RADIATION RESEARCH **157**, 371–377 (2002)
0033-7587/02 $5.00

Induction of Adaptive Response by Low-Dose Radiation in RIF Cells Transfected with Hspb1 (Hsp25) or Inducible Hspa (Hsp70)

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Lee, Y-J., Park, G-H., Cho, H-N., Cho, C-K., Park, Y-M., Lee, S-J. and Lee, Y-S. Induction of Adaptive Response by Low-Dose Radiation in RIF Cells Transfected with Hspb1 (Hsp25) or Inducible Hspa (Hsp70). ***Radiat. Res.*** **157, 371–377 (2002).**

An adaptive response results in a reduced effect of a high challenging dose of a stressor after a smaller, inducing dose has been applied a few hours earlier. Radiation-induced fibrosarcoma (RIF) cells did not show an adaptive response, i.e. a reduced effect from a high challenging dose (2 Gy) of a radiation after a priming dose (1 cGy) had been applied 4 or 7 h earlier, but cells of a thermoresistant clone (TR) derived from RIF cells did. Since the expression of inducible Hspa (also known as Hsp70) and Hspb1 (also known as Hsp25) was different in these two cell lines, the role of inducible Hspa and Hspb1 in the adaptive response was examined. When RIF cells were transfected with inducible Hspa or Hspb1, both radioresistance measured by clonogenic assays and a reduction of apoptosis were detected. The adaptive response was also acquired by these two cell lines. The inducible Hspa transfectant showed a more pronounced adaptive response than the Hspb1 transfectant. Based on these results, it appears that inducible Hspa and Hspb1 are at least partly responsible for the induction of the adaptive response in these cells. Moreover, when inducible Hspa or Hspb1 was transfected into RIF cells, coregulation of the two genes was detected. Heat-shock factor (Hsf) was found to be at least partially responsible for the induction of the adaptive response in these cells. © 2002 by Radiation Research Society

INTRODUCTION

Low doses of ionizing radiation can produce a stimulatory effect and can induce adaptive responses that reduce the harmful effects of subsequent exposure to high-dose radiation (*1*). Recent studies have demonstrated adaptive responses in chromosome aberrations (*2*), cell survival (*3*), sister chromatid exchanges (*4*), micronucleus induction (*5*), mutation (*6*) and neoplastic transformation (*5*). The mechanisms and conditions for the adaptive response to radiation have not been clarified, although one possible explanation relates to the induction of DNA repair processes in response to low doses of around 0.01 Gy. The induction of new proteins in response to low doses provides experimental support for this explanation (*7*). Our previous data showed that when normal cells were preirradiated with 1 cGy, they showed the adaptive response, but neoplastic cells did not. A reduction of apoptosis by low-dose preirradiation is another potential mechanism for this effect (*8*). This adaptive response appears to be most prevalent in radioresistant cell lines, since several radiosensitive cell lines failed to show an increase in radioresistance at doses beyond 0.5 Gy (*9*).

It is well established that members of the HSP family function as molecular chaperones and assist in the intracellular folding of newly synthesized proteins (*10*). Several investigators reported the induction of a member of the HSP70 protein family (now know as HSPA) during the adaptive response to oxidative stress produced by H_2O_2 (*11*). This induction occurred during the pretreatment of cells with a low concentration of H_2O_2. Low doses of X rays were found to activate the promoter of the human *HSP70B* gene (now known as *HSPA7*): Transcription was silent under control conditions but was highly induced by heat-shock treatment (*12*). The low dose of 4 cGy radiation that induced the adaptive response also increased *HSPA7* mRNA (*13*). The induction of an adaptive response by low-dose ionizing radiation also involved induction of PBP74/mortalin/Grp75, a member of the HSPA family (*14*). In the present study, we demonstrated that mouse RIF cells, which did not induce Hspa and Hspb1 (also known as Hsp25), did not exhibit an adaptive response after 0.01 Gy of low-dose preirradiation, whereas the thermoresistant TR cells, which expressed inducible Hspa and Hspb1, showed a response. In addition, when inducible Hspa and Hspb1 were transfected into RIF cells, the cells acquired radioresistance, suggesting that inducible Hspa and Hspb1 are important for the induction of the adaptive response and radioresistance.

