

INVOLVEMENT OF p27CIP/KIP IN HSP25 OR INDUCIBLE HSP70 MEDIATED ADAPTIVE RESPONSE BY LOW DOSE RADIATION

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Thermoresistant (TR) clones of radiation-induced fibrosarcoma (RIF) cells have been reported to show an adaptive response to 1cGy of low dose radiation, and HSP25 and inducible HSP70 are involved in this process. In this study, to further elucidate the mechanism by which HSP25 and inducible HSP70 regulate the adaptive response, HSP25 or inducible HSP70 overexpressed RIF cells were irradiated with 1cGy and the cell cycle was analyzed. HSP25 or inducible HSP70 overexpressed cells together with TR cells showed increased G1 phase after 1cGy irradiation, while RIF cells did not. [³H]-Thymidine and BrdU incorporation also indicated that both HSP25 and inducible HSP70 are involved in G1 arrest after 1cGy irradiation. Molecular analysis revealed upregulation of p27Cip/Kip protein in HSP25 and inducible HSP70 overexpressed cells, and cotransfection of p27Cip/Kip antisense abolished the induction of the adaptive response and 1cGy-mediated G1 arrest. The above results indicate that induction of an adaptive response by HSP25 and inducible HSP70 is mediated by upregulation of p27Cip/Kip protein, resulting in low dose radiation-induced G1 arrest.

KEYWORDS : Adaptive Response, Low Dose Radiation, Inducible HSP70, HSP25, p27CIP/KIP, G1 Arrest

1. INTRODUCTION

Adaptive responses that reduce the harmful effects of subsequent exposure to high-dose radiation [1] have been demonstrated in chromosome aberration [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 yet been clarified, although the continuous production of free radicals from radiation and other sources has been shown to stimulate cells to evolve a repair system for chromosome breaks [7]. Alteration of DNA molecules triggers the repair system, irrespective of the cause of damage, and frequent activation may increase the general repair capacity, cell cycle regulation systems [8], antioxidant defense systems [9], and molecular chaperone or stress-response systems [10]. Our previous data have shown that when cells were preirradiated with 1cGy, they showed increased clonogenic survival [11], and reduced apoptosis resulting from low-dose preirradiation was responsible for this adaptive response.

It has well been established that heat shock protein (HSP) families are molecular chaperones and assist intracellular folding of newly synthesized proteins [12]. Several studies showed the induction of a member of the HSP70 protein family during the adaptive response to oxidative stress and radiation [13] and this induction occurs during pretreatment of cells with a low concentration of H₂O₂. Low doses of X-rays were found to activate the promoter of inducible HSP70 gene: Transcription was silent under control conditions, but was highly induced by heat shock element [14] and the low dose of 4 cGy radiation that induces the adaptive response also increases inducible HSP70 mRNA [15]. The induction of an adaptive response by low dose radiation also involves induction of PBP74/mortalin/Gip75, a member of the HSP70 family [16]. However, the mechanism of HSP involvement in the induction of the adaptive response is not well defined. We previously demonstrated that mouse RIF cells, which did not induce HSP25 or inducible HSP70, did not exhibit an adaptive response after 1cGy preirradiation [17], whereas the thermoresistant TR cells, which expressed HSP25

and inducible HSP70, showed a response. Moreover, when inducible HSP70 or HSP25 was transfected to RIF cells, the cells acquired the adaptive response. In the present study, in order to elucidate the mechanisms involved in the induction of an adaptive response by HSP25 and inducible HSP70, we compared cell cycle distribution of HSP25 and inducible HSP70 transfected cells, respectively, after low dose radiation and found that p27Cip/Kip was responsible for induction of the adaptive response.

2. MATERIALS AND METHODS

2.1 Plasmids

The MFG retroviral vector was constructed by replacing the GFP sequence of MFG.GFP.IRES.puro in order to construct MFG-HSP25puro or MFG-HSP70puro [18]. The MFG.GFP.IRES.puro itself was used as a negative control throughout the experiment. The retroviral plasmids were introduced into a 293gpg retrovirus packaging cell line by transient transfection with Lipofectamine (Gibco/BRL). After 72 hrs, the supernatants were harvested and used for retroviral infection. The virus titers, measured in the NIH3T3 cell line by puromycin-resistant colony formation, were between 10^5 and 5×10^5 /ml. The infection and selection of the target cells by puromycin were performed as described previously [18].

