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2-step purification of the Ku DNA repair protein expressed in *Escherichia coli*

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Abstract

The Ku protein is involved in DNA double-strand break repair by non-homologous end-joining (NHEJ), which is crucial to the maintenance of genomic integrity in mammals. To study the role of Ku in NHEJ we developed a bicistronic *E. coli* expression system for the Ku70 and Ku80 subunits. Association of the Ku70 and Ku80 subunits buries a substantial amount of surface area ($\sim 9000 \text{Å}^2$ [1]), which suggests that heterodimerization may be important for protein stability. N-terminally his₆-tagged Ku80 was soluble in the presence, but not in the absence, of bicistronically expressed untagged Ku70. In a 2-step purification, metal chelating affinity chromatography was followed by step-gradient elution from heparin-agarose. Co-purification of equimolar amounts of his₆-tagged Ku80 and untagged Ku70 was observed, which indicated heterodimerization. Recombinant Ku bound dsDNA, activated the catalytic subunit of the DNA-dependent kinase (DNA-PKcs) and functioned in NHEJ reactions in vitro. Our results demonstrate that while the heterodimeric interface of Ku is extensive it is nonetheless possible to produce biologically active Ku protein in *E. coli*.

Keywords

Ku; DNA-PK; non-homologous end-joining; NHEJ

The repair of DNA double strand breaks (DSB) is critical to the maintenance of genomic integrity. The importance of DSB repair is highlighted by the fact that unrepaired DSBs can result in the loss of genetic information, chromosomal translocation and even cell death [2]. An important mechanism for DSB repair is non-homologous end-joining (NHEJ) – a homology independent process that is used for the repair DSBs during G0, G1 and early-S phases of the cell cycle [2]. As most of the cells in the mammalian body are terminally differentiated (G0), NHEJ is the major DSB repair pathway used by mammals.

Thus far, six mammalian NHEJ proteins have been described. Central to the DSB repair process is DNA Ligase IV and the proteins XRCC4 and XLF, which are thought to function as a complex in the ligation reaction [3–6] (Fig. 1C). Three other proteins – the Ku heterodimer (Fig. 1) and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) – comprise the DNA-dependent protein kinase (DNA-PK) [7]. Studies employing mouse models have demonstrated that genomic stability depends upon the fidelity of DSB repair through the NHEJ pathway and deficiencies in NHEJ have been linked to cancer [2,8].

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The DNA end-binding protein Ku protein plays an important role in mammalian NHEJ. Owing to its ring-shaped structure, Ku threads onto exposed DNA termini (Fig. 1A). Following DNA binding by Ku, DNA-PK is assembled and the DNA Ligase IV complex is recruited to the site of the break (Fig. 1C). Highlighting the central role of Ku in NHEJ, deficiencies in Ku have been shown to result in defects in growth, immunoglobulin gene rearrangement recombination, increased sensitivity to ionizing radiation and genomic instability, all of which can lead to tumor formation [2]. Because of the defined link between Ku, genomic stability and cancer we wish to examine the role of Ku in mammalian NHEJ.

Conventional purification of Ku from mammalian cell nuclear extract typically requires 5–6 chromatography steps and yields <10 µg/L of original culture. Purification of recombinant his₆-tagged Ku produced in insect cells requires fewer fractionation steps with higher yield (~1 mg/L of original culture [9] and Hanakahi and Cheung unpublished obs.). To study the role of Ku in NHEJ we have established an *E. coli* expression system for Ku, which will eliminate the need for eukaryotic cell culture. Heterodimerization of the Ku subunits buries ~9000 Å² [1] (Fig. 1); an unusually large amount when compared with most other 2 subunit multimerization events that typically bury <5000 Å² [10]. The substantial amount of surface area buried at the Ku heterodimerization interface suggested that folding of Ku in *E. coli* might be problematic [11]. As we show here, production of Ku in *E. coli* was possible using bicistronic expression coupled with compensation for sub-optimal codon usage. Purification of Ku from *E. coli* required only 2 affinity chromatography steps and yielded ~0.47 mg/L of original culture of active protein that bound dsDNA activated DNA-PKcs and functioned in mammalian NHEJ in vitro.

