Genetic Analysis of the DNA-dependent Protein Kinase Reveals an Inhibitory Role of Ku in Late S-G2 Phase DNA Double-strand Break Repair*

Toru Fukushima‡§, Minoru Takata‡§, Ciaran Morrison‡, Ryoko Araki‡**, Akira Fujimori‡, Masumi Abe**‡, Kouichi Tatsumi**‡, Maria Jasín‡§, Pawan Kumar Dhar‡, Eiichiro Sonoda‡, Tsutomu Chiba§, and Shunichi Takeda‡§§

From the CREST Research Project, Radiation Genetics, Faculty of Medicine, Kyoto University, Konoe Yoshida, Sakyoku, Kyoto 606-8501, Japan, the Institute of Cell and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JR, United Kingdom, the National Institute of Radiological Sciences, Image-ku Anagawa, Chiba 263-8555, Japan, the Cell Biology Program, Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, New York, New York 10021, and the Division of Gastroenterology and Hepatology, Department of Internal Medicine, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawara-cho, Sakyoku, Kyoto, 606-8507, Japan

Received for publication, July 5, 2001, and in revised form, September 26, 2001
Published, JBC Papers in Press, September 27, 2001, DOI 10.1074/jbc.M106295200

Two major complementary double-strand break (DSB) repair pathways exist in vertebrates, homologous recombination (HR), which involves Rad54, and non-homologous end-joining, which requires the DNA-dependent protein kinase (DNA-PK). DNA-PK comprises a catalytic subunit (DNA-PKcs) and a DNA-binding Ku70 and Ku80 heterodimer. To define the activities of individual DNA-PK components in DSB repair, we targeted the DNA-PKcs gene in chicken DT40 cells. DNA-PKcs deficiency caused a DSB repair defect that was, unexpectedly, suppressed by Ku70 disruption. We have shown previously that genetic ablation of Ku70 confers RAD54-dependent radiosensitivity on S-G2 phase cells, when sister chromatids are available for HR repair. To test whether direct interference by Ku70 with HR might explain the Ku70-/−/DNA-PKcs−/− radiosensitivity, we monitored HR activities directly in Ku- and DNA-PKcs-deficient cells. The frequency of intrachromosomal HR induced by the I-SceI restriction enzyme was increased in the absence of Ku but not of DNA-PKcs. Significantly, abrogation of HR activity by targeting RAD54 in Ku70−/− or DNA-PKcs−/− cells caused extreme radiosensitivity, suggesting that the relative radiosensitivity seen with loss of Ku70 was because of HR-dependent repair pathways. Our findings suggest that Ku can interfere with HR-mediated DSB repair, perhaps competing with HR for DSB recognition.

DNA double-strand breaks (DSBs) can occur during normal cell division or be induced by ionizing radiation (IR). Vertebrates possess two major, complementary DSB repair pathways, homologous DNA recombination (HR) and nonhomologous DNA end-joining (NHEJ). DSB repair by HR uses homologous sequence provided by either a homologous chromosome or sister chromatid, whereas NHEJ joins DNA ends through a process that is largely independent of terminal homologies and that can produce junctions of varying sequence. The HR pathway requires genes of the RAD52 epistasis group, whose products include Rad51 and Rad54. NHEJ is necessary for V(D)J recombination, which generates antibody diversity and is essential for the development of the immune system. The NHEJ pathway requires the DNA-dependent protein kinase, DNA-PK (1), XRCC4 (2), and ligase IV (3–5) proteins. The DNA-PK holoenzyme is a serine-threonine protein kinase comprising a large catalytic subunit (DNA-PKcs) and a heterodimeric component, Ku, which consists of the 70-kDa Ku70 and 80-kDa Ku80 proteins (6–8). DNA-PKcs is a member of a family of large proteins characterized by a carboxyl-terminal phosphatidylinositol 3-kinase-like domain (1, 9). The nature of its physiological target(s) in the NHEJ pathway is still uncertain. The Ku70/Ku80 heterodimer binds to DSBs, recruits DNA-PKcs, and eventually stimulates its kinase activity (1, 10).