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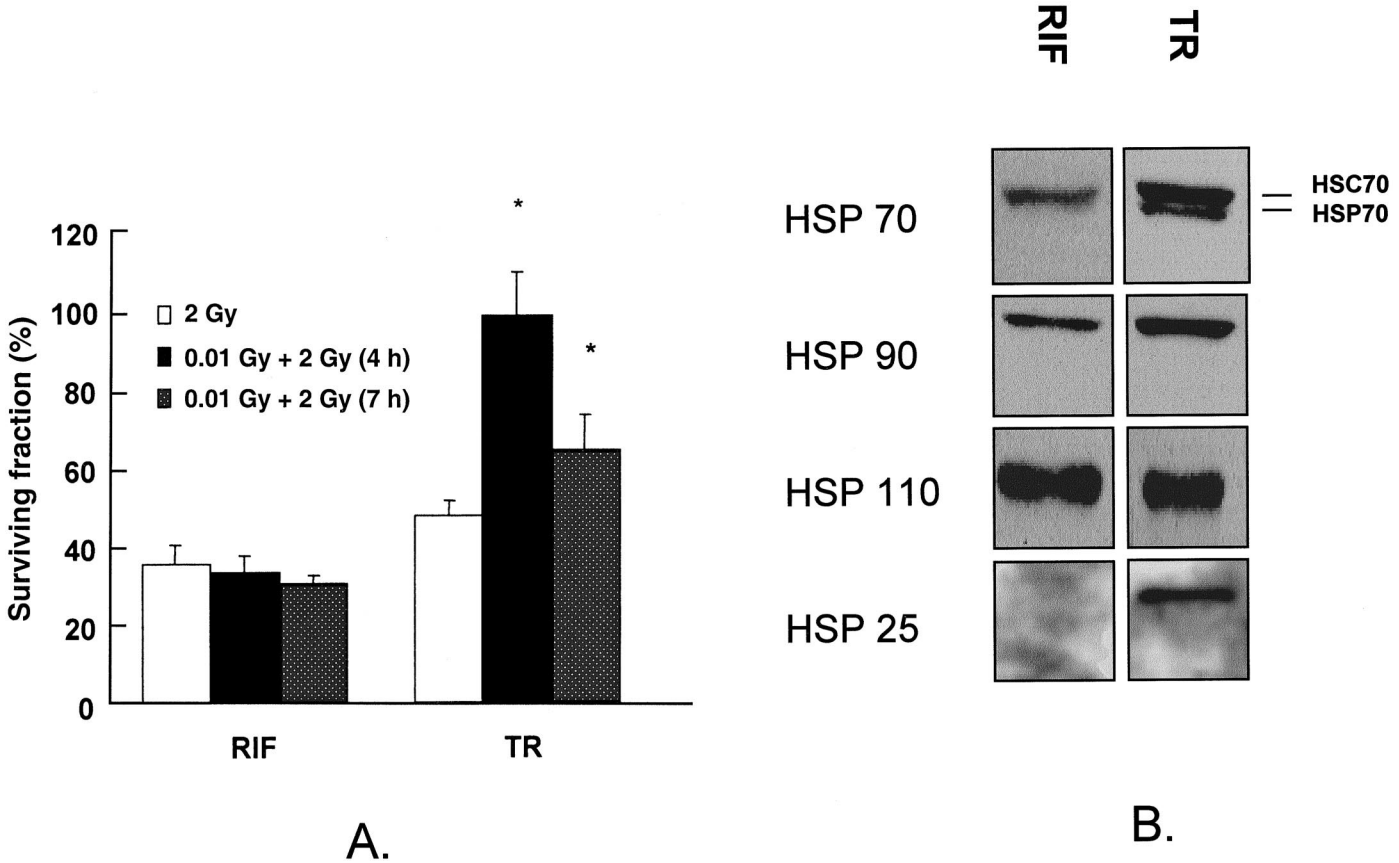

FIG. 1. Hspb1 (HSP25) and inducible Hspa (HSP70) are involved in the adaptive response induced by preirradiation with low-dose (0.01 Gy) radiation. Panel A: Induction of an adaptive response was observed using a clonogenic assay for survival. Radiation-induced fibrosarcoma (RIF) cells and cells of their thermoresistant clone (TR) were irradiated with 0.01 Gy; after 4 or 7 h, a high challenge dose of 2 Gy was administered. The number of colonies consisting of 50 or more cells was scored. Each point represents the mean ± SD of three independent experiments. $*P < 0.05$ compared to cells irradiated with 2 Gy alone. Panel B: Protein extracts of RIF and TR cells were prepared and assessed by Western blot analysis for Hspb1, Hspa, Hspc and Hsf110 as described in the Materials and Methods.

