Ku protein stimulates DNA end joining by mammalian DNA ligases: a direct role for Ku in repair of DNA double-strand breaks

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Ku protein binds to DNA ends and is a cofactor for the DNA-dependent protein kinase. Both of these components are involved in DNA double-strand break repair, but it has not been clear if they function indirectly, by sensing DNA damage and activating other factors, or if they are more directly involved in the processing and rejoining of DNA breaks. We demonstrate that intermolecular ligation of DNA fragments is highly dependent on Ku under conditions designed to mimic those existing in the cell. This effect of Ku is specific to eukaryotic DNA ligases. Ku protein, therefore, has an activity consistent with a direct role in rejoining DNA breaks and independent of DNA-dependent protein kinase.

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Introduction

DNA double-strand breaks (DSBs) result from a variety of exogenous DNA-damaging agents, such as ionizing radiation. They are repaired either by using an intact copy of the broken region as a template (homologous recombination) or by direct rejoining of the broken ends [non-homologous end joining (NHEJ), or illegitimate DSB repair]. DSBs are also produced as normal intermediates in V(D)J recombination, and mutational studies identify the NHEJ pathway as the primary means for resolving these intermediates (reviewed in Jeggo *et al.*, 1995; Roth *et al.*, 1995; Weaver, 1995).

Mammalian genes that have been implicated in the NHEJ pathway fall into four complementation groups; XRCC4, XRCC5, XRCC6 and XRCC7 (reviewed in Thompson and Jeggo, 1995). XRCC4 protein associates with mammalian DNA ligase IV in cells (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997), and may enhance DNA ligase IV activity (Grawunder *et al.*, 1997). The other three genes encode components of the DNA-activated protein kinase (DNA-PK) (reviewed in Lees-Miller, 1996). DNA-PK consists of Ku, a heterodimeric DNA-binding protein, and the catalytic subunit (DNA-PK_{CS}). The XRCC5 and XRCC6 genes encode the 86 and 70 kDa subunits of the Ku heterodimer, while the XRCC7 gene encodes DNA-PK_{CS}. Efficient DNA-PK activity requires association of DNA-PK_{CS} with Ku bound to DNA (Gottlieb and Jackson, 1993; Yaneva *et al.*, 1997). The

Ku heterodimer binds to ends of duplex DNA, including hairpins, nicks and forked structures (Falzon *et al.*, 1993). Once bound, Ku can translocate internally on the DNA fragment (de Vries *et al.*, 1989; Paillard and Strauss, 1991). Alternatively, Ku bound to one DNA fragment can transfer to a second DNA fragment when the two fragments have compatible ends (Bliss and Lane, 1997). Direct imaging has shown that two molecules of DNA-bound Ku can associate, resulting in formation of DNA loops (Cary *et al.*, 1997).

These activities have led to much speculation on the role of Ku in DSB repair. Ku may act solely as a sensor of DNA damage, binding to ends of a DSB and signaling events that indirectly affect DSB repair (e.g. cell cycle regulation) through induction of DNA-PK activity. Recent evidence demonstrating an interaction between DNA-PK and the c-abl tyrosine kinase (Kharbanda *et al.*, 1997) supports this possibility. Ku may also play a more direct role, including protection of ends from degradation, bridging of DNA ends prior to joining, or recruitment of other proteins that repair DSBs.

A direct involvement of Ku in DSB repair is suggested by the structures of junctions recovered from cells deficient in Ku. Repair of DSBs in several such systems [Ku-deficient mice (Bogue *et al.*, 1997), CHO cells (Pergola *et al.*, 1993) and yeast (Boulton and Jackson, 1996)] is inefficient, but the recovered junctions are remarkably homogeneous. They occur principally at short direct repeats of ~4 bp (microhomologies) flanking the DSB. Formation of these homology-directed junctions can result in large deletions (Boulton and Jackson, 1996). Processing of broken DNA can generate complementary overhangs at these homologies, resulting in increased ligation efficiency (Roth *et al.*, 1985). The increased proportion of junctions at microhomologies in Ku-deficient cells suggests that Ku might act to facilitate ligation in the absence of these homologies.

