Ceramide has emerged as a mediator of cell growth, differentiation, and apoptosis in many biological systems. Many kinds of stresses are reported to induce apoptosis with an increase of ceramide generation. Here we showed that the intracellular ceramide levels increased in parallel with heat shock (HS)-induced apoptosis in an intensity- and time-dependent manner, and synthetic N-acetylphosphosine (C2-ceramide) synergistically enhanced HS-induced apoptosis in HL-60 cells. In order to know the role of ceramide generation in HS-induced apoptosis, we examined the effects of C2-ceramide on the levels of mRNA and protein of heat shock proteins (HSPs). The increase of HSP-70 mRNA levels 1–2 h after HS at 42 °C for 30 min was suppressed by C2-ceramide in a dose-dependent manner. In comparison with HSP-70, the levels of HSP-60 and -90 mRNAs were faintly suppressed by C2-ceramide. Similarly, the increase in the protein levels of HSP-70 was significantly suppressed 4–8 h after HS by C2-ceramide in a dose-dependent manner. Additionally, in 293 cells, which are constitutively overexpressing HSP-70 gene, the levels of HSP-70 mRNA were suppressed by C2-ceramide in parallel with the increase of apoptotic cells. We next examined the mechanisms by which C2-ceramide suppressed HS-induced HSP-70 expression. The treatment with C2-ceramide did not affect both an activation of a nuclear transcription factor for HSP-70, heat shock factor-1, and an increased transcriptional rate of HSP-70 by HS, but increased the rates of HSP-70 mRNA degradation. In summary, ceramide may efficiently induce HS-induced apoptosis by suppressing anti-apoptotic HSP-70 through a post-transcriptional regulation.

It is now widely accepted that programmed cell suicide called apoptosis plays an important role in embryogenesis (1), metamorphosis (2), normal tissue turnover, tumorigenesis, and an elimination of damaged cells (3). Various kinds of extracellular stresses including ultraviolet (UV), irradiation, tumor necrosis factor (TNF)-α, anti-Fas-cross-linking, viral infection, and anti-cancer reagents are known to induce apoptosis in many cell systems (4–6). Cell death by apoptotic mechanism was generally executed mainly through a network of pro-apoptotic signals such as cascade of cysteine proteases named caspase family and caspase-related DNase (7, 8). Anti-apoptotic molecules such as Bcl-2 family, CrmA, and p35 blocked the execution of apoptosis by inhibiting pro-apoptotic signals (9–11). Recently inhibitor of apoptosis protein (IAP) family including XIAP, c-IAP1, and c-IAP2 was shown to act as anti-apoptotic signal by inhibiting caspase-3, -7, and -9 activity (12, 13). These results suggest that the extent of apoptosis induction is determined by the balance of intensity between pro-apoptotic and anti-apoptotic signals.

Heat shock (HS) is one of the important apoptosis-inducing stresses and is known to synthesize a set of proteins called heat shock proteins (HSPs). HSPs are involved in the transport, folding, and assembly of the proteins and play a role in keeping cell homeostasis by functioning as molecular chaperons (14). HSPs consist of a family including HSP-90, HSP-70, HSP-60, and other small HSPs (15). In terms of the role of HSP-70 on apoptosis, it was reported that the induction of thermotolerance correlated with the increase of HSP-70 proteins in Chinese hamster fibroblasts (16) and was blocked by the inhibition of HSP-70 expression in K-562 leukemia cells (17). HS-induced thermotolerance showed the resistance to apoptosis induction by pro-apoptotic stresses including TNF-α, UV, oxidative stress, and ceramide (18, 19). Moreover, the overexpression of HSP-70 induced the resistance to TNF-α-induced cytotoxicity (20) and to ischemic heart injury (21). In contrast, the same overexpression of HSP-70 was recently reported to enhance T cell receptor/CD3- and Fas-mediated apoptosis in Jurkat cells (22). However, it seems to be in general agreement with that HSP-70 plays a role in the induction of thermotolerance because there are many reports suggesting the anti-apoptotic effects of HSP-70 by showing the inhibition of caspase-3 activation and Jun N-terminal kinase (JNK)-related pathway (19, 23, 24).

