IDENTIFICATION OF Mg\(^{2+}\)-DEPENDENT NEUTRAL SPHINGOMYELINASE 1 AS A MEIDIATOR OF HEAT STRESS-INDUCED CERAMIDE GENERATION AND APOPTOSIS

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Running title: Neutral sphingomyelinase and heat-induced apoptosis

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A neutral sphingomyelinase (SMase) is involved in the induction of ceramide-mediated pro-apoptotic signaling under heat stress. Although ceramide is an important mediator of apoptosis, the neutral SMase that is activated under heat stress has not been identified. Our examination indicated that neutral SMase 1, which was isolated from zebrafish embryonic cultured ZE cells, was a mediator of stress-induced apoptosis. Mg\(^{2+}\)-dependent neutral SMase was cloned from a cDNA library from ZE cells using an Escherichia coli expression vector by an SMase assay against the substrate C\(_6\)-7-nitro-2-1, 3-benzoxadiazol-4-yl-sphingomyelin. The isolated cDNA clone encoded a polypeptide of 420 amino acids (putative molecular weight: 46.9 K) containing two predicted transmembrane domains in the C-terminal region. Bacterially expressed recombinant neutral SMase 1 hydrolyzed [choline-methyl-\(^{14}\)C]sphingomyelin optimally at pH 7.5 in the presence of Mg\(^{2+}\) ion. In the endogenous SMase in ZE cells, the enzyme was localized in the microsomal fraction. The overexpressed FLAG-tagged SMase was co-localized with a Glogi cytostaining marker by cytochemical observation. The loss of function of neutral SMase 1 by antisense phosphorothioate oligonucleotides in ZE cells repressed ceramide generation, caspase-3 activation, and apoptotic cell death under heat stress. Thus, neutral SMase 1 participates in an inducible ceramide-mediating pro-apoptotic signaling pathway that operates heat-induced apoptosis.

Sphingolipids have been implicated as bioactive molecules in animal cells; specifically, ceramide is a mediator of apoptosis (1,2), differentiation (3), and senescence (4,5). Ceramide is accumulated in response to heat shock in U937 (6), BAE (6), HL-60 (7), and NIH WT-3T3 (8) cells. Ceramide initiates apoptosis through the stress-activated protein kinase (SAPK/JNK) cascade in U937 and BAE cells (6). The exogenous cell-permeable ceramide analog N-acetylspingosine (C\(_2\)-ceramide) also induces \(\alpha\B-crystallin transcription, mirroring the effect of heat shock-produced ceramide in vivo (8). In heat-shocked HL-60 cells, elevated ceramide induces the expression of e-jun/e-fos and activated caspase-3, resulting in apoptosis (9).

Sphingomyelinases (SMases, EC3.1.4.12) hydrolyze sphingomyelin, which is a major component of the lipid bilayer of subcellular membranes, to generate ceramide and phosphorylcholine (10). To date, six types of SMases have been described, including the lysosomal acidic SMase (11-13), the cytosolic Zn\(^{2+}\)-dependent acidic SMase (14,15), the membrane neutral magnesium-dependent SMase (16-19), the cytosolic neutral magnesium-independent SMase (20), and the alkaline SMase (21-24).
These enzymes differ in their subcellular localization, tissue specificity, and enzymatic properties, especially optimum pH (25). SMases, especially acidic SMase and neutral SMase, are activated in response to growth factors, cytokines, chemotherapeutic agents, irradiation, nutrient removal, and stress (4,10,26). Recent research has resulted in the molecular identification of at least three distinct neutral SMases in human and mouse: neutral SMase 1, 2, and 3 (27-29). Although a neutral or acidic SMase is thought to mediate stress-induced ceramide generation and apoptosis, the enzyme (or enzymes) induced under stress condition that mediates apoptosis has not been identified. Molecular cloning and biochemical characterization of the SMase(s) are required to understand the molecular mechanisms of stress-induced apoptosis and sphingolipid metabolism.

We have focused on zebrafish cultured cells and embryos as experimental models for characterizing ceramide-induced apoptosis (30-32). Exogenous C2-ceramide induces apoptosis in Japanese flounder embryos (33), and the inhibition of neutral ceramidase in zebrafish embryos induces ceramide generation and apoptosis (34). Recently, we found a zebrafish Mg2+-dependent neutral SMase 1 that produces ceramide and causes thalidomide-induced vascular defects in zebrafish embryos (32). Thus, this enzyme is a candidate for the mediator of stress-induced ceramide generation and apoptosis.

In our preliminary studies, zebrafish cells showed high Mg2+-dependent neutral SMase activity, but the amino acid sequence of the fish enzyme was expected to show very low sequence homology with the known mammalian neutral SMases. In order to identify the fish SMase responsible for stress-induced ceramide generation and apoptosis, we carried out the cloning strategy using an Escherichia coli expression vector by neutral SMase assay.

Here, we report the expression cloning and biochemical features of zebrafish neutral SMase 1 as a key enzyme in stress-induced, as well as its activation and ceramide generation. This enzyme was found to be a mediator of heat-inducible apoptosis in zebrafish ZE cells.

**EXPERIMENTAL PROCEDURES**

Materials—Acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (Ac-DEVD-MCA) and benzoxycarboxyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) were purchased from the Peptide Institute (Osaka, Japan). C6-7-nitro-2,1,3-benzoxadiazol-4-yl (C6-NBD) sphingomyelin was obtained from Matreya (Pleasant Gap, PA). [choline-methyl-14C]sphingomyelin (2.0 GBq/mmol), [choline-methyl-14C]dipalmitoylphosphatidylcholine (2.11 GBq/mmol), [1-O-octadecyl-2H]lyso-platelet activating factor (5.99 TBq/mmol), and L-[U-14C]serine (5.7 GBq/mmol), protein A Sepharose, PVDF membranes, the ECL™ Western Blotting Detection kit, and horseradish peroxidase-labeled secondary antibodies were purchased from GE Healthcare (Piscataway, NJ). Mouse monoclonal anti-KDEL antibody and mouse monoclonal anti-58K Golgi protein antibody were purchased from Abcam (Cambridge, MA). Anti-transferrin receptor polyclonal antibody, anti-cadherin polyclonal antibody, and anti-aldolase monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PARP polyclonal antibody, anti-FLAG M2 monoclonal antibody, anti-actin monoclonal antibody, and p3XFLAG-CMV™-14 expression vector were purchased from Sigma-Aldrich. Xpress™ System Synthetic Oligonucleotides, goat anti-rabbit IgG Alexa Fluor 488-labeled antibody, and anti-mouse IgG Alexa Fluor 594-labeled antibody were purchased from Invitrogen (Carlsbad, CA). The Premix Taq (Ex Taq™ Version 2) and PrimeSTAR™ HS DNA polymerase were purchased from Takara Biomedicals (Shiga, Japan).

Cell Culture—Zebrafish embryonic cell line ZE cells and fathead minnow FHM cells were cultured in Leibovitz’s L-15 medium (Gibco Invitrogen, Carlsbad, CA) supplemented with 2% fetal calf serum (FCS;
JRH Biosciences) and 80 µg/ml kanamycin sulfate at 28.5°C (30,35). Human leukemia HL-60 cells, human kidney embryonic HEK293 cells, and COS-7 cells were cultured in RPMI1640 (Gibco Invitrogen) supplemented with 10% FCS (JRH Biosciences) and 80 µg/ml kanamycin sulfate at 37°C in a humidified atmosphere containing 5% CO₂.

