J. Biochem. 132, 271–278 (2002)

Hsp105α Enhances Stress-Induced Apoptosis but Not Necrosis in Mouse Embryonal F9 Cells[1]

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Received April 5, 2002; accepted June 4, 2002

Hsp105α, which belongs to the HSP105/110 family, is expressed at especially high levels in the brain in mammals and has been shown to prevent stress-induced apoptosis in neuronal cells. This protein is also expressed transiently at high levels during mouse embryogenesis, and is characteristically found in apoptotic cells and bodies in embryos. In the present study, to elucidate a role of Hsp105α in embryonal cells, we established Hsp105α-overexpressing F9 cells, and examined the effect of Hsp105α on cell death induced by etoposide, heat shock or cycloheximide. Apoptotic cell death was induced in cells treated with etoposide or heat shock, and necrotic cell death was induced in cells by cycloheximide. The apoptosis was enhanced by overexpression of HSP105α, whereas the necrosis was not affected by overexpression of HSP105α. Furthermore, Hsp105α seemed to modulate the stress-induced apoptosis at different steps of the apoptotic process depending on the stress stimuli. The present findings together with the previous observation on neuronal cells suggested that Hsp105 has opposite effects on stress-induced apoptosis depending on the cell type; a pro-apoptotic effect in embryonal cells and an anti-apoptotic effect in neuronal cells. The apoptosis-enhancing activity of Hsp105α may play an important role during embryogenesis.

Key words: apoptosis, embryogenesis, F9 cells, Hsp105α, necrosis.

Apoptosis defines a type of regulated cell death associated with various morphological features that include cell shrinkage, plasma membrane blebbing, and chromatin condensation, and is a fundamental and indispensable process during normal embryonic development, tissue homeostasis and regulation of the immune system (*1–3*). In addition, environmental stresses such as heat shock, radiation, chemical agents, and oxidative stress can also induce apoptosis (*4–6*). The induction of apoptosis by diverse stimuli is associated with activation of aspartate-specific cysteine proteases (caspases), which are activated in a proteolytic cascade to cleave specific substrates (*7*). Activated caspases cleave several substrates such as poly(ADP-ribose)polymerase (PARP) (*8, 9*) and DNA fragmentation factor 45/inhibitor for caspase-activated deoxyribonuclease (*10, 11*), and activate death effector molecules or trigger the characteristic structural changes of apoptotic cells.

Heat shock proteins (Hsps) comprise a set of highly conserved proteins that are induced in response to physiological and environmental stresses, and protect cells from the cytotoxic effects of aggregated proteins produced by various types of stress (*12*). These proteins are also expressed under physiological conditions, and play important roles in the synthesis, folding, translocation and assembly/disassembly of proteins as molecular chaperones. Mammalian Hsps are classified into several families on the basis of their apparent molecular masses and structures, such as HSP105/110, HSP90, HSP70, HSP60, HSP40, and HSP27. Recent studies have shown that Hsp90, Hsp70, and Hsp27 modulate the functions of several major components of apoptotic processes, such as the caspase cascade and c-Jun N-terminal kinase (JNK) signaling pathway (*13–18*).

Studies in our laboratory have focused on two members of a diverged subgroup of the HSP70 family (HSP105/110 family), Hsp105α and Hsp105β. The former is a constitutively expressed 105-kDa stress protein in most tissues of mammals and mammalian cell lines, and its expression is induced by a variety of stressors, whereas the latter is an alternatively spliced form of Hsp105α that is specifically induced by heat shock at 42°C. The predicted secondary structures of these proteins are composed of amino-terminal ATP binding, β-sheet, loop, and carboxyl-terminal α-helical domains, homologous to those of HSP70 family proteins (*19, 20*). Hsp105α and Hsp105β exist as complexes associated with Hsp70/Hsc70 (*21, 22*), negatively regulate the Hsp70/Hsc70 chaperone activity, and prevent aggregation of proteins caused by heat shock *in vitro* (*23*). Hsp105α protects neuronal PC12 cells from apoptosis induced by various stresses (*24*). Furthermore, we have also shown that Hsp105α increases transiently in most tissues of mouse embryos from gestational day 9 to 11, and is localized not only in various tissue cells but also in apoptotic cells and apoptotic bodies, suggesting that Hsp105α may play important roles in apoptosis during mouse embryogenesis (*25*).

[1] This study was supported in part by a grant from the Ministry of Education, Science, Sports and Culture of Japan (to T.H.).

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Abbreviations: Hsp, heat shock protein; PARP, poly(ADP-ribose)-polymerase; JNK, c-Jun N-terminal kinase; p38 MAPK, p38 mitogen–activated protein kinase.

In the present study, to elucidate the roles of Hsp105α in the stress-induced cell death in mouse embryonal cells, we examined the effect of Hsp105α on the cell death of embryonal cells induced by etoposide, heat shock or cycloheximide using the Hsp105α-overexpressing F9 cells, and showed that Hsp105α enhanced the apoptosis but not necrosis in F9 cells.

MATERIALS AND METHODS

Construction of a Mouse Hsp105α Expression Plasmid and Isolation of Hsp105α-Overexpressing Cells—Plasmid pcDNA105α was used to express mouse Hsp105α in F9 cells. To construct this plasmid, the mouse Hsp105α cDNA derived from the pB105-1 plasmid (*19*) was subcloned into the *Eco*RV–*Xba*I sites of mammalian expression vector pcDNA3 (Invitrogen).