Ceramide has been recognized as a lipid mediator in the induction of apoptosis (25), since a diverse array of stresses leading to apoptosis were reported to increase ceramide levels in many cell types (26). As a downstream signal of ceramide, the transcription factors including NF-κB and c-Myc (27), serine/threonine kinases (ceramide-activated protein kinase, MAP kinase, and JNK), and phosphatases (28–31) were reported to be related to apoptosis. Recently, we demonstrated that a transcription factor AP-1 was also required to ceramide-induced

buffered saline; PBS-T, phosphate-buffered saline with Tween 20; DGR, diacylglycerol kinase; DAPI, 4,6-diamidino-2-phenylindole; IAP, inhibitor of apoptosis protein.
apoptosis (32) and that the translocation of protein kinase C δ and ε to the cytosol from the membrane was necessary to TNF-α-, Fas cross-linking-, and ceramide-induced apoptosis (33). Among a family of cysteine protein proteases called caspase, caspase-3 has been recognized to function as an executioner of apoptosis, and ceramide was known to increase the degradation of poly(ADP-ribose) polymerase and the activity of caspase-3, probably by releasing cytochrome c from mitochondria and activating caspase-9 (34, 35). Since the overexpression of HSP-70 inhibited ceramide-induced apoptosis through the inhibition of caspase-3 like protease activation (23, 36), HSP-70 may exert its anti-apoptotic effects upstream of caspase-3 induced by ceramide. However, in contrast, the direct effect of ceramide on HSP-70 remains to be clarified.

In this report, therefore, we investigated the relation of ceramide with HSP-70 in HS-induced apoptosis. We here found that HS-increased ceramide levels were closely related to the intensity of apoptosis and that synthetic N-acetyl-sphingosine (C₂-ceramide) suppressed HS-induced HSP-70 at both mRNA and protein levels. In addition, HSP-70 overexpressing cells showed the decrease of HSP-70 expression as ceramide induced apoptosis, suggesting that anti-apoptotic HSP-70 function was suppressed by pro-apoptotic ceramide-mediated signals. We also investigated the mechanisms by which ceramide suppressed the expression of HSP-70 mRNA and showed the post-transcriptional regulation by ceramide without affecting a transcription factor HSF-1 and HSP-70 transcriptional rate.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human leukemia HL-60 cells were kindly given by Dr. M. Saito (Hokkaido University). Wn-x10 and Wn-113 WEHI-S cells, which were transfected by a vector and DNA construct containing HSP-70 gene, respectively, were a kind gift from Dr. Jaap de Vries (Danish Cancer Society). Human embryonic kidney 293 cells were a kind gift from Dr. S. Miyatake (Kyoto University). N-Acetylphosphosine (C₂-ceramide) were obtained from Matraya. Other chemicals were purchased from Sigma.

**Cell Culture**—The cells were grown in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (JRH Biosciences) and kanamycin sulfate (80 mg/ml), and incubated at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were counted by a hemocytometer, and the viability was always greater than 95% in all experiments as assayed by 0.025% trypan blue dye exclusion method. Before the experiments, the cells were washed with phosphate-buffered saline (PBS) and incubated overnight in RPMI 1640 medium supplemented with 2% fetal calf serum if not described particularly.

**Heat Shock Treatment**—Before the heat shock treatment the cells were resuspended at 5 × 10⁶ cells/ml in a preheated media, immersed in the water bath (Thermomider Mini-80, AITEC, Saitama, Japan) at various temperatures for the indicated durations, and then incubated at 37 °C in 5% CO₂ for the indicated times. After heat shock, the cytopsin specimens were obtained by the centrifuge at 190 × g, 10 min on the slide glass and stained with May-Giemsa method for morphological examination.

**Measurement of Morphological Changes and DNA Fragmentation of Apoptotic Cells by Flow Cytometer**—The cells were collected with heat shock and/or C₂-ceramide at the various conditions, harvested, and then stained with May-Giemsa or DAPI (4',6-diamidino-2-phenylindole) staining method. At least 200 cells in one determination were counted under the light or fluorescent microscopy.

Flow cytometric DNA analysis was performed for quantification of cell death by apoptosis. Apoptotic cells can be detected by DNA-specific fluorochrome staining due to diminishing DNA contents (37). The cells were harvested at a concentration of 2 × 10⁶ cells/ml, washed with PBS, and resuspended in PBS containing 0.5% paraformaldehyde and 0.5% saponin for fixing the cells. The cells were washed and resuspended in fluorochrome solution containing 50 μg/ml propidium iodide and 1 mg/ml RNase (Bachem, Torrance, CA). The fluorescence of propidium iodide was measured by FACScan (Becton Dickinson) and the cells showing hypodiploid pattern were judged as apoptotic cells.

