Ku70 Is Required for DNA Repair but Not for T Cell Antigen Receptor Gene Recombination In Vivo

By Honghai Ouyang,* Andre Nussenzweig,* Akihiro Kurimasa,4
Vera da Costa Soares,* Xiaoling Li,* Carlos Cordon-Cardo,*
Wen-hui Li,* Nye Cheong,* Michel Nussenzweig,†
George Iliakis,‡ David J. Chen,‡ and Gloria C. Li*†

From the *Department of Medical Physics and Department of Radiation Oncology, Memorial Sloan-Kettering Cancer Center, New York, 10021; ‡Los Alamos National Laboratory, Los Alamos, New Mexico 87545; †Thomas Jefferson University, Philadelphia, Pennsylvania 19107; ‡Rockefeller University, New York, 10021.

Summary

Ku is a complex of two proteins, Ku70 and Ku80, and functions as a heterodimer to bind DNA double-strand breaks (DSB) and activate DNA-dependent protein kinase. The role of the Ku70 subunit in DNA DSB repair, hypersensitivity to ionizing radiation, and V(D)J recombination was examined in mice that lack Ku70 (Ku70−/−). Like Ku80−/− mice, Ku70−/− mice showed a profound deficiency in DNA DSB repair and were proportional dwarfs. Surprisingly, in contrast to Ku80−/− mice in which both T and B lymphocyte development were arrested at an early stage, lack of Ku70 was compatible with T cell receptor gene recombination and the development of mature CD4+CD8− and CD4+CD8+ T cells. Our data shows, for the first time, that Ku70 plays an essential role in DNA DSB repair, but is not required for TCR V(D)J recombination. These results suggest that distinct but overlapping repair pathways may mediate DNA DSB repair and V(D)J recombination.

Two distinct processes involving DNA double-strand breaks (DSB)1 have been identified in mammalian cells: the repair of DNA damage induced by ionizing radiation and V(D)J recombination during T and B cell development. So far, all mammalian cell mutants defective in DNA DSB repair share the common phenotype of hypersensitivity to radiation and impaired ability to undergo V(D)J recombination (1–6). Cell fusion studies using DSB sensitivity to radiation and impaired ability to undergo DNA DSB repair have de-
TCR V(D)J recombination. The distinct phenotype of Ku70−/− mice should make them valuable tools for unraveling the mechanism(s) of DNA repair and recombination.

Materials and Methods

Target Disruption of Ku70 and Generation of Ku70−/− Mice. Mouse genomic Ku70 gene was isolated from a scos I cosmid library constructed from a mouse strain 129 embryonic stem (ES) cell lines 21. The replacement vector was constructed using a 1.5 kb 5′-fragment that contains the promoter locus with four GC boxes and exon 1, and an 8-κb EcoR V–EcoI fragment extending from intron 2 to intron 5 as indicated in Fig. 1 A. Homologous recombination results in a deletion of 336-bp exon 2 including the translational initiation codon.

The targeting vector was linearized with NotI and transfected into C57 ES cells by electroporation using a gene pulser (Bio Rad Labs., Hercules, CA). 300 ES cell clones were screened, and five clones carrying the mutation in Ku70 were identified by Southern blotting. Positive ES clones were injected separately into a C57BL/6 blastocysts to generate chimeric mice. One clone was successfully transmitted through the germline after chimeras were confirmed by a Ku–DNA-end–binding assay (gel mobility shift analysis). The genotype of the mice was first determined by tail PCR analysis which distinguishes endogenous from the targeted Ku70 allele, and subsequently confirmed by Southern blot analysis. The PCR reaction contained 1 μg genomic DNA; 0.6 μM (each) of primers HO-2: GGGCCAGCTATTCTGACTAT, HO-3: CCTACAGTGTACCCGGATCC, and HO-4: CGGAAACAGCTGTGTTGAGCC; 0.2 mM (each) deoxynucleoside triphosphate; 1.5 mM MgCl2, and 2.5 U of Taq polymerase. Cycling conditions were 94°C for 1 min, 64°C for 1 min, 72°C for 1 min (30 cycles), followed by an extension at 72°C for 10 min. Primers HO-2 and HO-4 give a product of the targeted allele that is ~380 bp; primers HO-2 and HO-4 yield a wild-type product of 407 bp.

