

# Heat Inactivation of Ku Autoantigen: Possible Role in Hyperthermic Radiosensitization<sup>1</sup>

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## Abstract

Heat shock prior, during, or immediately after ionizing radiation synergistically increases cell killing, a phenomenon termed hyperthermic radiosensitization. Recently, we have shown a constitutive DNA-binding factor in rodent cells that is inactivated by heat shock to be identical to Ku autoantigen. Ku, consisting of an  $M_r$  70,000 (Ku70) and an  $M_r$  86,000 (Ku80) subunit, is a heterodimeric nuclear protein and is the DNA-binding regulatory component of the mammalian DNA-dependent protein kinase DNA-PK. Recent genetic and biochemical studies indicate the involvement of Ku and DNA-PK in DNA double-strand break repair and V(D)J recombination. On the basis of these findings, we propose that heat-induced loss of the DNA-binding activity of Ku may lead to hyperthermic radiosensitization. To test this hypothesis, we examined and compared the DNA-binding activity of Ku, the DNA-PK kinase activity, and hyperthermic radiosensitization in rodent cells immediately after heat shock and during post-heat shock recovery at 37°C. Our results show that the heat-induced loss of Ku-DNA binding activity correlates well with an increased radiosensitivity of the heat-shocked cells, and furthermore, the loss of synergistic interaction between heat and radiation parallels the recovery of the DNA-binding activity of Ku. On the other hand, the heat-induced decrease of DNA-PK activity did not correlate with hyperthermic radiosensitization. Our data, for the first time, provide evidence for a role of Ku protein in modulating the cellular response to combined treatments of heat shock and ionizing radiation.

## Introduction

Heat shock prior, during, or immediately after ionizing radiation synergistically increases cell killing, a phenomenon termed hyperthermic radiosensitization. Although the molecular mechanisms are not very well understood, it seems to be generally believed that hyperthermic radiosensitization is due to heat-induced inhibition of repair of radiation-induced DNA damage. The combination of heat and radiation does not result in more DNA damage than observed for radiation alone (1-3). However, heat shock has been shown to inhibit the rejoining of radiation-induced DNA strand breaks (1, 2, 4). Two hypotheses have been postulated in the past to explain the thermal inhibition of DNA repair: (a) heat inactivates proteins/enzymes involved in DNA repair; and (b) heat alters the chromatin structure, rendering the damaged DNA less accessible for the repair enzyme complex(es). Indeed, some reports have shown a correlation between heat-induced inactivation of DNA polymerases (especially polymerase  $\beta$ ) and hyperthermic radiosensitization (5), whereas other studies demonstrated a good correlation between heat-induced nuclear protein

aggregation and hyperthermic radiosensitization (6). However, variation between the correlations from different experiments or cell lines suggests a lack of a more generalized relationship between DNA polymerase inactivation, nuclear protein aggregation, and thermal radiosensitization. Although DNA dsb<sup>3</sup> are thought to be the lethal lesions, in mammalian cells genes involved in the repair of radiation-induced dsb have been identified only recently (7, 8). Consequently, the effect of heat shock on the gene products involved in dsb repair has not yet been studied in detail.

Recently, it has been shown that the  $M_r$  86,000 subunit (Ku80) of Ku is the product of the human DNA dsb repair gene *XRCC5* (7, 8). Cells defective in Ku80 expression (e.g., *xrs-5* cells) are extremely sensitive to radiation and are deficient in dsb repair and V(D)J recombination. Transfection of these cells with Ku80 cDNA restores radiation resistance, dsb repair efficiency, and V(D)J recombination capability (7, 8). Ku is a heterodimeric nuclear protein, consisting of  $M_r$  70,000 (Ku70) and  $M_r$  86,000 (Ku80) polypeptides (9). *In vitro* studies have shown that this nuclear protein binds to the termini of double-stranded DNA and DNA ending in stem-loop structures, probably via the  $M_r$  70,000 subunit (10). By virtue of its DNA-binding activity, Ku serves as a regulatory component of the DNA-PK, which is activated by DNA-ends (11, 12). The other component of DNA-PK is the  $M_r$  460,000 catalytic subunit (DNA-PKcs; Refs. 13-15), which has been identified as the product of the murine gene *SCID* (16). Recently, we have shown that the Ku protein is identical to a heat-responsive, constitutive heat shock element binding factor described previously, CHBF (17), which appears to be involved in the regulation of the  $M_r$  70,000 heat shock protein (hsp70) expression (18).