Materials and Methods

Cloning pET70H80

PCR, with primers that incorporated a 5' ribosome-binding site (RBS) RBS70: 5'-GCACTAGTAATAATTTAAGGAGATATACATGTCAGGGTGGGAG-3' and Ku703' SpeI: 5'-GCACTAGTTCAGTCCTGGAAGTG-3', was used to amplify human Ku70 and the product was cloned into the XbaI site of pET14bKu80, which expressed his₆-tagged Ku80, (a generous gift from M. Lieber [12]) placing the RBS and Ku70 cDNA downstream of the T7 promoter and upstream of the pET14b RBS and the Ku80 translation start site (Fig. 2).

Expression and purification of recombinant Ku70/H80

BL21(DE3)pLysS Rosetta *E. coli* cells (Novagen) were transformed with the resulting construct, pET70H80, grown in 2 L of Luria Broth (LB) at 37 °C until the culture reached OD₆₀₀ 1.0. Cells induced at 37 °C were induced with 0.25 mM IPTG and cultured for 1 hour. Cells induced at low temperatures were chilled on ice to reduce culture temperature to the desired induction temperature then transferred to 25 °C or 18 °C and grown for 4 or 18 hours respectively. Cells were harvested by centrifugation (15 min, 4 °C, 8000 g) and 1 g wet weight of cells was resuspended in 25 ml of lysis buffer (R buffer (50 mM Tris, pH 8.0, 5% glycerol and 1 mM PMSF) with 1 M NaCl, 0.4 M (NH₄)₂OAc, 10 mM imidazole and 2 mM 2-mercaptoethanol (BME)). BME was used in place of DTT during metal chelating chromatography according to manufacturer's instructions. Cells were opened by 3 cycles of freezing on liquid nitrogen and thawing (10 min ice/water slurry) followed by sonication with a Branson Sonifier 450 (20% output at 25% duty cycle 5 x 30 sec.) and insoluble material was removed by centrifugation (30 min, 4 °C, 25,000 g). 2.5 ml of Talon metal-affinity resin (Clontech) was batch washed twice with lysis buffer and lysate (125 mg) was bound in batch by rocking at 4 °C for 30 min. After binding, the resin was collected by centrifugation (5 min, 4 °C, 700 g), washed in batch twice with lysis buffer, twice with R buffer containing 50 mM NaCl, resuspended in 10 ml of R buffer containing 50 mM NaCl, packed into a column and

eluted with R buffer containing 50 mM NaCl and 0.5 M imidazole. The eluted protein was loaded directly onto a 1 ml Hi-Trap heparin (GE) column that had been equilibrated in R buffer with 50 mM NaCl, 0.5 mM EDTA and 1 mM DTT then eluted with R buffer containing 1 M NaCl, 0.5 mM EDTA and 1 mM DTT. Peak fractions were pooled and dialyzed against R buffer with 20% glycerol, 50 mM NaCl, 0.5 mM EDTA and 1 mM DTT. Protein concentrations were determined by Bradford assay (BioRad) using BSA as the protein standard.

DNA binding assays

Double-strand DNA cellulose pull-down—20 μ l of a 50% native DNA cellulose (SIGMA) slurry was added to 100 μ l of 20 nM rKu in HEK buffer (20 mM HEPES, pH 7.6, 0.1 M KHAc, 0.5 mM EDTA), incubated for 1 hour at 4°C with turning, collected by centrifugation, washed with 3 x 1 ml HEK buffer and resuspended in 2x protein sample buffer. Samples were heated to 100°C for 5 min, resolved on SDS-PAGE and subject to western transfer. Ku70 was detected with anti-Ku70 rabbit polyclonal antibodies (1:1500 dilution). Ku80 was detected using anti-Ku80 mAb 111 (NeoMarkers) at 1 μ g/ml.

