A Possible Role of Ku in Mediating Sequential Repair of Closely Opposed Lesions*

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Ionizing radiation generates a wide spectrum of DNA damages including single-stranded breaks, base lesions, abasic sites, double-stranded breaks, multiple damage sites, and DNA-protein and DNA-DNA cross-links (1–2). The energy from low linear energy transfer-ionizing radiation is not dispersed uniformly in the absorbing medium but is dispersed along the tracks of the charge particles (3). These nonrandom ionized tracks, consisting of “spurs” and “blobs” when traversing a DNA molecule, will generate lesions that are clustered within a small region (3). These clustered lesions commonly referred to as multiple damage sites are the hallmarks of exposure to ionizing radiation (4–7). The composition of lesions within these clusters is not clear; however, they are expected to consist predominantly of a random mixture of abasic sites, base lesions, and strand breaks (6–7).

Recently, it was demonstrated that base lesions that are in close proximity to each other are repaired at a reduced rate as compared with isolated base lesions. Bacterial base excision repair enzymes, such as endonuclease III and formamidopyrimidine-N-glycosylase, were shown to be inhibited by nicks formed either directly opposite or closely opposed to a base lesion (8–12). The biological relevance of this inhibition is not clear; however, it was thought that this would help to reduce the formation of double strand breaks. In mammalian cells, double strand breaks are repaired primarily through the non-homologous end-joining reaction involving the DNA end-binding protein Ku and the catalytic subunit of a DNA-dependent protein kinase, p450 (13–17).

In this study, we show that Ku binds to a nick opposite of DHU, leading to an inhibition of endonuclease III activity. Furthermore, we show that the presence of Ku at the nick helps to prevent the formation of free double strand breaks by tethering the new ends generated by endonuclease III-induced cleavage. Based on these observations, we suggest that the formation of the DNA-Ku complex immediately after the enzymatic processing of the closely opposed lesion is important for channeling the double strand break directly to the end-joining reaction to avoid the possibility of aberrant recombination through the misjoining of different molecules containing double strand breaks.

MATERIALS AND METHODS

DNA Substrates—All oligonucleotides were obtained from Operon and purified by polyacrylamide gel (15%) electrophoresis as described previously (18). Oligonucleotides containing DHU were 5’-end-labeled with [γ-32P]ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase or 3’-end-labeled with [α-32P]cordycepin 5’-triphosphate (PerkinElmer Life Sciences) using terminal deoxynucleotidyltransferase following the instructions from the enzyme supplier (U.S. Biochemical Corp.). Labeled oligonucleotides containing DHU were annealed to the appropriate complementary strands at 1:1.5 ratio in 10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and 2 mM 2-mercaptoethanol by heating the mixture to 90 °C and cooling down gradually to room temperature. The following oligonucleotide duplexes were used in this study (Q represents dihydrouracil):

Duplex N:

5’-CACCGGAGTAGGGGTTGGGATGGCAGTGATGAGGGTGAGTTTC

GTGGCCGAGTAGGGGTTGGGATGGCAGTGATGAGGGTGAGTTTC

Duplex L:

5’-CACCGGAGTAGGGGTTGGGATGGCAGTGATGAGGGTGAGTTTC

GTGGCCGAGTAGGGGTTGGGATGGCAGTGATGAGGGTGAGTTTC

The abbreviations used are: Ku, Ku70/80 complex; DHU, dihydrouracil.
Duplex N contained a DHU that was placed three nucleotides 5’ to a nick on the opposite strand. It is expected that incubating duplex N with endonuclease III at room temperature will generate a double strand break. Duplex L differed from duplex N only in that it lacked a nick. Incubating duplex L with endonuclease III should generate a single strand break in the DNA.

Enzymes and Proteins—Escherichia coli endonuclease III was purified from an overproducing strain employing MonoS, MonoQ and phenyl-Sepharose column chromatography as described previously (19). Human endonuclease III was purified by Dr. Robindra Roy (20). Ku was purified by Dr. David Chen according to an earlier published procedure (21).

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Endonuclease III Assay—Endonuclease III was assayed in a standard reaction buffer (10 μl) containing 0.1 M KCl, 10 mM Tris-HCl, pH 7.5, 20 fmol of DNA substrate, and 20 fmol of endonuclease III. The reaction was performed at 37 °C for 10 min (22). The reaction was stopped with 10 μl of polyacrylamide gel-loading buffer (90% formamide, 1 mM EDTA, 0.1% xylene, and 0.1% bromphenol blue) and heated to 90 °C for 10 min. 3–5 μl of the reaction were loaded onto a 12.5% denaturing polyacrylamide gel and electrophoresed at 2000 V for 1.5 h. The polyacrylamide gel was then dried, and the amount of endonuclease III-induced nicks was estimated by using the STORM PhosphorImager (Molecular Dynamics).