2.2 Cell Culture

RIF (radiation-induced fibrosarcoma cells) and TR (a thermoresistant clone of RIF) (kindly provided by Dr. Young-Mee Park, Roswell Park Institute) were cultured in Dulbecco's minimal essential medium (DMEM) (GIBCO, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal bovine serum (FBS, GIBCO) and antibiotics at 37°C in a 5% CO₂ humidified incubator.

2.3 Irradiation

Cells were plated in 3.5, 6, or 10 cm diameter dishes and incubated at 37°C under humidified 5% CO₂-95% air in a culture medium until 70-80% confluent culture was obtained. Cells were then exposed to γ -rays with ¹³⁷Cs gamma-ray source (Cesium irradiator, Atomic Energy of Canada, Ltd., Canada) at a dose rate of 3.81 Gy/min. For low dose irradiation, a dose rate of 0.143 cGy/min was used.

2.4 Cell Cycle Analysis

For cell cycle analysis, cells were fixed in 80% ethanol for at least 18 h at 4°C. The fixed cells were then washed once with PBS-EDTA and resuspended in 1 ml of PBS. After the addition of 10 ml each of propidium iodide (PI, 5 mg/ml) and RNase (10 mg/ μ l), the samples were incubated for 30 min at 37°C and analyzed with a FACScan flow cytometer.

2.5 [³H] Thymidine Incorporation Assay

The cells were plated at a confluence of 70-80% in 10 cm diameter petri dishes. After irradiation, the cells were trypsinized, plated in 96 well plates (5×10^3 cells/well), and incubated for various periods. The cells were incubated with [³H] thymidine (37 kB1/well) for the last 4 h before harvesting, and the radioactivity of the cells was determined with a scintillation counter (Parkard, TRI-CARB 4530, Meriden, DT).

2.6 BrdU Incorporation Assay

BrdU incorporation was measured using a commercially available ELISA kit (Roche, Nutley, NJ) according to the manufacturer's instructions.

2.7 Detection of Apoptosis

Cells were cultured, harvested at the indicated times, and stained with propidium iodide (PI), according to the manufacturer's protocol, and were then analyzed using a FACScan flow cytometer.

2.8 Polyacrylamide Gel Electrophoresis and Western Blot

For polyacrylamide gel electrophoresis (PAGE) and the Western blot analysis, cells were solubilized with lysis buffer [120 mM NaCl, 40 mM Tris (pH 8.0), 0.1% NP40], the samples were boiled for 5 min, and an equal amount of protein (40 μ g/well) was analyzed on 10% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and processed for immunoblotting. Blots were further incubated with horseradish peroxidase-conjugated secondary antibody, diluted at 1:5,000, and specific bands were visualized by chemiluminescence (ECL, Amersham International). Autoradiographs were recorded onto X-Omat AR films (Eastman Kodak Co.).

2.9 Statistical Analysis

Statistical comparisons were made using a Student's t test (independent group) and a null hypothesis was rejected whenever a P value of 0.05 or less was found.

3. RESULTS

3.1 Induction of Adaptive Response by Low Dose Radiation

When cells were preirradiated with 1cGy before a high challenging dose of radiation (2 Gy), cell death induced by high dose radiation was significantly inhibited in TR cells, but not in the parental RIF cells (Fig. 1A). It was found that 2Gy radiation induced 32-37% G2/M phase arrest, which is a typical radiation induced cell cycle arrest, and part of G2/M arrested cells undergo mitotic cell death [19,20]. However, low dose preirradiated cells with 4 hrs