**Analysis of DNA Fragmentation**—DNA was isolated using a Apotasis Ladder detection kit (Wako, Osaka, Japan), electrophoresed through a 1.5% Nusieve agarose minigel (FMC Corp. Bio Products) in 40 mM Tris acetate and 1 mM EDTA at 100 V for 30 min, and visualized under UV light after ethidium bromide staining.

**cDNA Probes and Antibodies**—Human HSP-70 cDNA (pH 2.3) was obtained from Dr. R. Morimoto (Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL) and K. Nagata (Department of Cell Biology, Chest Disease Research Institute, Kyoto University, Kyoto, Japan). Human cDNAs of HSP-90 (pHS90αa) and β-actin were obtained from RIKEN DNA bank. Human HSP-60 probe was obtained by reverse transcription-polymerase chain reaction from the mRNA of the HL-60 cells using the 5'-primer (5'-AAGGGAGGCATGAGCAGGTCC-3') and 3'-primer (5'-CATAGGGCACCTCGTGAATTCCAC-3'), which encode of 138–243 amino acids. Monoclonal antibodies against human HSP-90 (SPA835), HSP-70 (SPA10) and HSP-60 (SPA806) were obtained from Stressgen Biotech Corp. (Victoria, British Columbia, Canada). Antiseras against HSP-1 and -2 were kind gifts of Dr. R. Morimoto. A peroxidase-conjugated anti-mouse immunoglobulin, a horseradish peroxidase-linked anti-rat immunoglobulin (NAS929) and [γ-32P]ATP were purchased from Tago, Inc. (Burlingame, CA), Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom), and Du Pont, respectively.

**Ceramide Quantitation**—After extracting the lipids according to the Bligh and Dyer method as described before (38), ceramide levels in the cells were measured enzymatically by using Escherichia coli diacylglycerol kinase (DGK) (39) and 9-cis-retinoic acid converting enzyme (DGAT) (40). The cells were sonicated in 2× SSC, 0.1% SDS at room temperature for 30 min and subsequently in 1× SSC, 0.15 mM NaCl, and 15 mM sodium citrate at 50 °C for 20 min. The membranes were exposed to Fuji x-ray films with the intensifying screens at ~80 °C for 1 or 2 days. Equal loading of RNA was confirmed by methylene blue staining of ribosomal RNAs in each sample.

**RNA Preparation and Northern Blotting**—Total RNA was prepared using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Twenty μg of total RNA was used for the Northern blotting and performed as described previously (32). Briefly, human HSP-60, -70, and -90 cDNA probes were labeled with [γ-32P]ATP using a multiprime labeling kit (Amersharm Pharmacia Biotech) according to the manufacturer's protocol. Hybridizations were performed at 42 °C for 24 h. Then the membranes were washed in 2× SSC, 0.1% SDS at room temperature for 5 min at 4 °C in order to obtain nuclei, and the supernatant was centrifuged at 100,000 × g for 20 min at 4 °C in a Beckman TL-100 s ultracentrifuge. The supernatant was collected and used as the cytosol fraction. Protein concentration was determined by using a protein assay kit (Bio-Rad).

**Western Blot Analysis**—The samples (50 μg) were denatured by boiling in Laemmli's sample buffer for 5 min, subjected to SDS-polyacrylamide gel electrophoresis using a 7.5% running gel, and electroblotted to Immobilon-P Transfer Membrane (Millipore) as described (33). Nonspecific binding was blocked by incubation of the membrane with PBS containing 5% skim milk and 0.1% Tween 20 for more than 1 h. The membrane was then washed in PBS containing 0.1% Tween 20 (PBS-T) for 15 and 5 min, and incubated with 1:2000 dilution of anti-HSP-90, -70, -60, actin, and HSF-1 and HSF-2 antibodies in PBS-T for 1 h. The membrane was washed in PBS-T for 15 and 5 min and then incubated with 1:4000 dilution of anti-mouse or rat immunoglobulin peroxidase conjugate in PBS-T for 1 h. After washing the membrane three times for 5 min each in PBS-T, detection was performed using ECL Western blotting detection reagents (Amersharm Pharmacia Biotech) according to the manufacturer's protocol.