We tested this possibility by adding Ku protein to intermolecular ligation assays. Different DNA ligases, while all proficient in sealing DNA nicks, vary widely in their ability to perform intermolecular ligation. Of the mammalian ligases, only DNA ligase I efficiently joins blunt ends (reviewed in Lindahl and Barnes, 1992). The two remaining genetically distinct mammalian DNA ligases, DNA ligase III (Elder *et al.*, 1992) [DNA ligase II is probably a fragment of ligase III (Wang *et al.*, 1994; Wei *et al.*, 1995)] and DNA ligase IV (in association with XRCC4) both require complementary overhangs of 4 bp or longer for efficient ligation (Robbins and Lindahl, 1996; Grawunder *et al.*, 1997).

Here we describe conditions where Ku greatly stimulates intermolecular DNA ligation, without affecting the joining of nicks. Ku reduces the requirement for complementary overhangs. The effect of Ku on ligation is specific for

mammalian DNA ligases, and is apparently due to the ability of Ku to bridge DNA ends while leaving them accessible for ligation.

Results

Ku protein stimulates end joining by DNA ligase I

We first used a 60 bp blunt-ended substrate to assess intermolecular end joining *in vitro*. Initial experiments were performed with highly purified recombinant human Ku and recombinant human DNA ligase I.

Ligation of this substrate by ligase I was stimulated >10-fold by addition of Ku protein. Stimulation increased with the amount of Ku protein added, until an optimum was reached when the number of Ku molecules added approximately equalled the number of DNA ends (Figure 1A). Addition of an excess of Ku molecules over DNA ends inhibited ligation (compare lanes 3 and 6, Figure 1A). The effect of Ku protein was due primarily to an increase in the initial rate of reaction; at early time points, ligation was as much as 200-fold faster in the presence of Ku (Figure 1B). Incubation of the substrate with Ku prior to addition of ligase did not increase the reaction rate further (Figure 1B).

We next assessed the ability of Ku to stimulate ligase I activity on substrates with different overlapping ends (Figure 1C and Table I). A substrate with a 2 bp overhang again required addition of Ku protein for efficient ligation. However, Ku had only modest effects on ligation of substrates with 4 bp complementary overhangs, and had no effect on ligation of nicked substrates (data not shown).

In the absence of Ku, efficient joining required a low, non-physiological salt concentration (25 mM) and a temperature no higher than 25°C. In contrast, ligation with Ku added was relatively insensitive to higher salt or an increase in temperature to 37°C (Figure 1C). Thus Ku is necessary for efficient joining by ligase I under the stringent conditions (short or no overlaps, physiological salt and temperature) required for end joining in cells.

The effect of Ku protein is specific to eukaryotic ligases

The joining of blunt or nearly blunt ends by ligases III or IV was also improved significantly by addition of Ku (Table I). Ligase IV required Ku for efficient ligation of the substrate with a 4 bp overhang as well. We note that XRCC4 protein, which associates with ligase IV (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997), is also thought to stimulate ligase IV activity (Grawunder *et al.*, 1997). XRCC4 co-purified with the ligase IV used here (T.Lindahl, personal communication), and indeed we observed no increase in activity upon addition of recombinant XRCC4 (data not shown).

Even with Ku protein added, ligases III and IV are more dependent on complementary overhangs than ligase I. Ligase I in the presence of Ku joins blunt ends 3-fold less well than a 4 bp overhang, while the corresponding factor for ligase III is 20-fold, and for ligase IV ~100-fold (Table I).

Although we observed strong stimulatory effects of Ku with these mammalian ligases, Ku had little or no effect on intermolecular joining by ligases from phage T4 and *Escherichia coli* (Table I). Under the more stringent

Fig. 1. Effect of Ku on activity of ligase I. (A) Ligation of a blunt end substrate after 3 h of incubation without Ku (lane 1) or in the presence of increasing amounts of Ku, in 20 ng increments, from 20 to 100 ng (lanes 2–6). (B) Time course of ligation. Sixty ng of Ku (as in A, lane 4) were added as noted in the figure. Pre-incubation was performed with Ku but without ligase. (C) Effect of conditions on stimulation by Ku. Bars represent the extent of ligation after 3 h with (stippled bars) or without (hatched bars) 60 ng of Ku. The salt concentration, temperature and length of complementary overhanging ends are varied as shown. Ten units (see Materials and methods) of ligase I were used in all experiments.

