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Characterization of stress sensitivity and chaperone activity of Hsp105 in mammalian cells

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ABSTRACT

Hsp105 is a major mammalian heat shock protein that belongs to the Hsp105/110 family, a diverged subgroup of the Hsp70 family. Hsp105 not only protects the thermal aggregation of proteins, but also regulates the Hsc70 chaperone system *in vitro*. Recently, it has been shown that Hsp105/110 family members act as nucleotide exchange factors for cytosolic Hsp70s. However, the biological functions of Hsp105/110 family proteins still remain to be clarified. Here, we examined the function of Hsp105 in mammalian cells, and showed that the sensitivity to various stresses was enhanced in the Hsp105-deficient cells compared with that in control cells. In addition, we found that deficiency of Hsp105 impaired the refolding of heat-denatured luciferase in mammalian cells. In contrast, overexpression of Hsp105α enhanced the ability to recover heat-inactivated luciferase in mammalian cells. Thus, Hsp105 may play an important role in the refolding of denatured proteins and protection against stress-induced cell death in mammalian cells.

1. Introduction

Heat shock proteins are a set of highly conserved proteins produced in response to physiological and environmental stresses that serve to protect cells from stress-induced damage by preventing protein denaturation and/or repairing such damage [1]. Mammalian heat shock proteins are classified into several families on the basis of their apparent molecular weight and function, such as HSP105/110, HSP90, HSP70, HSP60, HSP40, and HSP27. The HSP 70 family is the major and best-characterized group of heat shock proteins. Several different species of HSP70 family proteins are present in different compartments of eukaryotic cells and play important roles as molecular chaperones that prevent the irreversible aggregation of denatured proteins. Hsp70s also assist the folding, assembly, and translocation across the membrane of cellular proteins [2,3].

Hsp105α and Hsp105β are mammalian members of the Hsp105/110 family, a diverged subgroup of the Hsp70 family. Hsp105α is expressed constitutively and induced by various forms of stress, while Hsp105β is an alternatively spliced form of Hsp105α that is specifically produced following heat shock at 42 °C [4–6]. These proteins suppress the aggregation of denatured proteins caused by heat shock *in vitro*, as does Hsp70 [7]. Furthermore, Hsp105α and Hsp105β exist as complexes associated with Hsp70 and Hsc70 in mammalian cells [8], and regulate the Hsc70 chaperone system [7,9]. Recently, it has been shown that mammalian and yeast Hsp105/110 family members act as nucleotide exchange factors for cytosolic Hsp70s [10–12]. Furthermore, the yeast Hsp105/110 homolog, Sse1p, has been shown to be an important component of the folding machinery for newly synthesized proteins [10] and heat-denatured proteins [11]. However, human and yeast Hsp105/110 proteins show significant differences in their biochemical properties, such as thermostability and intrinsic ATPase activity [13]. In addition, several evolutionarily unrelated families of NEFs have been identified in mammalian cells, and it is unclear whether mammalian Hsp105/110 family proteins act as an important component of the protein folding machinery in mammalian cells.

Here, we examine the function of Hsp105α in mammalian cells using the Hsp105-deficient cells, and show that Hsp105α plays an important role in protein refolding and protection against stress-induced cell death in mammalian cells.

2. Materials and methods

2.1. Cell culture and stress treatments

Mouse fibroblast C3H10T1/2 cells were obtained from RIKEN Bioresource Center Cell Bank (Tsukuba, Japan) and were cultured

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in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 in air at 37 °C. Hsp105-deficient mouse embryonic fibroblasts (*hsp105*$^{-/-}$ MEFs) and wild-type mouse embryonic fibroblasts (*hsp105*$^{+/+}$ MEFs) were prepared from day 13.5 hsp105 knockout mouse embryos as described previously [14]. These cells were cultured in DMEM containing a higher concentration of glucose (4.5 mg/ml) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 in air at 37 °C.