**Nuclear Run-on Assay**—The cells (5 × 10⁷) were collected, resuspended in 4 ml of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40), and left on ice for 10 min. Nuclei were pelleted by centrifugation at 5000 × g for 5 min and resuspended in 100 μl of glycerol buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA). An equal volume of reaction

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buffer (100 mM KCl, 0.5 mM ATP, CTP, and GTP) was added to the nuclear suspension, and the reaction mixture was incubated with 100 μCi of [α-32P]UTP (3000 Ci/mmol) at 26 °C for 30 min. The reaction was then terminated by the addition of 100 μl of stop buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 20 mM MgCl₂, 150 units/ml RNAsin, 40 units/ml DNase) and incubated at 28 °C for 15 min. Then proteinase K (750 μg/ml) and 1% SDS were added and incubated at 37 °C for 30 min. RNA was isolated by phenol/chloroform extraction and precipitated in ethanol and 2.5 M ammonium acetate. Human HSP-70 cDNA or β-actin cDNA (100 μg each) was denatured in 100 μl of 0.1 N NaOH for 30 min, neutralized by addition of an equal volume of buffer containing 0.5 mM Tris-HCl, pH 7.0, and 3 mM NaCl, and then blotted onto proteinase K (750 μg/ml) and 1% SDS were added and incubated at 37 °C for 30 min. RNA was isolated by phenol/chloroform extraction and precipitated in ethanol and 2.5 M ammonium acetate. Human HSP-70 cDNA or β-actin cDNA (100 μg each) was denatured in 100 μl of 0.1 N NaOH for 30 min, neutralized by addition of an equal volume of buffer containing 0.5 mM Tris-HCl, pH 7.0, and 3 mM NaCl, and then blotted onto proteinase K (750 μg/ml) and 1% SDS were added and incubated at 37 °C for 30 min. RNA was isolated by phenol/chloroform extraction and precipitated in ethanol and 2.5 M ammonium acetate. Human HSP-70 cDNA or β-actin cDNA (100 μg each) was denatured in 100 μl of 0.1 N NaOH for 30 min, neutralized by addition of an equal volume of buffer containing 0.5 mM Tris-HCl, pH 7.0, and 3 mM NaCl, and then blotted onto proteinase K (750 μg/ml) and 1% SDS were added and incubated at 37 °C for 30 min. RNA was isolated by phenol/chloroform extraction and precipitated in ethanol and 2.5 M ammonium acetate. Human HSP-70 cDNA or β-actin cDNA (100 μg each) was denatured in 100 μl of 0.1 N NaOH for 30 min, neutralized by addition of an equal volume of buffer containing 0.5 mM Tris-HCl, pH 7.0, and 3 mM NaCl, and then blotted onto proteinase K (750 μg/ml) and 1% SDS were added and incubated at 37 °C for 30 min. RNA was isolated by phenol/chloroform extraction and precipitated in ethanol and 2.5 M ammonium acetate. Human HSP-70 cDNA or β-actin cDNA (100 μg each) was denatured in 100 μl of 0.1 N NaOH for 30 min, neutralized by addition of an equal volume of buffer containing 0.5 mM Tris-HCl, pH 7.0, and 3 mM NaCl, and then blotted onto
nylon membrane (Biodyne, Pall Corp.) using a slot-blot apparatus (Schleicher & Schuell). The membrane was prehybridized in hybridization buffer (5× Denhardt’s solution, 40% formamide, 4× SSC, 5 mM EDTA, 0.4% SDS, and 100 μg/ml yeast tRNA) at 42 °C for 5 h. Hybridization was performed with 10⁷ cpm of 32P-labeled RNA/ml of hybridization buffer at 42 °C for 72 h. Then the membrane was washed in 2× SSC with 0.1% SDS at 37 °C for 30 min, in 2× SSC containing 10 μg/ml RNase A at 37 °C for 30 min, and in 0.1× SSC with 0.1% SDS at 42 °C for 30 min. Signals were detected using a Fuji Imaging Analyzer (BAS2000; Fuji Photo Film Co., Minaiasigara, Kanagawa, Japan) after 3 h of exposure at room temperature.