conditions used above, intermolecular ligation with *E. coli* ligase was not detectable. Ligation efficiency with T4 DNA ligase was stimulated at most 3-fold in the presence of Ku, but remained poor. Under conditions that restored efficient ligation (more enzyme, lower salt or use of substrates with 4 bp overlaps), addition of Ku protein actually inhibited the activity of both *E. coli* and T4 ligases. For example, 1000 U (as defined in Materials and methods) of T4 ligase joined 25% of the 2 bp overlap substrate without Ku, but only 17% in the presence of Ku.

Ku promotes association of two DNA molecules

Ku stimulated ligation most effectively when juxtaposed ends cohered poorly (short overlap or none, high temperature). This suggests that Ku may function to 'bridge' the two molecules, resulting in a more stable intermediate for intermolecular ligation. We developed a pulldown assay,

Table 1. The effect of Ku on activity of different ligases for three substrates

Overhang	Ligase ^a	-Ku ^b	+Ku	Stimulation
0 bp	I	0.8	12	15
	III	<0.1	2.9	>29
	IV	<0.1	0.2	>2.0
	T4	0.2	0.2	1
	<i>E. coli</i>	<0.1	<0.1	NA
2 bp	I	0.1	13	130
	III	0.3	34	110
	IV	<0.1	3.5	>35
	T4	1.6	3.9	2.4
	<i>E. coli</i>	<0.1	<0.1	NA
4 bp	I	40	36	1
	III	28	61	2.2
	IV	0.8	25	31
	T4	53	21	0.4
	<i>E. coli</i>	57	32	0.6

^aData for experiments using 10 U of ligases I and III, 1 U of ligase IV and 100 U of T4 and *E. coli* ligases are shown (unit definition is given in Materials and methods) to permit comparison of data in the linear range.

^bLevels of ligation below 0.1% were not detectable. (-Ku) and (+Ku) columns list the percentage ligation in the absence or presence of 60 ng of Ku.

NA; not applicable.

performed in the absence of ligases, to test this possibility directly.

A DNA duplex was produced with a biotin group coupled to one end and a 2 bp overhang on the opposite end. The biotinylated DNA was then incubated with a duplex DNA substrate with a complementary 2 bp overhang that had been ³²P-labeled at its 5' end. Biotinylated DNA was recovered using streptavidin beads, and free ³²P-labeled DNA was removed by washing the streptavidin beads with an excess of reaction buffer. The interaction between two DNA molecules could thus be determined by the ability of streptavidin beads to recover the ³²P-labeled duplex.

As much of 8% of total ³²P-labeled DNA remained associated with the biotinylated DNA in the presence of Ku, in a manner dependent on the concentration of Ku (Figure 2). In control experiments, with either Ku or the biotin group omitted, the fraction of ³²P-labeled DNA pulled down was very small (0.2–0.4% of total counts). The bound fraction of DNA molecules detected in this assay is likely to under-represent the frequency of interacting molecules. If the complexes are short lived, they could have largely dissociated during the time taken to complete the washes, or a large proportion may have been physically disrupted by the wash procedure.

DNA ligases are relatively inactive on substrates with mismatched ends. We used the pulldown assay to determine if the ability of Ku to bridge two DNA molecules was also dependent on the presence of compatible ends. We repeated the experiment described above, except that neither end of the labeled DNA could pair with the ends present on the biotinylated DNA. The labeled DNA was still pulled down in the presence of Ku, with an efficiency similar to that observed when a complementary end was present. Ku, therefore, promotes bridging between two DNA molecules even when the DNA ends are not suitable partners for ligation.

Fig. 2. Pulldown assay for association of two DNA molecules. DNA substrates are described in the left column. Biotin groups are noted with a circle and ³²P labels with an asterisk. Each bar represents the fraction of labeled DNA recovered with streptavidin beads after incubation with the specified amount of Ku protein.