On the basis of the facts that: (a) Ku is involved in DNA damage repair; and (b) Ku-DNA binding activity is heat sensitive, we hypothesized that heat-induced loss of the DNA-binding activity of Ku may lead to hyperthermic radiosensitization. In this report, we present data on the effect of heat shock on the DNA-binding activity of Ku, the DNA-PK activity, and their relationship with hyperthermic radiosensitization. Our results demonstrate a good correlation between heat inactivation of Ku and hyperthermic radiosensitization, suggesting a role for Ku protein in modulating the cellular response to a combined treatment of heat shock and ionizing radiation.

## Materials and Methods

**Cell Cultures, Heat Shock,  $\gamma$ -Ray Irradiation, and Cell Survival Assay.** Rat fibroblasts (Rat-1) and TT Rat-1 cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics. Experiments were performed with unfed plateau-phase cells. Heat treatments were performed in hot water baths in specially designed incubators (19). Cells were irradiated at room temperature with <sup>137</sup>Cs  $\gamma$ -rays at a dose rate of 0.8 Gy/min. TT Rat-1 cells were obtained by heating the cells at 45°C for 15 min followed by incubation at

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<sup>3</sup> The abbreviations used are: dsb, double-strand break(s); DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; TT, thermotolerant; HSE, heat shock element; RPA, replication protein A; HSF, heat shock factor; CHO, Chinese hamster ovary.