F9 cells were transfected with the pcDNA105α or pcDNA3 empty vector by lipofection using Superfect reagent (Qiagen) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were maintained in complete medium containing 400 μg/ml geneticin (Life Technologies) for 3 weeks to select geneticin-resistant cells. The surviving cell clones were isolated and grown in complete medium containing 200 μg/ml geneticin, and then the expression levels of Hsp105α were determined by Western blotting using anti–mouse Hsp105 antibodies.

Cell Culture and Stress Treatments—Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (Nissui Pharmaceutical) supplemented with 10% fetal bovine serum (Life Technologies) under a humidified atmosphere of 5% CO_2 in air at 37°C.

For heat shock treatment, exponentially growing cells in culture dishes were treated in a water bath set at 44°C for 30–90 min, and then further incubated at 37°C for 24 h. For chemical stress treatments, cells were incubated in the medium containing 0.1–2 μg/ml cycloheximide or 0.125–2 μg/ml etoposide at 37°C for 24 h.

Cell Viability Assay—Heat-shocked cells were recovered by harvesting the dead cells suspended in the medium, followed by detachment of the remaining adherent cells by trypsinization. Both the suspended and adherent fractions were combined and centrifuged at 400 ×*g* for 5 min, and the cell pellets were resuspended in PBS. Aliquots of the cell suspension were mixed with an equal amount of 0.4% trypan blue. The rates of viable cells that excluded the dye in the total cell population were calculated.

To estimate cell viability after chemical stress treatments, cells were incubated in medium containing 50 μg/ml neutral red at 37°C for 3 h, and then 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 then the absorbance at 540 nm was measured.

DNA Fragmentation Analysis—Cells were lysed at 37°C for 30 min in 200 μl of lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 0.5 mg/ml RNase A, 0.5 mg/ml proteinase K), and the cell lysates were mixed with 300 μl of a NaI solution (6 M NaI, 10 mM Tris-HCl, pH8.0, 13 mM EDTA, 0.5% sodium *N*-lauroylsarcosine, 30 μg/ml glycogen) and then incubated at 60°C for 15 min. An equal volume of isopropanol was added to the mixtures, followed by vigorous shaking and standing for 15 min at room temperature. After centrifugation at 15,000 ×*g* for 15 min, the precipitate was successively washed with 50 and 100% isopropanol, dried in air, and resolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Aliquots of 5 μg of DNA were electrophoresed on 2% agarose gels and stained with 1 μg/ml ethidium bromide.

Morphological Examination of Apoptotic Cells—Cells were plated onto coverslips at a density of 1×10^5 cells/cm^2 and then grown at 37°C for 24 h. After treatment, the cells were washed with PBS, fixed with 3.7% formaldehyde for 30 min at room temperature, and then stained with 10 μM Hoechst 33342 for 10 min in the dark. After washing with PBS, the stained cells were observed under a fluorescence microscope (Zeiss). Cells were scored as apoptotic if they exhibited nuclear fragmentation and/or chromatin condensation. At least 200 cells were counted in each experiment, and data are presented as the means ± SD of three independent experiments.

Assay for PARP Cleavage—Cells were lysed with 200 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM phenyl methyl sulfonyl fluoride) on ice for 1 h. The lysate was then sonicated for 10 sec and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was recovered as the cell extract. Aliquots (20 μg of protein) of the cell extracts in SDS-sample buffer containing urea (62.5 mM Tris-HCl, pH6.8, 6 M urea, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.00125% bromophenol blue) were subjected to 7.5% SDS-PAGE, and then transferred to nitrocellulose membranes by electrotransfer. The membranes were blocked with 10% skim milk in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween 20 (TTBS), and then incubated with anti-PARP antibodies (Santa Cruz). Then, the membranes were incubated with horseradish peroxidase (HRP)–conjugated anti–rabbit IgG antibodies, and the antibody-antigen complexes were detected using the ECL-Western blot detection system (Amersham Pharmacia Biotech).

Activation of Caspase-3 and Caspase-9—Cells were lysed with 0.1% SDS and then 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, 0.00125% bromophenol blue) were subjected to 12.5% SDS-PAGE, and then analyzed by Western blotting using anti–caspase-9 antibodies which react with both procaspase-9 and active caspase-9 (Cell Signaling Technology), and anti–caspase-3 antibodies which react with active caspase-3 (Cell Signaling Technology), respectively. The antibody-antigen complexes were detected using the ECL-Western blot detection system (Amersham Pharmacia Biotech).

Release of Cytochrome c from Mitochondria—Release of cytochrome *c* was analyzed as described previously (*26*). Briefly, cell suspensions were mixed with an equal volume of 100 μg/ml digitonin in PBS, and then incubated at 25°C for 5 min. After centrifugation at 15,000 ×*g* for 2 min, the supernatants were recovered as cytosolic fractions. The pellets were dissolved in PBS containing 0.5% Triton X-100, and centrifuged, and then the supernatants were recovered as mitochondrial fractions. Both fractions were subjected to 15% SDS-PAGE, and analyzed by Western blotting using anti–cytochrome *c* antibodies (Santa Cruz).