When the inducible *Hspa* or *Hspb1* genes were transfected into RIF cells, co-regulation of the two genes was detected. Furthermore, we observed that heat-shock factor (Hsf) was also involved in these phenomena.

MATERIALS AND METHODS

Cell Cultures and Treatment

Mouse RIF and TR cells (a thermoresistant clone of RIF) (*15*) were propagated in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FCS, 1% antibiotic/antimycotic solution, and 2 m*M* L-glutamine (all from Gibco BRL, Gaithersburg, MD).

Vector Construction

For transfection of RIF and L929 cells with inducible Hspa, MFG.HSP70puro was used (*15*). To establish cell line, 1 μg of MFG.HSP70puro or the MFGpuro plasmids was introduced into the cells by lipofection (Lipofectamine, Gibco BRL) in serum-free medium. The cells were propagated in DMEM supplemented with 10% FCS, 1% antibiotic/antimycotic solution, 2 m*M* L-glutamine, and 2 μg/ml puromycin (all from Gibco BRL). The cell density was kept subconfluent, and the cells were passaged twice a week. Twenty-four hours after transfection, the medium was changed, and the cells were maintained in medium containing 10% serum and 2 μg/ml puromycin. Control cells were transfected with MFGpuro alone. Hspa-transfected cells were tested frequently for the expression of transfected Hspa by Western blot analysis and were found to express high levels of inducible Hspa protein. For transfection of RIF and L929 cells with Hspb1, clones were obtained by transfection with phsp6 (containing the complete genomic sequence for murine *Hsp25*) and pBC vector (Stratagene, La Jolla, CA) (*16*). Exponentially growing RIF cells were plated into 60-mm dishes at 4×10^5 cells per plate 2 days prior to transfection and were transfected with 20 μg purified plasmid DNA in the presence of LipofectACE Reagent (Life Technologies, Gaithersburg, MD) for 24 h at 37°C. Stable transfectants were then selected with 400 μg/ml geneticin for 1 week, followed by continued growth in the presence of 200 μg/ml geneticin to obtain colonies suitable for isolation. The clones with high levels of Hspb1 protein as determined by Western blotting were selected.

Irradiation

Cells were plated in sterile 10-cm dishes and incubated at 37°C in humidified, 5% CO_2/95% air in culture medium until 70–80% confluent. High-dose irradiation was performed with γ rays from a ^{60}Co Theratron-780 (Atomic Energy of Canada, Ltd., Canada) at a dose rate of 1.294 Gy/min, while a ^{137}Cs irradiator at a dose rate of 0.143 cGy/min was used for low-dose irradiation with 0.01 Gy.

Cell Fractionation

Cells (1×10^6) grown on 10-cm tissue culture dishes were washed once with ice-cold PBS and harvested with a scraper. Cell pellets were

