less robust recovery of DNA binding upon re-reduction found in Ku70/80ΔC when compared to wtKu under similar conditions. While the ability of diamide to reversibly

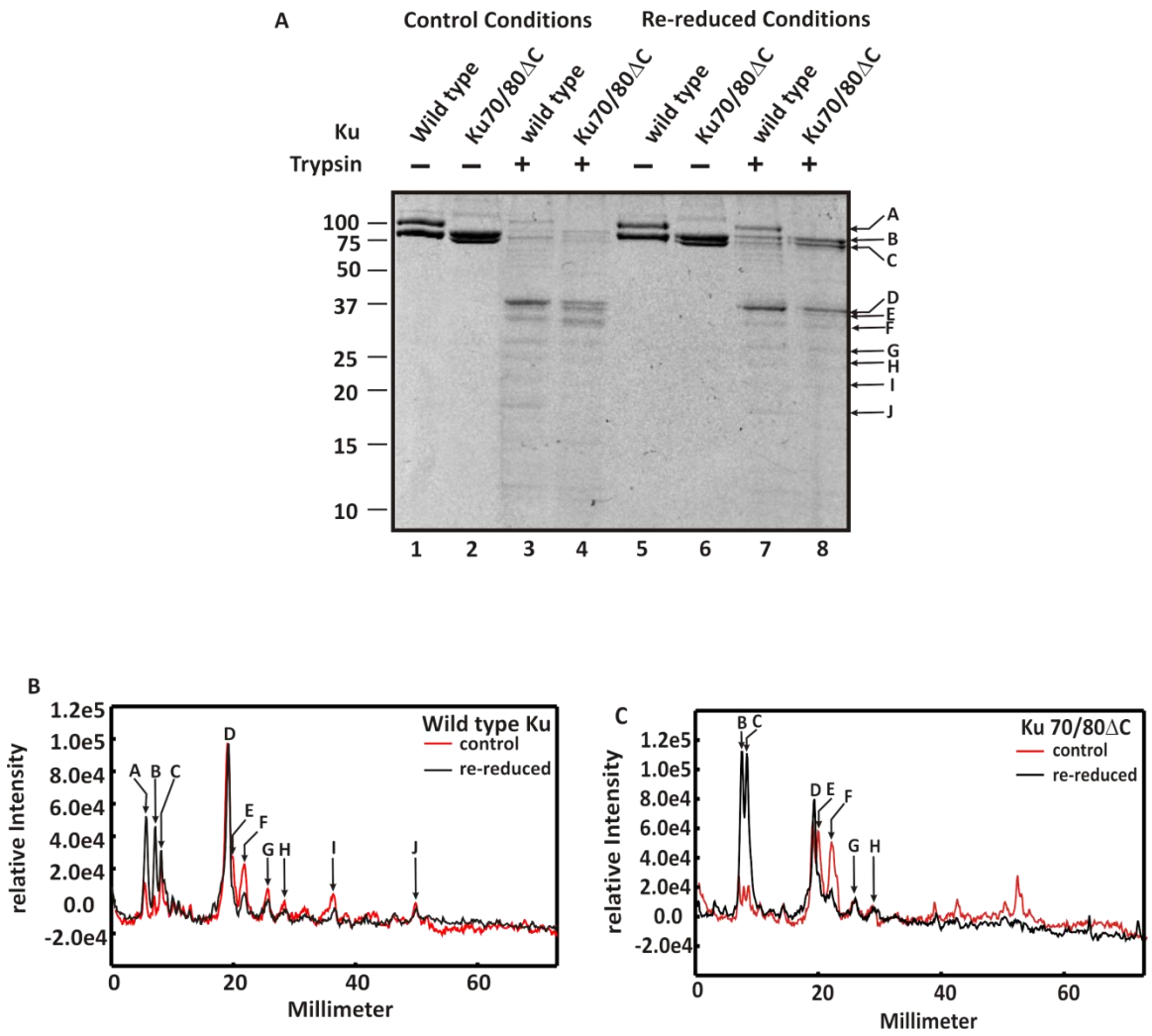


Figure 8. Effects of oxidation of wt and Ku70/80ΔC structure. A) Following oxidation and re-reduction as described in Figure 7 legend, wtKu (4 μg) and Ku70/80ΔC (4 μg) were subjected to limited tryptic digest. Reduced conditions were first oxidized with diamide then re-reduced with DTT. Reactions were terminated by the addition of SDS sample buffer and products separated by SDS-PAGE and visualized by Coomassie Blue staining. Band intensity was assessed via digitization of the gel image using Fuji Mulitgauge software. The intensity was normalized and plotted versus distance for the indicated lanes. B) wtKu digested with trypsin under control and re-reduced conditions, C) Ku70/80ΔC digested with trypsin under control and re-reduced conditions. Figure adapted from ref (52).

oxidize the Ku variant suggests that C638 is not involved in redox regulation, we have previously demonstrated that solution based, true equilibrium binding assays can often allow differences in activity to be determined that are not observed in an EMSA. Therefore, we assessed binding of wild type and Ku70/80ΔC to a 30-bp duplex DNA (30A and 30C Table 1) substrate using a fluorescence polarization solution based assay. The fluorescence polarization assay is read while the Ku/DNA complex is in solution associating and dissociating freely, where the EMSA assay does not allow for repeated dissociation and association activity. A 30-bp duplex with a single fluorescein label on the 5' terminus of 30C (Table 1) was used and Ku protein preparations were titrated into the binding reactions. Wild type and Ku70/80ΔC under reduced conditions displayed identical binding and, upon oxidation with diamide, minimal DNA binding was detected (Figure 7c). The oxidized Ku proteins were then re-reduced by the addition of excess DTT and titrated into binding reactions. In this analysis, wtKu regained complete DNA binding activity while the Ku70/80ΔC again regained significant DNA binding activity, though not complete. This data is consistent with the alteration in trypsin sensitivity observed following re-reduction after oxidation such that after re-reduction, both wild type and Ku70/80ΔC were less sensitive to trypsin digestion and suggest an alteration in structure that impacts DNA binding activity. Overall, these analyses provide insight into how Ku structural features impact redox regulation.

Ku80CTR Interaction with Ku70/80ΔC – To investigate the interaction between Ku80CTR and Ku70/80ΔC a variety of methods were utilized ranging from basic methods requiring

strong affinity, to more complex methods that are capable of detecting weak affinity interactions. The most basic and initial method was a pull down assay making use of the $[His]_6$-tag of Ku70 and its affinity for Ni resin. In this assay $[His]_6$-Ku70/80ΔC was pre-incubated with Ku80CTR that had the $[His]_6$-tag removed and bound to either Ni-NTA spin columns or batch agarose columns. Both methods resulted in Ku80CTR eluting from the column in the flow through fractions and not with Ku70/80ΔC (Figure 9). These results demonstrate there is no stable interaction under these conditions.