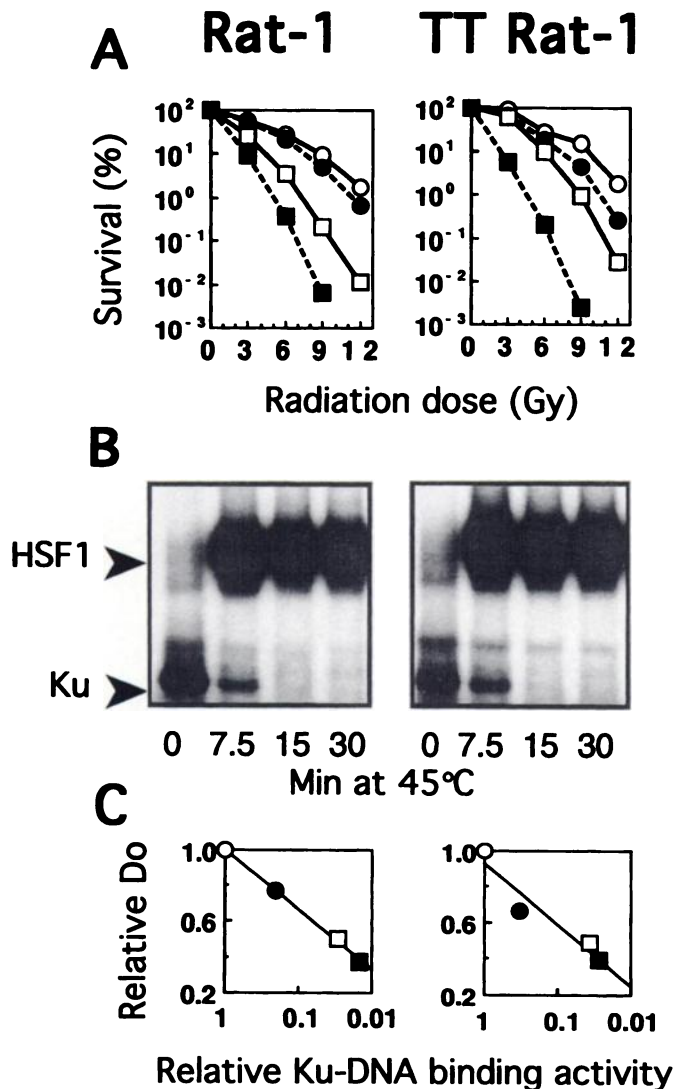


Fig. 1. Hyperthermic radiosensitization correlates with heat-induced inactivation of Ku-DNA binding activity in Rat-1 and TT Rat-1 cells. **A**, radiation survival curves of Rat-1 and TT Rat-1 cells irradiated immediately after heating. Heating times: ○, no heating; ●, 7.5 min at 45°C; □, 15 min at 45°C; and ■, 30 min at 45°C. Survival curves are corrected for heat-induced cell killing. **B**, gel mobility shift analysis of HSF1-DNA and Ku-DNA binding activities in Rat-1 and TT Rat-1 cells heated for the indicated times. **C**, correlation between the radiosensitivity of the heated cells ( $D_0$  values, the dose required to reduce the survival to  $1/e$  in the exponential part of the survival curves, calculated for the survival curves in **A**) and the relative Ku-DNA binding activity in the heated cells (quantification of autoradiograms in **B**). Symbols are as in **A**.

37°C for 16 h (19). Cell survival was determined using the colony formation assay, as described previously (19). Surviving fractions were normalized to the plating efficiency. Plating efficiencies for Rat-1 and TT Rat-1 are 60–80%.

**Preparation of Cell Extracts and Gel Mobility-Shift Assay.** Preparation of the cell extracts and the gel mobility-shift assay were performed as described (17). An equal amount of extracted proteins (50  $\mu$ g) from each sample was incubated with a  $^{32}$ P-labeled double-stranded oligonucleotide (5'-GGGC-CAAGAATCTTCCAGCAGTTTCGGG-3') containing the HSE of the rat hsp70 promoter. Protein-bound and -free oligonucleotides were electrophoretically separated on 4.5% native polyacrylamide gels in  $0.5 \times$  TBE buffer [44.5 mM Tris (pH 8.0), 1 mM EDTA, and 44.5 mM boric acid] for 2.5 h at 140 V. The gels were dried and autoradiographed with Kodak X-Omat film and a DuPont Cranex Lightning Plus intensifying screen at  $-80^\circ\text{C}$ . Gels were quantified using an AMBIS analysis system.

**Protein Kinase Assay.** For heat shock experiments, Rat-1 cells were heat shocked at 45°C for 7.5, 15, and 30 min; for recovery experiments, Rat-1 cells were heat shocked at 45°C for 30 min and returned to 37°C for 0, 4, 8, and

12 h. Cell extracts were prepared from isolated nuclei as described (15). The protein concentration of each of the extracts was adjusted to 7 mg/ml by dilution with TM buffer [50 mM Tris-HCl (pH 7.9), 12.5 mM  $\text{MgCl}_2$ , 1 mM EDTA, and 20% glycerol] containing 1 mM KCl, 1 mM DTT, 20  $\mu\text{g/ml}$  phenylmethylsulfonyl fluoride, 1  $\mu\text{g/ml}$  soybean trypsin inhibitor, and 1  $\mu\text{g/ml}$  each of leupeptin, pepstatin A, and aprotinin. The extracts were then incubated with sheared salmon sperm DNA that was covalently linked to agarose beads using cyanogen bromide-activated Sepharose CL6-B (0.05 mg of DNA/ml bead). DNA-protein complexes were formed on the DNA-beads as described (20). The DNA-protein complexes were then incubated alone or with 500 ng of the purified recombinant  $M_r$  32,000 subunit of human RPA for 10 min at 25°C. Protein kinase assays were performed for 60 min at 30°C by the addition of 12.5  $\mu\text{M}$  ATP containing 5  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ] ATP per reaction and terminated by boiling for 3 min in SDS-PAGE sample buffer. Phosphoproteins were resolved by 10% SDS-PAGE and visualized by autoradiography of the dried gel. Quantification of the gels was performed using an AMBIS analysis system.