**Heat Shock Treatment**–ZE cells were cultured in culture dishes at 5 X 10^5 cells/ml with preheated medium, placed in an incubator at 37°C or 38°C, and then allowed to recover at 28.5°C. Cell viability was determined using the trypan blue exclusion method (32). The cells were heat shocked at 37°C or 38°C for 0-11 h and then stained with 4',6-diamidino-2-phenylidole (DAPI) (7). At least 200 cells were counted under light microscopy, and cells with nuclear condensation and fragmentation were judged as apoptotic.

**Caspase-3 Assay**–Caspase-3 activity was measured using a synthetic substrate, Ac-DEVD-MCA, according to the method described previously (30). ZE cells were washed once with PBS and homogenized in lysis buffer (20 mM Hepes, pH 7.5, containing 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂, and 1 mM dithiothreitol) by passing through a 27-gauge needle. The lysate was centrifuged and the supernatant was collected. A portion of the supernatant was added to 180 µl of a caspase substrate mixture containing 20 mM Hepes, pH 7.5, 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂, 1 mM dithiothreitol, and 4 µM Ac-DEVD-MCA, and then incubated at 37°C for 60 min. The release of 7-amino-4-methylcoumarin was measured using a fluorescent spectrophotometer (Millipore) with excitation at 360 nm and emission at 450 nm. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad).

**Ceramide Measurement**–Lipids in the cells were extracted by the methods of Bligh and Dyer (36), and ceramide mass measurements using *Escherichia coli* diacylglycerol kinase (DGK) were according to the method described by Okazaki *et al.* (3). The solvent system used to separate ceramide-1-phosphate was chloroform/acetonemethanol/acetic acid/water (10:4:3:2:1). To calculate the ceramide content, the positive spots on thin-layer chromatography (TLC) plates were measured using a STORM 860 imaging analyzer (Molecular Dynamics, Tokyo, Japan).

**Sphingomyelin Quantification**–After heat shock treatment, 1 X 10^7 cells were harvested, and the lipids were extracted from the cells by the methods of Bligh and Dyer (36) and developed using a solvent system of chloroform/methanol/water (60:35:8, by volume) on a plastic TLC plate with standard sphingomyelin. The spots corresponding to sphingomyelin were scraped, and the lipids were extracted by the method of Bligh and Dyer (36). Inorganic phosphate in the extract was measured by the ammonium molybdate/ascorbic acid method (37) to calculate the sphingomyelin content.

**Metabolic Labeling of Ceramide with [14C]Serine**–The cells were wash with PBS, seed at 5 X 10^5/ml in Leibovitz’s L-15 medium supplemented with 370 MBq/ml L-[U-14C]serine, 2% FCS and incubated at 28.5°C for 3 days. The labeled cells were treated with heat shocked at 38°C for 1 h, and then recovered to incubate at 28.5°C for 3 h. After harvesting the cells, lipids extracted from the cells by the methods of Bligh and Dyer (36) were separated on TLC plates with a solvent system of methyl acetate, propanol-1, chloroform, methanol and 0.25% KCl (25:25:25:10:9, by volume), the spots corresponding to ceramide were visualized, and the relative radioactivity was determined by the STORM 860 analyzer system (Molecular Dynamics), corrected by the amount of phospholipids in each sample.

**Serine Palmitoyltransferase Assay**–The cells at 5 X 10^6/ml were homogenized in a lysis
buffer (20 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 1 X Complete® Protease inhibitor mixture (Roche Molecular Biochemicals)) by passing through a 27-gauge needle. The homogenate was centrifuged at 1300 g, and the supernatant was then ultra-centrifuged at 100,000 g. The pellets resuspended in 10 mM Tris-HCl buffer, pH 7.4, containing 30% glycerol were used for the enzyme assay as the microsomal fraction. Protein concentration was determined using a Bio-Rad Protein Assay kit (Bio-Rad).

Serine palmitoyltransferase activity was determined with the microsomal fractions prepared from both the heat-shocked and the control cells. The microsomal fraction containing 80 µg of protein was incubated in 100 mM HEPES buffer, pH 8.3, containing 5 mM dithiothreitol, 50 µM pyridoxal-5'-phosphate, 1 mM [14C]-labeled L-serine (370 MBq/mmol), and 200 µM palmitoyl CoA in a total volume of 200 µl at 37°C for 20 min with shaking. The reactions were stopped by addition of 1.5 ml of chloroform: methanol (1:2, by volume). Unlabeled sphinganine (25 µl of a 1-mg/ml ethanol solution) was added as a carrier, and extracted by phase separation using 1 ml chloroform and 2 ml of 0.5 M ammonia. After removal of the aqueous phase, the organic phase was washed with 2 ml water for two times to remove unincorporated radiolabeled serine. The resultant organic phase was dried under nitrogen, and the radioactivity of the produced 3-ketodihydrosphingosine was measured by liquid scintillation counting.

**SMase Assay**--The cells were lysed by through a 27-gauge needle in lysis buffer (10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin A, 0.15 unit/ml aprotinin, and 50 µg/ml leupeptin). The lysate was centrifuged at 10,000 g and 4°C for 10 min. The supernatant was used as an enzyme source. Supernatant protein (60 µg) was mixed in a reaction mixture (100 mM Tris-HCl, pH 7.5, containing 10 mM MgCl2, 5 mM DTT, 10 µM C6-NBD-sphingomyelin, and 0.1% Triton X-100) and incubated at 37°C for 1 h. The reaction was stopped by addition of 900 µl of H2O and 2 ml of chloroform/methanol (2:1, by volume), and then mixed well and centrifuged. The lower organic phase was collected, and the solvent was evaporated. Aliquots were applied to TLC plates. The solvent system used to separate C6-NBD-ceramide and C6-NBD-sphingomyelin was chloroform/methanol/12 mM MgCl2 in water (65:25:4, by volume). C6-NBD-ceramide was visualized by UV irradiation and measured using a TLC scanner with fluorometer (475 nm excitation; 525 nm emission).

**Expression Cloning of Neutral SMase cDNA from Zebrafish**--Total RNA was isolated from zebrafish embryonic cells using the TRizol reagent (Invitrogen), and poly(A)+ RNA was purified from total RNA using the Oligotex-dt30 mRNA purification kit (Takara Biomedicals, Shiga, Japan). Double-stranded cDNA was synthesized from the poly(A)+ RNA using a Superscript Choice System for cDNA Synthesis (Invitrogen). A cDNA library was prepared using the isopropyl-1-thio-β-D-galactopyranoside (IPTG)-inducible expression vector pET-28a (Novagen) in Escherichia coli BL21(DE3)pLysE cells (Novagen). The library contained 1 X 10^7 independent clones. Aliquots containing approximately 1000 E. coli clones from the positive pool that had high SMase activity were incubated on LB plates. Each E. coli clone was cultured in 4 ml and its SMase activity was measured. Finally, a positive
cDNA clone encoding neutral SMase from zebrafish was isolated and sequenced. The nucleotide sequence of the isolated zebrafish neutral SMase cDNA was deposited at the DDBJ/Genbank/EMBL database under accession number AB196165.

**Transfection of the Neutral SMase 1 cDNA into ZE cells**–The neutral SMase cDNA from zebrafish containing the complete ORF was amplified by polymerase chain reaction (PCR) using the isolated full-length cDNA as the template and sense primer 5’-TCAGGAGCGGACTGAAGCGGCATCA TGCA-3’ and antisense primer 5’-CCGTCGAGTCCTTTCAAACGGAGGA ATAA-3’. The PCR reaction was carried out with 25 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 2 min. The amplified product was subcloned downstream of the human cytomegalovirus promoter of the pTARGET Mammalian Expression Vector (Promega), according to the manufacturer’s protocol. The nucleotide sequence of the PCR product was confirmed by sequencing. This construct was named pTARGET-ZNSMase.