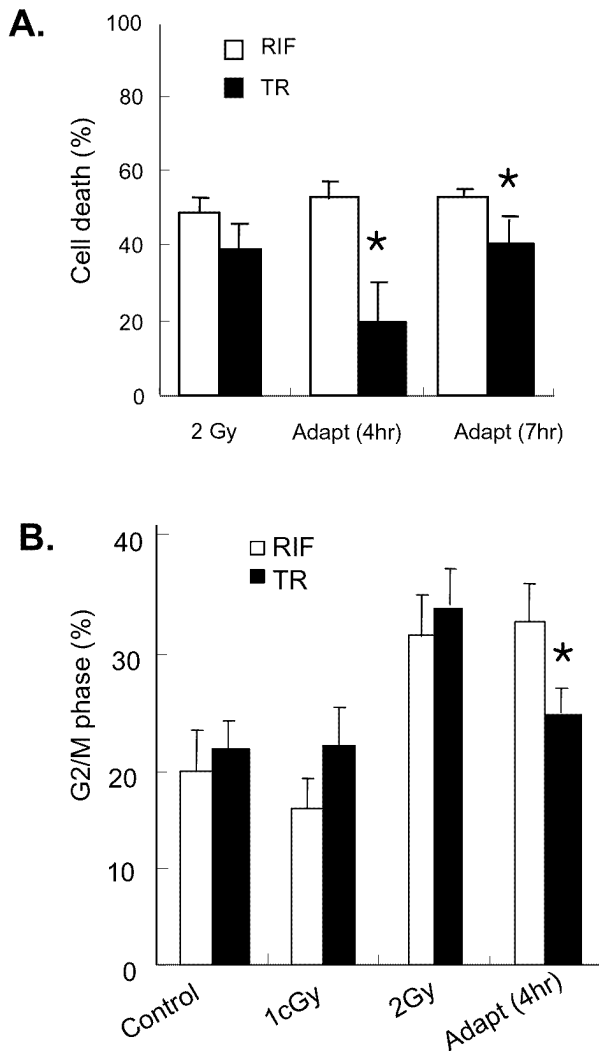


Fig. 1. Induction of Adaptive Response by Low Dose Radiation

RIF and TR cells were irradiated with 1cGy and a high challenge dose of 2 Gy was administered with 4 or 7 hrs intervals. (A) At 48hrs of high dose radiation, cell death was determined using propidium iodide (PI) staining. (B) At 12 hrs after radiation, flow cytometry analysis after PI staining was conducted. Each point represents mean \pm SD for three independent experiments. * $P < 0.05$ compared to cells irradiated with 2 Gy alone. Adapt (4hr): cells incubated for 4 hrs between 1cGy and 2Gy. Adapt (7hr): cells incubated for 4 hrs between 1cGy and 2Gy

interval between low and high challenging irradiation displayed a significant reduction in G2/M phase arrest, up to 25% in TR cells, whereas RIF cells showed no reduction (Fig. 1B). The difference in expression of HSP25 and inducible HSP70 in these two cell lines led us to speculate that HSP25 and inducible HSP70 might be involved in the induction of an adaptive response such as the reduction of cell death and G2/M arrest induced by high challenging dose radiation.

3.2 Involvement of HSP25 and Inducible HSP70 in Adaptive Response

Since expressions of HSP25 and inducible HSP70 were increased in TR cells, HSP25 and inducible HSP70, respectively, were transfected to RIF cells (Fig. 2A) and we determined whether there was any link between HSPs and the induction of an adaptive response. Cell death data revealed that HSP25 or inducible HSP70 overexpression acquired an adaptive response whereas their parent RIF cells did not show an adaptive response (Fig. 2B): Pretreatment of 1cGy radiation attenuated cell death by high dose radiation. Increased induction of G2/M arrest at 6 hr after 2Gy high dose radiation was significantly reduced by priming of 1cGy radiation when the time interval between low and high dose was 4 hrs (Fig. 2C). From the data, it has been determined that HSP25 and inducible HSP70 are involved in the induction of an adaptive response.