The second method, gel filtration chromatography, was slightly more complex and is more conducive to detecting lower affinity interactions. Gel filtration chromatography separates species based on overall molecular size, resulting in larger molecules eluting from the column first followed by smaller molecules. Preliminary work was performed to determine the elution volume of Ku70/80ΔC and Ku80CTR independent of the other protein. Individual proteins were pre-incubated in running buffer and applied to the column at 0.5 ml/min and 0.15 ml fractions were collected. Fractions were then analyzed by SDS-PAGE and visualized by Western blot probing (Figure 10a and b). Finally, Ku70/80ΔC and Ku80CTR were pre-incubated and applied to the gel filtration chromatography column in conditions matching those of the individual proteins. Fractions were again analyzed by SDS-PAGE and protein elution was visualized by western blot probing (Figure 10c). Ku80CTR elution volume did not decrease as a function of Ku70/80ΔC, as would have been the result if the molecular size increased as a result of an interaction. These results indicate that under the conditions employed for

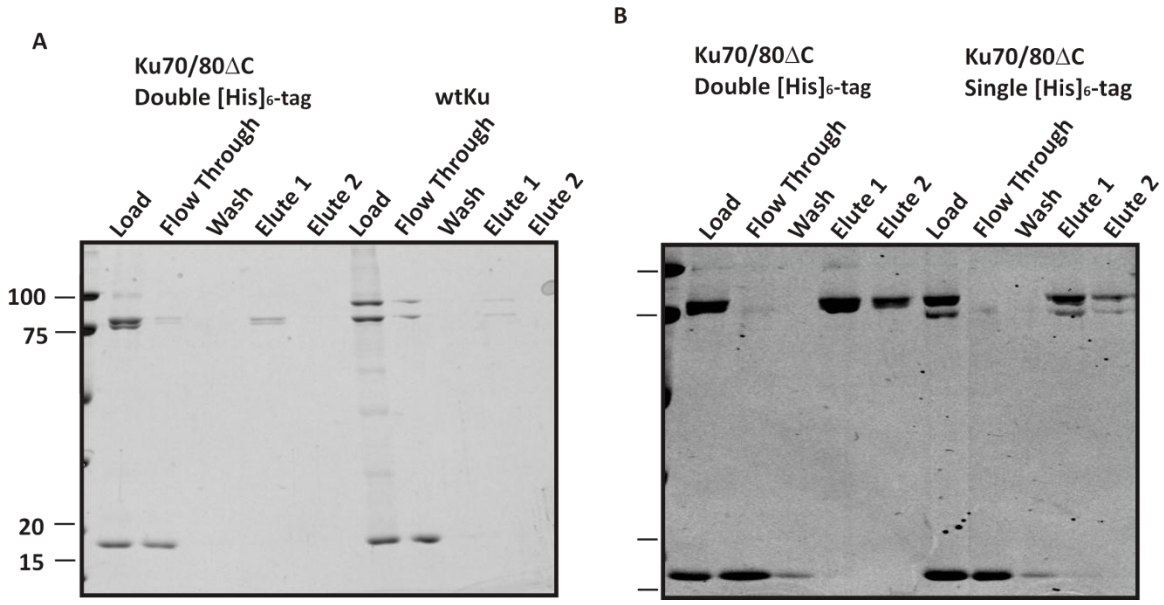


Figure 9. Ku70/80ΔC interaction with Ku80CTR analyzed via Ni-NTA pull-down assay. Ku70/80ΔC and Ku80CTR were incubated on ice for 20 minutes and applied to either Ni-NTA spin columns (A) or Ni-NTA agarose batch columns (B). Reactions were then separated via SDS-PAGE and visualized with Commassie Blue stain.

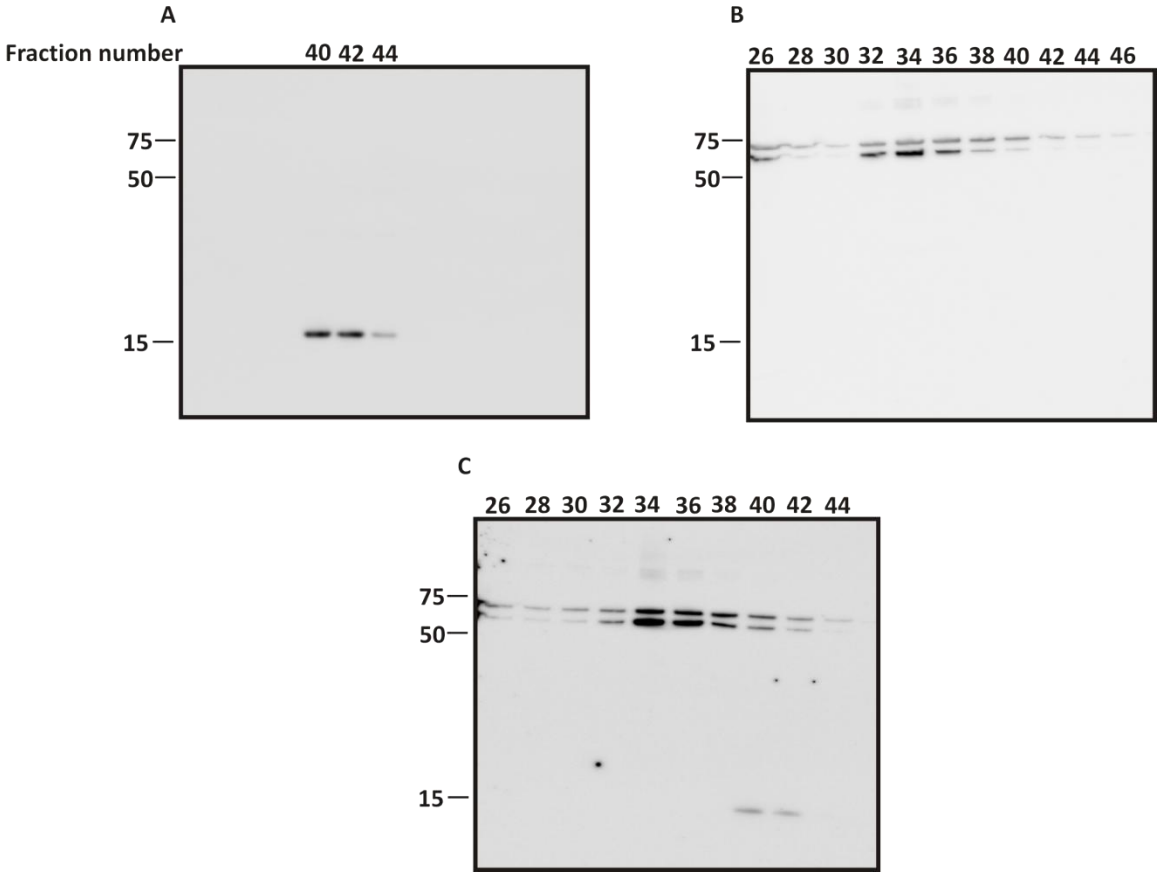


Figure 10. Ku70/80ΔC interaction with Ku80CTR in SEC250 gel filtration. Proteins were incubated on ice for 60 minutes before being applied to the gel filtration column and eluted at 0.5 ml/min. Samples were separated via SDS-PAGE, transferred to PVDF membrane for western blot analysis and visualized via subunit and protein specific antibodies (Table 2). (A) Ku80CTR alone. (B) Ku70/80ΔC alone. (C) Ku70/80ΔC and Ku80CTR combined (1:10 ratio).

gel filtration chromatography, no interaction between Ku80CTR and Ku70/80ΔC is observed.