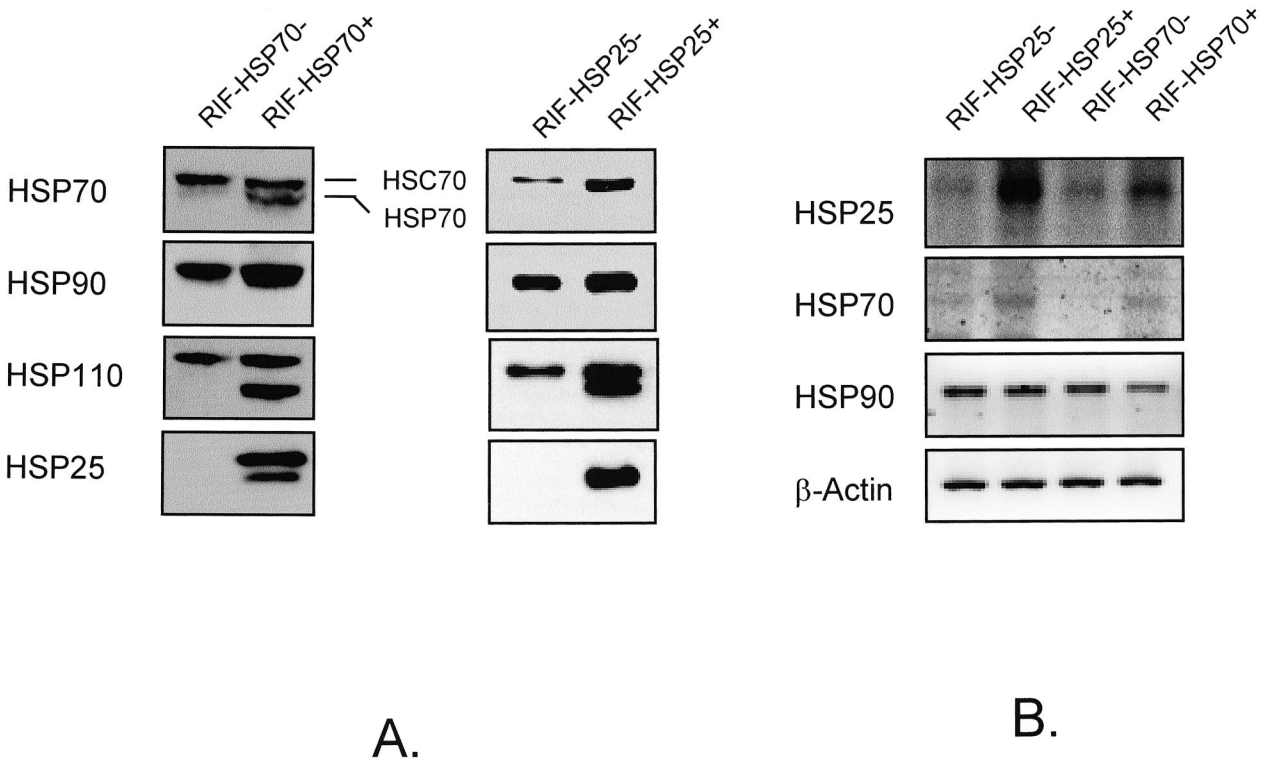

FIG. 2. Overexpression of inducible Hspa (HSP70) or Hspb1 (HSP25) in RIF cells was co-regulated. Panel A: Protein extracts of vector control cells and cells transfected with Hspb1 or inducible Hspa were prepared and assessed by Western blot analysis for Hspb1, Hspa, Hspc (HSP90) and Hsp110 (HSP110) as described in the Materials and Methods. Panel B: mRNA expression in vector-transfected cells and cells transfected with inducible Hspa. Total cellular RNA was extracted, reverse transcribed, and subjected to PCR. The products were electrophoresed on 1% agarose gel stained with ethidium bromide.

resuspended in hypotonic buffer (10 m*M* Hepes, 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 0.5% Nonidet P-40) and incubated at 4°C for 15 min. The samples were agitated every 5 min and then centrifuged at 12,000*g* for 30 s to collect the cytoplasmic fraction. The pellets were resuspended, incubated in nuclear extraction buffer (20 m*M* Hepes, 20% glycerol, 0.42 *M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA) for 30 min, and centrifuged at 12,000*g* for 20 min to collect the nuclear fraction. The protein concentration of supernatants was determined by Bio-Rad protein assay system.

Electrophoretic Mobility Shift DNA-Binding Assay of Hse

Two complementary single-strand DNA oligonucleotides, each 36 bases long and containing the Hse oligonucleotide, were annealed and used in the electrophoretic mobility shift DNA-binding assay. The DNA sequences were 5′-GAT CCT CGA AGG TTC GAG GAT CCT CGA AGG TTC GAG-3′ and 3′-GAG CTT CCA AGC TCC TAG GAG CTT CCA AGC TCC TAG-5′ (*17*). The Hse was terminally labeled with α-[^{32}P]ATP using T4 polynucleotide kinase, and 10 μg of nuclear protein was incubated with the [^{32}P]Hse at 30°C for 30 min. Subsequently, the Hsf-Hse binding complex was separated from unbound Hse on a 5% TBE gel. The gel was dried, and an autoradiogram was taken.

Reverse Transcriptase PCR (RT-PCR)

To measure the mRNA of Hspa, Hspb1, Hspc (also known as Hsp90), Hsf1, Hsf2 and β-actin, total RNA was isolated with the TRI™ reagent (MRC, Cincinnati, OH) according to the manufacturer's instructions. The reaction mixture contained 1× RT buffer, 1 μ*M* each of dNTPs, 2.5 U RNase, 0.5 μg of oligo (dT)-15 primer, 1 μg of total RNA, and 15 U of AMV reverse transcriptase (Promega, Madison, WI) in a final volume of 20 μl, and the mixture was incubated at 42°C for 20 min. The transcription reaction was terminated by heating the mixture at 95°C for 10 min and then chilling it on ice. Six respective pairs of primers specific for Hspa, Hspb1, Hspc, Hsf1, Hsf2 and β-actin were designed and synthesized as described previously (*18, 19*).