Discussion

Under appropriate conditions, Ku stimulates intermolecular joining by mammalian DNA ligases >100-fold. Stimulation is most significant under conditions of high salt and high temperature that parallel conditions in cells, particularly when substrates with ends overlapping by <4 bp are used. Recent *in vivo* experiments also support a role for Ku in the ligation step of NHEJ. The defects in end joining in yeast strains deficient in Ku or in a specific DNA ligase have been shown to be generally similar (Schar *et al.*, 1997; Teo and Jackson, 1997; Wilson *et al.*, 1997). Furthermore, a yeast strain defective in both genes is no more defective than either single mutant, suggesting that the two gene products operate in the same pathway (Teo and Jackson, 1997; Wilson *et al.*, 1997). The most striking similarity between the biochemical results described here and *in vivo* data is the close correlation between the ability of Ku to reduce dependence on long (4 bp and greater) overlapping ends, and the increased dependence on 4–7 bp microhomologies for end joining in Ku-deficient cells (Boulton and Jackson, 1996; Bogue *et al.*, 1997). Therefore, we suggest that the direct effects of Ku on ligation shown here represent a critical component of the function of Ku *in vivo*.

The ability of Ku to stimulate activity of all three genetically distinct mammalian DNA ligases suggests that these enzymes may be at least partially redundant in performing NHEJ. However, the combination of ligase I with Ku displays the least dependence on complementary overhangs; thus ligase I would be the most effective ligase in joining ends without microhomologies *in vivo*. This conclusion is supported further by our recent work with a cell-free model for the joining of intermediates in V(D)J recombination (Ramsden *et al.*, 1997). In this system, joining of coding ends was strongly dependent on a 5 bp microhomology unless the reaction was supplemented with ligase I (levels of Ku were already sufficient in this system; D.A.Ramsden, T.T.Paull and M.Gellert, unpublished observations). Other ligases (human ligases III and IV, and T4 DNA ligase) were not capable of relieving the dependence on microhomologies.

Other evidence argues that ligase IV is involved in NHEJ in mammals. Human DNA ligase IV is found in a complex with the XRCC4 gene product (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997), which when mutated

causes serious deficiencies in NHEJ (Jeggo, 1990; Taccioli *et al.*, 1993; Li *et al.*, 1995). However, ligase IV was unable to reduce the dependence on microhomologies in cell-free V(D)J recombination (Ramsden *et al.*, 1997), and its activity on blunt or nearly blunt ends in the presence of added XRCC4 and Ku remains relatively poor. Therefore, we suggest that if ligase IV performs NHEJ *in vivo*, association with other factors [e.g. a Sir4-like protein (Tsukamoto *et al.*, 1997)] is required to enable efficient overlap-independent ligation. Alternatively, XRCC4 may perform its role in NHEJ independently of its association with ligase IV.

Stimulation of ligation by Ku is probably due to the ability of Ku to bridge two DNA molecules and stabilize an intermolecular association, because the effect of Ku on ligation is most pronounced when juxtaposed ends are unstable (short overlaps and higher temperature). This stimulation is observed only with eukaryotic ligases, indicating that the ability to interact with the bridged intermediate is specific to eukaryotic ligases. The structure of Ku-bridged DNA ends may be such that eukaryotic ligases can 'fit' in and perform ligation, but prokaryotic ligases cannot.

The ability of Ku to bridge two DNA molecules was also shown directly using a pulldown assay. Several other experiments support this observation. Self-association of DNA-bound Ku, resulting in formation of DNA loops, has been observed by atomic force microscopy (Cary *et al.*, 1997), and gel shift experiments have shown that Ku can be transferred efficiently between two DNA molecules (Bliss and Lane, 1997). In the latter experiment, transfer of Ku between the two DNA molecules required the presence of complementary ends, but in our pulldown assay bridging was largely independent of complementarity. It is possible that intermolecular association occurs independently of base pairing, but the bridged intermediate is in a conformation permissive for transfer of Ku only when the ends are complementary.