Activation of JNK and p38 Mitogen-Activated Protein Kinase (p38 MAPK)—Phosphorylated JNK and p38 MAPK were detected by Western blotting using PhosphoPlus JNK (Thr183/Tyr185) and PhosphoPlus p38 (Thr180/Tyr182) antibody kits (Cell Signaling Technology), respectively. Cells were lysed with 0.1% SDS and then boiled for 5 min. Cell extracts (20 μg of protein) were subjected to 10% SDS-PAGE, and analyzed by Western blotting using phosphorylation state-specific anti-JNK or anti–p38 MAPK antibodies. Then, the membranes were incubated at 50°C for 30 min in stripping buffer (62.5 mM Tris HCl, pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol), and total JNK or p38 MAPK on the same membranes was detected using anti-JNK or anti-p38 antibodies, respectively.

RESULTS

Enhancement of Stress-Induced Apoptosis by Overexpression of Hsp105α in F9 Cells—We have shown that Hsp105α is expressed transiently at high levels during mouse embryogenesis and is localized in apoptotic cells (*25*). To determine the effect of Hsp105α on stress-induced cell death in mouse embryonal cells, we first established Hsp105α-overexpressing F9 cells. Here, we used two Hsp105α-overexpressing F9 cell lines, S3 and S23, in which the expression levels of Hsp105α were about 2- and 3-fold higher than that in parental F9 cells or cells transfected with the empty pcDNA3 vector (V1), respectively (Fig. 1). When the sensitivity of cells to etoposide, heat shock or cycloheximide was examined using the Hsp105α-overexpressing F9 cells, S3 and S23 cells were found to be more sensitive to etoposide and heat shock than F9 or V1 cells (Fig. 2). In contrast, the sensitivity to cycloheximide was not affected by overexpression of Hsp105α. Furthermore, overexpression of Hsp105α also enhanced the sensitivity to actinomycin D and serum deprivation (data not shown).

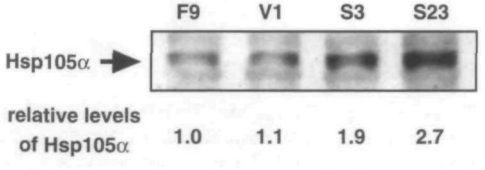

Fig. 1. **Levels of expression of Hsp105α in the parental F9 cells and Hsp105α– or vector-transfected cells.** Cell extracts (20 μg of protein) of parental F9 cells (F9) and cell clones stably transfected with either the pcDNA3 vector (V1) or pcDNA105α (S3 and S23) were separated by SDS-PAGE, and then the levels of Hsp105α were determined by Western blotting using anti-mouse Hsp105 antibodies. The relative levels of Hsp105α are expressed as the ratios of that of Hsp105α in parental F9 cells.

As cell death is classified into two morphologically and biochemical distinct modes, apoptosis and necrosis (*1*), we next examined whether apoptotic features such as DNA fragmentation and nuclear morphological changes were induced by these stress treatments. As shown in Fig. 3A, nucleosomal-length DNA fragmentation was observed in F9 cells treated with etoposide or heat shock, and Hoechst 33342 staining of these cells demonstrated that condensation of chromatin also prominently occurred with these treatments (Fig. 3B). The rate of apoptotic cells was approximately 2-fold higher among the Hsp105α-overexpressing cells than among F9 or V1 cells (Fig. 3C). In contrast, neither DNA fragmentation nor chromatin condensation was induced in F9 cells treated with cycloheximide (Fig. 3). Furthermore, as the cleavage of PARP was not detected in F9 cells treated with cycloheximide (data not shown), cycloheximide seemed to induce necrotic cell death. Thus, Hsp105α was suggested to enhance stress-induced apoptosis but not necrosis in embryonal F9 cells.

Overexpression of Hsp105α Enhances PARP Cleavage Induced by Heat Shock but Not Etoposide—A common event in the apoptotic pathway is the activation of caspases (*7*). These enzymes participate in a cascade that is triggered in response to pro-apoptotic signals and results in the cleavage of a set of substrate proteins. Caspase-3 is a major effector caspase, and PARP, a DNA repair-related enzyme, is an important substrate of caspase-3, and is cleaved from a 116-kDa protein into an 85-kDa fragment (*8, 9*). As shown in Fig. 4A, although cleavage of PARP seemed to be slightly increased by overproduction of Hsp105α, the low level cleavage of PARP induced in F9 cells by etoposide was not enhanced by overexpression of Hsp105α. In contrast, the amounts of full length 116-kDa PARP were markedly decreased in F9 cells treated with heat shock at 44°C, and the heat-induced cleavage of PARP was markedly enhanced by overexpression of Hsp105α (Fig. 4B).