Colony-Forming Assay

The clonogenicity was compared using a colony-forming assay, as described previously (*15, 16*). Five hundreds cells were seeded into 60-mm Petri dishes at densities to produce approximately 200 colonies per dish consisting of 50 or more cells per dish and were incubated for 7–14 days. Colonies were fixed with a mixture of 75% methanol and 25% acetic acid and stained with 0.4% trypan blue. The number of colonies consisting of 50 or more cells was scored.

Detection of Apoptosis

Cells were plated on glass slides and irradiated as indicated. The cells were fixed in 70% ethanol and washed with PBS. Cells were incubated with 1 μg/ml *bis*benzimide trihydrochloride in PBS (Hoechst No. 33258, Sigma) for 10 min at room temperature in the dark. Specimens were viewed by fluorescence microscopy using Olympus BX-40 microscope with a 100-W mercury lamp, and at least 200 cells were scored for each determination.

RESULTS

An Adaptive Response was Detected in TR Cells, but not in the Parental RIF Cells

When cells were preirradiated with 0.01 Gy before a high challenging dose of radiation, an adaptive response was de-

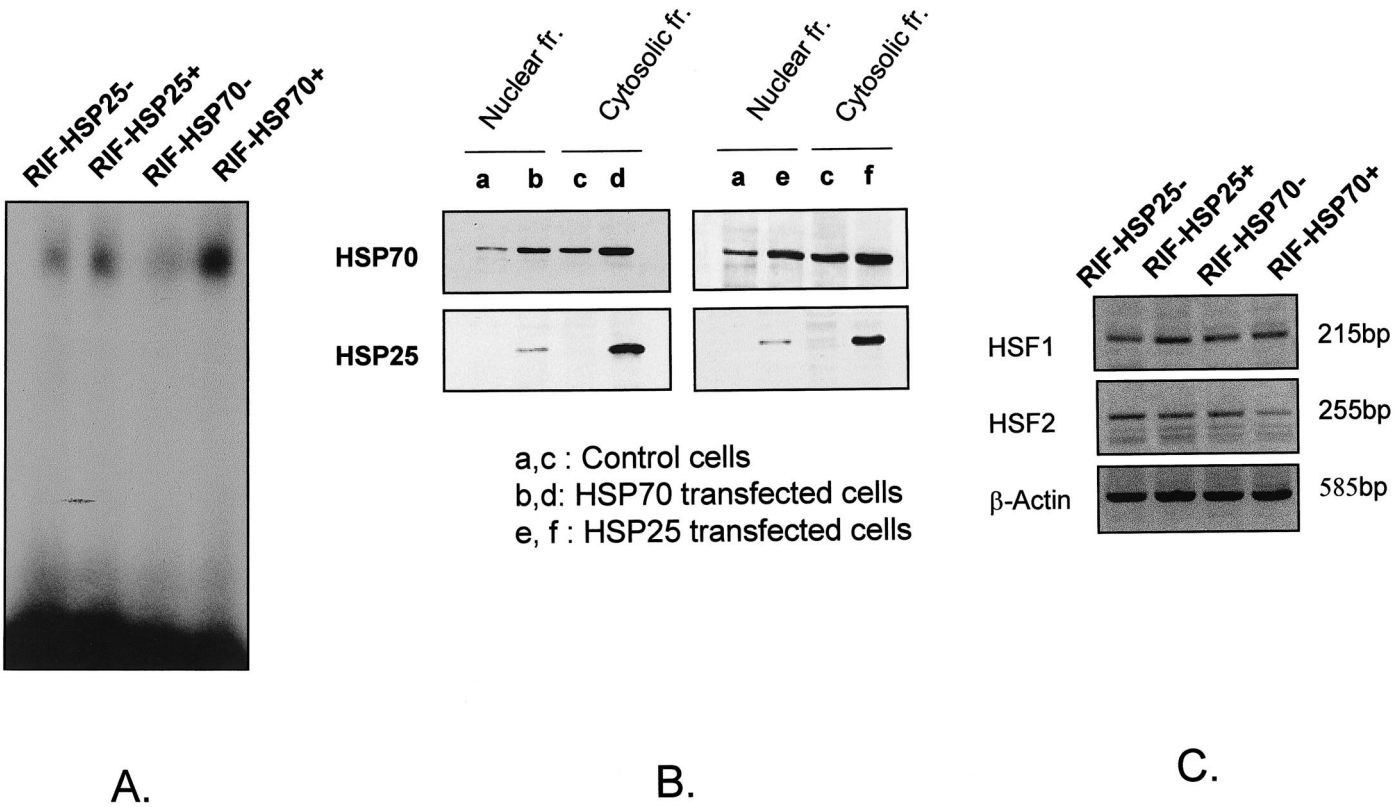

FIG. 3. Hsf activation was found in both Hspb1-transfected (HSP25) and inducible Hspa-transfected (HSP70) RIF cells. Panel A: Gel mobility shift analysis of Hse-binding activity in extracts of control vector and Hspb1-transfected or inducible Hspa-transfected cells. The nuclear and cytosol proteins were extracted and incubated with cold Hse or [^{32}P]Hse, and the Hsf bound to Hse was measured by a gel shift assay. Panel B: Nuclear and cytosol fraction was isolated and Hspb1 and Hspa expression was examined by Western blotting. Panel C: mRNA expression in vector-transfected cells and Hspb1- or inducible Hspa-transfected cells. Total cellular RNA was extracted, reverse transcribed, and subjected to PCR. The products were electrophoresed on 1% agarose gels stained with ethidium bromide.