Comparison of the properties of Ku described here with *in vivo* data on end joining in Ku-deficient cells suggests a model (Figure 3) where Ku acts like a previously postulated 'alignment factor' (Pfeiffer *et al.*, 1994). Introduction of a DSB would result in recruitment of a Ku heterodimer to each end. Subsequent association of the two heterodimers would stabilize the interaction between the two ends. If the broken ends are compatible, ligation would be rapid and efficient. However, if the ends are not a competent substrate for ligation [e.g. hairpins from V(D)J cleavage, non-complementary overhangs, etc.], one or both of the Ku heterodimers could translocate internally, continuing to stabilize the intermolecular association of the ends but permitting processing to occur until a substrate that is competent for ligation is produced. In cells with mutated Ku, there is a higher frequency of imprecise end joining (Boulton and Jackson, 1996; Liang and Jasin, 1996), which has led to arguments that Ku protects ends from degradation. In the model described above, processing of ends and ligation are competitive; Ku acts to increase the frequency of accurate end joining principally by increasing the rate of ligation, rather than by protecting ends from degradation.

Fig. 3. Suggested role of Ku in repair of a DSB. The DSB is a substrate for immediate ligation (left pathway) or requires processing (right pathway) prior to ligation.

Materials and methods

Proteins

Baculoviral isolates for the expression of human Ku86 and a hexahistidine-tagged variant of Ku70 protein were the gift of D.Capra (Ono *et al.*, 1994). Ku heterodimer was recovered from lysates of co-infected Sf-9 cells by immobilized metal affinity chromatography (IMAC) as previously described (van Gent *et al.*, 1995). The recovered material was then dialyzed against buffer K [25 mM Tris pH 8.0, 10% glycerol, 2 mM dithiothreitol (DTT), 150 mM KCl], loaded on a Mono Q column (Pharmacia) and eluted with a linear gradient from 150 to 500 mM KCl. Fractions containing Ku were again dialyzed against buffer K, loaded onto a native DNA-cellulose column (Pharmacia), washed for three column volumes at 300 mM KCl and eluted at 600 mM KCl. The final eluate was again dialyzed against buffer K, frozen and stored at -70°C . Ku purified from HeLa cells [as described by Chan *et al.* (1996) with minor modifications] stimulated ligation in a manner similar to recombinant Ku; thus the effect of Ku described here is dependent on neither the recombinant source nor the presence of the hexahistidine affinity tag. Ku was diluted in buffer K + 100 $\mu\text{g/ml}$ bovine serum albumin (BSA) (Pharmacia); reactions without Ku were made up with an equivalent volume of buffer K + 100 $\mu\text{g/ml}$ BSA.

Clones expressing histidine-tagged human ligases I and III in *E.coli* were the gift of T.Lindahl. Ligases were recovered from lysates by IMAC as previously described (van Gent *et al.*, 1995). Ligase I from IMAC was diluted 2-fold in buffer K and loaded on a HiTrap Blue column (Pharmacia). This column was washed with 500 mM KCl in buffer K for three column volumes before elution with 1 M KCl. Ligase I from the HiTrap Blue column was then dialyzed against buffer L (25 mM Tris pH 8.0, 40 mM KCl, 10% glycerol, 0.1 mM EDTA and 5 mM DTT), loaded onto a Mono Q column and eluted using a linear gradient of KCl from 40 to 500 mM. The final eluate was dialyzed against buffer L + 50% glycerol and stored at -20°C . Ligase III was dialyzed in buffer S (25 mM Tris pH 7.5, 120 mM NaCl, 10% glycerol, 0.1 mM EDTA and 5 mM DTT) after IMAC, loaded on a Mono S column (Pharmacia) and eluted with a linear gradient of NaCl from 120 to 350 mM. The peak fraction was stored at -70°C and was used without prior removal of salt, because the protein was diluted ~100-fold for assay. HeLa cell-derived ligase IV, the gift of T.Lindahl, was demonstrated to be free of other ligases as described (Robbins and Lindahl, 1996). XRCC4 co-purified with ligase IV using this protocol (as shown by immunoblotting, T.Lindahl, personal communication). Ligases from phage T4 and *E.coli* were from New England Biolabs. In all experiments with *E.coli* ligase, 26 μM NAD was substituted for ATP as the high energy cofactor. Ligases were diluted in buffer L + 100 $\mu\text{g/ml}$ BSA.