Gene disruption experiments in mice have probed the functional relationships within the NHEJ proteins. Although mice deficient in DNA-PK proteins consistently exhibit elevated radiosensitivity and impaired V(D)J recombination, a defect in Ku appears to disrupt end-joining more profoundly than one in DNA-PKcs-deficient mice, because neither V(D)J coding nor signal joints are observed in Ku-deficient mice (11, 12), whereas signal joints are found in DNA-PKcs-deficient mice (13, 14). Furthermore, the dwarf phenotype and apparent replicative senescence described for Ku70- and Ku80-deficient mice (11, 15–17), but not DNA-PKcs-deficient mice, raise the possibility that there exist other activities of Ku, such as controlling cell growth or telomeric function. Remarkably, mice deficient in either XRCC4 or ligase IV show even more severe phenotypes than DNA-PK-deficient mice, exhibiting early embryonic lethality and extensive chromosomal aberrations (18, 19). It is not clear whether these phenotypes can be explained solely by impaired end-joining.

* This work was supported in part by CREST, Japan Science and Technology Corporation (Saitama, Japan), and grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan (to M. T. and S. T.) and by grants from the Uehara Memorial Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, and the Naito Foundation. C. M. is the recipient of a Promising Scientist postdoctoral fellowship while in Kyoto. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Dept. of Immunology and Molecular Genetics, Kawasaki Medical School, Kurashiki, Japan.
‡§ To whom correspondence should be addressed. Tel.: 81-75-753-4410; Fax: 81-75-753-4419; E-mail: stakeda@rg1.rg.med.kyoto-u.ac.jp.
§§ The abbreviations used are: DBS, double-strand break; HR, homologous DNA recombination; NHEJ, non-homologous end-joining; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; PCR, polymerase chain reaction; ES, embryonic stem; Gy, gray.
NHEJ and HR play complementary roles in repairing DNA damaged by IR; NHEJ dominates during G1 to early S phase of the cell cycle, and HR is used in late S to G2 phases (20, 21). However, the overlap between these two pathways raises the possibility of competition between them. A recent model for vertebrate DSB repair proposed from a biochemical study that competition between NHEJ and HR proteins for the initial binding of a DNA lesion determines the eventual outcome of repair, i.e., whether the lesion is repaired by HR or by NHEJ (22). Ku, as the DNA-binding component of NHEJ, is a good candidate for the “switch” controlling entry to this pathway. To test this model and to gain further insight into the interplay between the DNA-PK subunits and HR, we engineered chicken DT40 cells with defective Ku70, DNA-PKcs, or both and examined their IR-induced DSB repair capacities at various cell-cycle stages. Furthermore, because a major prediction of the “competitive” model was that HR frequencies should be elevated in the absence of Ku, we tested the effects of Ku on cellular HR capability directly using expression of the restriction endonuclease I-SceI to generate DSBs.

**EXPERIMENTAL PROCEDURES**

**Construction of Targeting and Expression Vectors**—Chicken genomic DNA from the PRKDC locus was amplified using primer pairs designed to amplify between exons 61–63 and 65–69 (23) and then used to generate targeting vectors carrying neomycin, hygromycin, or histidinol resistance cassettes. Targeting with these constructs was expected to replace amino acids 2888 to 3012 of DNA-PKcs, which are upstream of the phosphatidylinositol 3-kinase homology domain. The previously described chicken KU70 targeting construct pKu70-puro (20) was modified to carry a blasticidin resistance cassette (pKu70-bsr) and a RAD54 vector carrying blasticidin resistance, pRad54-bsr, was cloned from pRad54-neo (24). To make the Ku70 expression constructs, the full-length chicken Ku70 cDNA was ligated into pAneo (25), yielding pAneo-GdKu70. The conditions of cell culture were described previously (26).