tected in TR cells, but not in the parental RIF cells (Fig. 1A). Since the Hsp expression profiles had been shown to be different in these two cell lines (*17*), we analyzed the basal expression of these proteins in control cells of both cell lines by Western blotting. Expression of Hspa and Hspb1 was increased dramatically in TR cells, along with a slight induction of Hspc and the truncated form of Hsp110, suggesting that the adaptive response is related to the expression of these proteins (Fig. 1B). We did not observe any change in the expression of any of the Hsp's after irradiation with 0.01 Gy or 4 Gy in either cell line (data not shown).

Overexpression of Inducible Hspa or Hspb1 in RIF Cells was Co-regulated

To examine the role of Hspb1 and inducible Hspa in the induction of an adaptive response, RIF cells, which do not show an adaptive response, were transfected with inducible Hspa or Hspb1. When Hspb1 was overexpressed, increased expression of inducible Hspa was found. Furthermore, when inducible Hspa was overexpressed, increased induction of Hspb1 was detected (Fig. 2A). Therefore, PCR analyses for Hspb1 and inducible Hspa were performed to determine whether the expression of these proteins was regulated at the mRNA level. As shown in Fig. 2B, increased *Hspb1* mRNA was detected in Hspb1-transfected cells, together with an increased level of inducible *Hspa* mRNA. Similarly, in the Hspa-transfected cells, both *Hspa* and *Hspb1* mRNA were increased. When L929 cells or NIH 3T3 cells were transfected with either Hspb1 or inducible Hspa, these phenomena were not detected (data not shown).

Hsf Activation was Found in both Hspb1-Transfected and Inducible Hspa-Transfected RIF Cells

Since heat-shock factor (Hsf) is known to bind to heat-shock element (Hse), to promote Hsp genes, and to induce the transcription and translation of these genes (*20*), nuclear fractions were incubated with ^{32}P-labeled Hse, and the Hsf-Hse complex was subjected to agarose gel electrophoresis. The amount of Hsf-Hse complex was found to be significantly increased in both Hspb1-transfected and inducible Hspa-transfected RIF cells, with more increase in the inducible Hspa-transfected cells (Fig. 3A). Studies with subcellular fractions showed that Hspa was present in both nuclear and cytosolic fractions, while Hspb1 was predominantly in the cytosolic fraction (Fig. 3B). No increase in the expression of either *Hsf1* mRNA or *Hsf2* mRNA was detected by PCR analysis (Fig. 3C).