Overexpression of Hsp105α Enhances Activation of Caspase-3 and Caspase-9 Induced by Heat Shock but Not Etoposide—Caspases are synthesized as inactive precursor

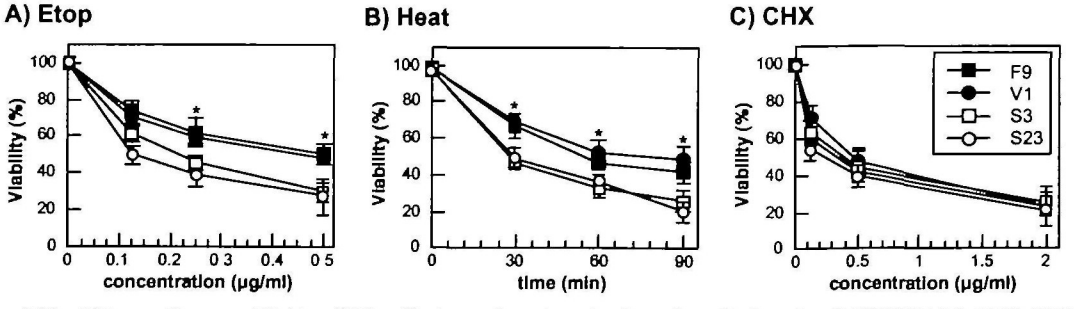

Fig. 2. **Effects of Hsp105α on the sensitivity of F9 cells to cycloheximide, etoposide, and heat shock.** F9, V1, S3, and S23 cells were exposed to 0.125–0.5 μg/ml etoposide (A) or 0.1–1.0 μg/ml cycloheximide (C) at 37°C for 24 h, or were treated at 44°C for 30–90 min and further incubated at 37°C for 24 h (B). Cell viability was then determined as described under "MATERIALS AND METHODS." The values presented as the means ± SD of three independent experiments. The significance of differences was assessed by means of the unpaired Student's *t*-test. A probability level (*p*) of less than 0.05 was considered statistically significant and is indicated by asterisks.

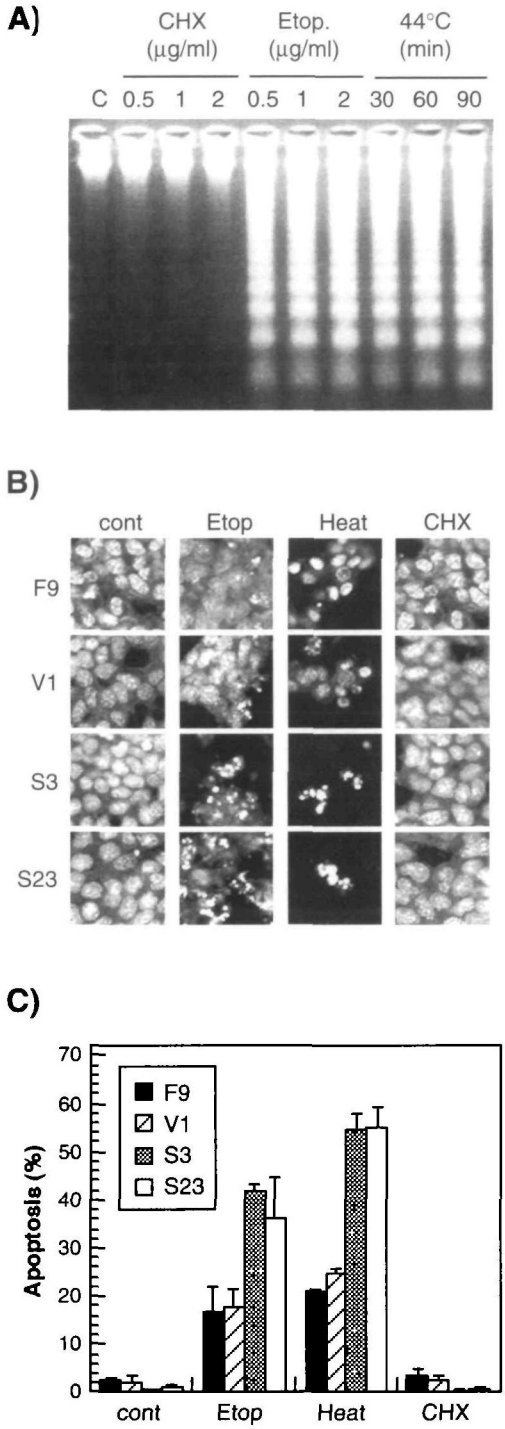

Fig. 3. **Enhancement of stress-induced apoptosis by Hsp105α.** A: DNA was extracted from F9 cells treated with cyclohexamide, etoposide and heat shock, and aliquots (5 μg each) of DNA were electrophoresed on a 2% agarose gel and visualized by staining with ethidium bromide. B: F9, V1, S3, and S23 cells were exposed to 1 μg/ml cyclohexamide or 1 μg/ml etoposide for 24 h, or were treated at 44°C for 15 min and further incubated at 37°C for 24 h. The cells were then fixed and stained with Hoechst 33342, and then the nuclear morphology of cells was observed under a fluorescence microscope. C: The rates of apoptotic cells were calculated for at least 200 cells in each experiment. The values shown are the means ± SD of three independent experiments.