A third method of detecting protein-protein interaction is through molecular crosslinking. This method allows for an extremely sensitive detection of protein-protein interaction by covalently binding together two molecules that are in close proximity, such as the potential proximity found in a protein-protein interaction, and making a transient interaction permanent. For this work, we utilized two unique crosslinking methods. The first system employed was photo-induced crosslinking of unmodified proteins, or PICUP, zero distance crosslinking (60;61). Zero distance crosslinking binds amino acid directly to amino acid with no additional linker distance tethering the two molecules together. Ku80CTR was pre-incubated with Ku70/80ΔC in the presence or absence of ruthenium, the catalyst for the PICUP reaction, and exposed to an intense white light for a pre-determined length of time. These reactions were then analyzed by SDS-PAGE and visualized by western blot probing first with an antibody to detect just Ku80CTR followed by Ku70 and Ku80ΔC subunits sequentially (Table 2). As evident in Figure 11, Ku80CTR migration shifted from 16 kDa to 150 kDa in the presence of Ku70/80ΔC. This migration shift was shown to increase in intensity as a function of Ku80CTR concentration and is consistent with the combined molecular weights of Ku70, Ku80ΔC, and Ku80CTR. These experiments were also conducted in the presence and absence of bovine serum albumin (BSA) to analyze the possibility of a non-specific interaction. In the presence of BSA there is no change in Ku80CTR migration pattern or the intensity of the band, which is indicative of a relatively specific interaction between

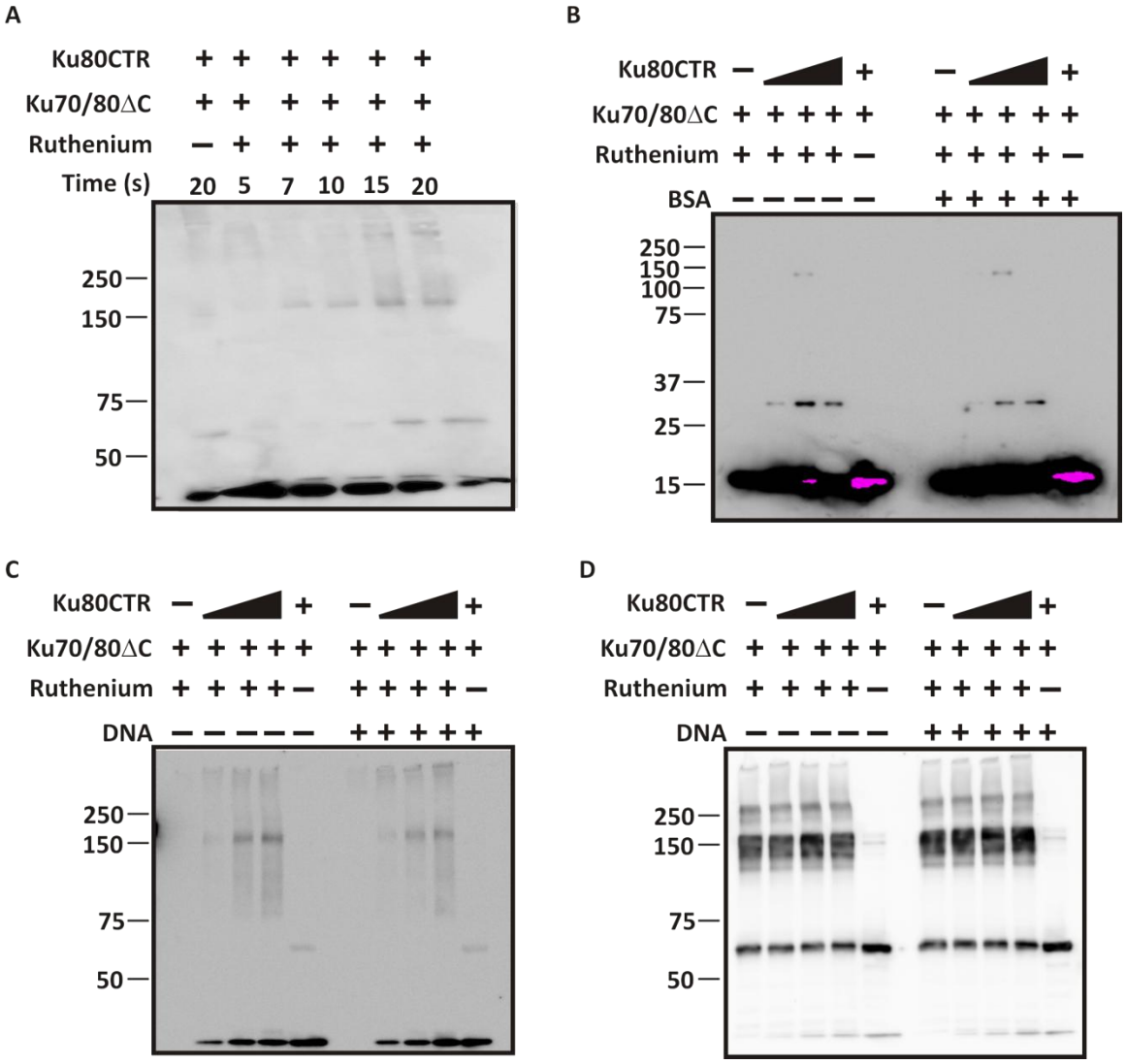
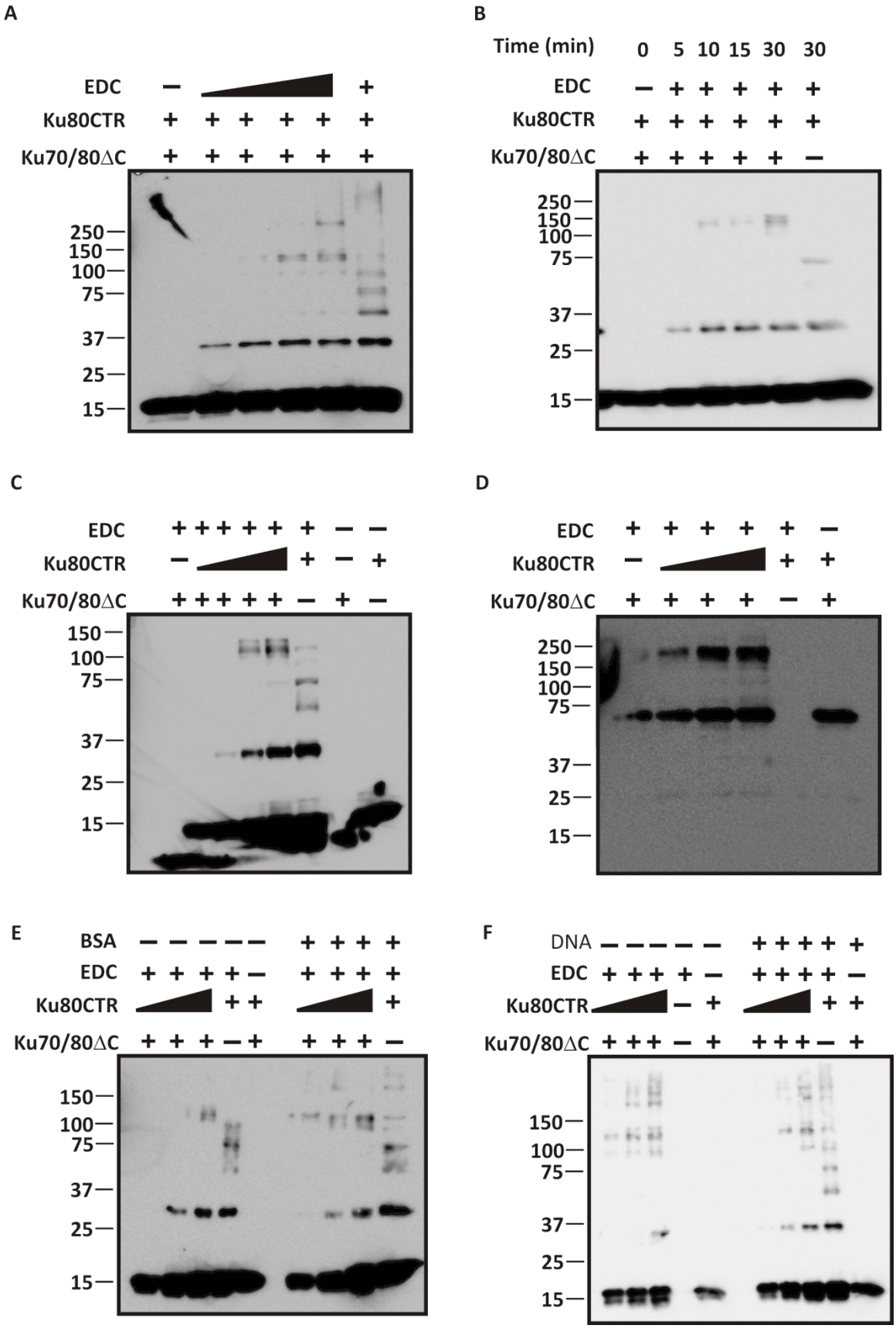