Staurosporine was dissolved in dimethylsulfoxide at a concentration of 1 mM, and diluted with culture medium to concentrations ranging from 0.05 to 0.8 μM. Cells were incubated in the

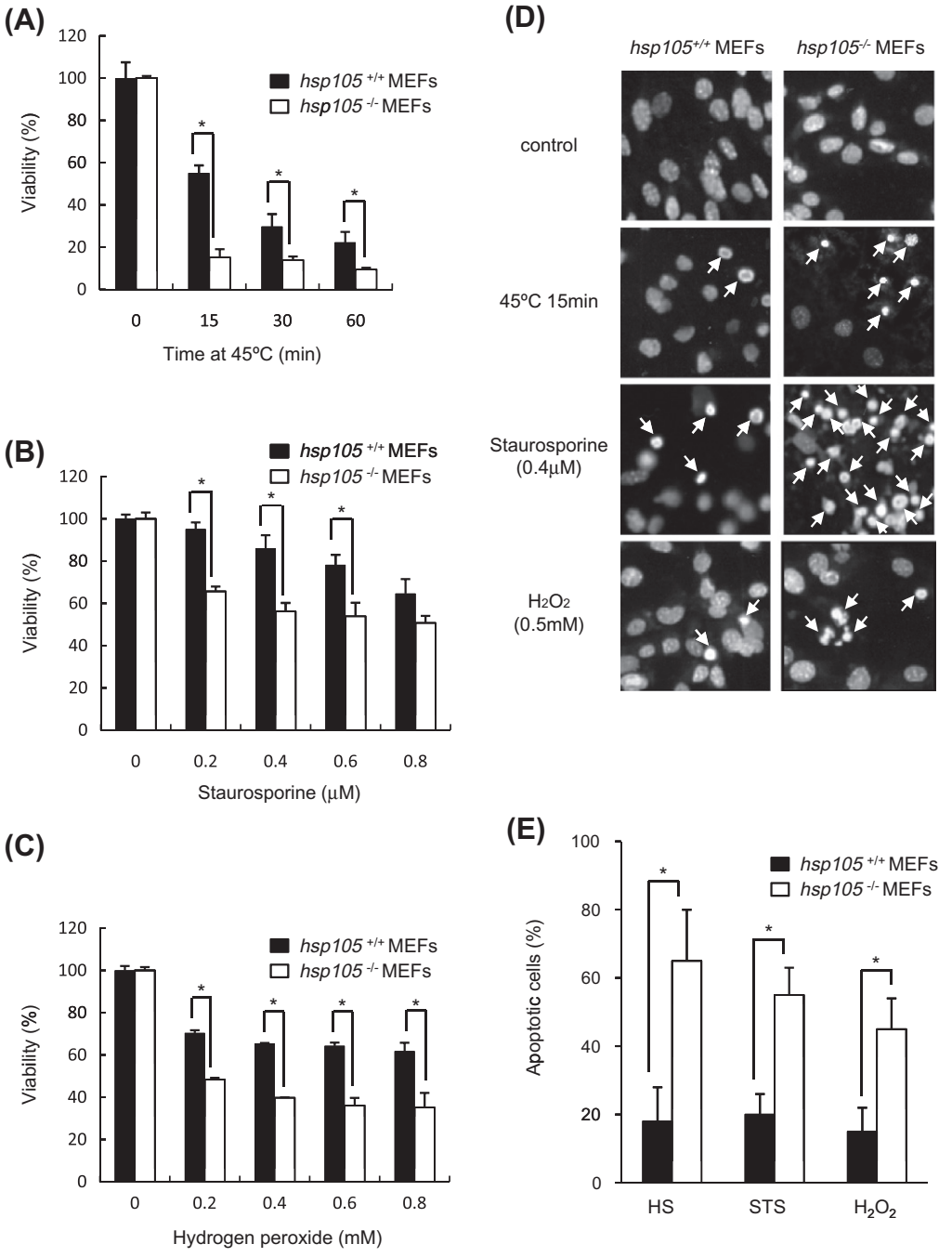

Fig. 1. Deficiency of Hsp105 enhances the sensitivity to various stresses in mouse embryonic fibroblasts. Wild-type (*hsp105*$^{+/+}$) MEFs and *hsp105*$^{-/-}$ MEFs were treated at 45 °C for 15–60 min and further incubated at 37 °C for 6 h (A), exposed to 0.2–0.8 μM staurosporine (B), or exposed to 0.2–0.8 mM H_2O_2 for 1 h and further incubated at 37 °C for 6 h (C). Cell viability was then determined by neutral red assay. Values represent the means ± SD of three independent experiments performed in triplicate. The significance of differences was assessed by an unpaired Student's *t*-test. $^*p < 0.05$ for viability in *hsp105*$^{+/+}$ MEFs vs. *hsp105*$^{-/-}$ MEFs. (D) *hsp105*$^{+/+}$ MEFs and *hsp105*$^{-/-}$ MEFs cells were grown on coverslips, treated at 45 °C for 15 min and further incubated at 37 °C for 6 h, exposed to 0.4 μM staurosporine for 6 h, or exposed to 0.5 mM H_2O_2 for 1 h and further incubated at 37 °C for 6 h. Cells were washed with PBS, fixed with 3.7% formaldehyde, and stained with 10 μM Hoechst 33342. Nuclear morphology of cells was observed using a fluorescence microscope. Arrows indicate the apoptotic cells. (E) Rates of apoptosis were calculated using at least 200 cells in each experiment. Values represent the means ± SD of four independent experiments. The significance of differences was assessed with an unpaired Student's *t*-test. $^*p < 0.01$ for viability in *hsp105*$^{+/+}$ MEFs vs. *hsp105*$^{-/-}$ MEFs.

medium containing staurosporine at 37 °C for 6 or 24 h. The final concentration of DMSO is less than 0.1%, and such concentration of DMSO did not affect the viability of cells (data not shown). For treatment with H_2O_2, cells were treated with 0.2–0.8 mM H_2O_2 in PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at 37 °C for 1 h, washed with PBS, and further incubated in fresh medium at 37 °C for 6 h. For heat shock treatment, cells were treated in a water bath set at 45 °C for 15–60 min and then incubated at 37 °C for 6 h.