3.3 Increase of Low Dose Radiation Mediated G1 Arrest by HSP25 and Inducible HSP70

Since the expressions of HSP25 and inducible HSP70 were increased in TR cells, HSP25 or inducible HSP70 was transfected to RIF cells using a retroviral vector system (Fig. 2A), and we assessed whether there was any link between HSPs and the induction of an adaptive response. Cell death data revealed that HSP25 or inducible HSP70 overexpression acquired an adaptive response, whereas their parent RIF cells did not show an adaptive response (Fig. 2B): Preirradiation of low dose radiation reduced cell death by high dose radiation. G2/M arrest at 6 hrs after high dose radiation (2Gy) was significantly reduced by priming of 1cGy radiation in HSP25 and inducible HSP70 overexpressed cells as well as in TR cells when the time interval between low and high dose was 4 hrs (Fig. 2C). The data indicate HSP25 and inducible HSP70 are involved in the induction of the adaptive response.

3.4 Increased Expression of p27Cip/Kip in HSP25 and Inducible HSP70 Overexpressed Cells

To examine the mechanisms involved in induction of the adaptive response, the cell cycle distribution was studied after low dose irradiation (1cGy). As seen in Fig 3A, dramatically reduced G1 phase from 6 hrs after low dose radiation was observed in the control and control vector (RIFMFG) alone transfected cells; in HSP25 and inducible HSP70 overexpressed cells, as well as TR cells, no difference was observed. [³H]-thymidine and bromodeoxyuridine (BrdU) incorporation data also suggest that control cells and vector alone transfected cells did not significantly change DNA synthesis by low dose radiation; however, in the case of HSP25 and inducible HSP70 overexpressed cells, reduced DNA synthesis (Fig. 3B and 3C) was observed, suggesting increased G1 phase arrest by 1cGy radiation in HSP25 and inducible HSP70 overexpressed cells.