Figure 11. Ku70/80ΔC interaction with Ku80CTR in PICUP assay. Reactions were prepared in the presence or absence of ruthenium to facilitate crosslinking. (A) Ku70/80ΔC (900 nM) and Ku80CTR (5.4 nM) were incubated with 3 μg BSA and ruthenium as indicated and exposed to intense white light for length of time indicated. Reactions were stopped with SDS loading dye and applied to SDS-PAGE, transferred to PVDF for western blot analysis and visualized with Ku80CTR specific antibody (Table 1). (B) Ku70/80ΔC (900 nM) and Ku80CTR (0-3.5 μM) were were incubated in the presence or absence of 3 μg BSA and ruthenium as indicated and exposed to intense white light for 20 seconds and continued on as described in panel A. (C) PICUP reactions were prepared as before in the presence or absence of 22 pmol 30 bp-DNA substrate as indicated. (D) Panel C probed with Ku80CTR specific antibody followed by Ku70 specific antibody (Table 2).

Ku80CTR and Ku70/80ΔC (Figure 11b). With the addition of DNA, there was also no change in the migration pattern of Ku80CTR or the intensity of the bands (Figure 11c). This result was surprising when considering the conformational change that is observed when Ku binds DNA. When antibodies that detect Ku70 and Ku80ΔC were sequentially added to the blot, a more intense band at 150 kDa was observed, confirming that Ku80CTR is in fact covalently bound to Ku70/80ΔC (Figure 11d). Upon comparison of the Ku80CTR specific band at 150 kDa and the Ku70 and Ku80ΔC antibody band at 150 kDa, it is apparent that the Ku80CTR specific band is much less intense. This decrease in intensity is evidence of a small percentage of crosslink reactions including Ku80CTR in the Ku70/80ΔC complex. This result is also apparent when considering the Ku80CTR specific band observed at 16 kDa compared to that observed at 150 kDa. The amount of Ku80CTR that was incorporated into the Ku70/80ΔC is far less than the Ku80CTR that remained un-crosslinked. From these results we conclude that there is a direct interaction between Ku80CTR and Ku70/80ΔC; however, the affinity is extremely low, which would explain why we did not see an interaction in the Ni- pulldown assay and gel filtration chromatography. This interaction is simply too weak to be observed in these less sensitive assays. This interaction is simply too weak to be observed in these less sensitive assays. There is also a band in Figure 11b at 32 kDa that is consistent with two Ku80CTR proteins forming a dimer. This band is appears in the presence or absence of Ku70/80ΔC and could be indicative of a Ku80CTR specific interaction.

The second form of crosslinking was 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) coupling. This form of protein-

protein crosslinking uses a carboxyl and amine-reactive small molecule, EDC, as a catalyst to react with a carboxyl group first that then forms an unstable intermediate that reacts with a primary amine to form an amide bond. This too is a zero distance crosslinker and covalently binds two molecules together that are found in the close proximity observed in a potential protein-protein interaction. Consistent with the PICUP results, the Ku80CTR migration pattern changed, but instead of 150 kDa, it shifted to 125 kDa (Figure 12). The intensity of this shift increased as a function of EDC concentration (Figure 12a) as well as Ku80CTR concentration (Figure 12c) and was completely dependent on the presence of Ku70/80ΔC. These experiments were also performed in the presence or absence of BSA, and surprisingly generated a change in band intensity (Figure 12e). The band remained completely dependent on the presence of Ku70/80ΔC, and the intensity increased as a function of Ku80CTR concentration. We attribute this change in band intensity to the phenomenon of molecular crowding increasing the local concentration of Ku80CTR for Ku70/80ΔC. These experiments were also conducted in the presence and absence of DNA and were consistent with the PICUP reactions, resulting in no change in Ku80CTR migration pattern or band intensity (Figure 12f). Again, the 125 kDa Ku80CTR specific band intensity is much lower than that of the 16 kDa Ku80CTR band and the band intensity generated from Ku70 and Ku80ΔC antibodies, indicating a small percentage of Ku80CTR incorporation into the Ku70/80ΔC complex. These data are all consistent with the observations found in the PICUP crosslinking reactions and indicate the existence of an interaction; however, the affinity of this interaction appears to be weak. There is also a band at 32 kDa when Ku80CTR is


A
EDC
Ku80CTR
Ku70/80ΔC
250
150
100
75
37
25
15
B
Time (min) 0 5 10 15 30 30
EDC
Ku80CTR
Ku70/80ΔC
250
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37
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15
C
EDC
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Ku70/80ΔC
150
100
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37
25
15
D
EDC
Ku80CTR
Ku70/80ΔC
250
150
100
75
37
25
15
E
BSA
EDC
Ku80CTR
Ku70/80ΔC
150
100
75
37
25
15
F
DNA
EDC
Ku80CTR
Ku70/80ΔC
150
100
75
37
25
15

Figure 12. Ku70/80ΔC interaction with Ku80CTR as assessed by EDC coupling. (A) Ku70/80ΔC (900 nM) and Ku80CTR (2.1 μM) were incubated with BSA (3 μg) and EDC (0-2 mM) for 30 minutes. Reactions were stopped with SDS loading dye, applied to SDS-PAGE, transferred to PVDF membrane for western blot analysis and visualized with a Ku80CTR specific antibody (Table 2). (B) Reactions were prepared as before and incubated for length of time indicated. Samples were visualized as before. (C) Ku70/80ΔC (900 nM) and Ku80CTR (0-3.5 μM) were incubated with BSA (3 μg) and EDC (0-2 mM) for 30 minutes. Samples were visualized with Ku80CTR specific antibody. (D) Samples were prepared as in panel C and visualized with Ku80 N-terminus specific antibody (Table 2). (E) Samples were prepared as before in the presence or absence of BSA (3 μg) as indicated and visualized with a Ku80CTR specific antibody (Table 2). (F) Samples were prepared as before in the presence of BSA (3 μg) and the presence or absence of 22 pmol 30 bp-DNA substrate as indicated. Samples were visualized with a Ku80CTR specific antibody (Table 2).

allowed to crosslink that is detected with only the Ku80CTR antibody. This band remains prominent despite the addition of BSA, DNA, and Ku70/80ΔC, suggesting that this is a specific interaction. The molecular migration of this band is consistent with 2 Ku80CTR molecules bound together.