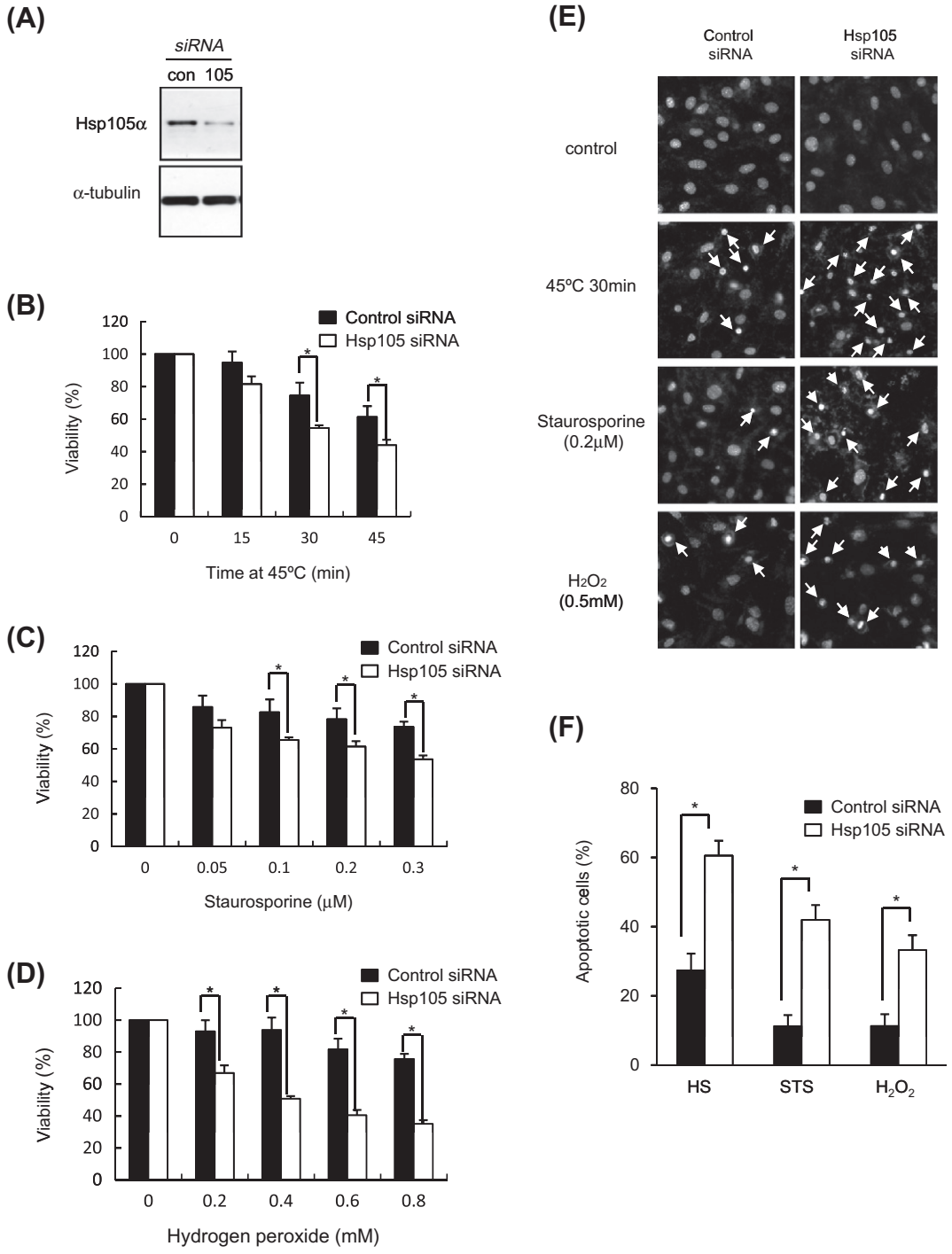

Fig. 2. Effect of Hsp105 knockdown on the sensitivity to various stresses in C3H10T1/2 cells. C3H10T1/2 cells were transfected with Hsp105 siRNA or RISC-free control siRNA, and incubated further at 37 °C for 72 h. Then, the cells were harvested and analyzed for expression of Hsp105α and luciferase with α-tubulin as a loading control by Western blotting using respective antibodies (A). After transfection, the cells were treated at 45 °C for 15–45 min and further incubated at 37 °C for 6 h (B), exposed to 0.05–0.3 μM staurosporine for 24 h (C), or exposed to 0.2–0.8 mM H_2O_2 for 1 h and further incubated at 37 °C for 6 h (D). Cell viability was then determined by neutral red assay. Values represent the means ± SD of four independent experiments performed in triplicate. The significance of differences was assessed by an unpaired Student's *t*-test. $^*p < 0.05$ for viability in control cells vs. hsp105 knockdown cells. (E) C3H10T1/2 cells were grown on coverslips, treated at 45 °C for 30 min and further incubated at 37 °C for 24 h, exposed to 0.2 μM staurosporine for 24 h, or exposed to 0.5 mM H_2O_2 for 1 h and further incubated at 37 °C for 24 h. Cells were washed with PBS, fixed with 3.7% formaldehyde, and stained with 10 μM Hoechst 33342. Nuclear morphology of cells was observed using