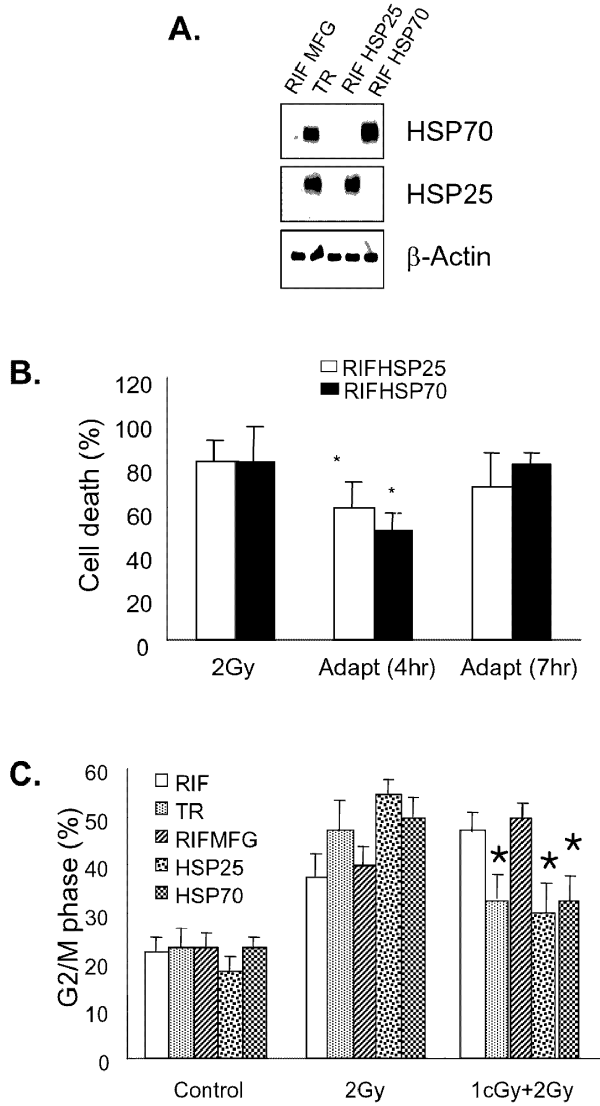


Fig. 2. Involvement of HSP25 and Inducible HSP70 in Adaptive Response

(A) Protein extracts of TR cells, vector control (MFG) RIF cells and RIF cells with HSP25 and inducible HSP70 transfection were prepared and assessed by Western blot analysis. (B) Vector control RIF cells and RIF cells with HSP25 and inducible HSP70 transfection were irradiated with 1cGy and a high challenge dose of 2 Gy was administered with 4 or 7 hrs intervals. At 48hrs of high dose radiation, cell death was determined using propidium iodide (PI) staining. (C) TR cells, vector control RIF cells, and RIF cells with HSP25 and inducible HSP70 transfection were irradiated with 1cGy and a high challenge dose of 2 Gy was administered with 4 hrs intervals. At 12hrs of high dose radiation, a flow cytometry analysis was performed after PI staining. Each point represents mean \pm SD for three independent experiments. $*P < 0.05$ compared to cells irradiated with 2 Gy alone. Adapt (4hr): cells incubated for 4 hrs between 1cGy and 2Gy. Adapt (7hr): cells incubated for 4 hrs between 1cGy and 2Gy. Relative inhibition of cell death is shown as % of unirradiated control cells

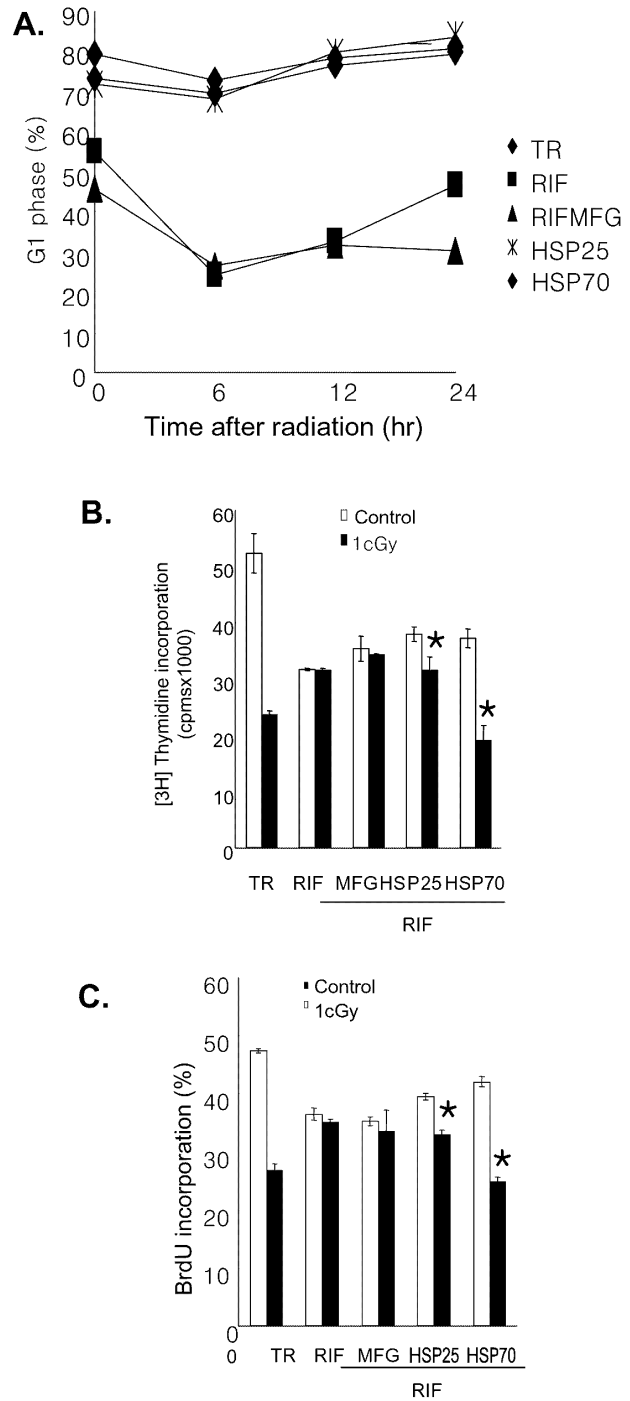


Fig. 3. Increase of Low Dose Radiation Mediated G1 Arrest by HSP25 and Inducible HSP70