Extreme C-Terminus Interaction Analysis by Proteolysis – To evaluate the interaction of the extreme C-terminus of Ku80 with the bulk of the Ku molecule, limited proteolysis was implemented. Previous work in our lab indicated that the extreme C-terminus of Ku80 was susceptible to tryptic cleavage and resulted in a 10 kDa band (62). Our current studies incorporated PICUP reactions in conjunction with limited proteolysis to further understand any interaction between Ku80 C-terminus with the rest of the Ku molecule. In this line of experiments, wtKu was allowed to undergo proteolysis in the presence or absence of crosslinking as well as DNA. When comparing lanes 2 to 4 and 6 to 8 in Figure 13 it is apparent that in the presence of a crosslinking agent the C-terminus of Ku80 is still susceptible to tryptic cleavage, but to a lesser degree. This observation is consistent with the observations seen in the two forms of crosslinking reactions mentioned above. There is an interaction, but it remains extremely weak. Also consistent with that seen in the PICUP and EDC coupling reactions, DNA does not appear to affect the ability of the Ku80 C-terminus to become incorporated into the Ku molecule through crosslinking as evidence of the lack of change in the 10 kDa band intensity (compare lane 4 to lane 8, Figure 13).

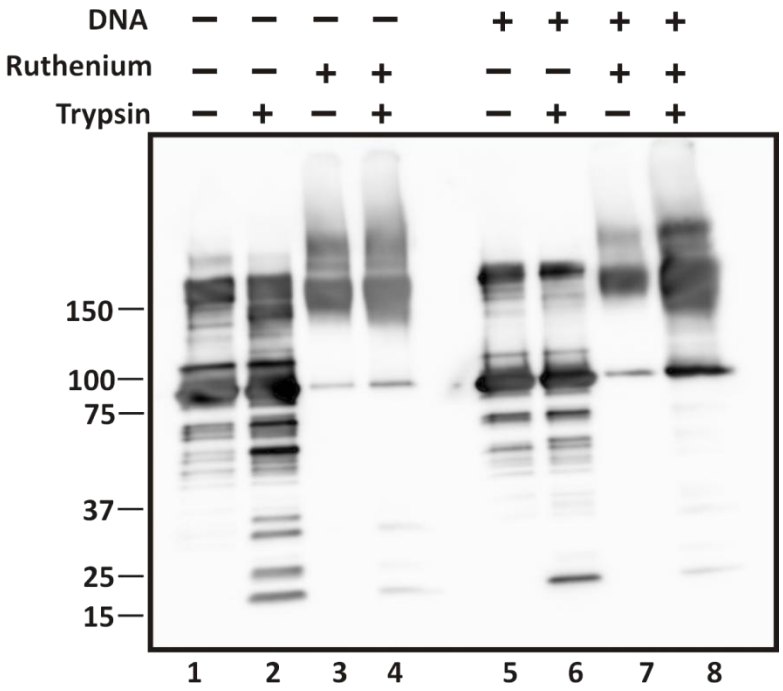

Figure 13. C-terminus of Ku80 interaction with the Ku heterodimer analyzed by crosslinking and limited proteolysis. wtKu (8 μg) was subjected to PICUP as described in Figure 11 legend. Reactions were stopped with DTT followed by incubation with trypsin (20 ng) for 1 hour. Products were separated by SDS-PAGE, transferred to PVDF membrane for western blot analysis and visualized with a Ku80 C-terminus specific antibody (Table 2).

As stated above, if the extreme C-terminus of Ku80 is not present, DNA-PK activation decreases dramatically (63). To further evaluate the role of the extreme C-terminus of Ku80, DNA-PK that was purified with endogenous Ku was also subjected to crosslinking and allowed to undergo proteolysis. Based on findings observed in Figure 14a, the DNA-PKcs does not appear to change the susceptibility of the extreme C-terminus of Ku80 to tryptic digestion (Figure 14a, lane 1). In the presence of crosslinking the extreme C-terminus of Ku80 remains susceptible to cleavage, but to a lesser degree which is consistent with the findings of the wtKu digestion with and without crosslinking (Figure 14a, compare lane 1 to 3 and 5 to 7). Also consistent with the wtKu digestion, the degree of Ku80 C-terminus digestion does not appear to be altered in the presence of DNA (Figure 14a, compare lanes 3 and 7). These experiments were taken a step further and DNA-PK/DNA was allowed to become phosphorylated. Similar results were observed, and the addition of ATP did not change the ability of trypsin to cleave the C-terminus of Ku80 (Figure 14b). These results indicate that if there is an interaction between DNA-PKcs and the C-terminus of Ku, it has a low affinity similar to that observed between Ku80CTR and Ku70/80ΔC and the conformational change associated with DNA binding and phosphorylation appears to have little to no affect on the C-terminus of Ku80 becoming incorporated into the heterotrimer.

DNA-PK Activation as a Function of Ku80CTR – to confirm previous results demonstrating a stimulation of activity as a function of the extreme C-terminus of Ku80, our Ku70/80ΔC mutant was incorporated into a kinase assay to measure DNA-PK kinase