Electrophoretic mobility shift assay (EMSA)—Synthetic oligonucleotide Ku shift Top: 5'-CTGAGAAAACCTGTGCGTCTTCGCGGC-AATTGAGAGGCATTCCATTAC-3' was radiolabeled, annealed to Ku shift Bottom: 5'-GTGAATGGAATGCCTCTCAATTGCCGCGAAGACGCACAGTTTTCTCAG-3' and gel purified. Binding to duplex DNA was carried out in 10 μ l reactions containing 50 mM Tris, pH 7.75, 100 mM KCl, 1 mM DTT, 5% glycerol, 100 μ g/ml bovine serum albumin, 5 nM 32 P-labeled duplex DNA and recombinant Ku as stated for 30 min at room temperature. Complexes were resolved on 5% (29:1 acrylamide:bis) native PAGE in 0.5 x TBE at 2.5 V/min at room temperature. The gel was dried and subject to autoradiography.

DNA-dependent protein kinase assay

The SignaTECT DNA-Dependent Protein Kinase Assay System (Promega) was used according to manufacturer's instructions. Briefly, reactions are carried out in the presence of gamma- 32 P ATP and a biotinylated p53 peptide is the protein kinase substrate. After the kinase reaction has been completed the radiolabeled substrate is captured on a streptavidin support, unincorporated 32 P is washed away and incorporated 32 P was quantified by scintillation counting. DNA-PKcs was purified as previously described [13].

In vitro NHEJ complementation

HeLa whole cell extracts were prepared as described [13]. For low salt Ku-depleted WCE no additional salt was added and the final salt concentration was 0.1 M KOAc. For high salt Ku-depleted WCE NaCl was added to 0.5 M, bringing the final salt composition to 0.5 M NaCl, 0.1 M KOAc. Ku anti-serum (Serotec) was used to immunodeplete Ku. Beads from the 100 μ l immunodepletion reactions were washed, resuspended in 100 μ l of HEK and used as the immunoprecipitate in complementation experiments. In vitro NHEJ assays were carried out as described [14,15]. Anti-XRCC4 antibodies (Serotec) and wortmannin (SIGMA) were used as shown. Ku was purified from HeLa cells as described [13].

Ku antibodies

Production of rabbit polyclonal anti-Ku70 antibodies. Full-length human his₆-Ku70 (pET11aKu70, a generous gift from M. Lieber, [12]) was expressed in BL21(DE3)pLysS Rosetta *E. coli* cells grown in LB at 37°C until the culture reached OD₆₀₀ 1.0 at which point cells were induced with 1 mM IPTG for 4 hours at 37°C. Crude lysate preparation and Talon affinity chromatography were carried out as described above. The eluted protein was loaded directly onto a 1 ml Hi-Trap Q (GE) column that had been equilibrated in R buffer with 50

mM NaCl, 0.5 mM EDTA and 1 mM DTT and eluted with a 50 mM to 1 M NaCl gradient in R buffer. Peak fractions were subject to gel purification to isolate Ku70, which was used to immunize two New Zealand White Rabbits (Covance). Rabbits were initially injected with 250 µg of Ku70, boosted 5 times over the course of 3 months with 125 µg per boost and production bleeds were collected. Rabbit polyclonal antibodies against Ku80 were purchased (Serotec).

Results and Discussion

Expression and purification of recombinant Ku

Attempts to express individual Ku subunits (Ku70 and Ku80) in *E. coli* BL21(DE3) were unsuccessful and full-length polypeptides were not observed (data not shown). Expression of Ku70 was successful in BL21(DE3)pLysS Rosetta *E. coli*, which compensates for sub-optimal codon usage, and we were able to express and purify Ku70 with an average yield of 3 mg/L of original culture (data not shown). Expression of Ku80 in BL21(DE3)pLysS Rosetta *E. coli* cells resulted in insoluble protein (data not shown). Co-expression of Ku70 and Ku80 from separate plasmids resulted in insoluble Ku80 and monomeric, full-length, soluble Ku70 (data not shown). Co-expression of Ku70 and Ku80 from the same plasmid using independent promoters to drive expression of the Ku subunits resulted in full-length, soluble Ku70 and soluble Ku80 that had undergone substantial proteolysis (data not shown). We believe that failure of the Ku subunits to heterodimerize caused insolubility and proteolysis of Ku80 [11].