RESULTS

Apoptosis and Generation of Ceramide Induced by HS, and Synergistic Effects of Ceramide on HS-induced Apoptosis—We examined whether the increase of HS-induced apoptosis was in parallel with ceramide generation in human leukemia HL-60

FIG. 2. Synergistic increase of apoptosis by simultaneous treatment with HS and exogenous ceramide. A, the cells were treated with various concentrations of C₂-ceramide (0, 2.5, 5, and 10 μM) in the presence or absence of HS at 42 °C for 30 min and 4 h after treatment the percents of apoptotic cells were counted under light microscopy. The numbers shown were the increased percentages of apoptotic cells as compared with those in the absence of HS. B–E, the cells were treated with or without 10 μM C₂-ceramide in the presence or absence of HS at 42 °C for 30 min, and 4 h after treatment the photos of apoptotic cells stained by May-Giemsa method were taken at the magnification of ×400. C, the cells were treated with or without 10 μM C₂-ceramide in the presence or absence of HS at 42 °C for 30 min, and 4 h after treatment the photos of apoptotic cells determined by fluorescence-activated cell sorter analysis as described under “Experimental Procedures.” E, the cells were treated with or without 10 μM C₂-ceramide in the presence or absence of HS at 42 °C for 30 min, and 4 h after treatment analysis of DNA fragmentation agarose gel was performed as described under “Experimental Procedures.” The results were the representative of three different experiments. Bars, 1 S.D. B–E, panel a, control; panel b, C₂-ceramide; panel c, HS; panel d, C₂-ceramide + HS.
cells. When the cells were incubated at 42 °C for the indicated duration (0–120 min) and returned to 37 °C, the increase of HS-induced apoptosis and ceramide generation paralleled with the intensity of HS treatment (Fig. 1, A and B). The percentages of the cells showing the morphological changes characteristic to apoptosis judged by May–Giemsa staining method were 16%, 56%, 69%, and 72% 5 h after HS at 42 °C for 15, 30, 60, and 120 min, respectively (Fig. 1A). The viable cell number did not change, even 4–24 h after HS treatment at 42 °C for 30 min (data not shown). When apoptotic cells were judged by DAPI staining, the percents were almost same as those by morphological changes (Table I). The intracellular ceramide levels similarly increased to 9.1, 10.5, 11.0, 13.0, and 15.6 pmol/nmol of phosphate immediately after the treatment at 42 °C for 5, 15, 30, 60, and 120 min, respectively, as compared with 7.7 pmol/nmol of phosphate of the control level (Fig. 1B). Ceramide levels increased by HS at 42 °C were in an incubation time-dependent manner (Fig. 1C). Four hours after HS treatment for 30 min, the cells showed 170% increase of ceramide as compared with the levels of ceramide immediately after HS. We usually use a DGK assay for measuring ceramide levels in the cells. To confirm the propriety of this method, we examined the changes of DGK activity and the amounts of phospholipids with or without HS at 42 °C for 30 min. As shown in Fig. 1D, C2-ceramide as an internal standard and phospholipids phosphate was not affected by HS, suggesting that the results measured by DGK assay and generalized by phospholipids were acceptable as intracellular mass levels of ceramide.

When the cells were treated with various concentrations of C2-ceramide for 4 h after HS at 42 °C for 30 min, the percents of apoptotic cells judged by May–Giemsa staining method were synergistically increased (Fig. 2, A and B). Simultaneous treatment with HS and 10 μM C2-ceramide increased apoptotic cells by 32% 4 h after treatment as compared with HS alone when 10 μM C2-ceramide alone increased apoptotic cells by 5%. As shown in Table I and Fig. 2 (C–E), significant synergistic effects of C2-ceramide on HS-induced apoptosis were confirmed by diverse assays for detection of apoptosis including DAPI nuclear staining, FACSscan analysis using propidium iodide, and DNA ladder detection method. The results suggested exogenous ceramide enhanced pro-apoptotic signaling or suppressed HS-induced anti-apoptotic signaling to exert synergistic induction of apoptosis with HS stress.