Western Blot Analysis and Gel Mobility Shift Assay. To confirm that the disruption of Ku70 produces a null mutation, Ku70 protein expression was measured by Western blotting using polyclonal antisera against intact mouse Ku70. The lack of Ku70 was also verified by a Ku–DNA-end–binding assay (gel mobility shift analysis). Cell extracts were prepared and gel mobility shift assays were performed as described (22). Equal amounts of cellular protein (50 μg) from Ku70+/+ (wild type), Ku70−/−, and Ku70−/− mouse embryo fibroblasts were incubated with a32P-labeled double-stranded oligonucleotide, 5′GGGCCAAGAATCTTCCAGCA- GGCGCTCGG-3′. The protein-bound and free oligonucleotides were electrophoretically separated on a 4.5% native polyacrylamide gel. Gel slabs are dried and autoradiographed with X-Omat film (Kodak, Rochester, NY).

Immunohistochromy. To determine the pathological changes, histological sections of various organs of Ku70−/−, Ku80−/−, and wild-type littermate mice were prepared and examined as previously described (23). Lymph nodes, spleens, and thymuses from 4-5-wk-old mice were fixed in 10% buffered formalin and embedded in paraffin, or embedded in (optimal cutting temperature) compound (Sakura Finetek, USA, Incorp., Torrance, CA) and frozen in liquid nitrogen at −70°C. Sections (5 μm) were stained with hematoxylin and eosin, and representative samples were selected for immunohistochromal analysis. Immunophenotyping was performed using an avidin-biotin immunoperoxidase technique (24). Primary antibodies included anti-CD3 (purified rabbit serum, 1:1,000; Dako Corp., Carpinteria, CA), anti-B220 (rat monocular, 1:1,000; Pharmingen, San Diego, CA), and anti-CD19 (rat monoclonal, 1:1,000; Pharmingen), and were incubated overnight at 4°C. Samples were subsequently incubated with biotinylated secondary antibodies (Vector Labs., Burlingame, CA) for 30 min (goat anti-rabbit, 1:100; rabbit anti-rat, 1:100), and then with avidin-biotin peroxidase (1:25 dilution; Vector Labs) for 30 min. Diaminobenzidine was used as the chromogen and hematoxylin as the counter stain. Wild-type lymphoid organs including thymus, spleen, and lymph nodes from different mice were used for titration of the antibodies and postive controls. Anti-CD3 and anti-CD19 antibodies were tested in both frozen and paraffin sections of wild-type lymphoid organs and showed the expected specific patterns of staining (data not shown). For negative controls, primary antibodies were substituted with class matched but unrelated antibodies at the same final working dilutions.

Cell Preparation and Flow Cytometric Analysis. For flow cytometry, single cell suspensions from lymphoid organs of 4–6-wk-old mutant and littermate control mice were prepared for staining as described previously (19) and analyzed on a FACScan® with Cell Quest software (Becton Dickinson, San Jose, CA). Cells were stained with combinations of PE-labeled anti-CD4 and FITC-labeled anti-CD8, or PE-labeled anti-B220 and FITC-labeled anti-CD43, or FITC–anti-1gM and PE–anti-B220 (Pharmingen), as needed. Bone marrow cells were harvested from femurs by syringe lavage, and cells from thymus and spleen were prepared by homogenization. Cells were collected and washed in PBS plus 5% FCS and counted using a hemacytometer. Samples from individual mice were analyzed separately. Dead cells were gated out by forward and side scatter properties. Experiments were performed at least three times and yielded consistent results.

DNA Preparation and Analysis of V(D)J Recombination Products. To determine whether a null mutation in Ku70 affects the recombination of antigen-receptor genes in T and B lymphocytes in vivo, we measured the immunoglobulin and T cell antigen receptor (TCR) rearrangements by PCR. DNA from bone marrow was amplified with primers specific to immunoglobulin D-JH and V-DJH rearrangements, and DNA from thymus was amplified with primers that detect V-DJHβ and Dβ5-Jβ rearrangement (20, 25–28).