When a single T7 promoter was used to drive bicistronic expression of untagged Ku70 and his₆-tagged Ku80 (expression vector pET70H80) in BL21(DE3)pLysS Rosetta *E. coli* we obtained soluble, full-length, heterodimerized recombinant Ku (rKu) (Fig. 2, top). Recombinant Ku was purified from *E. coli* lysate in a 2-step process described in Materials and Methods. Because Ku is a non-specific DNA binding protein metal-chelating affinity chromatography was carried out in high salt (1 M NaCl, 0.4 M (NH₄)₂OAc), which prevented Ku-DNA interaction and minimized co-purification of both DNA and *E. coli* DNA-binding proteins. Addition of 0.4 M (NH₄)₂OAc not only decreased co-purification of unwanted proteins but increased yield during the metal-chelating affinity chromatography step by approximately 2-fold. Full-length untagged- Ku70 and full-length his₆-Ku80 co-eluted (Fig. 2), which indicated heterodimerization of the Ku subunits.

Elution from the metal-chelating resin in low salt enabled direct binding to heparin-agarose without the need for dialysis. Recombinant Ku bound well to heparin-agarose and all of the observed contaminating proteins flowed through the column, making elution by step-gradient possible (Fig. 2). While rKu eluted from heparin-agarose at ~0.4 M NaCl, step-gradient elution with 1 M NaCl allowed collection of maximum concentration in minimum volume.

Recombinant Ku was dialyzed to reduce NaCl concentration for storage. A summary of the purification, which yielded 0.47 mg of rKu per L of original culture, is presented in Table 1. The low yield of this expression and purification system is likely the result of misfolding of Ku70 and Ku80. Western blot analysis using polyclonal antibodies directed against Ku70 and Ku80 showed that after sonication 25% of the total Ku70 and 20% of the total Ku80 are in the soluble fraction (data not shown). The insoluble fraction was found to contain both full length and proteolytic fragments of Ku70 and Ku80 (data not shown). Interestingly, only full-length Ku70 and Ku80 are found in the soluble fraction (data not shown). These data suggest that formation of the Ku70/80 heterodimer, and not expression of the full-length polypeptides, is the most likely cause of low yield in this system.

DNA binding and activation of DNA-PK by recombinant Ku

DNA-PK plays an important role in mammalian NHEJ (Fig. 1) [7]. As the DNA-binding subunit of DNA-PK, Ku plays an especially important role in protecting the exposed DNA

ends and regulating the protein kinase activity of DNA-PKcs. To assess the quality of our *E. coli* expressed rKu we examined the double-strand (ds) DNA-binding activity of rKu in an electrophoretic mobility shift assay (EMSA).

As shown in Fig. 3A, binding of rKu to the 48 bp substrate resulted in the step-wise formation of 2 distinct complexes with reduced electrophoretic mobility. Complex 1 is thought to result from binding of a single rKu to the dsDNA, while complex 2 is likely to contain 2 rKu per 48 bp duplex. Ku is thought to require a minimum of 20–25 bp for stable binding, and loading of multiple Ku molecules onto longer strands of duplex DNA has been observed [16]. It is worth noting that Figure 3A shows that at 7.7 nM Ku all of the 5 nM labeled DNA substrate has been bound, suggesting that a high percentage of the rKu is DNA-binding active.

Duplex DNA acts as an efficient competitor for rKu binding (Fig. 3B, left), which is abolished by the addition of 4-fold molar excess of unlabeled dsDNA. In contrast, single-stranded (ss) DNA is unable to compete for rKu binding as even a 40-fold molar excess of unlabeled ssDNA had no effect on rKu binding to the labeled dsDNA binding substrate (Fig. 3B). *E. coli* produced rKu was able to bind dsDNA cellulose prepared with calf thymus DNA (data not shown), which indicated a lack of sequence specificity in DNA recognition. Finally, covalently closed circular plasmid DNA did not act as an effective competitor for rKu binding (data not shown), which is consistent with the requirement of DNA ends for Ku binding. Taken together these data demonstrate that rKu produced in *E. coli* exhibits binding to dsDNA, regardless of DNA sequence, and that this binding requires accessible DNA termini. These findings are consistent with observations made with Ku purified from mammalian cells and further indicate that rKu produced in *E. coli* is properly folded and suitable for biochemical assays.