a fluorescence microscope. Arrows indicate the apoptotic cells. (F) Rates of apoptosis were calculated using at least 200 cells in each experiment. Values represent the means ± SD of four independent experiments. The significance of differences was assessed with an unpaired Student's *t*-test. $^*p < 0.01$ for viability in control cells vs. hsp105 knockdown cells.

2.2. Cell viability assay

Cells (1×10^4 cells/well) in 96-well plates were incubated at 37 °C for 3 h with 50 μg/ml neutral red, and fixed with 1% formaldehyde containing 1% $CaCl_2$ for 1 min. The dye incorporated into viable cells was extracted with 50% ethanol containing 1% acetic acid, and absorbance at 540 nm was measured.

2.3. Morphological examination of apoptotic cells

Cells grown on collagenized coverslips (2×10^4 cells/cm^2 in 24-well plates) were fixed with 3.7% formaldehyde for 30 min at room temperature. After washing with PBS, cells were stained with 10 μM Hoechst 33342 for 10 min in the dark and observed using a fluorescence microscope (Nikon).

2.4. Plasmids, transfection, and luciferase reactivation assay

Plasmid pGL3-control, encoding firefly luciferase, was purchased from Promega Corporation (Madison, WI, USA). For expression of mouse Hsp105α, we used pcDNA105α [15]. As a control, we used the empty vector pcDNA3 (Invitrogen, Carlsbad, CA, USA).