Oligonucleotides. Oligonucleotides for probes and PCR primers specific to TCR Vb/J rearrangements and immunoglobulin D-JH and V-DJH rearrangements are as follows. For TCR-β Vb/J rearrangements (19, 20): Vb/Jα, Vb/Jβ, Vb/Jγ, Vb/Jδ, and Vb/Jε rearrangements, and V-DJHβ rearrangements are as follows. For TCR-β Vb/Jα rearrangements: Vb/Jβ, Vb/Jγ, Vb/Jδ, and Vb/Jε rearrangements, and V-DJHβ rearrangements. Oligonucleotides for probes and PCR primers specific to TCR Vb/Jβ rearrangements and immunoglobulin D-JH and V-DJH rearrangements as follows. For TCR-β Vb/Jα rearrangements: Vb/Jβ, Vb/Jγ, Vb/Jδ, and Vb/Jε rearrangements, and V-DJHβ rearrangements. Oligonucleotides for probes and PCR primers specific to TCR Vb/Jβ rearrangements and immunoglobulin D-JH and V-DJH rearrangements are as follows. For TCR-β Vb/Jα rearrangements: Vb/Jβ, Vb/Jγ, Vb/Jδ, and Vb/Jε rearrangements, and V-DJHβ rearrangements.
pared dishes with 0.5% noble agar underlayer. The cells were immediately after radiation exposure, cells were diluted in 2 ml of 2.0 ml of the above media containing 0.5% noble agar (Difco source of colony-stimulating factor. 1 d before each experiment, GM-CSF (R & D Sys. Inc., Minneapolis, MN) was used as a activated FCS and 1% bovine serum albumin; in addition, 0.5 ng/ml of activity released from the well into the lane in irradiated and Levels of DNA DSB were quantified by calculating the fraction V/cm for 75 s in the reverse direction as described (31).

1.25 V/cm for 900 s in the direction of DNA migration, and 5.0 m for 8 min. The resulting pellet was resuspended and diluted to 106 cells/ml in MEM plus 15% FCS for further experiments.

TGF-beta releasable dishes were prepared and cells were counted using a hemacytometer and centrifuged at 1,000 rpm for 12 min. The resulting pellet was resuspended and diluted to ~106 cells/ml in IMEM plus 15% FCS for further experiments. To measure the survival of granulocyte-macrophage progenitors, the method of Van Zant et al. (29) was used with minor modifications (30). In brief, 8-MEM contained 30% heat-inactivated FCS and 1% bovine serum albumin; in addition, 0.5 ng/ml GM-CSF (R & D Sys. Inc., Minneapolis, MN) was used as a source of colony-stimulating factor. 1 d before each experiment, 0.5% noble agar and 0.5 ng/ml 50 cells were scored. The colony forming efficiency of CFU-GM was 60–100/105 CFU-GM). Only colonies containing ~50 cells were scored. The colony forming efficiency of CFU-GM was 60–100/105 nucleated cells for untreated controls. Surviving fraction was defined as the cloning efficiency of irradiated marrow cells relative to that of untreated controls. All experiments were performed at least twice and yielded consistent results.

Asymmetric Field Inversion Gel Electrophoresis. To determine the rate and extent of DNA DSB repair in Ku-deficient cells after exposure to ionizing radiation, primary embryo fibroblasts derived from Ku70+/−, Ku80−/− and wild-type littermate mice were used. Mouse embryo fibroblasts from day 13.5 embryos growing in replicate cultures for 3 d in the presence of 0.01 μCi/ml [3H]thymidine (New England Nuclear, Boston, MA) and 2.5 μM cold thymidine were exposed to 40 Gray (Gy) of x-rays and returned to 37°C. At various times thereafter, one dish was removed and trypsinsized on ice; single cell suspensions were made and embedded in an agarose plug at a final concentration of 3 × 108 cells/ml. Asymmetric field inversion gel electrophoresis (AFIGE) was carried out in 0.5% Seakem agarose (FMC Bioproducts, Rockland, ME; cast in the presence of 0.5 μg/ml ethidium bromide) in 0.5 × TBE (45 mM Tris, pH 8.2, 45 mM boric acid, 1 mM EDTA) at 10°C for 40 h by applying cycles of 1.25 V/cm for 900 s in the direction of DNA migration, and 5.0 V/cm for 75 s in the reverse direction as described (31).

Quantification and analysis for DNA DSB present were carried out in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Levels of DNA DSB were quantified by calculating the fraction of activity released from the well into the lane in irradiated and unirradiated samples, which equals the ratio of the radioactivity signal in the lane versus that of the entire sample (well plus lane).

Results

Targeted Disruption of Ku70 Gene. To study the role of Ku70 in vivo, we generated mice containing a germline disruption of the Ku70 gene. Murine genomic and wild-type littermate mice were isolated and a targeting vector was constructed (Fig. 1 A). Homologous replacement results in a deletion of 336-bp exon 2, including the translational initiation codon. Two targeted ES clones carrying the mutation in Ku70 were injected into C57BL/6 blastocysts to generate chimeric mice. One clone was successfully transmitted through the germline after chimeras were crossed with C57BL/6 females. No obvious defects were observed in Ku70+/− heterozygotes, and these Ku70+/− mice were subsequently used to generate Ku70−/− mice (Fig. 1 B). 25% of the offspring born from Ku70+/− × Ku70−/− crosses were Ku70−/−. Adult Ku70−/− mice are fertile, but give reduced litter size (two to four pups) as compared to the Ku70+/− or Ku70+/+ mice (about eight pups).