(A) Flow cytometric analysis after propidium iodide (PI) staining at indicated time points of 1cGy radiation, (B) [3H] thymidine incorporation and (C) Bromodeoxyuridine (BrdU) incorporation at 24 hr of 1cGy radiation were performed in TR cells, vector control RIF cells, and RIF cells with HSP25 and inducible HSP70 transfection at indicated time points after 1cGy radiation. Each point represents mean \pm SD for three independent experiments. $*P < 0.05$ compared to unirradiated control cells

3.5 Increased Expression of p27Cip/Kip in HSP25 and Inducible HSP70 Overexpressed Cells

In order to elucidate the mechanisms of G1 phase arrest by 1 cGy involved in HSP25 and inducible HSP70 overexpressed cells and to determine whether G1 phase arrest is related to the induction of an adaptive response, we examined expression levels of proteins that are related to G1 arrest in cell cycle regulation. Figure 4A shows that the expression of p27Cip/Kip was increased by HSP25 and inducible HSP70, respectively, while the protein of p21Waf expression did not change (data not shown). Next, in order to determine whether there is direct involvement of p27Cip/Kip in G1 phase arrest by low dose radiation, HSP25 and inducible HSP70 overexpressed cells as well as TR cells were treated with antisense of p27Cip/Kip. In addition, 2 clones, whose respective expression of p27Cip/Kip were downregulated (Fig. 4B), were isolated. As shown in Fig. 4C, low dose radiation mediated G1 phase arrest was abolished by antisense p27Cip/Kip treatment in HSP25 and inducible HSP70 overexpressed cells. When the induction of the adaptive response by low dose radiation was determined, the reduction of high dose radiation induced cell death by low dose preirradiation in HSP25 or inducible HSP70 transfected cells was restored to the control cell level by cotransfection of p27Cip/Kip antisense (Fig. 4D). Moreover, the inhibition of high dose radiation induced G2/M phase arrest by low dose pretreatment was also restored by antisense p27Cip/Kip treatment (Fig. 4E). The data indicate that increased expression of p27Cip/Kip is responsible for induction of the adaptive response and p27Cip/Kip mediated G1 arrest is related to the induction of the adaptive response by HSP25 and inducible HSP70.

4. DISCUSSION AND CONCLUSION

Adaptive response to ionizing radiation is a phenomenon whereby the harmful effects of a high dose exposure to ionizing radiation can be mitigated when cells are first exposed to a low dose of radiation. This radioresistance can occur even after radiation with doses as low as 0.5-10 cGy, and requires 3-4 hrs for full induction [16]. 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]: 1) The adaptation is a rapid process, being fully expressed 4-6 hrs after irradiation and persists for more than 20 hrs [22]. 2) It has a dose limitation, below ~0.1Gy for optimal expression. 3) In some systems, higher doses are incapable of inducing adaptation and rapidly destroy the adapted state that was previously induced by lower doses [3]. In other systems, relatively high doses delivered at a low dose rate induced an adaptive response [23]. However, the molecular mechanisms and signaling pathways

involved in the regulation of such a response remain unknown. The present study demonstrated that HSP25 and inducible HSP70 are responsible for the induction of an adaptive response, and that increased G1 phase arrest by high expression of p27Cip/Kip in HSP25 and inducible HSP70 overexpressed cells may be a key modulator of these phenomena.

RIF cells, in which HSP25 and inducible HSP70 are not expressed, did not exhibit an adaptive response to low-dose preirradiation with 1cGy, while their thermo-resistant TR cells, which expressed HSP25 and inducible HSP70, did exhibit an adaptive response (Fig. 1). When HSP25 and inducible HSP70 were transfected to RIF cells (Fig. 2A), inhibition of cell death was observed (Fig. 2B), as well as decreased radiation-induced G2/M phase arrest by low dose preirradiation (Fig. 2C). This suggests that the expression of both HSP25 and inducible HSP70 is important for the induction of the adaptive response. To examine the role of 1 cGy radiation in HSP25 and inducible HSP70 overexpressed cells, the cell cycle distribution was determined. Significantly increased G1 phase arrest was observed in HSP25 and inducible HSP70 overexpressed cells (Fig. 3A), and [³H]-thymidine and BrdU incorporation data also indicated reduced DNA synthesis in HSP25 and inducible HSP70 overexpressed cells (Figs. 3B and 3C). This suggests that 1 cGy radiation had a stronger effect on HSP25 and inducible HSP70 overexpressed cells, which resulted in increased G1 phase arrest by low dose radiation.