To determine the amount of double strand breaks induced by endonuclease III, at the end of 10 min of incubation, reactions were stopped with the gel-loading buffer and then assayed with 10% nondenaturing polyacrylamide gel. The double strand break assay is identical to the electrophoretic mobility shift assay described below.

**Electrophoretic Mobility Shift Assay**—The binding reaction (10 μl) was performed in a standard endonuclease III reaction mixture containing 0.1 M KCl, 10 mM Tris-HCl, pH 7.5, 20 fmol of DNA substrate, and increasing amounts of Ku (10–300 fmol). In some reactions, 20 fmol of endonuclease III were added. After 10 min at 37 °C, 5 μl of the reaction mixtures were added to 5 μl of polyacrylamide gel-loading buffer and heated to 90 °C for 10 min. The amount of endonuclease III-induced nicks was assayed as described in the previous section. The remainder (5 μl) of the binding reaction was subjected immediately to electrophoretic mobility shift assay as described below.

Electrophoretic mobility shift assay was performed with a 10% polyacrylamide gel containing acrylamide/N,N′-methylenebisacrylamide at 19.76/0.24 ratio as described previously (18). The gels were pre-electrophoresed in TBE buffer (89 mM Tris, 89 mM boric acid, pH 8.3 and 2.5 mM EDTA) at 300 V (4 °C) for 30 min. Samples were loaded onto the gel, and electrophoresis was continued at 300 V (4 °C) for an additional 150 min. After electrophoresis, the gels were dried under vacuum and exposed to x-ray film. The radioactive bands in the dried gels were quantified with the STORM PhosphorImager.

**RESULTS**

**Effect of Ku on Human and E. coli Endonuclease III Activities**—Dihydrouracil, a product of the anoxic radiolysis of deoxyribose, is readily recognized by human and *E. coli* endonuclease III (20). To examine the role of Ku in the repair of closely opposed lesions, duplex N that contained a DHU in close proximity opposite to a nick was used as a substrate for endonuclease III. Endonuclease III-induced cleavage of the DNA strand containing DHU of duplex N will generate an additional nick opposite to the pre-existing nick that is separated from each other by only three nucleotides. At room temperature, this will result in a double strand break that can be detected by electrophoresis in a nondenaturing polyacrylamide gel. Ku is known to bind to DNA ends and nicks (23–24), and thus it might interfere with endonuclease III activity on duplex N. In contrast, Ku would be expected to have little or no effect on the cleavage activity of endonuclease III on duplex L. Fig. 1 shows the effect of Ku on human endonuclease III activity on DNA duplex L in which the DHU-containing strands were labeled at their 5’ ends with 32P. Ku is known to bind to only DNA ends and nicks and has not been shown to have any endonuclease activity. Fig. 1A shows that incubating with increasing amounts of Ku did not lead to an appreciable nicking of duplex L (Fig. 1A, lanes 1–7). A small amount of background cleavage (approximately 5–10%) was frequently observed with the DNA preparations. Because Ku does not cleave DNA-containing dihydrouracil, no detectable double strand breaks were observed in the presence of increasing amounts of Ku (Fig. 1C, lanes 1–7). Double strand break was assayed by the formation of a species migrating faster than the free DNA substrate. Double strand break formation was assayed with nondenaturing polyacrylamide gel electrophoresis, an assay that is identical to the electrophoretic mobility shift assay used for detecting the various Ku-DNA complexes. Under these electrophoresis conditions, the various Ku-DNA complexes that migrated slower than the free DNA substrate were readily observed. Incubating duplex L with human endonuclease III led to a substantial cleavage occurring at the dihydrouracil lesion (Fig. 1B, lane 1); however, incubating endonuclease III with increasing concentrations of Ku had little or no effect on the human endonuclease activity on duplex L (Fig. 1B, lanes 2–7). Because cleavage of duplex L by human endonuclease III will only lead to a nick in a duplex DNA, no appreciable amount of double strand breaks was observed when duplex L was incubated with endonuclease III (Fig. 1D, lanes 1–7). However, when duplex N was used as a substrate, low concentrations of Ku (below 10 fmol) had a noticeable effect on the human endonuclease III activity (Fig. 2B, compare lane 1 with lanes 2–4); however, at higher concentrations of Ku (above 50 fmol), the inhibition of human endonuclease III activity was observed (Fig. 2B, compare lane 1 with lanes 5–7). At low concentrations of Ku, the observed endonucleolytic activity of human endonuclease III was also accompanied by a concomitant generation of free double strand breaks (Fig. 2D, compare lane 1 with lanes 2–4). Since higher concentrations of Ku (above 100 fmol) were found...
to inhibit the nicking of duplex N, the formation of free double strand breaks was also inhibited at higher Ku concentrations.