Having established that Ku is able to bind dsDNA we next examined the ability of rKu to activate DNA-PK. Expression of recombinant proteins in *E. coli* at low temperatures has been shown to improve protein stability. As shown in Fig. 3C, the temperature at which rKu was expressed had a significant impact on activation of DNA-PK. Preparations of rKu purified from cultures induced at 37°C, 25°C or 18°C were used in DNA-PK activation assays and maximum activity was observed with rKu purified from cells induced at 18°C. Control reactions carried out in the absence of either rKu or dsDNA demonstrated that both species were required for efficient activation of DNA-PK (data not shown). Data presented in Fig. 3 show that rKu produced in *E. coli* has the ability to recognize dsDNA and activate DNA-PK.

***E. coli* produced rKu can participate in NHEJ in vitro**

Ku-dependent mammalian NHEJ can be examined biochemically using whole cell extracts (WCE) prepared from cultured human cells [14] (Fig. 4). To be useful in the study of Ku in mammalian NHEJ it is crucial that rKu function in this in vitro NHEJ assay system. To determine if *E. coli* produced rKu is fully functional we carried out complementation analysis using Ku-depleted WCE. Anti-Ku antibodies were used to deplete endogenous Ku from HeLa WCE in the presence of 0.5 M NaCl. Immunodepletion of Ku under low-salt (0.1 M KOAc) conditions resulted in Ku-depleted WCE that was unable to carry out NHEJ in vitro and could be complemented by the immunoprecipitate, but could not be complemented by Ku purified from HeLa cells (Fig. 4A). Because Ku is thought to participate in protein-protein interactions with other NHEJ factors it is likely that under conditions of low ionic strength that Ku is associating with, and co-immunodepleting, factors important for NHEJ. Immunodepletion of Ku under conditions of high ionic strength (0.5 M NaCl (high-salt)) resulted in Ku-depleted WCE that could be complemented by both the immunoprecipitate and by Ku purified from HeLa cells (Fig. 4B). The increased salt was thought to abrogate protein-protein interactions with Ku and minimize depletion of NHEJ factors.

Addition of bacterially produced recombinant Ku to high salt Ku-depleted WCE restored in vitro NHEJ (Fig. 4C, lanes 4 and 9). To characterize the observed rKu-complemented ligation activity, end-joining was assayed in the presence of neutralizing anti-XRCC4 antibodies or in the presence of low concentrations of wortmannin (an inhibitor of DNA-PKcs and other PI3K-related protein kinases). Treatment with either anti-XRCC4 antibodies or wortmannin resulted in a significant decrease in ligation efficiency (Fig. 4C), which indicated a requirement for both XRCC4 and DNA-PKcs and identified the observed end-joining as bona fide mammalian NHEJ. Taken together these data demonstrate that recombinant Ku produced in *E. coli* is fully functional, can participate in mammalian NHEJ in vitro and can be used to study the role of Ku in mammalian NHEJ.

Conclusion

In conclusion, the Ku heterodimer is known to play an important role in mammalian DNA repair. Structurally, Ku is unusual in that heterodimerization of the Ku70 and Ku80 subunits buries a large amount of surface area ($\sim 9000\text{\AA}^2$ [1]). Perhaps as a result of the extensive interfacial surface, we found that soluble Ku could not be produced by independently expressing the two subunits. However, it was possible to express recombinant Ku (rKu) in *E. coli* using a bicistronic expression system. Purification of rKu was straightforward (Fig. 2). Purified rKu was found to have biologically relevant activity in that it bound dsDNA and activated the DNA-dependent protein kinase DNA-PK (Fig. 3). Most importantly purified rKu complemented DNA end-joining in Ku-depleted extracts in vitro (Fig. 4C). These data indicate that *E. coli* produced rKu is suitable for use in the biochemical analysis of mammalian NHEJ.