## Results

**Hyperthermic Radiosensitization and Heat Inactivation of Ku-DNA Binding Activity.** The objective of this set of experiments was to determine whether there is a correlation between heat-induced radiosensitization and heat-induced inactivation of Ku-DNA binding activity. We examined the effect of heat shock on radiation sensitivity. Rat-1 or TT Rat-1 cells were heat shocked at 45°C for various times and immediately exposed to graded doses of  $\gamma$ -rays. Cell survivals plotted as a function of radiation dose are presented in Fig. 1A, and the calculated  $D_0$  values are summarized in Table 1. It is clearly shown that the preheat treatment sensitizes cells to radiation; furthermore, there appears to be no significant difference in heat-induced radiosensitization between Rat-1 and TT Rat-1 cells.

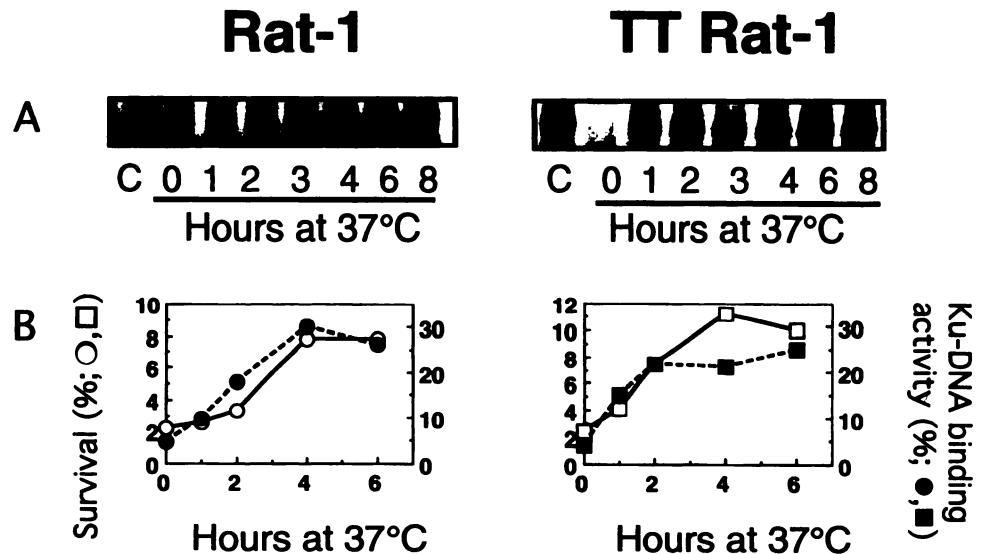
We then examined the effect of heat shock on Ku-DNA binding activity. Immediately after heating at 45°C for the indicated times, cells were trypsinized, and cell extracts were prepared and subjected to gel mobility shift analysis (Fig. 1B). With the oligonucleotide used in most experiments (containing HSE of the rat hsp70 promoter), two distinct DNA-binding activities were detected. The slower migrating complex is not present in unheated cells and is induced rapidly upon heat shock. This complex has been shown previously to correspond to the extensively studied HSE-binding activity of HSF1 (17). In contrast to that of HSF1, the faster migrating complex is present in unheated cells and is rapidly inactivated by heat shock. This complex is designated previously as the "constitutive HSE binding activity (CHBA)," and the protein component in this complex is referred to as CHBF (21). Recently, we purified CHBF to apparent homogeneity and found it to be identical to Ku autoantigen (17). Fig. 1B clearly shows that in both Rat-1 and TT Rat-1 cells, the Ku-DNA binding activity decreases in a heat dose-dependent manner. No significant difference was observed between the heat-induced reduction of the Ku-DNA binding activity in Rat-1 and TT Rat-1 cells. To verify that the heat inactivation of Ku-DNA binding activity is not HSE specific,

Table 1. Quantification of data from Fig. 1

	Heating time (min at 45°C)	Ku-DNA binding activity	$D_0$ (Gy)	TER <sup>a</sup> ( $D_0$ )	TER (%)
Rat-1	0	1.00	2.20	1.00	1.00
	7.5	0.21	1.70	1.29	1.14
	15	0.029	1.10	2.00	1.74
	30	0.015	0.82	2.68	2.51
TT Rat-1	0	1.00	2.20	1.00	1.00
	7.5	0.45	1.40	1.57	1.23
	15	0.037	1.00	2.20	1.46
	30	0.028	0.85	2.59	2.80

<sup>a</sup> TER, thermal enhancement ratio.