Similar results were also obtained with E. coli endonuclease III with the exception that increasing concentrations of Ku appeared to have a nonspecific inhibitory effect on duplex L (Fig. 3B, lanes 1–7) and duplex N (Fig. 4B, lanes 1–7). Like the results obtained for human endonuclease III, increasing concentrations of Ku did not lead to an appreciable production of double strand breaks with duplex L (Fig. 3D). As expected, increasing Ku concentrations lead to an increased inhibition of double strand breaks formation induced by E. coli endonuclease III (Fig. 4D).

The gel data presented in Figs. 1–4 are qualitative. To obtain quantitative analysis of these observations, these experiments were repeated three times, and the amount of endonuclease III-induced nicks and double strand breaks was quantified using a PhosphorImager. The average amount of nicks or double strand breaks generated by endonuclease III was plotted using a PhosphorImager. The average amount of nicks or double strand breaks formed as compared with the amount of nicks generated by E. coli endonuclease III (Fig. 5D).

Fig. 5 show that the amount of free double strand breaks did not correspond to the actual amount of nicks induced by human and E. coli endonuclease III in duplex N, suggesting that most of the double strand breaks were sequestered and bridged by Ku as DNA-protein complexes. Under nondenaturing conditions, it is clear that Ku forms various complexes with both duplexes N and L (Figs. 1–4, C and D). These DNA-Ku complexes were observed as slower migrating species than the substrate DNA. It is known that Ku will bind to the termini of a double-stranded DNA as a DNA-protein complex. In addition, Ku can translocate along the DNA (25), allowing additional Ku molecules to bind to the free DNA ends. As a result, Ku binds to a double-stranded DNA like beads on a string and generates multimeric Ku-DNA protein complexes. With Ku covering a 20–25-nucleotide footprint, it is likely that a 56-mer duplex DNA can interact with Ku, generating complexes containing 1, 2, or 3 Ku proteins (25, 26–28). Because endonuclease III reactions were performed at 37 °C, the binding assays were also performed at 37 °C. In the absence of endonuclease III incubating duplex oligonucleotides with Ku, three major slow migrating bands (complexes I, II, and III) were observed (Figs. 1–4, C and D, arrows marked I, II, and III). It is interesting to note that at 37 °C and at Ku protein concentrations below 50 fmol, little or no stable binding of the Ku complex to duplex oligonucleotides was observed (Figs. 1–4, C, lanes). However, when binding reactions were performed at 20 °C, the formation of complexes I and II can be readily observed even at 10 fmol of Kus. E. coli endonuclease III activity was inhibited ~30% for both substrates duplexes L and N (Fig. 5C). Similar to the results obtained for human endonuclease III, the increasing concentrations of Ku led to a much greater inhibition of the amount of free double strand breaks formed as compared with the amount of nicks generated by E. coli endonuclease III (Fig. 5D).
Ku (1:1 Ku/DNA ratio, data not shown). Similarly, the incubating duplex oligonucleotides with endonuclease III and increasing concentrations of Ku also generated three DNA-Ku complexes (Figs. 1–4, D).

Effect of Human and E. coli Endonucleases III on Ku Binding to DNA Containing DHU—To have a quantitative estimate on the interactions of Ku with duplexes N and L, each of the radioactive bands corresponding to the various forms of Ku-DNA complexes was quantified using a PhosphorImager and plotted as a function of Ku concentrations. Fig. 6 shows that when duplex L was used as a DNA substrate, little or no stable binding of Ku to DNA was observed at Ku concentrations below 50 fmol. At 300 fmol of Ku, ~30% of the DNA was bound with two molecules of Ku (complex II), presumably one at each end of the DNA. A smaller amount of complex I was observed. It is interesting to note that under the reaction conditions used, the amount of complex II formed was higher than the amount of complex I. Because duplex N is 56 nucleotides long, it is possible that the binding of more Ku molecules to the DNA might promote interactions among the Ku molecules, leading to increased stability for the DNA-Ku complex II as compared with DNA-Ku complex I. Only a small amount of complex III was formed even at concentrations above 200 fmol. In the presence of human endonuclease III (Fig. 6B), the interaction of Ku with the DNA substrate led to an appreciable increase in complex II, and as much as 50% of labeled DNA was bound as complex II. A similar increase in the level of complex II was also observed with E. coli endonuclease III (Fig. 6C). It is possible that the binding of endonuclease III at the nick (generated by the action of endonuclease III) or DHU lesion helps to stabilize complex II, thus leading to an apparent increase in the amount of complex III (D).

FIG. 5. Effect of Ku on human and E. coli endonuclease III activity. A, 20 fmol of duplex L were incubated with 20 fmol of human endonuclease III and an increasing amount of Ku in a standard endonuclease III buffer at 37 °C for 10 min. The amount of single strand breaks (C) and double strand breaks (■) induced by human endonuclease III was measured by electrophoresis on a 15% denaturing and 10% nondenaturing polyacrylamide gel, respectively. Each data point is the average of three independent experiments. B, 20 fmol of duplex N were incubated with 20 fmol of human endonuclease III and an increasing amount of Ku in a standard endonuclease III buffer at 37 °C for 10 min. The symbols used are the same as in A.