**Gene Targeting**—For gene targeting of the chicken DNA-PKcs locus, 10⁷ cells were electroporated with 30 μg of the linearized DNA-PKcs (PRKDC) targeting vectors conferring neomycin, hygromycin, and then histidinol resistance. Drug-resistant colonies were selected in the presence of 2.0 mg/ml G418, 2.5 mg/ml hygromycin (Calbiochem), and 1
mg/ml histidinol (Sigma). Genomic DNA was extracted from each clone by standard procedures, and clones that had undergone targeted recombination were identified by PCR or Southern blot analysis. The number of targeting events per analyzed clones following transfection of targeting vectors containing the neomycin, hygromycin, and histidinol resistance genes is 3/4, 4/24, and 3/21, respectively. To generate \( \text{Ku70}^{-/-} / \text{DNA-PKcs}^{-/-} \) double mutants, a \( \text{DNA-PKcs}^{-/-} \) clone was sequentially transfected with pKu70-puro and pKu70-bar. The

Fig. 2. Synchronization of DT40 cells with nocodazole and cell-cycle stage-specific sensitivity to \( \gamma \)-irradiation of cells deficient in NHEJ components. A, indicated cells were synchronized with nocodazole for 7 h or left untreated. At the indicated time points after the removal of nocodazole cells were labeled with BrdUrd (BrdU), harvested, fixed, and subjected to analysis by flow cytometry (20). Numbers indicate the percentage of cells in the gated regions. 4 (B) or nine (C) h after the removal of nocodazole, most cells are synchronized in G1–early S phase or late S–G2 phase, respectively. At these two time points, the radiosensitivity of synchronized populations was examined, as described for Fig. 2. Error bars show the mean ± S.D. for at least three separate experiments. Two independently targeted clones of each genotype show the same sensitivity to \( \gamma \)-rays (data not shown).
Inhibition of S-G₂ Phase HR Repair by Ku

number of targeting events per analyzed puro- and bsr-resistant clones is 16/37 and 3/81, respectively. To make DNA-PKcs⁻/⁻/RAD54⁻/⁻ double mutants, the DNA-PKcs⁻/⁻ clone was sequentially transfected with pRAD54-puro and pRAD54-bsr. The number of targeting events per analyzed puro- and bsr-resistant clones is 3/40 and 2/85, respectively.

**Colony Formation Assays and Cell-cycle Analysis**—Serially diluted cells were plated in triplicate onto 6-well clusters with 5 ml/well of 1.5% chicken serum, and 10⁻⁵ M medium/F-12 (Life Technologies, Inc.), 15% fetal calf serum, (w/v) methylcellulose (Aldrich) plates containing Dulbecco's modified Eagle's medium/F-12 (Life Technologies, Inc.), 15% fetal calf serum, 1.5% chicken serum, and 10⁻⁵ M β-mercaptoethanol. Subsequently, γ-irradiation of the cells was performed using Cs¹³⁷ (0.02 Gy; Gamma cell 40; Atomic Energy of Canada Limited Industrial Products, Ontario, Canada). Colonies were counted at 7 days after irradiation treatment. Percentage survival was determined relative to numbers of colonies from untreated cells. For cell-cycle analyses, cells were labeled for 10 min with 20 µCi BrdUrd (Amersham Pharmacia Biotech). They were then harvested and fixed at 4 °C overnight with 70% ethanol and successively incubated as follows: (i) in 4 N HCl, 0.5% Triton X-100 for 30 min at room temperature; (ii) in fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (Pharmingen, San Diego, CA) for 1 h at room temperature; (iii) in 5 µg/ml phosphatidylinositol in phosphate-buffered saline. Between each incubation, cells were washed with phosphate-buffered saline containing 2% fetal calf serum and 0.1% sodium azide. Subsequent flow cytometric analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA). Fluorescence data were displayed as dot plots using Cell Quest software (Becton Dickinson).