For the neutral SMase assay, ZE cells were cultured at a density of 1 X 10⁶ cells per 60-mm dish in 5 ml of Leibovitz’s L-15 medium supplemented with 2% FCS. At approximately 90% confluence, each dish of cells was transiently transfected with 5 µg of DNA of pTARGET-ZNSMase vector or mock vector with the FuGENE 6 Transfection Reagent (Roche), according to the manufacturer’s protocol. At 24 h after transfection, the cells were washed twice with PBS and homogenized in lysis buffer for neutral SMase assay.

**Preparation of Recombinant Neutral SMase**–The neutral SMase 1 cDNA was amplified by PCR using the sense primer 5’-CATATGGCACCACAGCAGCGGCA ACTG-3’ and antisense primer 5’-CATATGTATTTACTCCGTTGAAGGA CT-3’, each containing an Nde I site, and the amplimer was subcloned into the pGEM-T Easy plasmid vector by the TA-Cloning method (Promega). The cloned nucleotide sequence was confirmed by sequencing and subcloned into the Nde I site in the multiple-cloning site of the pET-16b vector (Novagen) to fuse a His₁₀-tag sequence to the N-terminus of the neutral SMase 1 ORF. This construct was named pETZNNSMase 1. Neutral SMase 1 was expressed in Escherichia coli BL21(DE3)pLysE cells (Novagen) transformed with pETZNNSMase 1. The cells were inoculated in 100 ml of LB broth and grown overnight at 30°C in a shaker at 200 rpm. The culture was transferred to 1000 ml of fresh LB medium with 100 µg/ml ampicillin in a 5 l flask, and the above incubation conditions were continued until turbidity at 600 nm reached 0.8. IPTG was added to a final concentration of 1 mM, and the culture was grown for a further 4 h to induce the expression of the transgene. Bacterial cells were collected by centrifugation at 4000 g for 15 min. The N-terminal His-tagged neutral SMase was purified from the bacterial extract by affinity chromatography with His Trap HP (GE Healthcare), according to the manufacturer’s protocol. The bacterial cells were suspended in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 10% glycerol, 1 X Complete protease inhibitor cocktail, 5 mM MgCl₂, 60 mM imidazole, 0.1% TritonX-100, 1 mM EDTA, and 1 mg/ml lysozyme) by passing through a 22-gauge needle. All subsequent procedures were carried out at 4°C. The supernatant was collected by centrifuging the lysate at 10,000 g for 30 min and was dialyzed against wash buffer (50 mM Tris-HCl, pH 7.5, containing 300 mM sodium chloride, 10% glycerol, 5 mM MgCl₂, 60 mM imidazole, 0.1% TritonX-100, and 1 mM EDTA). The dialyzed sample was loaded onto a 5-ml His Trap HP column (GE Healthcare) that had been equilibrated in wash buffer. After sample loading, the column was washed with 100 ml of wash buffer, followed by a 0-100 % linear gradient of elution buffer (50 mM Tris-HCl, pH 7.5, containing 300 mM sodium chloride, 10% glycerol, 5 mM MgCl₂, 800 mM imidazole, 0.1% TritonX-100, and 1 mM EDTA). The
flow rate was 1 ml/min and 2-ml fractions were collected. The His Trap HP fractions that had neutral SMase activity were pooled and dialyzed against gel filtration buffer (25 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 5 mM MgCl₂, 0.1% TritonX-100 and 1 mM EDTA) and then loaded onto a Sephacryl S-100 column (HR 16/60, GE Healthcare) that had been equilibrated in gel filtration buffer. The enzyme was eluted at 1 ml/min with 150 ml of gel filtration buffer.

**Preparation of Rabbit Polyclonal Antibody against Zebrafish Neutral SMase**—To generate a polyclonal antibody against zebrafish neutral SMase 1, the purified recombinant protein was used for immunization in the rabbit. Specific IgG was purified from the serum by an affinity chromatography on a protein A Sepharose column (GE Healthcare).

**SMase Assay of the Recombinant Enzyme**—The SMase activity was determined using radiolabeled substrate by mixed micelle assay system as described previously (20). Briefly, [¹⁴C]sphingomyelin and bovine brain sphingomyelin were placed in a glass tube and dried under nitrogen. The reaction mixture containing Tris-HCl, pH 7.5, 5 nmol of [¹⁴C]sphingomyelin (100,000 dpm), 5 mM DTT, 0.1% Triton X-100, and 5 mM MgCl₂ was prepared by sonication for 5 min. After addition of 1 µl of enzyme solution to the reaction mixture, the corresponding solution was incubated for 30 min at 37°C. The reaction was stopped by addition of 0.2 ml of water and 1.5 ml of chloroform/methanol (2:1, by volume). After vortexing and two phases separation by centrifugation, 0.2 ml of the upper aqueous phase was removed and added to 2 ml of scintillation solution for radioactivity counting. The reaction was linear with incubation times up to 3 h. The amount of enzyme added to the reaction mixtures was chosen such that < 10% hydrolysis of the substrate occurred. An appropriate blank containing denatured enzyme was run with each reaction and subtracted from the experimental samples. To examine the effect of magnesium ion on the activity of the purified enzyme, the reaction mixtures were used in the presence or absence of magnesium. For assaying effects of different pH on the SMase activity, the following buffers were used at final concentration of 100 mM: sodium acetate (pH 4 and 5), PIPES (pH 6 and 7), Tris (pH 7.5, 8, 8.5, and 9). Hydrolyzing activity against phosphatidylcholine of the purified enzyme was measured with 10 nmol [¹⁴C]phosphatidylcholine (100,000 dpm) instead of sphingomyelin in the mixed micelle solution for SMase assay. Hydrolyzing activity against lysophosphatidylcholine was determined using radiolabeled substrate in a mixed micelle assay system, as described previously (38). The recombinant enzyme was added to 100 µl of reaction mixture containing 100 mM Tris-HCl, pH 7.5, containing 5 mM DTT, 5 mM MgCl₂ and 10 nmol [³H]lyso-PAF (200,000 dpm). The mixture was incubated for 30 min at 37°C. The lipid was extracted by the method of Bligh and Dyer (36) and separated by TLC in solvent system chloroform/methanol/15 mM CaCl₂ in water (60:35:8, by volume). To calculate the monoalkylglycerol content, the TLC plate was exposed to imaging film. The radioactivity of the positive spots scraped from the TLC plate was determined by liquid scintillation counting.

**Construction of Neutral SMase Variant**—An SMase-FLAG-tag fusion construct and an alanine substitution mutant were created by PCR using zebrafish neutral SMase 1 cDNA as the template using appropriate combinations of the forward and reverse oligonucleotides primers, and introduced into p3XFLAG-CMV™-14 expression vector (Sigma-Aldrich). All the nucleotide sequences of the vector constructs were confirmed by DNA sequencing and used for the assays.

**Generation of Zebrafish ZE and Human HEK293 Stable Transfectants**—ZE cells were
culture in Leibovitz’s L-15 medium containing 10% FCS. To obtain stable transfectants, 1 X 10^6 ZE cells were transfe

ected with 2 μg of DNA of SMase-FLAG-tag fusion constructs (wild-type) and alanine substitution mutants (H272A mutant) using FuGENE 6 Transfection Regent (Roche) according to the manufacturer’s instruction and selected in the presence of 0.4 mg/ml genetecin (Invitrogen). HEK293 cells were cultured in RPMI1640 medium containing 10% FCS. 5 X 10^6 HEK293 cells were transfected with 4 μg of DNA of wild-type vector and H272A mutant vector using FuGENE 6 Transfection Regent and selected in the presence of 0.8 mg/ml geneticin. Both ZE and HEK293 cell lines overexpressing wild type of neutral SMase 1 and its alanine substitution mutant were generated.