Fig. 2. Loss of interaction between heat and radiation after a 15 min 45°C heat treatment. **A**, gel mobility shift analysis of the Ku-DNA binding activity during post heat-shock recovery at 37°C. Rat-1 and TT Rat-1 were heat shocked at 45°C for 15 min and returned to 37°C for different times before preparation of the cell extracts. *Lane C*, control, unheated cells. **B**, comparison of the recovery of the Ku-DNA binding activity (●, ■) and the loss of interaction between the heat and the radiation treatment (*Survival*; ○, □). The Ku-DNA binding activity is quantified using the AM-BIS imaging system and expressed as a percentage of the activity in control Rat-1 cells. The survival after the combined treatments is expressed as the percentage of surviving cells, corrected for the plating efficiency of control cells.



similar experiments were performed using other oligonucleotides of the same length but not containing the HSE sequence. In this case, the sequence-specific HSF1-HSE binding complex in heat-shocked cells was absent, whereas the effect of heat shock on the Ku-DNA binding activity was identical to that presented in Fig. 1B (data not shown). The quantification of the gels shown in Fig. 1B is summarized in Table 1.

Fig. 1C shows the relative Ku-DNA binding activity, plotted against the  $D_{010}$  values of the radiation survival curves obtained immediately after heat shock. A good correlation is observed between the Ku-DNA binding activity and the radiosensitivity ( $D_{010}$ ) for both Rat-1 and TT Rat-1 cells, suggesting a causal relationship between the heat-induced inactivation of the Ku-DNA binding activity and hyperthermic radiosensitization.

**Recovery of Ku-DNA Binding Activity after Heat Shock.** Heat shock immediately followed by ionizing irradiation kills cells synergistically; however, this heat-induced radiosensitization gradually decreases as the interval between heat and radiation treatments increases. In the following sets of experiments, we examined whether a correlation exists between the recovery of Ku-DNA binding activity after heat shock and the loss of synergistic interaction, in terms of cell killing, between heat and radiation. Rat-1 and TT Rat-1 cells were heat shocked for 15 min at 45°C, followed by incubation at 37°C for 0–8 h. One group of cells was used to determine the Ku-DNA binding activity, whereas the other group of cells was given an additional 6 Gy of  $\gamma$ -rays and assayed for survival after the combined treatment (Fig. 2). The Ku-DNA binding activity, which is reduced to a nearly undetectable level immediately after the heat treatment, gradually recovers during the post-heat shock recovery at 37°C (Fig. 2, upper panels); the recovery is faster in TT cells compared to nontolerant Rat-1 cells. Quantification of the autoradiograms is plotted in the lower panels of Fig. 2. In these graphs, the relative DNA-binding activity of Ku is compared with the loss of synergistic interaction between heat and radiation (percentage of cells surviving the combined treatments) during post-heat shock recovery. Our data clearly show that the recovery of the DNA-binding activity of Ku parallels the loss of synergistic interaction between heat and radiation.

**Heat Inactivation of DNA-PK Activity.** To examine whether heat-induced inactivation of Ku-DNA binding resulted in the disruption of the Ku-dependent activation of the DNA-PK, we determined the DNA-PK activity in Rat-1 cells heat shocked at 45°C for 0–60 min. Cell extracts were prepared, and DNA-PK activities were ana-

lyzed by examining the phosphorylation of the  $M_r$  32,000 subunit of human RPA as described in "Materials and Methods" (20). We found that the DNA-PK activity appeared unaffected in cells heated at 45°C for up to 15 min, a heat dose that reduced the Ku-DNA binding with more than 95% (Fig. 1B). DNA-PK activity was significantly reduced, however, in cells treated for 30 min at 45°C and was nearly undetectable after heating at 45°C for 60 min (Fig. 3). Similar results were found for heat-shocked TT Rat-1 cells (data not shown). Thus, the heat-induced loss of DNA-PK activity did not correlate with the heat-induced inactivation of Ku-DNA binding or with hyperthermic radiosensitization.