To confirm that the disruption produced a null mutation, Ku70 protein expression was analyzed by both Western blotting (Fig. 1 C) and a DNA end binding assay (Fig. 1 D). Ku70 immunoreactivity was undetectable (Fig. 1 C), and there was no Ku–DNA-end–binding activity in Ku70−/− fibroblasts (Fig. 1 D). The Ku80 subunit of the Ku heterodimer was found, but at much reduced levels (Fig. 1 C), suggesting that the stability of Ku80 is compromised by the absence of Ku70. These observations are consistent with the finding that the level of Ku70 was significantly reduced in Ku80−/− fibroblasts and Ku80−/− ES cells (19). Taken together, these data suggest that the stability of either component of Ku is compromised by the absence of the other.

Newborn Ku70−/− mice were 40–60% smaller than their Ku70+/− and Ku70+/+ littermates. During a 5-mo observation period, Ku70−/− mice grew and maintained body weight at 40–60% of controls. Thus, Ku70−/− mice, like Ku80−/− mice, are proportional dwarfs (19).

Development of B Lymphocytes, but Not T Lymphocytes, Is Blocked at Early Stage in Ku70−/− Mice. Examination of vari-

Figure 1. Inactivation of Ku70 by homologous recombination. (A) Diagrammatic representation of the Ku70 locus (top), the targeting construct (middle), and the targeted allele and hybridization probe (bottom). EcoR I (E) restriction sites used to detect the targeted gene are indicated (21). (B) Southern blot of EcoR I-digested tail DNA from control wild-type (WT), heterozygous (+/−), and homozygous (−/−) Ku70−/− targeted mice. The wild-type and mutant fragments are 13 and 5.7 kb, respectively. (C) Western blot analysis showing that Ku70 protein is not expressed in Ku70−/− cells. Whole cell lysates prepared from mouse ear fibroblasts (50 μg) and mouse embryo fibroblasts (100 μg) were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with polyclonal antibodies against full-length rodent Ku80 (top) and Ku70 (bottom), respectively. (D) Gel mobility shift assay (22) showing the lack of DNA end-binding activity in Ku70−/− cells. Ku-DNA binding complex is indicated by arrow on the right.
ous organs from Ku70−/− mice showed abnormalities only in the lymphoid system (Fig. 2 A). Spleen and lymph nodes were disproportionately smaller by 5–10-fold relative to controls. In particular, splenic white pulp nodules were significantly reduced. Immunohistochemistry on deparaffinized tissue sections revealed that the splenic white pulp contained cells that stained with anti-CD3 (i.e., CD3-positive T cells), but there were no CD19-positive B cells (Fig. 2 A, k and n).

Figure 2.
Table 1. Lymphoid Cellularity of Ku70−/− Mice

| Tissue and genotype | Total (x 10⁶) | B220⁺ | CD4⁺CD8⁺ |
|---------------------|---------------|-------|----------|
| **Thymus**          |               |       |          |
| wild type (n = 4)   | 155 ± 42      | -     | 104 ± 28 |
| Ku70−/− (n = 3)     | 2.98 ± 0.91   | -     | 0.6 ± 0.2|
| Ku80−/− (n = 2)     | 1.0 ± 0.5     | -     | -        |
| **Bone marrow**     |               |       |          |
| wild type (n = 4)   | 11.9 ± 3.3    | 5.5 ± 1.5 | -        |
| Ku70−/− (n = 3)     | 7.2 ± 2.9     | 1.1 ± 0.4 | -        |
| Ku80−/− (n = 2)     | 9.0 ± 3.0     | -     | -        |
| **Spleen**          |               |       |          |
| wild type (n = 4)   | 53 ± 20       | 29 ± 11 | -        |
| Ku70−/− (n = 3)     | 6.5 ± 1.3     | 0.4 ± 0.2 | -        |
| Ku80−/− (n = 2)     | 1.2 ± 0.5     | -     | -        |

Data shown are arithmetic means ± standard deviations from two to four individuals of each genotype analyzed at 4–6 wk of age. Cell numbers are shown per femur for bone marrow, and per whole organ for spleen and thymus.