**Immunofluorescence Microscopy**–ZE cells and HEK293 cells transfected with cultured on a cover glass were fixed with 4% paraformaldehyde in PBS for 15 min. After being rinsed with PBS, cells were permealized with 0.1% Triton X-100 in PBS for 3 min at room temperature. After treatment with PBS containing 1% BSA and 2% FBS for 1 h, the samples were incubated with both an anti-zebrafish SMase 1 rabbit IgG (1:2000) and a subcellular marker antibody, anti-KDEL mouse IgG (Abcam; 1: 200) or anti-58K mouse IgG (Abcam; 1: 200), in blocking buffer at 4°C for overnight. The cells were washed with PBS for three times for 15 min, and were incubated with a goat anti-rabbit IgG Alexa Fluor 488-labeled secondary antibody (Invitrogen; 1: 200) and anti-mouse IgG Alexa Fluor 594-labeled secondary antibody (Invitrogen; 1: 200) for 3 h. In non-permeation stain, the HEK293 transfected cells were cultured on a cover glass and then fixed with 4% paraformaldehyde in PBS for 15 min. After treatment with PBS containing 1% BSA and 2% FBS for 1 h, the samples were incubated with anti-FLAG antibody (1:5000) and with anti-cadherin antibody (Santa Cruz; 1: 1000) containing blocking buffer at 4°C for 1 h. The cells washed for three times with PBS for 15 min were incubated with both goat anti-mouse IgG Alexa Fluor 594-labeled antibody (Invitrogen; 1: 200) and anti-rabbit IgG Alexa Fluor 488-labeled antibody (Invitrogen; 1: 200) for 3 h. The cells were washed for three times with PBS for 15 min, and counter-stained with DAPI. All fluorescence images were observed with a fluorescence microscope (Edge Scientific Instruments, R400).

**Subcellular Fractionation**–Subcellular fractionation was performed by the modified method described previously (20). The cells (5 X 10^6/ml) were homogenized in a lysis buffer (20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethysulfonylfluoride, 10 μg/ml pepstatin A, 0.15 units/ml aprotinin, and 50 μg/ml leupeptin) by passing through a 27-gauge needle. The lysate was centrifuged at 1300 g for 15 min at 4°C. The obtained pellet was used as the nuclei fraction. The supernatant was centrifuged at 100,000 g for 1 h at 4°C. The collected supernatant was used as the cytosolic fraction. The pellet was resuspended in the lysis buffer by homogenization and sonication, and then centrifuged at 12,000 g for 1 h at 4°C. The obtained pellet was used as the microsomal fraction.

**Western Blotting**–The proteins extracted from the cytosolic and microsomal fractions were separated by SDS-PAGE and electroblotted onto PVDF membrane as described by Yabu et al (30). The anti-zebrafish neutral SMase 1 polyclonal, anti-aldolase polyclonal, anti-actin monoclonal, anti-PRAP polyclonal, anti-transferrin polyclonal, and anti-FLAG monoclonal antibodies were used as the primary antibody and signals were detected with the secondary antibody, with the ECL™ Western blotting detection kit according to the manufacturer’s protocol (GE Healthcare).

**Neutral SMase Antisense Oligonucleotides**–The phosphorothioate
oligonucleotide was synthesized to block the translation of neutral SMase 1: antisense neutral SMase 1, 5'-CGGGCTGCTGTGGTGCCATGA TGCC-3'; sense neutral SMase 1, 5'-GGCATCATGGCACCACAGCAGCCCG-3'. The cells were incubated with sense or antisense neutral SMase (0-10 \( \mu \)M) at a concentration 5 X 10^5 cells/ml in Leibovitz’s L-15 medium supplemented with 2% FCS for 48 h before heat shock treatment.

**Statistical Analysis**—Statistical values are expressed as the mean with standard deviation. Differences among groups were analyzed by one-way ANOVA, followed by Bonferroni’s post-hoc \( t \) test. Comparisons between two experimental groups were based on two-tailed \( t \) test.

**RESULTS**

Caspase-3 Dependent Apoptosis Induced by Heat Shock—Zebrafish ZE cells and embryos exhibit typical apoptotic features such as membrane blebbing, nuclear and cytoplasmic shrinkage and nuclear DNA fragmentation (30). We examined whether such apoptotic processes are mediated by ceramide generation and caspase-3 activation under heat stress. When ZE cells were incubated at 37ºC or 38ºC for 1 h and returned to 28.5ºC, cell survival decreased and apoptotic cell numbers increased in a time- and dose-dependent manner (Fig. 1A and 1B). The percentages of cells that showed the morphological changes characteristic of apoptosis (7), judged by DAPI nuclear staining, were 25% and 35% at 6 h after heat shock treatment for 1 h at 37ºC and 38ºC, respectively (Fig. 1C). After heat shock at 39ºC for 1 h, all cells died by 1 h in recovery (data not shown). When apoptotic cells were judged by DAPI staining, the percentages of apoptotic cells were similar to those determined by morphological changes. When caspase-3 activity for Ac-DEVD-MCA was assayed after heat shock treatment at 37ºC or 38ºC for 1 h, a peak of caspase-3 activity was found at 4 h after heat treatment, which then returned to control levels (Fig. 1C). A specific caspase inhibitor, Z-VAD-fmk, which reportedly prevents heat-induced apoptosis, actually inhibited heat-induced apoptosis in ZE cells (Fig. 2A and 2B). In heat-induced apoptosis, active caspase-3 cleaved PARP (Fig. 2C). Thus, heat-induced apoptosis in ZE cells was mediated by a pathway involving caspase-3 activation.

When the intercellular ceramide content was measured using a diacylglycerol kinase assay, heat shock transiently generated ceramide; a peak of approximately twice the basal ceramide levels induced after heat shock for 3 h (Fig. 3A). A decrease in cellular sphingomyelin levels almost paralleled the increase in cellular ceramide levels after heat shock for 3 h (Fig. 3B).

The biochemical mechanism of ceramide generation by de novo biosynthesis under heat stress condition was investigated in ZE cells. Firstly, we examined whether ceramide biosynthesis was inhibited by an inhibitor of ceramide synthase, \( i.e. \) fumonisin B1. When the cells were radiolabeled with \([^{14}C]\)serine following pretreatment with fumonisin B1, the attempt was unsuccessful in the case of both ceramide and sphingomyelin under normal condition (data not shown), indicating fumonisin B1 inhibited ceramide generation by ceramide synthase in ZE cells. On the contrary, when ZE cells were radiolabeled with \([^{14}C]\)serine, the labeled ceramide was induced by heat stress, but the treatment of fumonisin B1 did not repress the heat-induced ceramide generation (Fig. 3D and 3E). These findings revealed that heat-induced ceramide generation is not mediated by ceramide synthase. Thus, heat shock affected the sphingomyelin cycle with increased cellular ceramide levels and decreased cellular sphingomyelin levels.