## Discussion

Inhibition of the repair of DNA dsb as a possible cause for hyperthermic radiosensitization has been suggested previously by Iliakis and Seamer (22). These investigators observed significantly reduced hyperthermic radiosensitization in *xrs-5* cells compared to parental CHO cells. The *xrs-5* cell line is deficient in Ku80 expression, lacks Ku-DNA binding activity, is defective in DNA dsb repair, and is

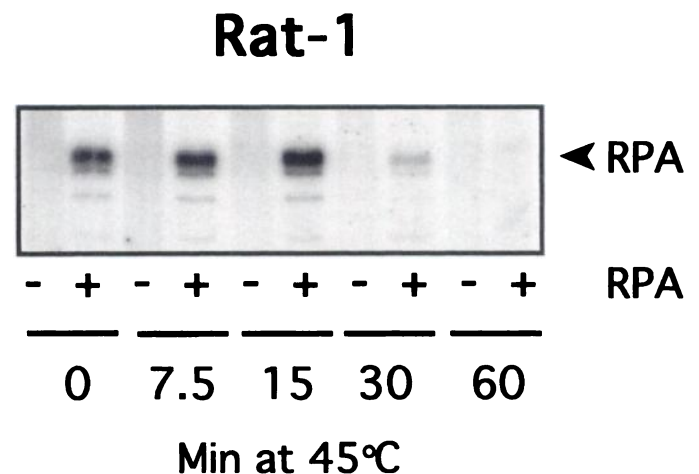


Fig. 3. Loss of DNA-PK activity in heated Rat-1 cells. Rat-1 cells were heated for the indicated times at 45°C, and cellular extracts were prepared as described in "Materials and Methods." Protein kinase assays were performed in the presence (+) or absence (-) of the  $M_r$  32,000 subunit of human RPA. Phosphoproteins were resolved by 10% SDS-PAGE and visualized by autoradiography of the dried gel. Similar results were obtained for TT Rat-1 cells (data not shown).

extremely sensitive to ionizing radiation (6, 7). Our results show that in plateau-phase Rat-1 cells, a good correlation exists between heat-induced radiosensitization and heat-induced inactivation of Ku-DNA binding. Collectively, these data suggest that heat inactivation of Ku-DNA binding, which should lead to a deficiency in DNA dsb repair, may be responsible for hyperthermic radiosensitization. In contrast, xrs-5 cells are proficient in DNA single-strand break repair, and hyperthermia inhibits repair of DNA single-strand breaks in these cells to the same extent as in the parental CHO cells (23). Thus, it appears that inhibition of single-strand break repair is not associated with hyperthermic radiosensitization, and Ku protein appears not to be involved in the repair of radiation-induced DNA single-strand breaks.

Although heating for 15 min at 45°C almost completely abolished Ku-DNA binding, no significant decrease of DNA-PK activity was observed (compare Figs. 1B and 3). In both Rat-1 and TT Rat-1 cells, loss of 95% of the Ku-DNA binding activity did not reduce the DNA-PK activity. This apparent discrepancy can be explained in two ways: (a) mild heat treatments only partially inactivate DNA-PK, disrupting the Ku-DNA binding activity but leaving the kinase activity intact. However, Ku-mediated DNA binding has been shown to be an absolute requirement for DNA-PK activity (11, 12), and furthermore, our assay for DNA-PK activity (pull-down assay) requires the Ku-DNA binding-dependent assembly of DNA-PK on double-stranded DNA, making this explanation less probable; and (b) a more plausible explanation is that the presence of a much larger pool of Ku protein relative to DNA-PKs in the cell accounts for this apparent discrepancy. Heating for 15 min at 45°C does not reduce DNA-PK activity, suggesting that the DNA-PKs are relatively heat stable. Therefore, it is likely that the loss of DNA-PK activity as observed after prolonged heat treatments reflects a reduction of the amount of DNA binding-competent Ku protein to substoichiometric levels, as well as inactivation of the DNA-PKs.

In conclusion, our study lends additional support to the concept that heat-induced inhibition of repair of DNA dsb is the cause of hyperthermic radiosensitization. Furthermore, our data, for the first time, provide evidence suggesting that heat inactivation of Ku may play an important role in this process. On the other hand, loss of DNA-PK activity does not seem to be involved in hyperthermic radiosensitization.

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