**Western Blot Analysis**—Western blot analysis of DNA-PKcs and Ku70 was performed as described previously (26). Briefly, 10⁶ cells were lysed in 20 µl of SDS lysis buffer. Following sonication and boiling, the lysates (10 µl/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis gel. After transfer to a nylon membrane, proteins were detected by anti-DNA-PKcs antisera (Neomarkers, Fremont, CA) and horseradish peroxidase-conjugated anti-mouse IgG antibody and Super Signal chemiluminescent substrate (Pierce, Rockford, IL). The same lysates (10 µl/lane) were separated by 5% SDS-polyacrylamide gel electrophoresis gel. After transfer to a nylon membrane, proteins were detected by anti-DNA-PKcs antisera (Neomarkers, Fremont, CA) and horseradish peroxidase-conjugated anti-mouse IgG antibody and Super Signal chemiluminescent substrate (Pierce, Rockford, IL). The same lysates (10 µl/lane) were separated by 5% SDS-polyacrylamide gel electrophoresis gel. After transfer to a nylon membrane, proteins were detected by anti-DNA-PKcs antisera (Neomarkers, Fremont, CA) and horseradish peroxidase-conjugated anti-mouse IgG antibody and Super Signal chemiluminescent substrate (Pierce, Rockford, IL).

**Measurement of Recombination Frequencies by an I-SceI-induced DSB Repair System**—Modified SCneo was inserted into the previously described OVALBUMIN gene construct and then targeted into the OVALBUMIN locus in wild-type, KU70⁻/⁻, and DNA-PKcs⁻/⁻ DT40 cells. In transient transfections, 5 × 10⁶ cells suspended in 20 µl of phosphate-buffered saline were mixed with each of the following plasmid DNA (30 µg) without linearization: pBluescript SK, I-SceI expression vector (pCBASce), and chicken Ku70 expression vector and chicken DNA-PKcs expression vector (pCBASce), and chicken Ku70 expression vector and chicken DNA-PKcs expression vector (pCBASce). The same lysates (10 µl/lane) were separated by 5% SDS-polyacrylamide gel electrophoresis gel. After transfer to a nylon membrane, proteins were detected by anti-DNA-PKcs antisera (Neomarkers, Fremont, CA) and horseradish peroxidase-conjugated anti-mouse IgG antibody and Super Signal chemiluminescent substrate (Pierce, Rockford, IL). The same lysates (10 µl/lane) were separated by 5% SDS-polyacrylamide gel electrophoresis gel. After transfer to a nylon membrane, proteins were detected by anti-DNA-PKcs antisera (Neomarkers, Fremont, CA) and horseradish peroxidase-conjugated anti-mouse IgG antibody and Super Signal chemiluminescent substrate (Pierce, Rockford, IL).

**RESULTS AND DISCUSSION**

**Generation of DNA-PKcs⁻/⁻/⁻ and KU70⁻/⁻/DNA-PKcs⁻/⁻/⁻ Cells**—To investigate competition between the two DSB repair pathways, we generated chicken DT40 cells (28, 29) with defective Ku70, DNA-PKcs, or both and examined their HR-mediated DSB repair capability and radiosensitivity. The chicken DNA-PKcs gene (Prkdc) lies on chromosome 2, which is trisomic in DT40 cells. Thus, three disruption constructs, DNA-PKcs-neo, DNA-PKcs-hyg, and DNA-PKcs-his (Fig. 1A) were sequentially transfected to generate DNA-PKcs⁻/−/− clones. Targeted integration was verified by genomic PCR and Southern blot analysis (Fig. 1B). One DNA-PKcs⁻/−/− clone was transfected subsequently with two KU70 disruption constructs to generate a KU70⁻/−/−/DNA-PKcs⁻/−/− clone, and the disruption of the DNA-PKcs and KU70 genes was verified by Western blotting using antibodies to human DNA-PKcs and chicken Ku70, respectively (Fig. 1C). The proliferative properties of KU70⁻/−/−, DNA-PKcs⁻/−/−, and KU70⁻/−/−/DNA-PKcs⁻/−/− cells were indistinguishable from those of wild-type cells, as monitored by growth curves and by cell-cycle analysis (data not shown).
DNA-PKcs
PKcs
that of Ku70 in the DNA ligase IV-deficient cells makes them relatively more radioresistant with a survival profile that matches that of Ku70 cells (30). This result supports our data that Ku proteins suppress homologous recombination in late S-G2 phase.

