Ku Proteins Join DNA Fragments as Shown by Atomic Force Microscopy

Dalong Pang, Sunghan Yoo, William S. Dynan, Mira Jung, and Anatoly Dritschilo

Abstract

The binding of the Ku protein to DNA was investigated using the atomic force microscope. Ku was found to bind predominantly to the ends of double-stranded DNA. Experiments with plasmid DNA revealed that Ku does not bind to circular plasmids but does bind to plasmids that have been linearized by treatment with ionizing radiation. The binding of Ku to poly(dG-dC)-poly(dG-dC) poly(dG-dC) and to a 400-bp DNA EcoRI fragment resulted in a shift in the fragment size distribution to include longer fragments, with internally binding Ku. Furthermore, we observed images consistent with fragments joined together by Ku, showing an interaction with two ends of DNA. These observations suggest that Ku may play a role in physically orienting DNA for ligation by binding the ends of adjacent DNA molecules.

Introduction

The autoantigen protein Ku is a relatively abundant nuclear DNA-binding protein. It was first found as a target of autoantibodies in patients with sclerodermapolymyositis overlap syndrome and was identified as a nonhistone DNA-binding heterodimeric protein with Mr 70,000 and Mr 80,000 (Mr 86,000) subunits (1, 2). Ku protein binds to DNA ends, nicks, gaps, and regions of transition between single- and double-stranded structures (3–7). These binding properties suggested a role for Ku protein in DNA repair (8). Consistent with this, dsb1-repair deficient murine cells in the XRCC5 complementation group have been shown to specifically lack the Ku86 subunit (9–12).

The exact role of Ku protein in dsb repair remains under investigation. In higher eukaryotes, Ku protein serves as a regulatory component of the DNA-dependent protein kinase (13, 14). Activation of the kinase may provide an intracellular signal relating to DNA damage or repair. In addition to its role in kinase activation, Ku protein may also participate directly in repair, for example, by physically protecting broken DNA from degradation or by orienting DNA ends for religation. Such a direct role is suggested by experiments in Saccharomyces cerevisiae, which appears to lack a homologue of the DNA-PK catalytic subunit. S. cerevisiae Ku protein is required for precise religation of the cohesive ends of linearized plasmids. In the absence of Ku protein, plasmid recircularization occurs at lower frequency and entails loss of terminal sequences (15, 16).

Previous investigations of Ku protein-DNA interaction have relied primarily on gel electrophoretic mobility shift assays, which separate DNA probes into different bands based on the presence of bound Ku protein. These assays can only detect high-affinity interactions and, in addition, are often unable to resolve higher-order complexes involving multiple DNA fragments. In the present study, we have used AFM to study the binding interactions of Ku proteins with DNA. The use of AFM has provided images of the DNA, the Ku protein, and the sites of binding of Ku on DNA for individual DNA fragments. In addition, the high resolution capacity of AFM and its ability to image the structures of biological macromolecules have allowed us to examine the geometric conformations and to measure the three-dimensional sizes of the DNA and protein macromolecules. Our observations suggest that the ends of broken DNA molecules are preferentially bound by the Ku protein and are physically held together, suggesting a role for Ku in broken DNA orientation in its function in DNA repair.

Materials and Methods

DNA Samples and Ku Protein. Three different DNAs were used in this study: pUC19 ds plasmid DNA; relaxed circular or broken linear fragments of pUC19 produced by ionizing radiation; poly(dG-dC)-poly(dG-dC) and 400-bp restriction enzyme-digested, linear double-stranded DNA fragments. The pUC19 plasmid was purchased from New England Biolabs (Beverly, MA). The plasmids were 2742 bp in length and were diluted in buffer containing 10 mM HEPES and 1 mM MgCl2 to a concentration of 5 ng/μl. The original plasmids were in relaxed circular conformation, as observed with the AFM. No linear fragments were observed. To study the binding of Ku to DNA, we used the pUC19 samples irradiated with 18 MeV electrons for a dose of 150 Gy (17). As a result, a large percentage of the plasmids were broken into linear fragments, with a small fraction remaining in circular form.

The poly(dG-dC) used in these experiments was purchased from Pharmacia Biotech, Inc. (Piscataway, NJ). This sample was diluted in 10 mM HEPES and 1 mM MgCl2 to a concentration of 5 ng/μl. Examination with the AFM revealed a uniform structural conformation along the fragments; no blunting was observed at the ends. The 400-bp EcoRI enzyme-digested linear DNA fragments were prepared in our laboratory.

Ku protein was purified from recombinant baculovirus-infected insect cells essentially as described (18) with the addition of a Superdex S-300 gel filtration step to assure that the Ku protein was in the active heterodimeric form (19). Purified Ku protein was stored frozen in a buffer containing 50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 100 mM KCl, 20% glycerol, 0.02% Tween 20, 3 mM DTT, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml soybean trypsin inhibitor, and 10 μg/ml phenylmethylsulfonyl fluoride. The stock concentration was 12 ng/μl, and for purposes of AFM imaging, the Ku protein was diluted to a concentration of 2.5 ng/μl.

DNA Irradiation. For DNA irradiation, we used a medical linear accelerator (Varian 2100 C/D). The electron energy was set at 18 MeV, and DNA was irradiated at room temperature for a dose of 150 Gy.