Activation of Neutral SMase in Heat-Induced Apoptosis—To confirm the importance of SMase-dependent ceramide generation in the heat-induced apoptosis, we measured the activities of ceramide-related enzymes, such as Mg\(^{2+}\)-dependent neutral
SMase, acid SMase, sphingomyelin synthase, glucosylceramide synthase, acid ceramidase, and serine palmitoyltransferase. Heat shock treatment induced the activity of Mg\(^{2+}\)-dependent neutral SMase in a time- and dose-dependent manner (Fig. 3C). Heat shock treatment also induced the activity of sphingomyelin synthase (Fig. 3G). In contrast, the activities of acid SMase, sphingomyelin synthase, glucosylceramide synthase, acid ceramidase, and serine palmitoyltransferase in ZE cells were unaffected by heat shock (Fig. 3F, 3G, 3H, and 3J). In addition, the Mg\(^{2+}\)-independent neutral SMase activity was not detected in ZE cells. Mg\(^{2+}\)-dependent neutral SMase activity was increased from 1.58 to 2.56 nmol/mg/h or 3.25 nmol/mg/h by heat shock for 1 h at 37°C or 38°C, respectively. Heat shock induced Mg\(^{2+}\)-dependent neutral SMase activity in ZE cells as early as 1 h after exposure and reached a peak of activity 2 h after heat shock (Fig. 3C). Sphingomyelin synthase activity was slowly increased from 45.5 to 66.3 pmol/mg/h by heat shock for 1 h at 38°C (Fig. 3H). Thus, ceramide is generated via sphingomyelin hydrolysis, catalyzed by mainly activated Mg\(^{2+}\)-dependent neutral SMase as an early event in the induction of apoptosis after heat shock.

**Expression Cloning of cDNA Encoding Mg\(^{2+}\)-Dependent Neutral SMase**—ZE cells had approximately 14-times higher Mg\(^{2+}\)-dependent neutral SMase activity than other fish and mammalian cell lines (Table 1). The ZE cell line has been studied previously for apoptotic responses under stresses such as UV irradiation, heat shock, cold shock and starvation (35). We used ZE cells to characterize the neutral SMase. To isolate cDNA encoding neutral SMase, we screened bacterial clones that showed a neutral SMase activity in ZE cells that was higher than other fish and mammalian cell lines (Table 1). The ZE cell line has been studied previously for apoptotic responses under stresses such as UV irradiation, heat shock, cold shock and starvation (35). We used ZE cells to characterize the neutral SMase. To isolate cDNA encoding neutral SMase, we screened bacterial clones that showed a neutral SMase activity against C\(_6\)-7-nitro-2,1,3-benzoaziadiazol-4-yl-sphingomyelin as a substrate from a cDNA library from ZE cells in an *Escherichia coli* pET-28a expression vector. A single cDNA clone showing neutral SMase activity was isolated. This clone had a 1260-bp ORF that encoded a protein of 420 amino acids, with a predicted molecular weight of 46.9 K (Fig. 4A). A FASTA search of the SWISS-PROT/PIR protein sequence database revealed that zebrafish neutral SMase possessed significant homology with the human neutral SMase 1 (29). The amino acid sequence identity of the neutral SMase 1 with human and mouse homologs were 44% and 46%, respectively (Fig. 4A). According to Tomiuk et al. (29), conserved amino acid residues in neutral SMase 1 were identified as the putative magnesium-binding glutamine residue (Gln-49) (39), the substrate-binding asparagine residue (Asn-180) (40), and the histidine residue (His-272) of the active site, deduced from studies of the *Bacillus cereus* IAM 1208 enzyme (41) (Fig. 4A).

The secondary structure of the neutral SMase1 was predicted by the SMART program (42). Two transmembrane domains were found in the C-terminal region, suggesting that this protein binds to cellular membranes (Fig. 4A).

To determine the enzyme activity of the cloned Mg\(^{2+}\)-dependent neutral SMase, a cDNA expression construct under the control of the human cytomegalovirus promoter (pTRAGET-ZSMase) was introduced into COS-7 cells and ZE cells, and SMase activity in the cell lysate was assayed. The transfectants showed 10.9 and 7.8 times higher sphingomyelin-hydrolyzing activity than did those of the empty vector in COS-7 and ZE cells, respectively (Table 2). We showed that overexpression of the cloned enzyme in ZE cells expressed transgene products (Fig. 4E). The transfectant of wild-type SMase had a higher activity of Mg\(^{2+}\)-dependent neutral SMase than transfectants with H529A mutant or non-transfectant (control) (Fig. 4F).

A recombinant protein with the His-tag sequence at the N-terminus was produced, using the expression vector pET-16b carrying the neutral SMase cDNA. The affinity purification by His-Tag chromatography of the recombinant protein yielded a high-purity protein as indicated by a single protein band with molecular weight of 49.4 K on SDS-PAGE (Fig. 4B).
purified recombinant Mg\(^{2+}\)-dependent neutral SMase showed high activity towards the substrate [choline-methyl-\(^{14}\)C]sphingomyelin, whereas the enzyme had no activity against the phospholipid [choline-methyl-\(^{14}\)C]phosphatidylcholine and very little activity against [1-O-octadecyl-9,10-\(^{3}\)H]lyso-platelet activating factor (Table 1). The activity was absolutely dependent on magnesium ions (Fig. 4C), optimally at pH 7.5 (Fig. 4D). The isolated clone encoded a membrane-bound magnesium-dependent neutral SMase that was designated “ neutral SMase 1: SMPD2” (29,43).

**Subcellular Localization of Neutral SMase 1**—To examine the subcellular localization of the neutral SMase 1, we constructed an SMase-FLAG fusion, and established its stable transfectants in the both ZE and HEK293 cell lines. The transfectants overexpressed wild-type SMase 1 were double-stained with anti-zebrafish neutral SMase 1 antibody together with anti-58K protein antibody (Golgi maker) or anti-KDEL protein antibody (ER maker) by Immunofluorescence microscopy. Subcellular localization of SMase 1 with that of the Golgi maker (Fig. 5A and 5B) showed that the SMase 1 exhibits major signals in the Golgi (Fig. 5C). Consequently, staining with ER (Fig. 5D and 5E), SMase 1 was not colocalized in the ER (Fig. 5F). In the case of HEK293 transfectants, the zebrafish SMase 1 was also colocalized in both the Golgi and ER (Fig. 5G-L). When we used a non-permeation treatment without 0.1% Triton-X 100 in the staining steps to detect the SMase 1 in the cell membrane, the enzyme was localized differentially with the cell membrane cadherin, revealing disappearance of the SMase 1 in the cell membrane (Fig. 5M-O).

Subcellular localization of the SMase 1 was also examined in ZE cells by centrifuge fractionation. The activities of neutral SMase in the microsomal fraction in both the ZE cells and SMase 1-overexpressed transfectant were higher than those of the nuclear and cytosolic fractions (Table 4). Western blotting showed that the SMase 1 was detected in the microsomal fraction but not in the cytosolic fraction (Fig. 5P). Therefore, the neutral SMase 1 was present in the microsomal fraction.

**Knockdown Experiment with Antisense Oligonucleotides**—To examine whether ceramide generation by Mg\(^{2+}\)-dependent neutral SMase was involved in heat-induced apoptosis, the phosphorothioate oligonucleotide for neutral SMase 1 was used to repress the levels of this protein. The activity of Mg\(^{2+}\)-dependent neutral SMase after 48 h of antisense oligonucleotide treatment was decreased in an oligonucleotide-dependent manner (Fig. 6A). Treatment with an antisense oligonucleotide for Mg\(^{2+}\)-dependent neutral SMase reduced not only the basal activity of Mg\(^{2+}\)-dependent neutral SMase from 1.58 to 0.25 nmol/mg/h but also heat shock-activated neutral SMase from 3.12 to 0.25 nmol/mg/h (Fig. 6B and 6C). The treatment with the antisense oligonucleotide repressed induced endogenous ceramide under heat shock (Fig. 6D and 6E), but did not affect the basal level of endogenous ceramide (Fig. 6D). Antisense oligonucleotide treatment repressed the decrease in cellular sphingomyelin content (Fig. 6F), the increase in caspase-3 activity (Fig. 6G), and the induction of apoptosis under heat stress (Fig. 6H and 6I), whereas treatment with the corresponding sense oligonucleotides had no such effect. Thus, the Mg\(^{2+}\)-dependent neutral SMase deficiency induced by antisense oligonucleotide treatment repressed heat-induced apoptosis. The enzyme was activated and catalyzed sphingomyelin processing to generate ceramide under heat shock.