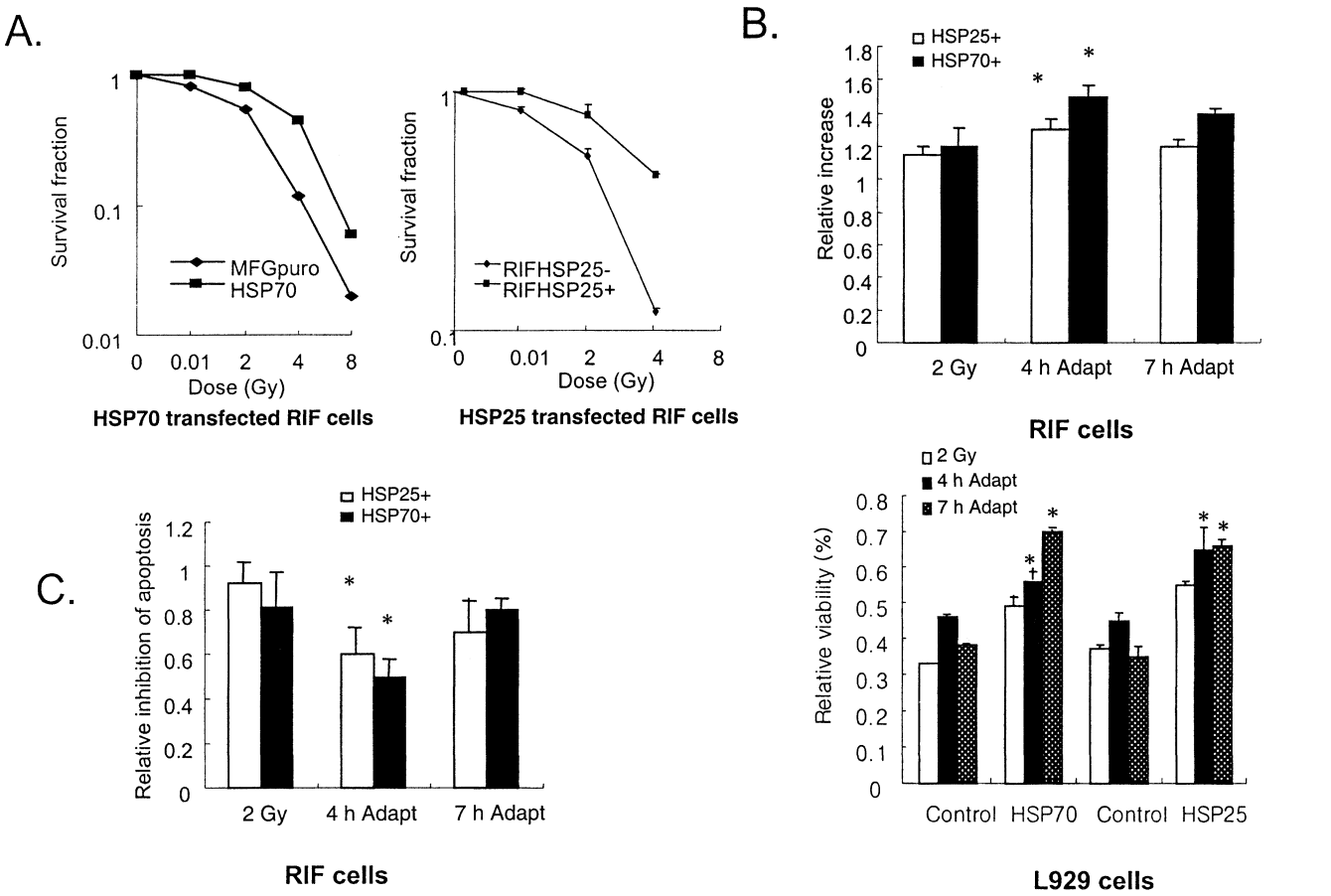

FIG. 4. An adaptive response was acquired in RIF cells transected with inducible Hspa (HSP70) or Hspb1 (HSP25). Panel A: The surviving fractions of vector-transfected control cells and Hspb1- or Hspa-transfected cells were assayed using a colony-forming assay. Panel B: The induction of an adaptive response was observed using a clonogenic cell survival assay. Cells from Hspb1- or Hspa-transfected RIF or L929 cells were irradiated with 0.01 Gy. After 4 or 7 h, a high challenge dose of 2 Gy was administered. The relative increase is the ratio of the surviving fraction in transfected cells to that in vector-transfected control cells. Each point represents the mean ± SD for three independent experiments. *$P < 0.05$ compared to cells irradiated with 2 Gy alone. 4 h Adapt: cells incubated for 4 h between 0.01 Gy and 2 Gy. 7 h Adapt: cells incubated for 7 h between 0.01 Gy and 2 Gy. Panel C: DNA fragmentation measured by Hoechst 33258 staining after irradiation. Points are means ± SD from three independent experiments. *$P < 0.05$ compared to cells irradiated with 2 Gy alone. 4 h Adapt: cells incubated for between 0.01 Gy and 2 Gy. 7 h Adapt: cells incubated for 7 h between 0.01 Gy and 2 Gy.