The reactivation of thermally denatured luciferase in mammalian cells was measured, according to the method reported by Michels et al. [16]. Briefly, *hsp105*$^{+/+}$ MEFs and *hsp105*$^{-/-}$ MEFs were grown in φ 35-mm dishes to 70–80% confluence and were transiently transfected with 1 μg of pGL3-control using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. To overexpress Hsp105α in C3H10T1/2 cells, the cells were co-transfected with 1 μg of pGL3-control and 1.5 μg of pcDNA105α using Lipofectamine 2000. For the knockdown of Hsp105α and/or Hsp70 expression, 20 pmol of the Hsp105α siRNA (Dharmacon, Chicago, IL, USA; 5′-GCA AAU CAC UCA UGC AAA CUU-3′), Hsp70 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or RISC-free control siRNA (Dharmacon) was co-transfected into C3H10T1/2 cells with 1 μg of pGL3-control using Lipofectamine 2000. The following day, cells were trypsinized and divided equally into four wells of 4-well plate. At 72 h after transfection, the cells were preincubated in a medium containing 10 μg/ml cycloheximide for 30 min at 37 °C to inhibit new protein synthesis, then heat-shocked at 42 °C for 60 min and incubated further at 37 °C. At various time points, duplicate samples were taken for the measurements of luciferase activity as described previously [17]. The protein concentration of the cell lysates was also determined for normalization of protein content, and the relative activity is expressed as ratio to the luciferase activity of cells before heat shock.

2.5. Western blotting

Cells (4×10^5 cells/35-mm dish) were lysed with 0.1% SDS and boiled for 5 min. Aliquots (20 μg of protein) of cell extracts in SDS-sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.00125% bromophenol blue) were subjected to SDS–polyacrylamide gel electrophoresis, then transferred onto nitrocellulose membranes by electrotransfer. The membranes were blocked with 5% skim milk in Tris-buffered saline (20 mM Tris–HCl, pH7.6, 137 mM NaCl) containing 0.1% Tween 20, and incubated with anti-mouse Hsp105 [18], anti-human Hsp70 (clone BRM-22, Sigma, St. Louis, MI, USA), anti-α-tubulin (clone DM 1A, Sigma), or anti-luciferase (Sigma) antibody for 16 h at 4 °C. After being washed with TTBS, the membrane was further incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. Then, the antibody–antigen complexes were detected using the Western blot luminal reagent (Santa Cruz Biotechnology).

3. Results and discussion

We showed previously that Hsp105α and Hsp105β suppress the apoptosis induced by staurosporine or H_2O_2 using HeLa cells with doxycycline-regulated expression of Hsp105α or Hsp105β, which suggested their important role in the protection against stress-induced apoptosis in mammalian cells [19,20]. However, *hsp105/110* knockout mice are resistant to ischemic injury, and the role of Hsp105 in the cellular response to a variety of stresses is not fully understood. In the present study, we first compared the sensitivity to various stresses of Hsp105-deficient cells. As shown in Fig. 1A, *hsp105*$^{-/-}$ MEFs were more sensitive to heat shock than *hsp105*$^{+/+}$ MEFs. In addition, deficiency of Hsp105 also enhanced the sensitivity to staurosporine and H_2O_2 (Fig. 1B and C). To determine whether the decrease in the viability of cells is due to apoptosis, we next examined whether nuclear morphological changes were induced by these treatments. As shown in Fig. 1D, apoptotic

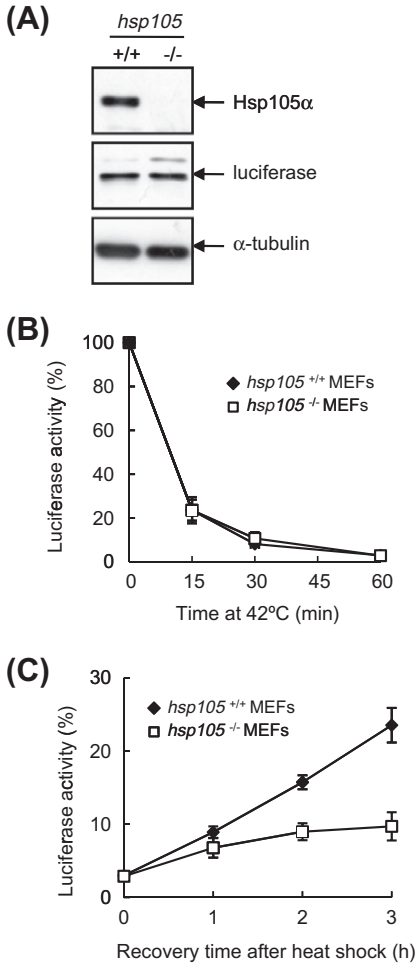

Fig. 3. Deficiency of Hsp105 impairs recovery of heat-inactivated luciferase in mouse embryonic fibroblasts. (A) *hsp105*$^{+/+}$ MEFs and *hsp105*$^{-/-}$ MEFs were transiently transfected with pGL3-control (encoding firefly luciferase), and incubated further at 37 °C for 72 h. Then, the cells were harvested and analyzed for expression of Hsp105α, luciferase, and α-tubulin by Western blotting using respective antibodies. (B and C) *hsp105*$^{+/+}$ MEFs and *hsp105*$^{-/-}$ MEFs were transfected as described above. At 72 h after transfection, the cells were treated with cycloheximide to inhibit new protein synthesis, and heated at 42 °C for the indicated time (B) or allowed to recover at 37 °C for 0–3 h after heat shock at 42 °C for 60 min (C). Samples were taken at the indicated time points, and luciferase activity was measured and plotted relative to the activity prior to heat shock (=100%). Values represent the means ± SD of three independent experiments performed in duplicate.