**DISCUSSION**

Ceramide is a lipid signaling molecule in stress-induced apoptosis; it is generated by the hydrolysis of sphingomyelin through the action of an SMase that is activated by stresses such as heat, UV and γ-irradiation, Fas ligand and TNF-α, serum
withdrawal, and hypoxia (4,26,44,45). At least three distinct neutral SMases have been identified in human and mouse: neutral SMase 1, 2, and 3 (27-29). However, the neutral SMase(s) responsible for stress-induced ceramide generation and apoptosis has not been identified. To characterize the molecular mechanism of stress-induced ceramide generation and apoptosis, we cloned a membrane-bound Mg$^{2+}$-dependent neutral SMase 1 from zebrafish ZE cells and showed that this enzyme was activated in response to heat shock and generated ceramide and induced apoptosis. The isolated neutral SMase 1 showed similar enzymatic characteristics to the known mammalian neutral SMase 1s. Both the zebrafish and mammalian enzymes share common structural features such as a magnesium-binding site, a substrate-binding site, and an active center His residue. They also have two transmembrane domains in the C-terminal region, indicating that the neutral SMase 1 is membrane bound.

Furthermore, the activity and substrate specificity of the enzyme are similar to those of the mammalian neutral SMase 1s (43). The recombinant zebrafish neutral SMase 1 had specific hydrolyzing activity against sphingomyelin and produced ceramide. In addition, the enzyme was responsible for heat-inducible ceramide generation and apoptosis. The human enzyme also possesses Mg$^{2+}$-dependent neutral SMase activity (43). However, the mechanism responsible for ceramide generation and induction of apoptosis has not been well characterized.

The over-expression of the human neutral SMase 1 has no effect on sphingomyelin metabolism, but decreases levels of lyso-PAF, suggesting that it has a lyso-PAF phospholipase C activity (38). In knockout mice, the loss of function of neutral SMase 1 results in neither lipid storage abnormalities nor deficient sphingomyelin metabolism (46). The over-expression of human neutral SMase 1 in human Jurkat cells results in no effect on CD95/Fas receptor-induced ceramide production or apoptosis, suggesting that it is not a candidate enzyme for the execution phase of death receptor-induced apoptosis (47). On the other hand, zebrafish neutral SMase 1 showed specific hydrolyzing activity against sphingomyelin and only low lyso-PAF phospholipase C activity, indicating that the SMase 1 primarily regulates ceramide generation on sphingomyelin metabolism.

The specific inactivation of neutral SMase 1 by antisense RNA represses in ceramide-mediated apoptosis triggered by T cell receptor ligation (48). The loss of function of neutral SMase 1 represses amyloid-β peptide-induced ceramide generation and apoptosis in rat oligodendrocytes (49). Thus, the known mammalian neutral SMase 1s have closely related biochemical characteristics to the zebrafish enzyme and its biological significance in ceramide metabolism is suggested.

Because the zebrafish neutral SMase 1 had sphingomyelin-hydrolyzing activity and apoptosis-inducing ability in vivo and in vitro, such functions in mammalian neutral SMase 1 should be confirmed with regard to ceramide metabolism and the induction of apoptosis.

Several lines of evidence support the induction of ceramide generation and stress-induced apoptosis in ZE cells, under heat stress at 37-38°C, similar to heat-shocked human HL-60 cells (7). Heat shock and following recovery at 28.5°C caused apoptosis in a dose- and time-dependent manner in ZE cells. This apoptotic process showed enhanced caspase-3 activity during the recovery after heat shock, and the apoptosis could be rescued by the presence of the caspase inhibitor Z-VAD-fmk, indicating that the induced apoptosis in ZE cells was regulated by a caspase-3-dependent mechanism. Heat shock (8) and Fas ligand and TNF-α have been shown to activate neutral and/or acid SMase in generating ceramide (4,26,50). Heat shock induced apoptosis with ceramide generation in parallel with SMase activation and decreased in sphingomyelin content in a temperature-
and time-dependent manner in zebrafish cells (Fig. 3). The temporally produced ceramide may be the pro-apoptotic signal for apoptosis in the stressed cells, and the ceramide was reduced to the normal physiological levels by the activation of sphingomyelin synthase during the recovery after heat shock (Fig. 3H). In contrast, the activities of other ceramide-related enzymes, such as acid SMase, glucosylceramide synthase, acid ceramidase, and serine palmitoyltransferase, were unaffected in ZE cells by heat shock (Fig. 3F, 3G, 3I, and 3J). These findings support an important function of the neutral SMase in heat shock-induced ceramide generation. Unlike our present findings, de novo biosynthesis for ceramide generation has been reported in bacteria and yeast, while heat shock induced ceramide generation from dihydroshingosine by the action of ceramide synthase and serine palmitoyltransferase (51). In zebrafish cells, ceramide generation was not affected by fumonisin B1 under the prescribed heat-shock conditions (Fig. 3D and 3E), indicating that ceramide synthase is not responsible for heat-induced ceramide generation in the fish cells.

Ceramide is thought to be a signaling molecule upstream to bel-2, caspase-9, and caspase-3 (7,52-55). In the fish cells, caspase-3 activation was followed by ceramide generation of the heat shock. Thus, it is required that the mechanism of caspase-3 activation induced by the elevated ceramide. Other pro-apoptotic signaling molecules, such as apaf-1 and unknown ceramide-binding protein, may induce the caspases cascade.

Hostetler and Yazaki (17) reported that the activity of a microsomal neutral SMase in rat liver was different from the enzyme in the cell membrane fraction. The Mg²⁺-dependent neutral SMase was suggested to be localized at the cytosolic side of the cell membrane during apoptosis (56-58). The subcellular localization of human and mouse neutral SMase 1 was also shown in the ER membranes (29,43). In addition, the zebrafish enzyme was localized mainly in the Golgi by immunocytochemistry, and distributed in the microsomal fraction by subcellular fractionation (Fig. 5). The structural prediction of zebrafish neutral SMase 1 indicates that the enzyme possesses two transmembrane domains in the C-terminal region and an intracellular catalytic domain (Fig. 4A). Therefore, the enzyme may mainly bind to the Golgi membranes.

The localization of neutral SMase 1 in the Golgi supports cellular functions of the enzyme in ceramide generation for vesicle-membrane fusion and endocytosis. SMase has been reported to induce aggregation and fusion by ceramide release (59) and disturb the lipid bilayer structure in favor of a nonlamellar and micellar phase (60). Thus, SMase activation and ceramide generation may be induced by local vesicle formation and fusion under heat shock conditions. Sphingomyelin and phosphatidylcholine are asymmetrically distributed in the outer leaflet and the aminophospholipids are in the inner leaflet of the lipid bilayer (61,62). Since the neutral SMase is suggested to bind to cellular membranes, heat shock may induce the temporal loss of the asymmetric phospholipids distribution and the SMase activation. In addition, the neutral SMase 1 activity is regulated by glutathione levels (63). The enzyme activities were inhibited by glutathione at 1 mM in the ZE cells in vitro and in vivo (T. Yabu, unpublished). Therefore, the depletion of glutathione may be an important mechanism in activation of the SMase.