An Adaptive Response was Acquired by Inducible Hspa- or Hspb1-Transfected RIF Cells, and a More Pronounced Adaptive Response was Acquired by Hspb1- or Hspa-Transfected L929 Cells

To determine whether there was any link between Hsp's and the induction of an adaptive response, a clonogenic cell survival assay was performed. Overexpression of Hspb1 or inducible Hspa induced radioresistance (Fig. 4A). Nuclear staining with Hoechst 33254 also revealed that radiation-induced apoptosis in cells that overexpressed Hspa and Hspb1 was decreased by 10–20% from the levels in cells that had been transfected with the control vector (data not shown). An adaptive response was also found in both inducible Hspa-transfected RIF cells and Hspb1-transfected RIF cells (Fig. 4B). When Hspb1 or inducible Hspa was transfected into L929 cells, an increased induction of an adaptive response was shown; L929 cells that were not transfected with Hspb1 or Hspa also showed an adaptive response in our system (*8*). Similarly, when the cells were preirradiated with 0.01 Gy, apoptosis was reduced in both inducible Hspa-transfected and Hspb1-transfected cells, compared to the transfected cells treated with only the high challenging dose. This effect was also more pronounced in cells transfected with inducible Hspa than in those transfected with Hspb1 (Fig. 4).

DISCUSSION

The present study showed that mouse RIF cells, in which inducible Hspa and Hspb1 were not expressed, did not exhibit an adaptive response to low-dose preirradiation with 1 cGy, while the thermoresistant TR cells, which expressed inducible Hspa and Hspb1, did (Fig. 1). Also, when inducible Hspa or Hspb1 was transfected into RIF cells or into L929 cells, the cells acquired increased radioresistance and a radioadaptive response was induced (Fig. 4), suggesting

that expression of both inducible Hspa and Hspb1 is important for the induction of the adaptive response as well as for radioresistance.

The radioadaptive response was first described by Olivieri *et al.* in 1984 (*21*) in cultured human lymphocytes and was later confirmed by others in a wide variety of animal and plant cells. It has been characterized as follows: (1) The adaptation is a rapid process, being fully expressed 4–6 h after irradiation, and it persists for more than 20 h (*22*). (2) It has a dose limitation below ~0.1 Gy for an optimal expression. (3) In some systems, higher doses are incapable of inducing adaptation and rapidly destroy an adapted state that was induced previously by lower doses (*4*). In other systems, relatively high doses delivered at a low dose rate induced an adaptive response (*23*). However, the molecular mechanism(s) and signaling pathway(s) affecting the regulation of such a response remain unknown.

We showed in the present study that overexpression of Hspb1 induced the expression of inducible Hspa protein and that overexpression of inducible Hspa also induced Hspb1 protein; these expressions were transcriptionally regulated by each other (Fig. 2). However, we do not know the mechanism(s) by which these two proteins regulate each other or what major factor(s) is involved. NIH 3T3 or L929 cells transfected with inducible Hspa or Hspb1 did not exhibit such phenomena (data not shown); therefore, they may be unique in RIF cells.

Hsf is involved in the initiation and regulation of Hsp expression. Our data showed that transfection with inducible Hspa or Hspb1 increased Hsf-Hse binding, the increase being greater in inducible Hspa-transfectant cells (Fig. 3), and suggested that Hsf might be responsible for the increased transcription of inducible *Hspa* or *Hspb1* genes. We are not certain how exogenous *Hspa* or *Hspb1* genes could facilitate Hsf-Hse binding activity. One possibility might be that gene transfection activated Hsf. However, Hsf activation was not found after transfection with inducible Hspa or Hspb1 in L929 and NIH 3T3 cells (data not shown). Another possibility might be that RIF cells provide a unique environment for the induction of Hsp's, and the expression of inducible Hspa or Hspb1 facilitated Hsf activation, and the activated Hsf then affected each other's expression. The possibility that Hsf activation was also involved in the expression of Hspc cannot be excluded because Hspc expression was also increased in Hspb1- or Hspa-transfected RIF cells.

There are reports showing that cells irradiated with low doses exhibit various responses by synthesizing proteins such as Hsp's (*13*). In our system, increased induction of Hsp's was not detected by radiation in Western blotting; however, this might have been because the amount of protein was too small to be detected. Indeed, an increase in Hspa was detected only when the cells were exposed to 400 or 1000 Gy (*24*). Therefore, to elucidate the function of Hspb1 and inducible Hspa in the induction of an adaptive response, we transfected inducible Hspa or Hspb1 into RIF cells that did not induce these proteins and found no adaptive response, and also found that these transfected cells acquired the adaptive response. From the results, it was concluded from these results that inducible Hspa and Hspb1 were responsible at least in part for the induction of an adaptive response in RIF cells.

ACKNOWLEDGMENT

This work was supported by a National Nuclear R&D Program from the Ministry of Science and Technology of Korea.

Received: January 12, 2001; accepted: September 4, 2001

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