Ligation assays

The relative activity of each ligase was assessed in a buffer containing 25 mM Tris pH 8.0, 20 mM KCl, 10 mM MgCl_2 , 1 mM ATP, 5 mM DTT, 0.05% Triton X-100 and 100 $\mu\text{g/ml}$ BSA, on a 50 bp nicked substrate. One unit was defined as the amount of enzyme necessary to ligate 50% of 200 fmol of this substrate in 15 min. This was equivalent to 0.5 ng of ligase I, 0.8 ng of ligase III, ~4 ng of ligase IV and 0.3 cohesive end units (New England Biolabs) of T4 and *E.coli* ligase.

Unless otherwise noted, intermolecular ligations were performed in a buffer containing 25 mM Tris pH 8.0, 120 mM KCl, 10 mM MgCl₂, 1 mM ATP, 5 mM DTT, 0.05% Triton X-100 (Aldrich), 100 µg/ml BSA and 10% (w/v) PEG (average M_r 8 kDa). No ligation activity was observed with addition of Ku alone (assayed from 1 ng to 1 µg; data not shown). In the absence of PEG, ligation was also stimulated 10- to 100-fold by the presence of Ku, but ligation was both less efficient and slower. Ligations were performed with 100 fmol of oligonucleotide substrate at 25°C for 3 h in a reaction volume of 10 µl. Ligations were stopped by addition of an equal volume of stop buffer (10 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.2% SDS) and heating for 2 min at 85°C. When present, PEG was removed by extraction with two volumes of chloroform. Reaction products were analyzed by electrophoresis on denaturing 8% polyacrylamide gels, and quantified using a phosphorimager and Image QuANT v4.1 software (Molecular Dynamics). Replicate assays showed little experimental variation; for example, in three experiments using ligase I and blunt-ended substrates, we observed $11 \pm 0.5\%$ (standard deviation) ligation in the presence of Ku and $0.8 \pm 0.2\%$ ligation in the absence of Ku.

Intermolecular ligation assays were performed with a 60 bp duplex where the identity of the 5'-³²P-labeled strand was kept constant throughout, but complementary strands possessed different 3' ends and a 4 nt non-palindromic 5' overhang. The blunt-end substrate had no 3' overhang, and head-to-head self-ligation was measured. Because the product of this ligation contains two ³²P labels, activity was corrected by dividing the amount of product by 2. To test the effect of different overhangs, non-palindromic 2 and 4 nt 3' overhangs were used, and ligation to a second DNA duplex (30 bp) with a complementary end was measured.

Pulldown assays

For the pulldown assays, a biotin group was attached to the 3' end of one strand of a 60 bp duplex with a tri-ethylene glycol spacer (Glen Research). The opposite end had a 2 nt 3' overhang. A second DNA duplex was ³²P-labeled at its 5' end, and also possessed a 2 nt 3' overhang. The sequence of this overhang was varied to be complementary or not, as noted in the figures. Ku protein, 100 fmol of biotinylated substrate and 25 fmol of the labeled substrate were incubated in 10 µl of standard reaction buffer but without PEG for 15 min at room temperature. Magnetic streptavidin beads (Promega) were washed three times and concentrated 6-fold in reaction buffer. Five µl of the concentrated beads were added to the reaction mix for 2 min. The pellet was collected with a magnet and washed twice with 100 µl of reaction buffer. The counts in each pellet ('bound') were recovered by resuspending the pellet and spotting the material on a GF/C disk (Whatman), and measured by liquid scintillation. 'Free' counts were recovered from the first supernatant and counted in the same manner. For the standard assay (complementary ends), the average percentage bound for replicate assays was $0.45 \pm 0.25\%$ (standard deviation) in the absence of Ku and $7.1 \pm 0.83\%$ in the presence of 60 ng of Ku.

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Note added in proof

Bridging of DNA molecules by Ku protein has also been observed by Pang *et al.* [Pang,D., Yoo,S., Dynan,W.S., Jung,M. and Dritschilo,A. (1997) Ku proteins join DNA fragments as shown by atomic force microscopy. *Cancer Res.*, **57**, 1412–1415.]