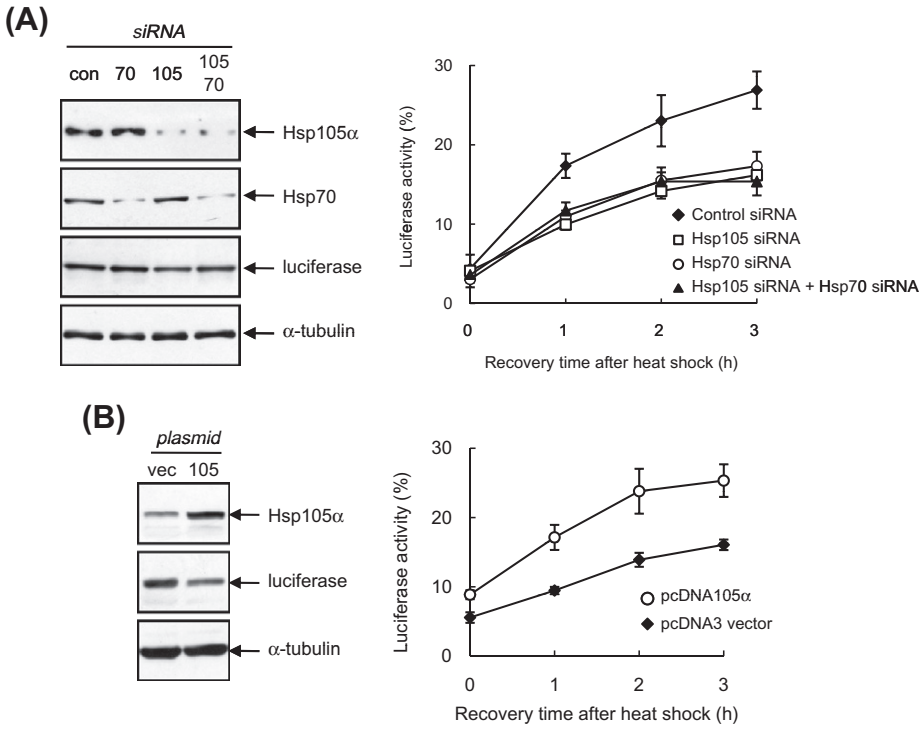

Fig. 4. Role of Hsp105 in the recovery of heat-inactivated luciferase in C3H10T1/2 cells. (A) C3H10T1/2 cells were transfected with pGL3-control together with Hsp105 siRNA and/or Hsp70 siRNA, and incubated further at 37 °C for 72 h. Then, the cells were harvested and analyzed for the expression of Hsp105α, Hsp70, and luciferase with α-tubulin as a loading control by Western blotting using respective antibodies. At 72 h after transfection, the cells were treated with cycloheximide to inhibit new protein synthesis, heated at 42 °C for 60 min, and allowed to recover at 37 °C for 0–3 h. Samples were taken at the indicated time points, and luciferase activity was measured and plotted relative to the activity prior to heat shock (=100%). Values represent the means ± SD of four independent experiments performed in duplicate. (B) C3H10T1/2 cells were transfected with pGL3-control together with pcDNA105α or pcDNA3 empty vector, and incubated further at 37 °C for 72 h. Then, the cells were harvested and analyzed for the expression of Hsp105α and luciferase with α-tubulin as a loading control by Western blotting using respective antibodies. At 72 h after transfection, the cells were treated with cycloheximide to inhibit new protein synthesis, heated at 42 °C for 60 min, and allowed to recover at 37 °C for 0 to 3 h. Samples were taken at the indicated time points, and luciferase activity was measured and plotted relative to the activity prior to heat shock (=100%). Values represent the means ± SD of three independent experiments performed in duplicate.

