Molecular Cloning and Functional Analysis of Zebrafish Neutral Ceramidase*§

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Almost all observations on the functions of neutral ceramidase have been carried out at cellular levels but not at an individual level. Here, we report the molecular cloning of zebrafish neutral ceramidase (znCD) and its functional analysis during embryogenesis. We isolated a cDNA clone encoding znCD by 5′ and 3′ rapid amplification of cDNA ends-PCR. It possessed an open reading frame of 2,229 base pairs encoding 743 amino acids. A possible signal/anchor sequence near the N terminus and four potential O-glycosylation and eight potential N-glycosylation sites were found in the putative sequence. The enzyme activity at neutral pH increased markedly after transformation of Chinese hamster CHOP and zebrafish BRF41 cells with the cDNA. The overexpressed enzyme was found to be distributed in endoplasmic reticulum/Golgi compartments as well as the plasma membranes. The antisense morpholinol oligonucleotide (AMO), which was designed based on the sequence of znCD mRNA, successfully blocked the translation of znCD in a wheat germ in vitro translation system. The knockdown of znCD with AMO led to an increase in the number of zebrafish embryos with severe morphological and cellular abnormalities such as abnormal morphogenesis in the head and tail, pericardiac edema, defect of blood cell circulation, and an increase of apoptotic cells, especially in the head and neural tube regions, at 36 h post-fertilization. The ceramide level in AMO-injected embryos increased significantly compared with that in control embryos. Simultaneous injection of both AMO and synthetic znCD mRNA into one-cell-stage embryos rescued znCD activity and blood cell circulation. These results indicate that znCD is essential for the metabolism of ceramide and the early development of zebrafish.

Sphingolipids and their metabolites are multifunctional components of eukaryotic cell membranes that are involved in a variety of biological processes. Ceramide (Cer) has been implicated as a novel lipid modulator in signal transduction pathways involved in cell growth, differentiation, and apoptosis (1, 2). The metabolite of Cer, sphingosine-1-phosphate (S1P), which is produced through the phosphorylation of sphingosine (Sph) by sphingosine kinase, promotes the proliferation and migration of endothelial cells through the activation of G protein-coupled receptors from the S1P (Edg) family (3–5). Ceramidase (CDase, EC 3.5.1.23), which catalyzes the hydrolysis of the N-acyl linkage of Cer, serves to control the intracellular levels of Sph/Cer and possibly S1P and then regulate the cell functions. It is noteworthy that Sph is not produced through de novo synthesis but is thought to be generated from Cer through hydrolysis by CDase (6). Three CDase isoforms (acidic, neutral, and alkaline enzymes), which differ mainly in the catalytic pH optimum, have been reported. Interestingly, a recent study revealed that the three isoforms can also be distinguished by their primary structure, suggesting that they are derived from different ancestral genes (7). Neutral CDase, which shows an optimum pH of 6.5–8.5, has been cloned from bacteria (8), Drosophila (9), mouse (7), rat (10), and human (11). Recently, we reported the molecular cloning of the neutral CDase homologue of slime mold, which exhibits maximal activity at around pH 3 (12). Slime mold CDase is the first exception to the rule linking optimum pH and the primary structure of CDases. The neutral CDase seems to regulate the balance of Cer/Sph/S1P in response to varius stimuli, including cytokines (13, 14) and growth factor (15). Acharya et al. reported that targeted expression of neutral CDase rescued retinal degeneration in arrestin and phospholipase C mutants of Drosophila, correlating with a decrease in Cer (16). Very recently, they reported that neutral ceramidase could modulate the endocytosis of rhodopsin in Drosophila photoreceptors (17). These findings indicate that neutral CDase could keep a normal level of Cer in cells, and Cer metabolism seems to be important for the functions of photoreceptors in Drosophila.

Interestingly, neutral CDases of bacteria (18) and Drosophila (9) were solely detached from the cells as a soluble form, whereas those of mammalian origins were mainly recovered in membrane fractions (19). Recently, we clarified the reason for this discrepancy at the molecular level, i.e. the latter possessess a mucin-like domain (mucin box) near the N terminus that is glycosylated with O-glycans, whereas the former completely lacks this specific domain. A deletion mutant CDase that lacks the mucin box and an Ala replacement mutant CDase in which all Ser or Thr residues in the mucin box are replaced by Ala

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¶ The abbreviations used are: Cer, ceramide; AMO, antisense morpholinol oligonucleotide; CDase, ceramidase; CHOP cells, Chinese hamster ovary cells that express polyoma LT antigen; hpf, hours post-fertilization; HRP, horseradish peroxidase; Mops, 4-morpholinepropanesulfonic acid; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; ORF, open reading frame; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; Sph, sphingosine; S1P, sphingosine 1-phosphate; znCD, zebrafish neutral ceramidase.

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were not retained in the cell membranes, indicating that O-glycosylation of the mucin box is required to keep the enzyme at the plasma membrane. The mammalian CDase was found to be expressed on plasma membranes as a type II integral protein in which the N-terminal hydrophobic sequence just before the mucin box serves as an anchor to the membranes, and on some occasions the enzyme was detached from the cell via processing of the anchor (20).