Since the expression of p27Cip/Kip was increased by HSP25 and inducible HSP70 overexpressed cells (Fig. 4A) and p27Cip/Kip is an inhibitor of a broad range of the cell cycle, the role of p27Cip/Kip in the induction of the adaptive response was examined. When p27Cip/Kip antisense was transfected and 2 stable clones were selected (Fig. 4B), increased G1 phase by HSP25 and inducible HSP70 was abrogated (Fig. 4C). In our experiment, expression of p21Waf, another broad spectrum of cell cycle inhibitors, was not altered by HSP25 or inducible HSP70 overexpression. In addition, the induction of the adaptive response by HSP25 and inducible HSP70 was abolished by cotransfection of p27Cip/Kip antisense, indicating that increased expression of p27Cip/Kip by HSP25 and inducible HSP70 is responsible for the adaptive response (Fig. 3D). Moreover, the reduction of radiation-induced G2/M phase arrest by HSP25 and inducible HSP70 overexpressed cells was restored by antisense cotransfection of p27Cip/Kip (Fig. 4). At present, it is not clear how increased G1 arrest by p27Cip/Kip in HSP25 or inducible HSP70 overexpressed cells is involved in reduced G2/M phase arrest by high dose radiation when 1cGy is pretreated. One possibility is that increased G1 arrest might reduce G2/M phase arrest.

Cell cycle progression depends on the activity of a series of cyclin dependent kinase (CDK) complexes [24]. The activity of the CDKs depends on the phosphorylation state, binding of cyclins, and the presence of CDK inhibitors