Neutral SMase 1 plays a critical role for zebrafish development. Knockdown for SMase 1 gene showed blockage of epiboly formation and neurogenesis, hence generation of abnormal phenotype. Such phenotypic arrest was rescued by microinjection with exogenous ceramide to the normal phenotype. Thus, the SMase 1 and its production of ceramide are essential for programmed cell death and differentiation (T. Yabu, unpublished results). The enzyme expressed in the notochord at segmentation stages and depletion of ceramide by the SMase knockdown resulted in thalidomide-
induced ceramide generation and vascular defect (32). In contrast to ceramide, sphingosine-1-phosphate inhibits SMase-dependent ceramide generation and restores thalidomide-induced vascular defect with an increase of expression of VEGF receptors. Therefore, the neutral SMase 1 is an essential mediator for normal development and embryogenesis as well as a pro-apoptotic signal in stress-induced apoptosis.
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Abbreviations are used: SMase, sphingomyelinase; Ac-DEVD-MCA, Acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide; Z-VAD-fmk, benzylloxycarbonyl-Val-Ala-Asp-fluoromethylketone; C6-NBD-sphingomyelin, C6-7-nitro-2-1,3-benzoxadiazol-4-yl-sphingomyelin; FCS, fetal calf serum; [14C]sphingomyelin, [N-methyl-14C]sphingomyelin; [14C]phosphatidylcholine, L-3-phosphatidyl[N-methyl-14C]choline,1,2-dipalmitoyl; [3H]lyso-PAF, [1-O-octadecyl-3H]lyso-platelet activating factor; TLC, thin layer chromatography; ZE cells, zebrafish embryonic cells; PBS, phosphate-based saline.
**Figure Legends**

**Figure 1. Dose- and time-dependent apoptosis induced by heat stress.** (A) Effect of heat shock on cell survival in zebrafish embryonic (ZE) cells. Control cells were maintained in Leibovitz’s L-15 medium supplemented with 2% fetal calf serum at 28.5°C. The cells were heat-shocked at 37°C or 38°C for 1 h at an initial concentration of 1 X 10⁶ cells/ml, allowed to recover at 28.5°C for 0-11 h, and harvested. Viable cell numbers were counted using the trypan blue dye exclusion method. (B) The DAPI staining assessment of cell survival indicated dose- and time-dependent apoptotic effects of heat stress. (C) Caspase-3 activation was induced by heat stress. Caspase-3 activity was measured using Ac-DEVD-MCA hydrolyzing activity in the cells after heat shock treatment. Each value indicates the mean three independent experiments with an error bar of standard deviation.

**Figure 2. Heat-induced apoptosis by caspase-3 activation.** (A) Inhibition of heat induced-cell death by a caspase inhibitor. ZE cells were treated with 0, 20, or 40 µM Z-VAD-fmk for 1 h before heat shock at 38°C for 1 h and allowed to recover at 28.5°C for 0-11 h. The cells were heat-shocked at 38°C for 1 h at an initial cell number of 1 X 10⁶ cells/ml, allowed to recover at 28.5°C for 0-11 h, and harvested. (B) The DAPI staining assessment of nuclear fragmentation indicated dose- and time-dependent suppressive effects on heat-induced apoptosis. Values and bars indicate the means and standard deviations, respectively, of three independent experiments. (C) Suppression of cleavage of PARP by caspase inhibitor. The cells were treated with or without 20 µM Z-VAD-fmk for 1 h before heat shock treatment and then harvested after 4 h. The protein levels of PRAP and actin were detected with Western blotting method as described under “Experimental Procedures”.

**Figure 3. Increasing ceramide content, decreasing sphingomyelin content, and activation of magnesium-dependent neutral SMase by heat stress in zebrafish embryonic (ZE) cells.** (A) Changes in ceramide levels after heat shock. (B) Changes in sphingomyelin levels after heat shock. ZE cells were treated at 37°C or 38°C for 1 h and allowed to recover at 28.5°C for 0-11 h. Cellular lipids were extracted at the indicated times, and ceramide and sphingomyelin contents were quantified using the DGK assay and phosphate measurement after TLC separation, respectively. (C) Changes in the activity of a magnesium-dependent neutral SMase. The activities were measured against C₅-NBD-sphingomyelin as a substrate. (D) Effects of fumonisin B1 on the ceramide production induced by heat shocked in ZE cells. After ZE cells were radio-labeled with 370 MBq/ml L-[U-¹⁴C]serine at 28.5°C for 48 h, the cells were
incubated at 28.5°C for 1 h (lane 2), and then heat-shocked at 38°C for 1 h and allowed to recover at 28.5°C for 2 h in the presence of 100 µM fumonisin B1 (lane 1), in the absence of fumonisin B1 (lane 3). (E) The lipids were extracted and separated on TLC plate. A STORM 860 imaging analyzer as described in “Experimental Procedures” detected signals. Changes in the activities of ceramide-metabolizing enzymes, such as (F) acidic SMase, (G) glucosylceramide synthase, (H) sphingomyelin synthase, (I) acidic ceramidase, and (J) serine palmitoyltransferase were measured. ZE cells were heat shocked at the indicated temperatures for 1 h at an initial cell number of 1 X 10^6 cells/ml, allowed to recover at 28.5°C for 0-11 h, and harvested. Each value indicates the mean of three independent experiments with an error bar of standard deviation. *P < 0.05 versus control.

**Figure 4. Expression cloning of zebrafish cDNA for Mg^{2+}-dependent neutral SMase activity.** (A) Predicted amino acid sequence of zebrafish Mg^{2+}-dependent neutral SMase 1. The two putative transmembrane domains identified by the SMART program are boxed. The putative Mg^{2+}-complexing glutamine residue (Δ), the asparagine residue involved in substrate binding (#), and the catalytic base histidine residue (*) are shown. Proteins with significant amino acid sequence homology were identified using a FASTA search of the Genbank database. The sequences of zebrafish neutral SMase 1 (AB196165) was aligned with the deduced amino acid sequences of homologous proteins from human (NM_009213), and mouse (NM_009213). Identical amino acid residues in two or three proteins were shaded in black. (B) Polyacrylamide gel electrophoresis of the purified recombinant enzyme. SDS-PAGE (10% gel) was performed after reduction of the sample. The gel was stained with Coomassie Brilliant Blue R-250. (C) Effect of Mg^{2+} ions on recombinant neutral SMase activity. (D) The pH dependence of neutral SMase activity. The activity of purified recombinant enzyme was measured at 37ºC for 30 min at varying pH to estimate the optimum pH. The pH was adjusted by the addition of the following buffers at a final concentration of 100 mM: acetate (pH 4 and 5), PIPES (pH 6, 6.5, and 7) and Tris (pH 7.5, 8, 8.5, and 9). Each value indicates the mean of three independent experiments at each pH. (E) ZE cell lines stably transfected with the wild-type or H272A mutant constructs were established and the activities of their lysates were assayed. The expressed proteins in the cell lines were detected with anti-FLAG antibody or anti-actin antibody by Western blotting as described under “Experimental Procedures.” (F) The neutral SMase activity against in each line was shown. Values and bars indicate the means and standard deviations of three independent experiments.