In this study, we cloned the neutral CDase from zebrafish, a well-accepted vertebrate model, and examined the functions of the enzyme during embryogenesis by knockdown of the enzyme using the antisense morpholino oligonucleotide (AMO). It was revealed that the enzyme, which possesses a mucin box, is essential for the metabolism of Cer and the early development of zebrafish.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chinese hamster CHOP cells and zebrafish BRF41 cells were donated by Dr. K. Nara (Mitsubishi Kagaku Institute of Life Sciences, Tokyo Japan) and Dr. H. Mitani (Department of Biological Sciences, University of Tokyo), respectively. The pcS2 + vector and Cer kinase cDNA, pC/Ch/CEK, was provided by Dr. S. Takada (National Institute for Basic Biology, Okazaki, Japan) and Dr. T. Kakuma (Sankyo Co.), respectively. The anti-Rab6 antibody was provided by Dr. S. Tanaka (Shizuoka University, Shizuoka, Japan). HRP-labeled anti-mouse IgG antibody was purchased from Nacalai Tesque (Tokyo, Japan). Cy3-labeled anti-mouse IgG antibody and the anti-calnexin antibody were obtained from Sigma. ECL Plus and Cy3-labeled anti-rabbit IgG (Amersham Biosciences). The anti-FLAG antibody was purchased from Invitrogen. The anti-neutral rat CDase antibody was raised in a rabbit using recombinant CDase as the antigen (10). C12-NBD-Cer was prepared as described (21). All other reagents were of the highest purity available.

**Cloning of the cDNA Encoding Neutral CDase**—To obtain a DNA fragment encoding zebrafish neutral CDase (znCD), PCR amplification was performed using sense and antisense oligonucleotide primers based on the cDNA sequence of mouse neutral CDase (AB037111); the sense primer (5'-TCCCCGGGAAATACAACCTAGT-3') and antisense primer (5'-TCTGAAGTAGTATGATCGA3') were used for PCR with the cDNA library of adult zebrafish as a template in a GeneAmp PCR System 9700 (Applied Biosystems) using AmpliTaq Gold (Applied Biosystems). The cycling parameters for PCR were 94 °C for 30 s, 72 °C for 1 min, and the cycle number was 35. An amplified 228-bp PCR product containing the putative CDase sequence was subcloned into the pGEM-T Easy vector (Promega) and then sequenced. Further 5' -and 3'-znCD cDNA sequences were obtained by reverse transcription PCR from adult zebrafish mRNA by using the SMART RACE cDNA amplification kit (BD Biosciences) according to the manufacturer's protocol. The specific primer sequence used for 5'-RACE, 5'-AGAGGTGTTAGGGCCAAAAATGGTGGTC-3', was based on the fragment mentioned above. Similarly, the specific primer sequence used for 3'-RACE, 5'-AGGGCGGGACGTGTAAACCAGCTCTGTC-3', was based on the clone obtained by 5'-RACE. To obtain a cDNA containing the putative znCD ORF, PCR was performed using a sense primer with a KpnI site (5'-GGGTTACATCGGCTGGATCTAGTCTG-3') or a BamHI site (5'-GGGGTACCATCGGCTGGATCTAGTCTG-3') and a pcDNA3.1(+) vector (Invitrogen) and pcS2 + vectors to generate pcDNA/znCD and pcS2/znCD, respectively.

**Cell Culture and Transfection**—CHOP cells, Chinese hamster ovary cells that express a polyclonal LT antigen to support the replication of a polyoma LT antigen to support the replication of pcDNA/znCD and pCS/znCD, respectively.

**Preparation of the Enzyme from Cultured Cells and Embryos**—To prepare cell lysates, cells attached to the culture plate were scraped off and collected in a 1.5-mL tube by centrifugation. The cell pellets were rinsed with PBS, suspended in lysis buffer (10 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100, 1 µg/ml chymostatin, leupeptin, and pepstatin A, and 2 mM EDTA), and then lysed by sonication. The enzymes from embryos, embryos were washed with PBS, suspended in lysis buffer, and then lysed by sonication.

**Protein Assay, SDS-PAGE, and Western Blotting**—Protein content was determined by the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as a standard. SDS-PAGE was carried out according to the method of Laemmli (23). Protein transfer onto a polyvinylidene difluoride membrane was performed using Trans-Blot SD (Bio-Rad) according to the method described in a previous study (24). After treatment with 3% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h, the membrane was incubated with the primary antibody overnight at 4 °C. After a wash with Tris-buffered saline containing 0.1% Tween 20, 0.1% bovine serum albumin, 0.1% Tween 20, and 0.05% Tween 20, the ECL reaction was performed for 2–3 min as recommended by the manufacturer, and chemiluminescent signals were visualized on an ECL™ Mini-camera (Amersham Biosciences).