A possible complication was a potential aberrant cell-cycle checkpoint response in the mutants described here. Because DT40 cells are p53-deficient, they lack a G1/S arrest. In yeast, Ku70 proteins are involved in G2/M arrest after DNA damage (31). However, after treatment with ionizing radiation, all cell lines described in this paper showed similar G2/M arrest and inhibition of DNA synthesis (data not shown), indicating that our results are not because of any difference in cell-cycle checkpoint.

HR Deficiency in Ku70 and DNA-PKcs Cells Causes Severe Radioresistance—If the model that the different IR sensitivities of Ku70 and DNA-PKcs cells are because of different HR efficiencies is correct, disruption of HR-mediated repair in the two mutants should result in identical IR sensitivity in them. To test this hypothesis, we disrupted HR-mediated DSBR repair by targeting the Rad54 gene in Ku70 and DNA-PKcs cells, thus generating Ku70-/-/Rad54-/- and DNA-PKcs-/-/Rad54-/- clones. Consistent with our model, both clones exhibited the same high level of radiosensitivity (Fig. 4). Therefore, the increased IR tolerance of Ku70-/- cells in late S to G2 phase requires functional HR-mediated repair. This conclusion supports the idea that Ku can interfere with HR-mediated DSBR repair.

Ku70, but Not DNA-PKcs, Deficiency Elevates HR Frequencies in an Artificial Substrate—To investigate directly the Ku protein’s suppression of HR-mediated repair, we measured DSBR-induced HR using the SCneo substrate (27), which we integrated into the OVALBUMIN locus of wild-type, Ku70-/-, and DNA-PKcs-/- cells by gene targeting. In each clone containing the modified SCneo, a single DSBR was introduced at the I-SceI site by transient expression of the I-SceI endonuclease. Because HR generates a functional neomycin resistance (neo*) gene from SCneo, the number of HR events can be determined by counting the number of neo-resistant colonies (27, 32, 33). A Ku70-/- clone transfected with an I-SceI expression vector showed significantly higher HR frequencies than a wild-type clone (paired t test, p = 0.0047 < 0.01; see Table I) and a DNA-PKcs-/- cell line (p = 0.0409 < 0.05). Notably, the expression of Ku70 reduced the recombination frequency by 25—40% in Ku70-/- cells but not in wild-type DT40s (Table II). The higher level of HR in Ku70-/- cells correlates with the increased late S to G2 phase radiotolerance seen in Ku-deficient cells relative to wild-type cells (Fig. 3C). Therefore, Ku70 expression decreases the frequency of DSBR-induced HR, providing a mechanistic explanation for the radiotolerance induced by Ku deficiency.

To define how the association of Ku with DSBs interferes with HR, we measured the induction of IR-induced subnuclear Rad51 foci and gene targeting efficiency in the presence and absence of Ku. We found no delay in kinetics of Rad51 focus formation in response to IR in Ku70-/- cells when compared with wild-type and DNA-PKcs-/- cells (data not shown). Thus, the Ku proteins do not necessarily affect the assembly of Rad51 in nucleoprotein filaments at DSBRs. There was no significant difference in gene targeting efficiencies in wild-type and Ku70-/- cells, whereas a slight reduction of random integration frequencies was observed in the absence of Ku70 (data not shown). Thus, the Ku proteins do not suppress all HR reactions but specifically interfere with HR-mediated DSBR repair. Presumably, the association of Ku with DSBRs may disturb D-loop formation, i.e. interaction of the nucleoprotein filaments with homologous sequences and/or inhibit DNA synthesis following D-loop formation.