Suppression of HSP-70 mRNA and Protein Levels Increased by Heat Shock in the Presence of Ceramide—In order to investigate the mechanism by which ceramide synergistically enhances the induction of apoptosis with HS, we examined the effect of ceramide on mRNA expression of HSP-70 since HSP-70 has been recognized as a cell protecting molecule in response to stress (32, 33). We therefore examined whether ceramide affected the activation of HSF-1 to suppress the HS-increased levels of HSP-70 mRNA. HS at 42 °C for 30 min increased the transcriptional rate of HSP-70 but not that of β-actin mRNA 1 h after treatment, and the increased rate of HS-70 was not significantly suppressed by C2-ceramide. These results suggested that the mechanisms by which ceramide decreased mRNA levels of HSP-70 were not related to its transcriptional regulation through HSF-1.

Effects of Ceramide on HS-Activated Nuclear Transcription Factor, Heat Shock Factor-1, and Transcriptional Rate of HSP-70 mRNA—What, finally, are the mechanisms by which HSP-70 mRNA levels are suppressed by ceramide? HSP-70 was reported to be transcriptionally regulated by a nuclear transcription factor, heat shock factor (HSF)-1 (34). We therefore examined whether ceramide affected the activation of HSF-1 to suppress the HS-increased levels of HSP-70 mRNA. HS at 42 °C for 30 min was confirmed to activate HSF-1 by showing the translocation of HSF-1 from the cytosol to the nucleus (40) (Fig. 5A). Equal amounts of loading of HSF-1 and its specific change was confirmed by no significant change of HSF-2 in the nucleus, which did not increase by HS (41). When the cells were treated with 10 μM C2-ceramide, translocation to the nucleus of HSF-1 induced by HS was not affected at all.

We further performed a nuclear run-on assay to examine whether the increased transcriptional rate of HSP-70 mRNA was directly suppressed by ceramide (Fig. 5B). HS at 42 °C for 30 min increased the transcriptional rate of HSP-70 but not that of β-actin mRNA 1 h after treatment, and the increased rate of HS-70 was not significantly suppressed by C2-ceramide. These results suggested that the mechanisms by which ceramide decreased mRNA levels of HSP-70 were not related to its transcriptional regulation through HSF-1.

Effect of Ceramide on Post-transcriptional Regulation of HS-Increased HSP-70—The levels of HSP-70 mRNA were reported to increase not only transcriptionally by HSF-1 but also post-transcriptionally by 12-O-tetradecanoylphorbol-13-acetate
(42), which competed with ceramide in the induction of apoptosis (33). We therefore investigated whether ceramide post-transcriptionally increased the decay of HS-induced HSP-70 mRNA. When the cells were treated with HS at 42 °C for 30 min in the presence or absence of 10 μM C2-ceramide and harvested at the indicated times (0, 1, 2, and 4 h) after HS, B, dose dependence of suppression of HS70 mRNA levels in HL-60 cells. The cells were treated with various concentrations of C2-ceramide (0, 2.5, 5, and 10 μM) before HS at 42 °C for 30 min and harvested 2 h after HS. C, time and dose dependence of suppression of constitutively expressed HSP-70 mRNA levels in 293 cells. The cells were treated with indicated concentrations of C2-ceramide (20, 30, 40, 60, and 80 μM) for indicated times (0, 1, 2, and 4 h). The mRNA levels of HSP-70, -60, and -90 and β-actin were detected by Northern blotting method as described under “Experimental Procedures,” and the amounts of 28S ribosomal RNA were stained to confirm the equal amounts of loading. The results were the representative of at least three independent experiments.