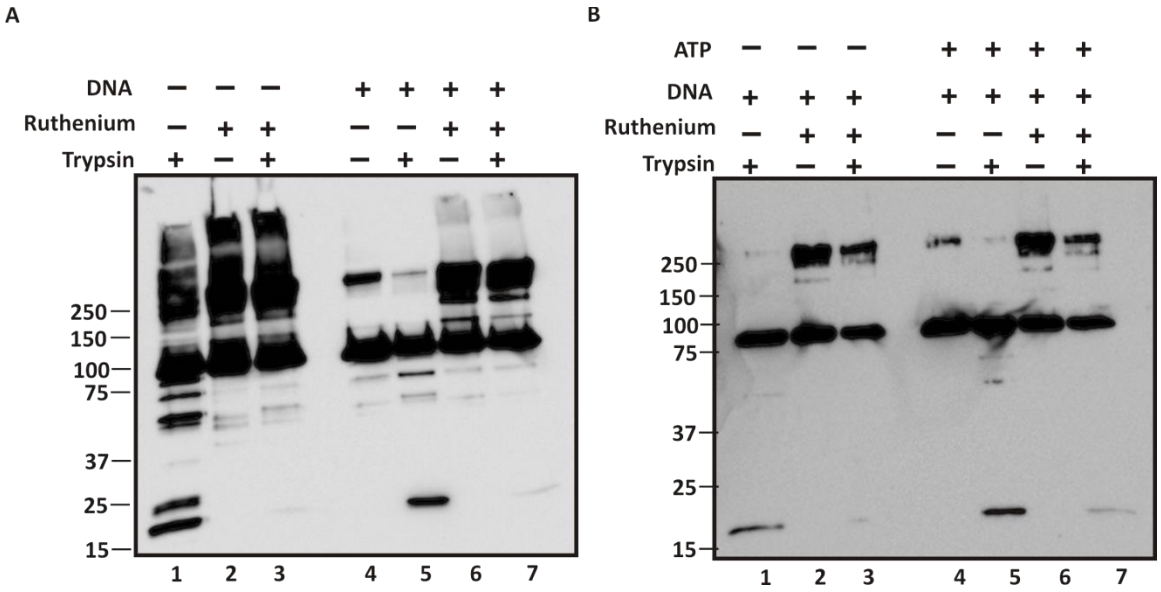

Figure 14. C-terminus of Ku80 interaction with the DNA-PK heterotrimer analyzed by crosslinking and limited proteolysis. DNA-PK (2.7 μg) was subjected to PICUP as described in Figure 11. Reactions were stopped with DTT followed by incubation with trypsin (20 ng) for 1 hour. Products were separated by SDS-PAGE, transferred to PVDF membrane for western blot analysis and visualized with a Ku80 C-terminus specific antibody (Table 2). (A) Reactions performed in the presence or absence of 26 pmol 30 bp-DNA substrate as indicated. (B) Reactions performed with 26 pmol 30 bp-DNA substrate and the presence or absence of ATP as indicated.

activity. DNA-PKcs was purified away from a majority of endogenous Ku, and these preparations were then supplemented with either wtKu, Ku70/80ΔC, Ku80CTR or a mixture of Ku70/80ΔC and Ku80CTR. Consistent with previous results, wtKu was able to stimulate DNA-PKcs kinase activity, while Ku70/80ΔC was able to stimulate kinase activity only slightly above background levels (Figure 15). Ku80CTR was not able to stimulate kinase activity and remained at background levels, and when Ku70/80ΔC was supplemented with Ku80CTR the activity observed was, again, only slightly above background levels (Figure 15). It is apparent that the extreme C-terminus of Ku80 greatly stimulates DNA-PK kinase activity, which is consistent with previous work performed in other labs. Also, the lack of recovery of kinase activity when Ku80CTR was added back to Ku70/80ΔC indicates that Ku80CTR, when it is not tethered to the Ku molecule, is not capable of stimulating kinase activity and the presence of Ku70/80ΔC does not appear to aid in kinase activity recovery.

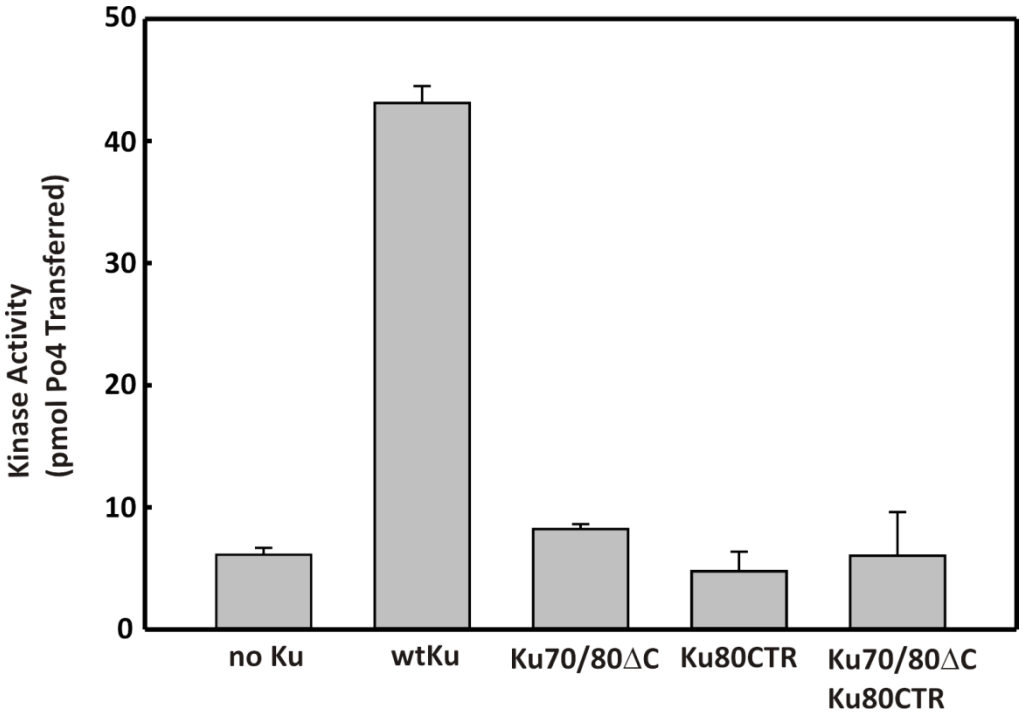

Figure 15. Effect of Ku80 C-terminus on DNA-PK activation. DNA-PK kinase assays were performed as described in Materials and Methods in the presence of wtKu (1 pmol), Ku70/80ΔC (1 pmol) or Ku80CTR (10 pmol) as indicated. Results are presented as pmoles of ^{32}P transferred to a synthetic peptide in a 15 minute reaction. Data presented is the average and standard deviation of three analyses.

Discussion

It is well understood that DNA double strand breaks are a result of IR, radiomimetic agents, and endogenous DNA-damaging agents among other sources. It is also understood that without the extreme C-terminus of Ku80, DNA-PK kinase activity is drastically decreased (64;65). NMR structural studies of the Ku80 C-terminus have shown that this is a highly flexible, relatively unstructured region that contains six alpha helixes at the extreme C-terminus (Figure 2b) (66;67). Often times, flexible regions function as a linker to facilitate protein-protein interactions, and with these interactions, proteins can undergo a conformational change. With this knowledge, we set out to determine if there is a protein-protein interaction between the C-terminus of Ku80 and the rest of the Ku molecule in hopes to gain some insight into the mechanism of DNA-PK regulation. To this end, three mutant constructs were generated, one containing only the extreme C-terminus of Ku80 and the other two containing full length Ku70 paired with a truncated form of Ku80 with and without an N-terminal $[His]_6$-tag. Upon DNA binding experiments, we verified that Ku70/80ΔC was able to bind DNA with no significant loss in affinity when compared to wtKu (Figure 9). These results were not surprising in that the DNA binding region of Ku was not affected by the removal of the Ku80 C-terminus as well as the fact that the crystal structure of Ku was solved in the presence and absence of DNA and it too is missing the C-terminus of Ku80 (Figure 2a) (68). This data was also supported by the additional DNA binding analysis of wtKu and Ku70/80ΔC that included an EMSA of wtKu and Ku70/80ΔC at a fixed concentration with

Ku80CTR titrated back in with both constructs resulting in no change in DNA binding activity (Figure 6c). Overall we can confirm that the deletion of the final 182 amino acids of Ku80 does not affect Ku's DNA binding ability, and any changes we see in regulation of Ku70/80ΔC when compared to wild type are a direct result of the removal of this region and not a reflection of a general loss of protein function.