**Figure 5. Subcellular localization of neutral SMase 1.** ZE cells (A-F) and HEK293 cells (G-
O) stably transfected and overexpressed FLAG-tagged SMase were fixed and permealized with 0.1% Triton X-100. The cells were double-stained with rabbit anti-zebrafish neutral SMase 1 antibody together with anti-58K protein (the Golgi maker) antibody (A-C, G-I) or anti-KDEL protein (the ER maker) antibody (D-F, J-L), following fluorescent secondary antibodies. In the case of observation for the localization in the cell membrane, the cells overexpressed with FLAG-tagged SMase 1 were fixed and stained without Triton X-100 by non-permeation treatment (M-O). The cells were double-stained with mouse anti-FLAG antibody together with rabbit anti-cadherin (the cell membrane marker) antibody, following fluorescent secondary antibodies. The signals for the SMase 1 (A, D, G, J and N), the Golgi (B and H), the ER (E and K) and the cell membrane (M) were observed. The overlay images (C, F, I, L, and O) indicate that SMase 1 and the subcellular marker were colocalized in either the same place or adjacent to one another. Bar = 10 µm. (P) Subcellular fractionation of zebrafish neutral SMase 1. The whole lysate of ZE cells (lane 1) was fractionated into the cytosolic fraction (lane 2) and microsomal fraction (lane 3) by ultracentrifuge. These fractions were applied to Western blotting using antibodies against zebrafish SMase 1, aldolase (the cytosolic marker), and transferrin receptor (the cell membrane marker).

Figure 6. The antisense oligonucleotide against zebrafish Mg$^{2+}$-dependent neutral SMase 1 mRNA blocked heat shock-mediated apoptosis and ceramide generation. (A) ZE cells were pretreated with 0-10 µM antisense or 10 µM sense oligonucleotide against neutral SMase 1 for 48 h. The neutral SMase activity in oligonucleotide-treated cells was measured according to the methods as described in “Experimental Procedures.” The cells were pretreated with 0-10 µM antisense or 10 µM sense oligonucleotide against neutral SMase 1 for 48 h and with or without 20 µM Z-VAD-fmk for 1 h before heat shock treatment. ZE cells were heat shocked at 38°C for 1 h, allowed to recover at 28.5°C, and harvested after 0, 1, 2, 3, 6, and 12 h. (B and C) Mg$^{2+}$-dependent neutral SMase activity, (D and E) ceramide, (F) sphingomyelin content, (G) caspase-3 activity, (H) DAPI assay, and (I) cell viability. Each value indicates the mean of three independent experiments with an error bar of standard deviation. *P < 0.05 versus sense oligonucleotide-treated cells.
Table 1

Comparison of Mg²⁺-dependent neutral SMase activity in various cell types.

| Cell type    | Neutral SMase activity * (pmol/mg/h) |
|--------------|--------------------------------------|
| ZE cells     | 1585.5 ± 19                          |
| FHM cells    | 1345.2 ± 11                          |
| HEK293 cells | 134.7 ± 8                            |
| HL-60 cells  | 110.2 ± 5                            |

*Mean ± standard deviation (n=3).*
Table 2

Transient over-expression of the cloned SMase in COS-7 and ZE cells.

| Cell type                        | Neutral SMase activity *(pmol/mg/h) |
|----------------------------------|-------------------------------------|
| **COS-7 cells**                  |                                     |
| Untransfected                    | 120.2 ± 2.3                         |
| Mock-transfected                 | 123.3 ± 2.4                         |
| pTRAGET-ZNSMase                  | 1321.7 ± 4.5                        |
| **ZE cells**                     |                                     |
| Untransfected                    | 1585.5 ± 19                         |
| Mock-transfected                 | 1567.2 ± 23                         |
| pTRAGET-ZNSMase-transfected      | 12491.2 ± 24.6                      |

*Mean ± standard deviation (n=3).*
Table 3

Substrate specificity of the cloned recombinant SMase.

| Substrate                        | Activitya (µmol/mg/h) |
|---------------------------------|------------------------|
| \[^{14}\text{C}]\text{sphingomyelin} | 27 ± 0.76 b            |
| \[^{14}\text{C}]\text{phosphatidylcholine} | 0 b                   |
| \[^{3}\text{H}]\text{lyso-PAF}        | 1.5 ± 0.8 c            |

aMean ± standard deviation (n=3). bThe values in parentheses were determined in the presence of 0.1% Triton X-100 in the assay. cThe values in parentheses was determined in the absence of detergent in the assay.
Table 4

Neutral SMase activity in the subcellular fractions in ZE cells.

| subcellular fraction         | Neutral SMase activity a (pmol/mg/h) |
|------------------------------|--------------------------------------|
| ZE cells                     |                                      |
| Whole cells                  | 1590 ± 2                             |
| Nuclear fraction             | 551 ± 1                              |
| Cytosolic fraction           | 0                                    |
| Microsomal fraction          | 33800 ± 2                            |
| pTRAGET-ZNSMase-transfected ZE cells |                           |
| Whole cells                  | 12500 ± 25                           |
| Nuclear fraction             | 1008 ± 12                            |
| Cytosolic fraction           | 0                                    |
| Microsomal fraction          | 58300 ± 100                          |

*aMean ± standard deviation (n=3).*
Figure 1A-C

A

Cell viability

% cell survival

Time (h)

Control (28.5°C)

Heat shock (37°C, 1 h)

Heat shock (38°C, 1 h)

B

% apoptotic cells

Time (h)

C

Ac-DEVD-MCA hydrolyzing activity (units/mg)

Time (h)
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Figure 2A-C

A

Heat shock (38 °C, 1h)

Heat shock (38 °C, 1h) + 20 μM Z-VAD-fmk

Heat shock (38 °C, 1h) + 40 μM Z-VAD-fmk

Cell viability

% cell survival

Time (h)

0 3 6 9 12

B

% apoptotic cells

Time (h)

0 3 6 9 12

C

Control
Heat shock
Heat shock + 20 μM Z-VAD-fmk

PARP (116 K)

Actin (85 K)
Figure 3F-J

- **F**: Acid sphingomyelase activity (pmol/mg/h) over time (h) for Control and Heat shock (38°C, 1h).
- **I**: Acid ceramidase activity (pmol/mg/h) over time (h) for Control and Heat shock (38°C, 1h).
- **G**: Glucosylceramide synthase activity (pmol/mg/h) over time (h) for Control and Heat shock (38°C, 1h).
- **J**: Serine palmitoyltransferase activity (pmol/mg/h) over time (h) for Control and Heat shock (38°C, 1h).
- **H**: Sphingomyelin synthase activity (pmol/mg/h) over time (h) for Control and Heat shock (38°C, 1h).
Figure 4E and 4F

**E**

Control

Wt-type

H272A mutant

SMase

Actin

**F**

Neutral SMase activity (pmol/mg/h)

Control

Wt-type

H272A mutant
Figure 6A-C

**A**

![Graph showing C₆-NBD-sphingomyelin hydrolyzing activity (nmol/mg/h) for different conditions.

**B**

![Graph showing time course of C₆-NBD-sphingomyelin hydrolyzing activity (nmol/mg/h) with time.

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**Heat shock + 10 µM Sense**

**Heat shock + 10 µM Antisense**

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Figure 6D-G

D

Heat shock + 10 μM Sense
Heat shock + 10 μM Antisense

Ceramide content (pmol ceramide/nmol phosphate)

Time (h)

0  3  6  9  12

E

Ceramide content (pmol ceramide/nmol phosphate)

Heat shock
10 μM Antisense
10 μM Sense
20 μM Z-VAD-fmk

F

Sphingomyelin content (pmol sphingomyelin/nmol phosphate)

Time (h)

0  3  6  9  12

G

Ac-DEVD-MCA hydrolyzing activity (units/μg)

Heat shock
10 μM Antisense
10 μM Sense
20 μM Z-VAD-fmk
Figure 6H and 6I

Graph H:
- Open circles: Heat shock + 10 μM Sense
- Filled circles: Heat shock + 10 μM Antisense

Graph I:
- Bars represent % apoptotic cells
- Control, 1 μM Antisense, 5 μM Antisense, 10 μM Antisense, Heat shock
- Data points at 3, 6, 9, and 12 hours
- Significant difference indicated by *
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