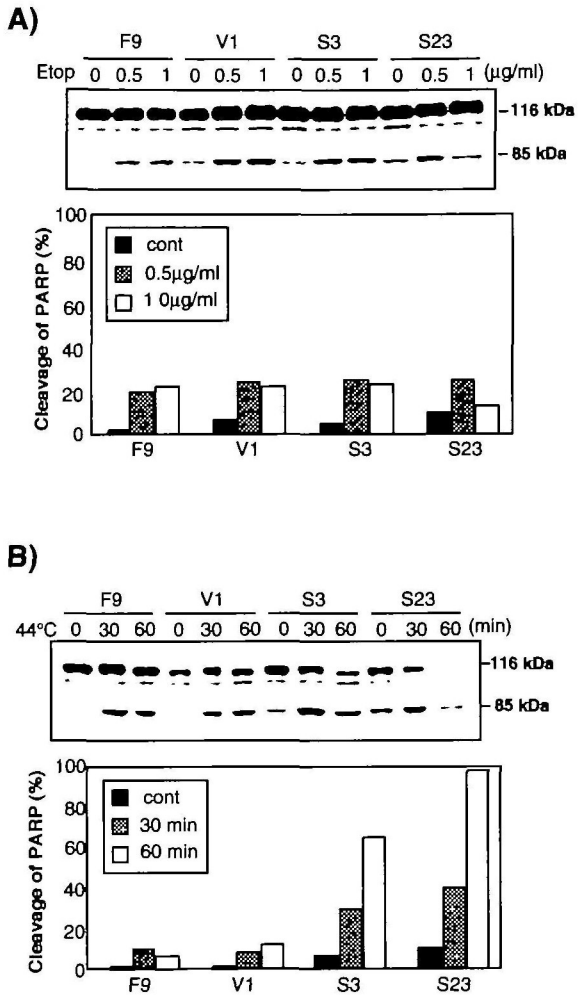

Fig. 4. **Effects of Hsp105α on the PARP cleavage caused by treatment with etoposide or heat shock.** F9, V1, S3, and S23 cells were exposed to etoposide for 24 h (A), or were treated at 44°C for 30 min and further incubated at 37°C for 24 h (B). Aliquots (20 μg of protein) of cell extracts were separated by SDS-PAGE, and then intact PARP (116 kDa) and the cleaved fragment (85 kDa) were detected by Western blotting using anti-PARP antibodies. The densities of the bands of 116-kDa PARP and the 85-kDa fragment were determined by densitometry. The rates of cleaved PARP are shown for etoposide-treated cells. For heat-shocked cells, the rates of decrease of 116-kDa PARP are shown. The values shown are the means of two independent experiments.

molecules (pro-caspases), and are converted through proteolytic cleavage into active forms in response to an apoptotic stimulus (27). As overexpression of HSP105α enhanced the PARP cleavage, we next examined whether the activation of pro-caspase-3 and upstream pro-caspase-9 was enhanced by overexpression of HSP105α in F9 cells. As shown in Fig. 5, B and D, the active forms of caspase-3 (p17) and caspase–9 (p39 and p37) were clearly detected in F9 cells treated with heat shock. The increases in both active caspases were enhanced in the Hsp105α-overexpressing cells compared to in F9 or V1 cells by heat shock. Thus, Hsp105α was suggested to enhance the activation of pro-caspase-9, and then subsequently the activation of caspase-3 during apoptosis induced by heat shock. In contrast, the cleaved forms of caspase-3 and caspase–9 were

not clearly detected in these cells treated with etoposide (Fig. 5, A and C).

Overexpression of Hsp105α does Not Affect the Release of Cytochrome c from Mitochondria Induced by Etoposide and Heat Shock—In mammalian cells, one of the main pathways that activates caspase-9 is one *via* mitochondria. When mitochondria receive appropriate signals from a variety of stresses or are damaged irreversively, pro-apoptotic molecules such as cytochrome *c* are released from the mitochondria into the cytosol (*28–30*). In the cytosol, cytochrome *c* forms a complex with Apaf-1 and procaspase-9, and activates caspase-9, which in turn converts procaspase-3 into its active form, and executes apoptosis (*31–33*). We next examined whether cytochrome *c* is released from mitochondria by etoposide or heat shock (Fig. 6). When F9 cells were fractionated into mitochondrial and cytosolic frac-