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Abbreviations used

DSB	double strand break
NHEJ	non-homologous end-joining
DNA-PK	DNA-dependent protein kinase
rKu	recombinant Ku
WCE	whole cell extract

Fig. 1.

Space-filling model of Ku showing Ku70 (red) and Ku80 (gold) generated using Protein Databank accession number 1JEY [1]. A) Side-view. Ring-structure of Ku capable of encircling 2 turns of dsDNA, which passes thru the opening of the preformed ring [1]. B) Top-view. Model in A has been rotated 90° toward the reader to show the large amount of subunit-subunit contact made in this heterodimer. C) Model for mammalian NHEJ. The Ku heterodimer (orange and red) binds DNA ends exposed by a double-strand break, recruits DNA-PKcs and forms the catalytically active protein kinase DNA-PK. DNA Ligase IV is recruited as part of a complex with XRCC4 and XLF and requires inward translocation of Ku to permit binding of DNA Ligase IV complex [17]. End-ligation is catalyzed by DNA Ligase IV.

Fig. 2.

Expression and purification of recombinant Ku. Top) Schematic diagram of bicistronic Ku expression construct pKu70H80 used to express the rKu heterodimer. Bottom) Coomassie stained gel of 50 μ g crude bacterial lysate (L) and 630 ng each Talon (T) and Heparin (H) eluate. Details of purification are presented in Materials and Methods and Table 1.

Fig. 3. Recombinant Ku binds dsDNA and activates DNA-PK. A) EMSA analysis of rKu binding to dsDNA using 5 nM radiolabeled dsDNA. A) dsDNA binding by rKu at 0.9, 2.6, 7.7, 23 and 69 nM. B) rKu binding in the presence of dsDNA (1.25, 5 and 20 nM) or ssDNA (50 and 200 nM). C) Activation of DNA-PK by rKu purified from cultured induced to express rKu at 37°C, 25°C or 18°C. (-) indicates no rKu.

Fig. 4.

E. coli produced rKu participates in NHEJ in vitro. A) Whole cell extract (WCE; lane 1) and low salt Ku-depleted WCE (LS Ku-dep WCE; lanes 2 – 8) were prepared and NHEJ reactions were carried out as described in Materials and Methods. 1, 2 or 3 μ l of the immunoprecipitate was used to complement low salt Ku-depleted WCE (IP; lanes 3 – 5). Ku purified from HeLa cells was used at 12.8, 38.4 and 115.2 nM (HeLa Ku; lanes 6 – 8). B) High salt Ku-depleted WCE (HS Ku-dep WCE; lanes 3 – 7) was used in NHEJ reactions. 3 μ l of the immunoprecipitate was used to complement high salt Ku-depleted WCE (IP; lane 4). Ku purified from HeLa cells was used as in (A) (HeLa Ku; lanes 5 – 7). C) High salt Ku-depleted WCE (HS Ku-dep WCE; lanes 3 – 12) was used in NHEJ reactions. Recombinant Ku (rKu) was used at 140 nM. Anti-

XRCC4 antibody (a-XRCC4; 1:6250, 1:1250, 1:250 and 1:50; lanes 5 – 8) and Wortmannin (wort; 0.3, 1.0 and 3.0 μ M; lanes 10 – 12) were added where indicated. (–) indicates no treatment

Table 1Purification of recombinant Ku from *E. coli*.

Purification step	Total protein (mg)	rKu (mg)	Fold purification	Yield (%)	Purity (%)
Bacterial lysate	125 ¹	1.6 ²	--	--	1.28
Talon Metal Chelating (Cobalt)	1 ¹	0.94 ²	125	60	93.8
Hi-trap Heparin	0.47 ¹	0.47	2.14	29.4	~100

Purification steps and estimated yield from 1g of wet weight cells.

-- does not apply;

¹ determined by Bradford analysis;² estimated by western blot