**DISCUSSION**

We have shown here that HS treatment induced apoptotic cell death in human leukemia HL-60 cells in a temperature-dependent manner, and that the intensity of apoptosis was in parallel with the increase of ceramide generation. The simultaneous treatment with HS and exogenous C2-ceramide induced synergistic effects on the induction of apoptosis as shown in Fig. 2 (A–E) and Table I, suggesting that exogenous ceramide not only mediated pro-apoptotic signals but also suppressed anti-apoptotic signals caused by HS to exert a synergistic and efficient apoptosis. Therefore, we examined the effects of ceramide on HS-induced HSP-70 expression and protein synthesis because HSP-70 seemed to function as “chaperonin” and protect the cells against HS-induced apoptosis (14). The results showed that ceramide suppressed HS-induced HSP-70 mRNA and protein levels but did not affect those of HSP-60 and -90 significantly (Figs. 3 and 4), suggesting the specific anti-apoptotic role of HSP-70 in HS-induced apoptosis. Our present data are consistent with the previous works showing that a transgenic mouse overexpressed by HSP-70 increased the resistance of the heart to ischemic injury (21), and that HSP-70-overexpressed Wn-113 WEHI-S cells showed the tolerance to the cytotoxicity caused by TNF-α, UV, and hydrogen peroxide (20). As shown in Fig. 3C, constitutively overexpressing HSP-70 in 293 cells significantly decreased, as apoptosis was induced by ceramide. These results strongly suggested that HSP-70 played a cell protective role in ceramide-induced apoptosis as well as HS-induced apoptosis, even though recently the overexpression of HSP-70 was reported to enhance T cell receptor/CD3- and Fas-mediated apoptosis in Jurkat cells. Taken together, ceramide seems to exert efficiently its pro-apoptotic effects by suppressing anti-apoptotic signals related to HSP-70 in HS-induced apoptosis.
What are the mechanisms by which ceramide suppresses the levels of HSP-70 mRNA expression and protein synthesis? One possibility is a transcriptional regulation of HSP-70 mRNA through a transcriptional factor HSF-1 (43, 44). HS was reported to induce the phosphorylation, nuclear translocation, oligomerization and DNA binding activity to heat shock element of HSF-1 and to increase HSP-70 expression transcriptionally. A calcium ionophore, A23187, suppressed HS-induced HSP-70 expression and synthesis because of the inhibition of HSF-1 phosphorylation in K-562 cells (17). We also found that HS induced the activation of HSF-1 by inducing the phosphorylation and nuclear translocation in HL-60 cells, but ceramide could not affect HS-induced activation of HSF-1 at all (Fig. 5A), suggesting that ceramide suppressed HSP-70 expression by the different way from a HSF-1-related transcriptional regulation.

To examine this possibility directly, we performed a run-on assay for HSP-70 mRNA expression. As shown in Fig. 5B, a transcriptional rate of HSP-70 mRNA increased by HS was not suppressed by ceramide. These results suggested that the suppression of HSP-70 expression by ceramide was not owing to a transcriptional regulation through a transcription factor such as HSF-1, but owing to other different mechanisms.

Besides a transcriptional regulation, another possible mechanism to suppress HSP-70 expression is post-transcriptional. In fact, HSP-70 expression increased by 12-O-tetradecanoylphorbol-13-acetate treatment owing to a post-transcriptional inhibition of mRNA degradation in the peripheral blood monocytes (42). It was, therefore, examined whether ceramide post-transcriptionally suppressed HS-increased HSP-70 expression in HL-60 cells. As shown in Fig. 6, when a novel transcription of HSP-70 mRNA was inhibited after the treatment with actinomycin D the degradation of HSP-70 mRNA was faster as compared with that of the control without actinomycin D, showing the existence of the mechanism to degrade HSP-70 mRNA in HL-60 cells. Moreover, the addition of ceramide after treatment with actinomycin D caused more rapid and significant disappearance of HSP-70 mRNA than did no addition of ceramide. These results suggested that ceramide suppressed HSP-70 expression caused by HS through a post-transcriptional regulation.

Ceramide has been recognized as a lipid messenger to mediate apoptotic signals. Many signals, including ceramide-activated kinases and protein phosphatases, transcription factors such as Myc and NF-κB, interleukin 1β-converting enzyme-like cysteine proteases called caspases, JNK, and MAP kinases have been reported as indispensable pro-apoptotic molecules in ceramide-induced apoptosis (28–31). We also reported that jun/AP-1 signaling and the cytosolic translocation of protein kinase Cδ were required to the induction of apoptosis by ceramide (32,
33). Whereas many pro-apoptotic signals were reported to play a role in ceramide-induced apoptosis, ceramide was recently shown to compete with anti-apoptotic bcl-2 (45) and cell survival signal related to phosphatidylinositol-3 and Akt kinases (46). We here added HSP-70 to the list of anti-apoptotic signals, which are suppressed by ceramide, and proposed that HSP-70 and ceramide seemed to be balanced for the determination of how and what kind ceramide-mediating apoptotic signals play a role in post-transcriptional regulation of HSP-70.

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Suppression of Heat Shock Protein-70 by Ceramide in Heat Shock-induced HL-60 Cell Apoptosis

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