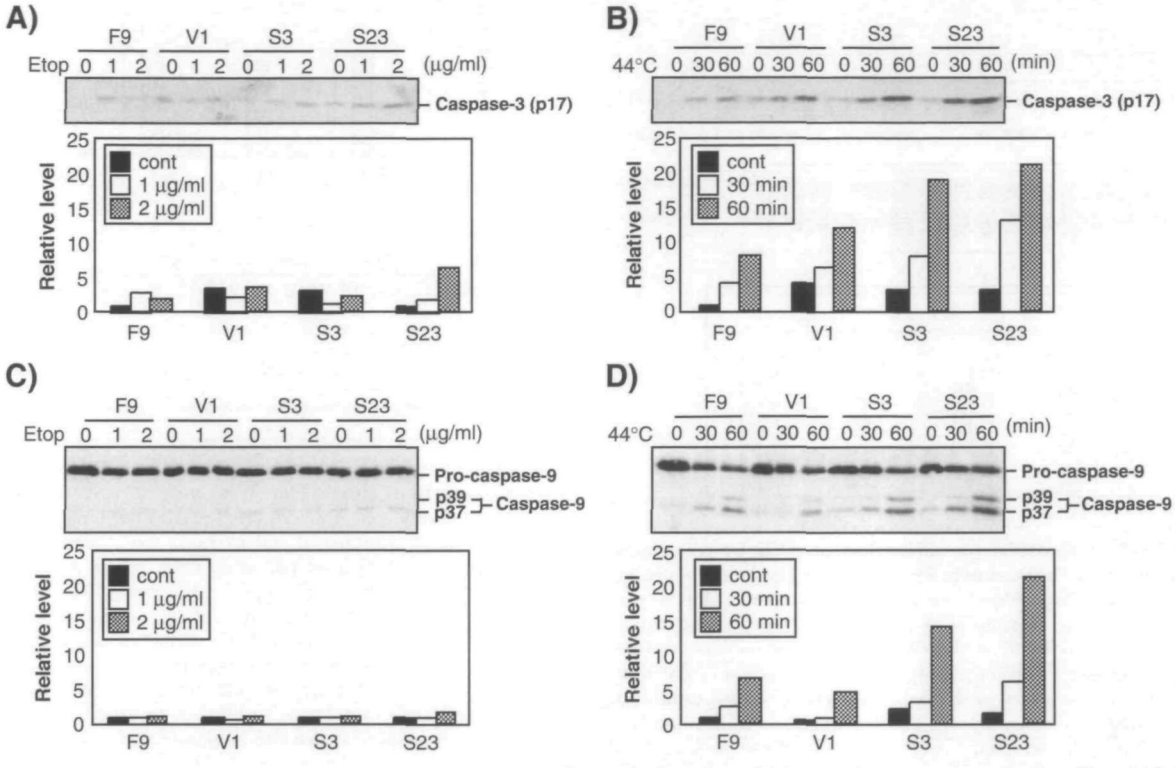

Fig. 5. **Effects of Hsp105α on the cleavage of pro-caspase-3 and pro-caspase-9 caused by treatment with etoposide or heat shock.** F9, V1, S3, and S23 cells were exposed to 1 or 2 μg/ml etoposide for 24 h (A and C), or were treated at 44°C for 30 or 60 min and further incubated for 24 h at 37°C (B and D). Aliquots (20 μg of protein) of cell extracts were separated by SDS-PAGE, and then analyzed by Western blotting using anti–active form specific caspase-3 antibodies (A and B) or anti–caspase-9 antibodies (C and D). The active forms of caspase-3 and caspase-9 are indicated as p17 and p37/p39, respectively. The densities of the bands of activated caspase-3 and caspase-9 were determined by densitometry, and the relative levels of activated caspase-3 and caspase-9 are shown as fractions of the untreated F9 cells, respectively.

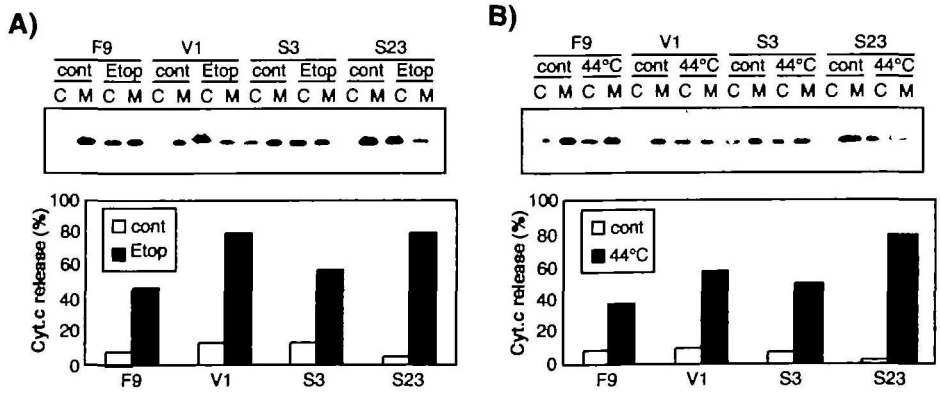

Fig. 6. **Effect of HSP105α on the release of cytochrome *c* from mitochondlia caused by treatment with etoposide or heat shock.** F9, V1, S3, and S23 cells were exposed to 2 μg/ml etoposide for 24 h (A), or were treated at 44°C for 30 min and further incubated for 24 h at 37°C (B). The cells were then fractionated into cytosolic and mitochondrial fractions. Both fractions were subjected to 15% SDS-PAGE, and then analyzed by Western blotting using anti-cytochrome *c* antibodies. The densities of the bands of cytochrome *c* were determined by densitometry, and the rates of cytochrome *c* release from mitochondria are shown. The values shown are the means of two independent experiments. C, cytosolic fraction; M, mitochondrial fraction.