The Ku70−/− thymus was also disproportionally smaller and contained 50–100-fold fewer lymphocytes than Ku70+/+ littermates (3 × 10⁶ in the former versus 2 × 10⁶ in the latter; measured in three mice of each genotype). In contrast to the Ku80−/− mice, the Ku70−/− thymus displayed normal appearing cortical-medullary junctions (Fig. 2 A, g and j). Overall, the lymphoid tissues and organs of Ku70−/− mice are somewhat disorganized and much smaller than Ku70+/+ mice (Table 1); yet, they are relatively more developed and slightly larger than in Ku80−/− mice.

To further examine the immunological defect in Ku70−/− mice, cells from thymus, bone marrow, and spleen were analyzed using monoclonal antibodies specific for lymphocyte surface markers and flow cytometry (19). Consistent with the immunohistological data, there was a complete block in B cell development at the B220⁺CD43⁺ stage in the bone marrow, and there were no mature B cells in the spleen (Fig. 2 B). In contrast, thymocytes developed through the CD4⁺CD8⁺ double-positive stage and matured into CD4⁺CD8⁻ and CD4⁻CD8⁺ single-positive (SP), TCR-β-positive cells (Fig. 2, B and C). In six 4-wk-old Ku70−/− mice analyzed, the percentage of CD4⁺CD8⁻ double-negative thymocytes ranged from 11 to 62%, and the CD4⁺CD8⁻ double-positive cells varied from 35 to 73%. CD4⁻CD8⁺ (1–11%) and CD4⁺CD8⁻ (1–3%) SP cells were also detected in the thymus. Furthermore, CD4⁺ CD8⁻ or CD4⁻CD8⁻ SP T cells were found in the spleen in 67% of the mice studied (Fig. 2 B), which expressed surface TCR-β (Fig. 2 C) and CD3 (data not shown). Thus, in contrast to the early arrest of both T and B cell development in Ku80−/− mice (Fig. 2 B), lack of Ku70 is compatible with the maturation of T cells.
Figure 3. TCR and immunoglobulin gene rearrangement in Ku70−/− mice. (A) Recombination of V558L, V17183 to DJ1, and DJ1 to JH, gene segment (26). 100 ng DNA was used for Ku70−/− (lanes 7 and 8), Ku80−/− (lanes 1–3), and sid mice (lanes 4–6), and 1, 10, and 100 ng for wild-type (WT) mice (lanes 9–11). For IVS controls, DNA was diluted 100-fold before PCR. (B) PCR analysis of TCR gene rearrangements. Thymus DNA was assayed for recombination of V8-J2,2 and D2-J51 rearrangements (26). 100 ng DNA was used for Ku70−/− (lanes 2 and 7), Ku80−/− (lanes 1), and Ku70−/− mice (lanes 7), and 1, 10, and 100 ng for wild-type mice (lanes 4–6). Controls include a 1-kb germline interval amplified in the D2-J51 rearrangements (26). Multiple lanes underneath each genotype label (Ku70−/−, Ku80−/−, and SCID) represent different individual animals.

Discussion

Absence of Ku70 results in radiation hypersensitivity and proportional dwarfism, as well as deficiencies in DNA DSB repair and V(D)J recombination. Thus, Ku70−/− mice resemble Ku80−/− mice in several respects, but the two mutations differ in their effects on T and B cell development. Absence of Ku70 was compatible with TCR gene rearrangement and development of mature CD4+CD8+ and CD4−CD8+ T cells, whereas mature T cells were absent in Ku80−/− mice. In contrast, B cells failed to complete anti-
What could account for the differences we find in TCR and immunoglobulin gene rearrangements in the Ku70−/− mice? One implication of our findings is that there are alternative Ku70-independent rescue pathways that are compatible with completion of V(D)J recombination in T cells. It is likely at the critical phase of T cell maturation, other DNA repair activity may be stimulated (33, 34) and can functionally complement the Ku70 gene in T cell-specific V(D)J recombination. Since Ku80−/− mice are deficient in both T and B lymphocyte development, it is plausible that these yet to be identified alternative DNA repair pathways include Ku80. The much reduced level of Ku80 protein in Ku70−/− cells may in part account for the hypocellularity of Ku70−/− thymuses.

In summary, our studies provide direct evidence supporting the involvement of Ku70 in the repair of DNA DSB and V(D)J recombination and the presence of a Ku70-independent rescue pathway(s) in TCR V(D)J rearrangement. The distinct phenotype of Ku70−/− mice should make them valuable tools for unraveling the mechanism(s) of DNA repair and recombination.
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Address correspondence to G. C. Li, Department of Medical Physics and Department of Radiation Oncology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave./Box 72, New York, NY 10021. Phone: 212-639-6028; FAX: 212-639-2611; E-mail: g-li@ski.mskcc.org

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