HR and NHEJ Activities in DT40 and Mammalian Cell Lines—Although genetic analyses of DNA repair in DT40 cells have recapitulated consistently the results gleaned from mammalian systems, there seems to be a qualitative difference in the relative usage of the two DSBR repair pathways in late S to G2 phase, with HR apparently playing a more important role in DSBR repair in DT40 cells than in mammalian cell lines (20, 34). In contrast, deletion of the Ku genes in mammalian cell lines including murine embryonic stem (ES) cells consistently increases their IR sensitivity (13, 35). However, asynchronous Ku70-deficient ES cells show a biphasic survival curve with increasing radiation dose, being highly radiosensitive up to 2 Gy and less sensitive at doses of 2—4 Gy. This biphasic curve likely reflects two distinct fractions with different IR sensitivities, as observed in Ku70-deficient DT40 cells, although no data exist for synchronized ES cells. DNA-PKcs-null mutant ES cells exhibit no radiosensitivity, suggesting that DNA-PKcs is not required for end-joining in ES cells (13). However, because Gao et al. (13) also showed that primary fibroblasts derived from DNA-PKcs-null mice were significantly more radiosensitive than those from wild-type mice, as has been observed in other DNA-PKcs-deficient mammalian cell lines, the requirement for DNA-PKcs in NHEJ may vary between tissues.
and cell lines. Therefore, the occasional discrepancies of between the results of IR sensitivity we have obtained using DT40 and those obtained in mammalian cells may actually prove useful in choosing appropriate experimental models for investigating NHEJ. Finally, more prominent enhancement of HR caused by defective Ku70 than by DNA-PKcs deficiency has been also observed in mammalian cells.2

Accumulating evidence points to the use of differential HR versus NHEJ depending on the cell-cycle phase, although cell type may further influence the competitive balance between these two complementary DSB repair pathways. Defective Rad51 focus formation following IR in G1 phase implies the presence of regulatory mechanisms that can specifically suppress HR at the step of formation of nucleoprotein filaments (36). A defect in this suppression might lead to ectopic recombination and heteroallelic recombination, possibly causing the loss of heterozygosity and eventually tumorigenesis. Conversely, a defect in suppressive regulation of the end-joining pathway could interfere with other repair pathways, as well as the HR-mediated repair pathway (37). As an example of this, we found that Ku70-deficient DT40 cells are rather more tolerant than wild-type cells of the chemotherapeutic DNA damage-inducing agent cisplatin, which does not directly induce HR caused by defective Ku70 than by DNA-PKcs deficiency has proved useful in choosing appropriate experimental models for investigating the manuscript.

Acknowledgments—We thank Dr. T. Honjo (Kyoto, Japan) and D. J. Chen (Lawrence Berkeley National Laboratory) for critically reading the manuscript.

REFERENCES
1. Smith, G. C. M., and Jackson, S. P. (1999) Genes Dev. 13, 916–934
2. Li, Z., Gerev, T., Gao, Y., Cheng, H. L., Seed, B., Stamato, T. D., Taccioli, G. E., and Alt, F. W. (1995) Cell 83, 1079–1089
3. Critchlow, S. E., Bowater, R. P., and Jackson, S. P. (1997) Curr. Biol. 7, 588–598
4. Grawunder, U., Wilm, M., Wu, X., Kulesza, P., Wilson, T. D., Mann, M., and Lieber, M. R. (1997) Nature 388, 492–495
5. Wilson, T. E., Grawunder, U., and Lieber, M. R. (1997) Nature 388, 495–498
6. Mimori, T., and Hardin, J. A. (1986) J. Biol. Chem. 261, 10375–10379
7. Mimori, T., Ohsone, Y., Hama, N., Sawa, A., Akizuki, M., Homma, M., Griffith, A. J., and Hardin, J. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1777–1781
8. Yaneva, M., kowalewski, T., and Lieber, M. R. (1997) EMBO J. 16, 5098–5112

2 A. J. Pierce, Memorial Sloan-Kettering Cancer Center, New York, NY, personal communication.
Genetic Analysis of the DNA-dependent Protein Kinase Reveals an Inhibitory Role of Ku in Late S–G2 Phase DNA Double-strand Break Repair
Toru Fukushima, Minoru Takata, Ciaran Morrison, Ryoko Araki, Akira Fujimori, Masumi Abe, Kouichi Tatsumi, Maria Jasin, Pawan Kumar Dhar, Eiichiro Sonoda, Tsutomu Chiba and Shunichi Takeda

J. Biol. Chem. 2001, 276:44413-44418.
doi: 10.1074/jbc.M106295200 originally published online September 27, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106295200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 17 of which can be accessed free at http://www.jbc.org/content/276/48/44413.full.html#ref-list-1