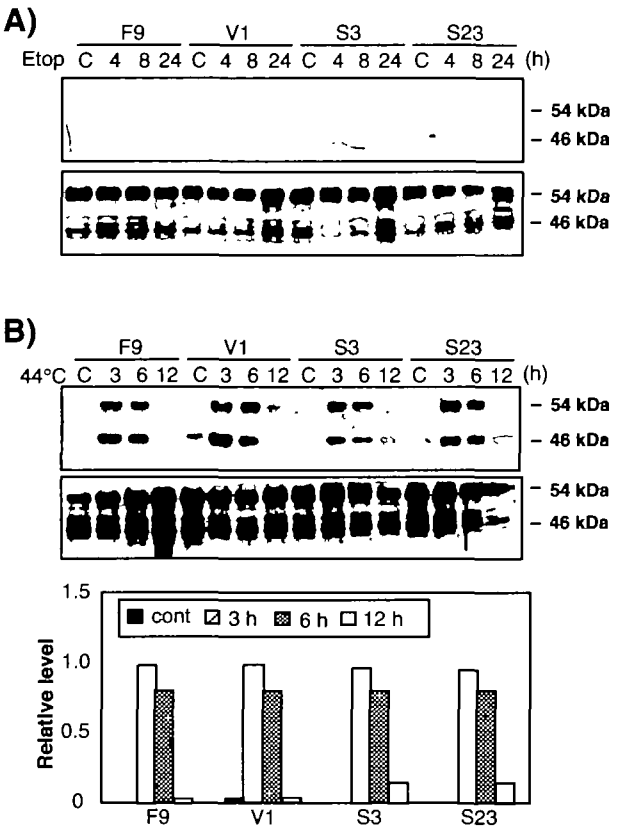

Fig. 7. **Effect of Hsp105α on activation of JNK by treatment with etoposide or heat shock.** F9, V1, S3, and S23 cells were exposed to 1 μg/ml etoposide (A), or were treated at 44°C for 30 min (B). Aliquots (20 μg of protein) of cell extracts were separated by SDS-PAGE. Activated and total JNK were detected by Western blotting. The upper and lower panels represent activated and total JNK, respectively. The densities of the bands of activated and total JNK were determined by densitometry, and the densities of the bands of activated JNK were corrected as to the densities of the bands of total JNK. The relative levels of activated JNK are shown as fractions of the maximal JNK activation levels at 3h after heat shock. The values shown are the means of two independent experiments.

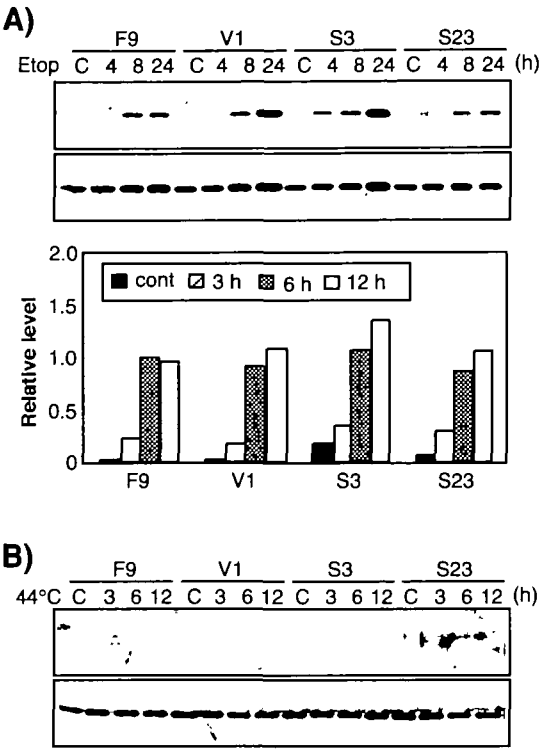

Fig. 8. **Effect of Hsp105α on activation of p38 by treatment with etoposide or heat shock.** F9, V1, S3, and S23 cells were exposed to 1 μg/ml etoposide (A), or were treated at 44°C for 30 min (B). Aliquots (20 μg of protein) of cell extracts were separated by SDS-PAGE. Activated and total p38 were detected by Western blotting. The upper and lower panels represent activated and total p38, respectively. The densities of the bands of activated and total p38 were determined by densitometry, and the densities of the bands of activated p38 were corrected as to the densities of the bands of total p38. The relative levels of activated p38 are shown as fractions of the maximal p38 activation levels in F9 cells treated with etoposide for 24 h. The values shown are the means of two independent experiments.

tions, and cytochrome *c* was detected by Western blotting analysis, a large amount of cytochrome *c* was found in mitochondria with only a small amount in the cytosolic fraction of control cells. The amount of cytochrome *c* released into the cytosol was markedly increased by treatment with etoposide or heat shock. However, the amounts of cytochrome *c* released in control cells were not different from those in S3 and S23, suggesting that Hsp105α did not affect the release of cytochrome *c* from mitochondria induced by etoposide or heat shock in F9 cells.

Effect of Overexpression of Hsp105α on Activation of JNK and p38 MAPK after Treatment with Heat or Etoposide— Transmission of signals from external stresses is accompanied by the activation of JNK and p38 MAPK (*34*), and these signaling pathways have been implicated as key regulators of stress-induced apoptosis in many cell types (*35–39*). We next examined whether these pathways are involved in the apoptosis induced by etoposide or heat shock in F9 cells. As shown in Fig. 7, JNK was activated by heat shock but not etoposide. JNK activation lasted for at least 6 h after heat shock and had decreased to the basal levels by 12 h. However, overexpression of Hsp105α did not enhance or suppress the activation of JNK. Furthermore, although p38 MAPK was activated by etoposide but not heat shock, overexpression of Hsp105α did not affect the activation of this signaling pathway (Fig. 8). Therefore, Hsp105α seemed to enhance the stress-induced apoptosis at steps downstream of the activation of these stress-activated kinases in embryonal F9 cells.