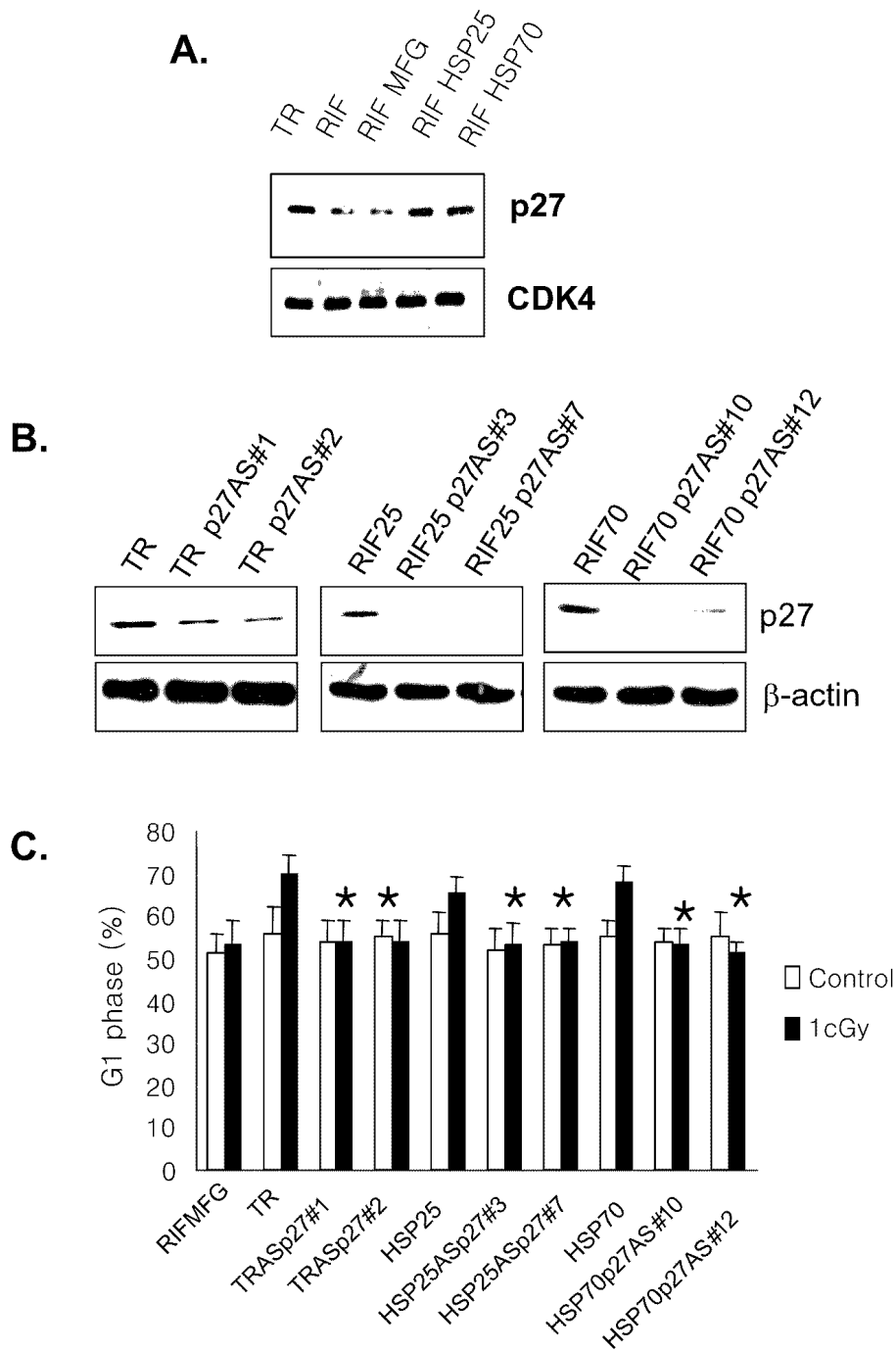


Fig. 4. Increased Expression of p27Cip/Kip in HSP25 and Inducible HSP70 Overexpressed Cells

(A) Protein extracts of TR cells, vector control (MFG) RIF cells and RIF cells with HSP25 and inducible HSP70 transfection were prepared and assessed by Western blot analysis. Two stable clones after p27Cip/Kip antisense transfection were isolated from TR cells, vector control (MFG) RIF cells, and RIF cells with HSP25 and inducible HSP70 transfection, and protein extracts were prepared and assessed by Western blotting analysis (B). Flow cytometry analysis at 12 hr of 1cGy radiation was performed (C). Cells were irradiated with 1cGy and a high challenge dose of 2 Gy was administered with 4 or 7 hrs intervals. At 48hrs of high dose radiation, cell death was determined using PI staining (D). At 12 hrs after radiation, flow cytometry analysis after PI staining was performed (E). Each point represents mean \pm SD for three independent experiments. * $P < 0.05$ compared to cells irradiated with 2 Gy alone.

(CDKIs) [25,26]. At least two families of CDKIs can modulate CDK activity during G1/S phase transition, the p27Cip/Kip family and the INK4 family [27,28]. p27Cip/Kip family can bind and regulate cyclin A, D, and E-dependent kinases [29,30], and p27Cip/Kip is directly involved in cell cycle restriction point control. Various antimitogens such as radiation induce p27Cip/Kip protein, allowing it to associate with and inhibit the cyclin E/CDK complex [33]. p27Kip/Cip knockout mice exhibit multiple organ hyperplasia and have 2-fold higher testes than their wild-type littermates [31,32]. A higher pituitary tumor incidence was reported in p27Cip/Kip knockout mice [33,34], indicating that the loss of p27Cip/Kip may contribute to oncogenesis and tumor progression. Our data demonstrate that induction of an adaptive response in cells carrying HSP25 and inducible HSP70 is caused by an increase in p27CIP/Kip through reduction of apoptosis and G2/M phase arrest. However, further study is needed to elucidate the mechanism by which HSP25 and inducible HSP70 regulate p27Cip/Kip.

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