Previous work has shown that Ku/DNA interaction has a preference for reduced conditions (69-71). It is also known that Ku, DNA-PK, and NHEJ are affected by cellular redox conditions (72-74). In general, redox conditions have a direct effect on the disulfide bonds that exist between two cysteine residues. These bonds are known to play a key role in protein folding and stability; however, the crystal structure of Ku does not reveal any disulfide bonds (75). Other modifications exist that are a function of redox conditions such as glutathione conjugates affecting DNA binding of Ku. Previous work studied the effect of ROS on cells through treatment with glucose and glucose oxidase, and a decrease in DNA binding activity was observed (76). Previous work in our lab determined that there are seven cysteine residues readily accessible to solutions, one being located in the C-terminus of Ku80 (data not shown). These residues would be affected by sulfenic acid modificationand could account for the reduction in DNA binding activity. The data presents strong evidence that C638 does not play a major role in redox regulation of Ku, but the incomplete re-reduction of Ku70/80ΔC observed if Figure 8 suggests that C638 may play at least a minor role in redox dependent changes in Ku structure.

A very common assay to demonstrate a protein-protein interaction is a form of a pull down assay, this type of assay generally requires a relatively strong affinity between the two molecules in question. Here, we have made use of $[His]_6$ affinity for Ni-NTA resin in a pull down assay to analyze any interaction between Ku70/80ΔC and Ku80CTR. In our experiments, we were able to remove the $[His]_6$-tag from Ku80CTR to more than 90% efficiency (Figure 5), while retaining the $[His]_6$-tag on Ku70. This allowed us to bind only the Ku70/80ΔC molecule to the Ni-NTA matrix in the presence and absence of Ku80CTR. If there was an interaction, we would have seen Ku80CTR elute from either the spin or the batch columns in conjunction with Ku70/80ΔC. Unfortunately these were not the results that were obtained; there were no detectable levels of Ku80CTR in the elution fractions (Figure 9). We took this assay a step further and visualized the same samples with the more sensitive western blot analysis and again we did not see Ku80CTR in conjunction with Ku70/80ΔC (data not shown). The possibility remains that the level of Ku80CTR remained below our detection limit or the affinity is too weak to withstand the more harsh conditions of a pull down assay.

We next implemented gel filtration and obtained similar results as the pull down assay. This assay allows for detection of lower affinity interactions as the conditions of separation are less harsh. Again, we did not observe any decrease in the elution volume of Ku80CTR as would have been expected if Ku80CTR was interacting with Ku70/80ΔC. However, the concentration of protein eluted from this column was fairly low and to detect Ku80CTR at the highest concentration required the sensitivity of western blot analysis (Figure 10). Attempts were also made to influence protein-protein interaction

by way of raising and lowering the salt concentration from 200 mM NaCl to as low as 50 mM and as high as 400 mM. With the lower concentration of salt there was no change in Ku80CTR elution volume alone or in the presence of Ku70/80ΔC (data not shown). With the higher salt concentration, Ku70/80ΔC would precipitate causing it to be incapable of eluting from the gel filtration column. However, it is not believed this would have driven the protein-protein interaction to detectable levels. Based on the evidence collected the interaction between Ku80CTR and Ku70/80ΔC is weak at best, and although salt concentrations are a factor in protein-protein interactions, we do not believe a drastic enough increase in affinity would be achieved by simply altering the salt concentration.

Through crosslinking, evidence was collected that lead us to the conclusion that there is an interaction between Ku70/80ΔC and Ku80CTR; however, the affinity remains low. With zero distance crosslink methods employed in this study two molecules must be in close proximity, such as that found when two molecules have a specific interaction. With the PICUP crosslinking method, we were able to detect a shift in Ku80CTR from 16 kDa to 150 kDa that is completely dependent on the presence of Ku70/80ΔC (Figure 11). It is possible to achieve the proximity required for crosslinking through random interaction, but the number of random interactions is not frequent enough to reach detectable limits with western blot analysis. The specificity of this interaction was supported with the presence or absence of BSA not altering the banding pattern or intensity as well as an increase of intensity as the concentration of Ku80CTR

increased; however, the overall intensity of the 150 kDa Ku80CTR specific band was not high.

The low band intensity observed in the PICUP assay could be a result of an already low affinity interaction, which is supported by the results of the Ni pull down assay and gel filtration chromatography, the possibility also exists that the low intensity could be a result of a less than perfect assay. The PICUP method makes use of photo-activated metal ligand that facilitates the oxidation of either a tyrosine or tryptophan residue. This activated residue then forms a covalent bond with either a nucleophilic or aromatic side chain of an adjacent protein or residue. Upon examination of the crystal structure of Ku (77) and the NMR structure of Ku80CTR (78) it is apparent that there is not a large abundance of tryptophan or tyrosine residues, and many of those that do exist are buried within the Ku70/80ΔC and Ku80CTR molecules. With a majority of the tryptophan and tyrosine residues located in Ku70/80ΔC, there is only one of each residue found in Ku80CTR both located in the cluster of six alpha helixes near the C-terminus of the molecule. The possibility of an inefficient reaction was disproved with the additional crosslinking assay that employed EDC. The EDC coupling assay facilitates crosslinking using EDC as a catalyst that activates a carboxyl group to produce an intermediate that then forms an amide bond with an amino group. We calculated that both Ku70/80ΔC and Ku80CTR have these residues in abundance and the crosslinking reaction will not be limited to only a few buried residues. Similar to the results obtained through the PICUP assay, EDC coupling resulted in a shift in Ku80CTR migration from 16 kDa to 125 kDa (Figure 12). This specific shift was also dependent on the presence of

Ku70/80ΔC and increased in intensity as the concentration of Ku80CTR increased. Also similar to the PICUP reactions, EDC coupling reactions did not show a change in band intensity with the addition of DNA, a result that was a bit of a surprise. Previous work has shown that with the addition of DNA, Ku undergoes a fairly significant conformational change (79-81) and that DNA is required for DNA-PK activation (82;83), therefore, it would stand to reason that the Ku80CTR would be a part of this conformational change. However, the results of our PICUP and EDC crosslinking experiments show that under these conditions, Ku80CTR interaction with Ku70/80ΔC does not change in the presence of DNA.