DISCUSSION

Hsp105α is expressed in most tissues, but its levels are especially high in the brain in adult mammals such as rats, mice and humans (*19, 22*). Furthermore, we showed that the levels of Hsp105α increase transiently in embryonic tissues during mouse embryogenesis, and this protein is localized not only in various tissues, but also in apoptotic cells and apoptotic bodies in the interdigital regions of limbs (*25*). In the present study, we showed that Hsp105α enhanced the sensitivity of embryonal cells to apoptosis induced by stress in F9 cells. This finding is in clear contrast

to the previous findings that Hsp105α plays an important role in the protection of neuronal PC12 cells against stress-induced apoptosis (*24*), and that Hsp110 (hamster homologue of Hsp105α) confers substantial heat resistance on rat fibroblasts and human epithelial carcinoma cells (*40*). However, Hsp105α did not enhance the non-apoptotic cell death, necrosis, induced by cycloheximide. The present findings together with previous observations on neuronal cells suggested that Hsp105α has opposite effects on stress-induced apoptosis depending on the cell type; a pro-apoptotic effect in embryonal cells and an anti-apoptotic effect in neuronal cells. These observations constitute the first evidence that Hsp105α can function as an enhancer or suppressor of apoptosis depending on the cell type in mammals.

Apoptosis is an active process resulting in characteristic morphological changes such as cell shrinkage, condensation of chromatin and membrane blebbing (*1–3*). The common pathway of apoptosis involves a family of proteases known as the caspases, which are activated in a proteolytic cascade to cleave specific substrates (*7*). The release of cytochrome *c* from mitochondria triggers the formation of an apoptosome complex with Apaf-1 and pro-caspase-9, and activates caspase-9, which then in turn activates downstream effector caspases such as caspase-3 (*28–30*). Active caspase-3 cleaves several substrates such as PARP (*8, 9*), and activates death effector molecules or triggers the structural changes characteristic of apoptotic cells. Here, we showed that overexprssion of Hsp105α enhanced the cleavage of PARP, and the activation of caspase-3 and caspase-9, but had no effect on the release of cytochrome *c* from mitochondria in F9 cells treated with heat shock. Thus, it is probable that HSP105α enhances the heat-induced apoptosis at a step of activation of caspase-9.

In tumor cells, topoisomerase II inhibitor etoposide can induce apoptosis in a tumor suppressor protein p53-dependent manner. During etoposide-induced apoptosis, pro-apoptotic protein Bax is up-regulated, and the release of cytochrome *c* from mitochondria, and the activation of caspases such as pro-caspases-9 and –3 are enhanced (*41, 42*). Furthermore, overexpression of a dominant negative caspase-9 suppresses etoposide-induced apoptosis in ovarian cancer cells (*41*), and caspase-3 inhibitor DEVD-CHO also suppresses the apoptosis in human glioma cells (*43*). However, we did not detect the cleaved and active forms of caspase-3 and caspase–9 in F9 cells treated with etoposide. Furthermore, although low levels of cleavage of PARP were observed in cells treated with etoposide, overexpression of Hsp105α did not enhance the cleavage of PARP in F9 cells. Thus, Hsp105α seemed to enhance etoposide-induced apoptosis in a caspase-3–independent manner or at steps downstream of caspase-3 in F9 cells.

Transmission of signals from external stresses is also accompanied by the activation of two families of kinases, JNK and p38 MAPK (*34*). In many cell types, JNK activation has been shown to be essential for the signal transduction that leads to apoptosis in response to heat shock, UV-irradiation anticancer drugs and certain other stimuli (*35–37*). Furthermore, the activation of p38 MAPK is involved in the apoptosis induced by heat shock and anticancer drugs in mouse mocrophage-like Raw264.7 cells and cardiomyocytes (*38, 39*). In the present study, although JNK and p38 MAPK were activated by heat shock and etoposide, respectively, overexpression of Hsp105α did not enhance the activation of JNK and p38 MAPK in F9 cells, indicating that these signaling pathways are not involved in the enhancement of stress-induced apoptosis by Hsp105α in embryonal cells.

Several Hsps have been shown to modulate the pathway of apoptosis positively or negatively. Hsp60 with or without Hsp10 directly stimulates apoptosis by promoting the proteolytic maturation of caspase-3 (*44, 45*). In contrast, Hsp70, Hsp90 and Hsp27 exert negative influences on apoptotic signaling. Hsp70 prevents apoptosis due to a variety of stressors through suppression of apoptosome formation (*13, 14*) and JNK activation (*16–18*). Interestingly, a recent study demonstrated that the chaperone activity of Hsp70 is required for protection against heat-induced apoptosis (*46*). Hsp105α exists as complexes associated with Hsp70/Hsc70 (*21, 22, 47*) and suppresses the Hsc70 chaperone activity *in vitro* (*23*). Hsp105α may stimulate stress-induced apoptosis through negative regulation of Hsp70/Hsc70 chaperone activity, although further studies are necessary to elucidate the precise mechanisms by which Hsp105 enhances stress-induced apoptosis in embryonal cells.

In conclusion, the present study demonstrated that Hsp105α enhances the apoptosis induced by a variety of stresses but not necrosis in embryonal cells. The apoptosis-enhancing activity of Hsp105α, which modulates the balance between cell death and survival, may play an important role during embryogenesis.

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