morphology such as nuclear condensation and chromatin fragmentation was prominently observed by Hoechst 33342 staining in these cells. Furthermore, the rates of apoptotic cells in *hsp105*$^{-/-}$ MEFs were significantly increased compared with those in *hsp105*$^{+/+}$ MEFs (Fig. 1E). To further confirm the contribution of Hsp105 to the stress-induced apoptosis, Hsp105 siRNA was transfected into mouse C3H10T1/2 cells to knockdown endogenous Hsp105α expression. Transfection of Hsp105 siRNA significantly reduced endogenous Hsp105α expression in C3H10T1/2 cells (Fig. 2A). As shown in Fig. 2B–D, down-regulation of Hsp105α expression also enhanced the sensitivity to heat shock, staurosporine, and H_2O_2. In addition, the apoptotic cells, in which nuclear condensation and chromatin fragmentation were observed, were significantly increased by Hsp105α knockdown (Fig. 2E and F). These results suggest that Hsp105 is necessary for suppression of stress-induced apoptosis in mammalian cells.

Hsp105/110 family members suppress the aggregation of heat-denatured proteins *in vitro* [7,21,22]. In addition, the yeast Hsp105/110 homolog, Sse1p, has been shown to be an important component of the folding machinery for newly synthesized proteins [10] and heat-denatured proteins [11]. However, human and yeast Hsp105/110 proteins show significant differences in their biochemical properties, such as thermostability and intrinsic ATPase activity [13]. To assess the role of Hsp105 in the refolding of denatured proteins *in vivo*, plasmid pGL3-control encoding firefly luciferase was transiently transfected into *hsp105*$^{+/+}$ MEFs and *hsp105*$^{-/-}$ MEFs (Fig. 3A). Firefly luciferase is a monomeric thermolabile protein, and the heat-inactivated luciferase in mammalian cells is capable of partially reactivating depending on the chaperone activity of Hsp70 [23–26]. At 72 h after transfection of pGL3-control, the cells were treated with cycloheximide to prevent new protein synthesis, and then heat-shocked at 42 °C. As shown in Fig. 3B, the luciferase activity decayed in a time-dependent manner, but deficiency of Hsp105 had no effect on the heat-inactivation of luciferase. However, the heat-inactivated luciferase was recovered to about 25% of the initial enzyme activity in *hsp105*$^{+/+}$ MEFs after incubation at 37 °C for 3 h, whereas in *hsp105*$^{-/-}$ MEFs the reactivation of luciferase reached only about 10%. To confirm the contribution of Hsp105 to the refolding of heat-denatured luciferase in mammalian cells, Hsp105 siRNA was co-transfected with pGL3-control into mouse C3H10T1/2 cells to knockdown endogenous Hsp105α expression. In agreement with the above studies, down-regulation of Hsp105α expression diminished the recovery of heat-inactivated luciferase (Fig. 4A). Similar effects were detected in human HEK293 cells (data not shown). In contrast, when Hsp105α was overexpressed, the recovery of heat-inactivated luciferase was increased compared with that in cells transfected with pcDNA3 vector alone (Fig. 4B). These results suggest that Hsp105/110 family members also play an important role in the refolding of heat-denatured proteins as a component of the protein folding machinery in mammalian cells as well as in yeast.

Hsp105/110 family members exist as complexes associated with Hsp70 family members [8,27,28]. Recently, it has been shown that Hsp105/110 family members act as nucleotide exchange factors for cytosolic Hsp70s *in vitro*, suggesting that Hsp105/110 family members cooperate with Hsp70 in the refolding of denatured proteins [10–12]. Therefore, we next examined whether the knockdown of Hsp105α had additional effects on the refolding of heat-

denatured luciferase in Hsp70-deficient cells. As shown in Fig. 4A, down-regulation of Hsp70 expression diminished the recovery of heat-inactivated luciferase similar to that of Hsp105α-knockdown cells. However, the knockdown of Hsp70 had no additional effects on the refolding of heat-denatured luciferase in Hsp105α-knockdown cells. These results are consistent with the idea that Hsp70 cooperates with Hsp105α in refolding the denatured proteins [10–12,29].

In this study, we showed that Hsp105α is an important element of the Hsp70 chaperone machinery for refolding of denatured proteins and protection against stress-induced cell death in mammalian cells. These findings may provide an important insight into the puzzle of the chaperone system in mammalian cells.

Acknowledgments

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