Surprisingly, the band intensity in the EDC coupling assays did show a mild decrease when BSA was removed from the reaction, which is not consistent with the PICUP assay. The banding pattern was, however, the same when compared to the EDC coupling experiments that did include BSA and was completely dependent on the presence of Ku70/80ΔC. We believe this variation to be a result of molecular crowding. With the presence of BSA the overall protein concentration of the reaction is increased thus increasing the local concentration of Ku80CTR and Ku70/80ΔC and helping to drive the interaction; however, an EMSA examining Ku70/80ΔC binding DNA was not affected by the presence of BSA in the reaction (data not shown). We believe that the molecular crowding phenomenon is able to produce a visible change in band intensity when performing EDC coupling reactions due to the fact that it is not a strong interaction and is easily persuaded. The interaction between Ku70/80ΔC and DNA was not influenced by the presence of BSA because this is known to be a very strong interaction and is

functioning at its peak ability without the aid of BSA. We do not believe there is a non-specific interaction between Ku80CTR and BSA as the banding pattern does not change, only the intensity. Also, if this were a non-specific interaction involving BSA, the band would completely disappear in the absence of BSA, which is not evident. Further evidence to support a specific interaction for Ku70/80ΔC can be found when analyzing Figure 12, specifically those lanes that contain only crosslinked Ku80CTR either with or without BSA. When Ku70/80ΔC is not present Ku80CTR appears to be crosslinking to other Ku80CTR molecules generating a ladder pattern of homopolymers. When Ku70/80ΔC is included in the reaction, either in the presence or absence of BSA, this Ku80CTR ladder is disrupted leaving only the homodimer. From this evidence we believe that the Ku80CTR shift we are seeing in the PICUP and EDC crosslinking assays is a result of a specific interaction with Ku70/80ΔC and not simply random chemical crosslinking.

For our final step in understanding the interaction between Ku80CTR and Ku70/80ΔC, we combined limited proteolysis with chemical crosslinking and observed only a slight change in the susceptibility of the C-terminus of Ku80 to cleavage. For these studies we crosslinked wtKu in an attempt to increase the local concentration, and possibly interaction, of the C-terminus of Ku80 with the bulk of the molecule by allowing the C-terminus to be physically connected to the molecule through the naturally occurring peptide bond. The results of these studies were completely consistent with the crosslinking studies and resulted in only a moderate decrease in C-terminus susceptibility to proteolysis, a result that was not influenced by the presence or absence

of DNA. Knowing that the C-terminus of Ku80 is required for DNA-PK activation, it is thought that with the addition of DNA-PKcs to the crosslinked tryptic digest reaction, the cleavage of the Ku80 C-terminus would be altered. Surprisingly, this was not the result. Previous work has also shown that DNA is required for DNA-PK activation and upon phosphorylation, DNA-PK undergoes a conformational change (84). With the addition of DNA and ATP we were able to verify that DNA-PK became phosphorylated (data not shown), but the C-terminus of Ku80 did not change in its susceptibility to be cleaved from the molecule. The results from these experiments lead us to believe that any affinity the C-terminus has for DNA-PKcs is similar to its affinity for Ku and is weak. It is possible that the C-terminus of Ku80 is interacting only with the Ku molecule and the addition of active DNA-PKcs does affect the C-terminus interaction. To rule out this possibility, further studies are currently underway replacing DNA-PKcs for Ku70/80ΔC in the crosslinking experiments presented here.

In light of more recent publications (85;86) there remains the possibility that the C-terminus of Ku functions in the synaptic complex that is believed to be formed between two DNA-PK heterotrimer molecules on separate broken ends of DNA (Figure 3). This possibility would help to explain the requirement for Ku80 C-terminus in the activation of DNA-PK, if DNA-PK undergoes trans-autophosphorylation. In this model, the C-terminus of Ku80 primarily interacts with a C-terminus of an adjacent molecule tethering the two complexes together. This model is consistent with the result seen in the PICUP assay and EDC coupling that produced a prominent band at 32 kDa which we suspect contains two Ku80CTR molecules. This model is also consistent with the results

we obtained in the kinase assay. The addition of Ku80CTR was not able to rescue the kinase activity of DNA-PK, if the model proposed with the C-terminus of Ku being involved in the synaptic complex is correct, it would not be able to rescue the kinase activity because it is not physically connected to the Ku80 molecule to function as a tether between two adjacent DNA-PK/DNA complexes.

Through this research and other ongoing research in this and other labs, it has become apparent that non-homologous end joining is a very complex pathway that is required for efficient DSB repair and ultimately cell survival. It has also become apparent that to fully understand the capability of a pathway, you must first understand the basic mechanisms of that pathway. This research has focused on the basic mechanisms of regulation of the Ku molecule in the NHEJ pathway. In the field of NHEJ research, we have discovered that Ku regulation is influenced by redox conditions, and in our lab we have determined that the C-terminus of Ku80 plays at best a minor role in this source of regulation. Through the removal of the C-terminus of Ku80, including cysteine 638, we have shown that Ku70/80ΔC retains the ability to bind DNA with similar affinity as wtKu, but when oxidized followed by re-reduction it does not appear to recover this ability to the same degree as wild type. This suggests that C638, and possibly the C-terminus of Ku, functions in protecting a portion of the Ku molecule when under oxidized conditions that cause irreversible damage to the structure of the molecule and ultimately affecting the DNA binding capability. The C-terminus of Ku80 has also been implicated in activation of DNA-PKcs. The work examined here sought to further understand the mechanism of this activation regulation through protein-protein

interaction of Ku80 C-terminus with the Ku molecule. By separating the C-terminus of Ku80 from the Ku molecule we were successful in demonstrating that an interaction does exist, but this interaction appears to be weak in affinity. Assays such as Ni-pull down and gel filtration chromatography, which require relatively stronger affinity, did not reveal any detectable interaction under the conditions evaluated. The more sensitive crosslinking assays were capable of a positive interaction, but a majority of the Ku80CTR molecules present were not incorporated into the Ku70/80ΔC crosslinked molecule allowing us to determine that there is an interaction, but it is only a weak interaction. This result was confirmed with the crosslinking assays that were combined with limited proteolysis. The C-terminus of Ku80 remained susceptible to tryptic digest, but to a lesser degree than when the molecule was not chemically crosslinked. Finally the kinase assay that did not show any rescue of kinase activity when Ku80CTR was supplemented with Ku70/80ΔC supports that this interaction is weak at best. Although there is still more work to be done to fully understand the function of the C-terminus of Ku80. The work provided here is conclusive in that the interaction between the C-terminus of Ku80 and the rest of the Ku molecule is weak; however, it does exist and plays a significant role in the NHEJ pathway.

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Curriculum Vitae

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Research Experience

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Project: Elucidating the Role of Redox Effects and the Ku80 C-Terminal Region Protein-Protein interaction on Human Ku Regulation, A DNA Repair Protein

- Results: Able to show using biochemical techniques such as gel filtration chromatography, electrophoretic mobility shift assay, western blot and other techniques that C-terminus of Ku80 is involved in redox regulation of DNA-PK as well as involved in a low affinity interaction with the Ku molecule

Publications

Bennett, S.M., Pawelczak, K.S, Woods, D.S., and Turchi, J.J. Analysis of the C-terminal Domain of Ku80 Reveals Interactions With Both Ku and DNA-PKcs. *In preparation*

Pawelczak, K.S., Bennett, S.M., and Turchi, J.J. Coordination of DNA-PK Activation and Nuclease Processing of DNA Termini in NHEJ. *Invited review in preparation*.

Bennett, S.M., Neher, T.M., Shatilla, A., and Turchi, J.J. 2009. Molecular Analysis of Ku Redox Regulation. **10**: 86-96 BMC *Molecular Biology.*

Jewell, J.L., Oh, E., Bennett, S.M., Meroueh, S.O., and Thurmond, D.C. 2008 The Tyrosine Phosphorylation of Munc18c Induces a Switch in Binding Specificity From Syntaxin 4 to Doc2beta. **31**: 21734-46 *Journal of Biological Chemistry*.