FIG. 6. Effect of endonuclease III on the formation of Ku-duplex L complexes. Complexes of Ku formed with duplex L were resolved by electrophoresis in a 10% nondenaturing polyacrylamide gel. A, 20 fmol of duplex L were incubated with an increasing amount of Ku in a standard endonuclease III buffer at 37 °C for 10 min. B, 20 fmol of duplex L were incubated with 20 fmol of human endonuclease III and an increasing amount of Ku in a standard endonuclease III buffer at 37 °C for 10 min. C, 20 fmol of duplex L were incubated with 20 fmol of E. coli endonuclease III and an increasing amount of Ku in a standard endonuclease III buffer at 37 °C for 10 min. The symbols used are same as in A. A–C, the amount of free DNA remaining (□) and the various forms of Ku70/80-duplex L complexes (complex I (○), complex II (■), and complex III (△)).

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FIG. 7. Proposed role of Ku70/80 in the repair of closely opposed base lesions.
II when endonuclease III is present. However, the levels of complexes II and I were less affected by the presence of either human or E. coli endonuclease III (Fig. 6, B and C).

In the absence of endonuclease III, the binding of Ku to duplex N was similar to that observed for duplex L. No appreciable stable binding of Ku to duplex N was observed at concentrations below 50 fmol. Three species of Ku-DNA complex were observed at higher concentrations of Ku (data not shown). The formation of form I and II complexes was readily observed, whereas the form III complex was only observed at relatively high concentrations of Ku. Similarly, incubating duplex N with human endonuclease III and increasing the amounts of Ku led to a significant increase in the formation of complex II and a slight increase in that of complex I (data not shown) while substantially inhibiting the formation of free double strand breaks. The parallel between the formation of complex II and the reduction in the number of free double strand breaks suggests that the double strand breaks formed were sequestered by Ku and migrated as DNA-protein complexes. Similar results were also observed with E. coli endonuclease III (data not shown).

**DISCUSSION**

It was shown earlier that base excision repair enzymes, such as endonucleases III and VIII and formamidopyrimidine-N-glycosylase, can efficiently remove 1 of 2 closely opposed base lesions (8–9) and produce a nick close to the remaining lesion on the opposite strand. The removal of the remaining base lesion is thereby inhibited. This inhibition was thought to be important for the possibility of generating free double strand breaks by these repair enzymes. However, prolonged incubation or the use of excess amounts of these N-glycosylases can eventually convert all closely opposed lesions in DNA to frank double strand breaks.

In humans, double strand breaks are primarily repaired by the nonhomologous end-joining pathway involving Ku (13–17). Although free double strand breaks are repaired readily in mammalian cells, it is nevertheless desirable to avoid this formation to reduce the frequency of misjoining, which might result in translocation mutagenesis. In the processing of closely opposed base lesions, a nick is generated in close proximity opposite to the remaining base lesion. We showed that Ku binds to duplex N and inhibits endonuclease III activity on DHU lesion opposite to a nick, suggesting that Ku might have a role in modulating the repair of closely opposed base lesions. It has been estimated that a human cell has as many as 500,000 molecules of Ku (24). Thus, it is believed that the abundant Ku is always in excess over endonuclease III and other repair N-glycosylases. Our results suggest that in vivo Ku will inhibit the action of endonuclease III on a lesion opposite to a nick. It is also interesting to point out that Ku was demonstrated to inhibit the binding of nucleotide excision repair proteins to a linear DNA (27). We suggest that the binding of Ku to the nicked DNA will either help to recruit DNA polymerase and ligase to the nick or provide extended time for these enzymes to repair the intermediary nick. A model for the role of Ku in the mediation of repair of the closely opposing lesion is depicted in Fig. 7. It has been shown that the binding of Ku to a nick does not inhibit the ligation reaction catalyzed by either DNA ligase II or III. In fact, the binding of Ku enhances these reactions (14). Recently, Ku-DNA complex was shown to recruit DNA ligase IV and also interact directly with the human DNA ligase IV (29, 30). However, it is not known whether the binding of Ku to a nick will affect the rate of repair synthesis by DNA polymerase once the nick is repaired, Ku should dissociate from the DNA, allowing further excision of the remaining base lesion by human endonuclease III. However, if the removal of the remaining lesion occurs before all the nicks are repaired, any double strand DNA breaks generated will be sequestered by Ku and channeled directly into the end-joining repair pathway. In that case, no intermediary free double strand breaks are formed, reducing the possibility of the misjoining of DNA ends.

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