AFM. The atomic force microscope uses a microcantilever system to detect the atomic and/or molecular forces between the probe tip and the sample. We used the NanoScope-III (Digital Instruments, Santa Barbara, CA) in its tapping mode in air at room temperature. The cantilevers are commercially available and are fabricated from silicon with spring constants of 16 to 88 N/m and a resonant frequency of 300 to 400 kHz. The scan rate was set at 5 Hz with an information density of 512 × 512 pixels.

For AFM imaging of the DNA and Ku samples, 1 μl of sample was deposited onto an atomically flat mica surface and rinsed gently with distilled water to remove excess molecules. We obtained approximately 20 images of each sample. The lengths of DNA fragments were determined by using the same sample analysis software incorporated into the NanoScope-III.
**Results**

We first imaged the pUC19 plasmid DNA. Intact pUC19 exists as a circular plasmid, and no Ku-DNA binding was seen (data not shown). Exposure of the DNA to 150 Gy of ionizing radiation resulted in broken linearized DNA as well as residual, intact plasmids. Ku-DNA binding was observed (Fig. 1A), consistent with requirements published previously for DNA ends for Ku binding activity. A total of 20 images were obtained, revealing 90 molecules (both linear and circular), with 23% of the linear fragments having Ku bound to one end and 4% binding internally. Binding to both ends were not observed in these images. Also, Ku was not observed bound to residual, intact plasmids.

We next investigated the binding of Ku polyd GC (Fig. 1B). Among the 120 polyd GC fragments analyzed, 20% have Ku bound to one end and 4% binding internally. Binding to both ends were not observed in these images. Also, Ku was not observed bound to residual, intact plasmids.
end, 13% to internal sites, and 3% to both ends. Furthermore, the molecular size distributions of control polyd GC and Ku-polyd GC showed a shift to larger size fragments in the Ku-polyd GC, suggesting the possibility of Ku joining smaller fragments together (Fig. 2A).

Ku has been reported to bind to double-stranded DNA ends with high affinity and to subsequently translocate to interval sequences (3, 4, 6). The finding of primary end binding is consistent with such a hypothesis. However, the observation that the fragment length distribution of poly GC was shifted to larger sizes suggested that Ku may be physically joining the ends of fragments. The variable lengths of poly GC precluded a direct resolution of these two possibilities by image analysis.

To distinguish between internal binding or the joining of two or more fragments by Ku, we used DNA that was digested with EcoRI to fragments of 400-base length (136 ± 20 nm). The presence of DNA fragments of lengths in multiples of 136 nm and showing internal Ku-DNA binding would be supportive for a role for Ku in joining broken DNA ends.

Among the 95 fragments analyzed, we found that 25% have Ku bound to only one end, 5% to both ends, and 13% with binding between the ends. We measured the lengths of fragments to which internal binding was observed and found that 64% include lengths of 136 nm. The lengths of the remaining 36% of fragments are equal to or less than 136 nm. Fig. 1C shows such an example. To further evaluate the observed fragment joining, we then measured the fragment length distribution of the Ku-400-bp sample and the control 400-bp sample. Comparison of the two distributions clearly revealed a shift of the fragments in the Ku-400-bp sample toward longer lengths (Fig. 2B). This observation reinforces the indication that the observed internal binding represents fragments joined by Ku, in support of a role for Ku in DNA end binding and fragment joining in vitro. Furthermore, the size of the Ku protein is consistent with one heterodimer at each DNA junction.

Discussion

AFM is a relatively new imaging technique with nanomolar resolution on soft biological macromolecules. As a complement to gel electrophoresis and filter techniques, AFM offers the capability for measuring individual molecules and providing high resolution structural information. The resolution of 4 nm, which is only twice the diameter of a DNA molecule, permits the measurement of short DNA fragments and distinction of proteins by size.

We have used AFM to image the binding of Ku proteins to DNA. These data confirm the previously described end-binding capacity of Ku as the predominant interaction with DNA (3). Ku binds to the free ends of double-stranded DNA generated by ionizing radiation and by restriction with the endonuclease EcoRI. Ku also binds to polyd GC ends with comparable affinity.

Ku binds primarily to one end of the DNA molecules. The fraction of events that result in the presence of Ku at internal DNA sites is consistent with the joining of the ends of smaller fragments. The previously suggested hypothesis that Ku plays a role in the repair of DNA double-stranded breaks is supported by our observations that two or more DNA fragments can be held together in vitro. Ku was rarely seen (~4%) at both ends of DNA in these experiments. This may be the result of molecular ratios of protein to DNA that were used in sample preparation. The relative concentrations of DNA templates and the Ku protein make it less probable that both ends of the DNA fragments are bound. Also, the two ends of a DNA template may not be identical in their attraction to Ku. Additional investigations will need to resolve this observation.

An alternative explanation for the observed results is that the Ku protein might be nonspecifically aggregated. We regard this as unlikely, however, because the preparation of Ku protein that was used for these experiments had been purified by gel filtration chromatography to obtain the active heterodimeric form (see "Materials and Methods").

It will be of interest to extend the studies described here by using mutant forms of Ku protein and by testing different DNA fragments to determine whether apposition of DNA fragments observed by AFM faithfully reflects the initial steps of in vivo dsb repair.

Acknowledgments

We thank Dr. Ying Zhang for assistance in sample preparation, the Institute of Materials Science of The George Washington University for unlimited access to the NanoScope-III, and Elaine North and Sandra Hawkins for assistance in manuscript preparation.

References


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