**CDase Assay**—CDase activity was measured using C12-NBD-Cer as a substrate as described (18, 21). Briefly, 400 pmol (for cell lysates) or 2 nmol (for embryo lysates) of C12-NBD-Cer was incubated at 37 °C for 1 h with an appropriate amount of enzyme in 40 µl of reaction mix (50 mM Tris-HCl buffer, pH 7.5, containing 0.05% Triton X-100). For embryo lysates, C12-NBD-Cer was dissolved in methanol and then added to the sample to dissolve the substrate completely (final concentration of methanol, 10%). The reaction was stopped by adding 100 µl of chloroform/methanol (2:1; v/v), and the lower layer was applied to a silica Gel 60 TLC plate (Merck), which was then developed with chloroform/methanol/25% ammonia (90:30:0.5; v/v). The NBD-dodecanoic acid released and the C12-NBD-Cer remaining were quantified with a Shi-madzu CS-9300 chromatoscanner (Shimadzu, Japan). One enzyme unit was defined as the amount capable of catalyzing the release of 1 nmol of NBD-dodecanoic acid per minute under the conditions described above. A value of 10−3 and 10−6 units of the enzyme was expressed as 1 milliunit and 1 microunit, respectively, in this study.

**Sphingomyelinase Assay**—The activity of sphingomyelinase was measured using C12-sphingomyelin (Shirey, Inc.) as a substrate. Five hundred picomoles of C6-NBD-sphingomyelin was incubated at 37 °C for 1 h with 80 µg of embryo lysate in 20 µl of reaction mix (50 mM acetate buffer, pH 4.5, containing 0.5% Triton X-100).

**Immunoprecipitation of znCD**—Anti-neutral CDase antibody at a dilution of 1:100 was incubated at 4 °C for 2 h with 10 µl of protein A-agarose (Santa Cruz Biotechnology) in 100 µl of reaction buffer (10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Triton X-100, and 0.1% bovine serum albumin). After five washes with reaction buffer, the protein A-agarose-conjugated antibody was resuspended in 900 µl of reaction buffer to which 100 µl of denatured sample (described below) was added, and then incubation was conducted at 4 °C overnight. Before immunoprecipitation, the sample was denatured by boiling for 5 min in 100 µl of SDS sample buffer (20 mM Tris-HCl, pH 7.5, containing 1% SDS and 1% 2-mercaptoethanol). The precipitate was spun down by centrifugation, washed with reaction buffer five times, and then suspended in 20 µl of SDS sample buffer. After boiling at 100 °C for 5 min, the sample was subjected to SDS-PAGE followed by Western blotting as described above.

**Immunocytochemistry of znCD**—Transfected cells were cultured on cover glass and then fixed with 3% paraformaldehyde in PBS for 15 min. After being rinsed with PBS and 50 mM NH4Cl in PBS, cells were permeabilized, if necessary, by 0.1% Triton X-100 in PBS. After treatment with 5% skim milk in PBS (blocking buffer) for 15 min, the samples were incubated with an anti-neutral antibody with blocking buffer at 4 °C overnight followed by incubation with Cy3-labeled anti-mouse IgG antibody at room temperature for 2 h. Samples were observed with a confocal laser-scanning microscope (Digital Eclipse C1, Nikon, Japan).

**Immunohistochemistry of znCD**—Zebrafish intestine was fixed with 3% paraformaldehyde in PBS at 4 °C overnight, rinsed with PBS, and then infiltrated with 20% sucrose in PBS at 4 °C overnight. The materials were embedded in OCT compound (Sakura Finetechnical, Japan), rapidly frozen using liquid nitrogen, and stored at −80 °C. The frozen materials were cut into 9-µm-thick sections using a Cryostat (Leica CM1850, Germany) and mounted on poly-L-lysine-coated glass slides. After treatment with 3% hydrogen peroxide at room temperature for 20 min, the samples were incubated with the anti-neutral rat CDase antiserum diluted 1:100 with a blocking buffer at 4 °C overnight followed by Cy3-labeled anti-rabbit IgG (Amersham Biosciences) at room temperature for 1.5 h. Immunostained samples were incubated with
fluorescein isothiocyanate-conjugated phalloidin (Sigma) to visualize actin filaments

**In Vitro Translation of znCD—**In vitro transcription and translation of zebrafish neutral CDase was performed by using the PROTEIOS wheat germ cell-free protein synthesis kit (Toyo-bo). For the preparation of mRNA, a zebrafish with a myc tag construct was generated by PCR using pcDNA/znCD as a template. The sense primer with a KpnI site mentioned above, and the antisense primer with a SalI site (5'-GGGGTTCTCAGCTCAGTGATGATGATGATG-3') and then cloned into a pcDV vector named pEU/znCD. In vitro transcription was performed using pEU/znCD as a template and T7 RNA polymerase according to the manufacturer's protocol. In *vitro* translation was performed according to the manufacturer's protocol with the following modifications. In a 25 μl reaction, 7 μg of transcribed mRNA of the enzyme and various amounts of AMOs (1-50 μg at the final concentration) were added to the reaction mixture that contained all of the required components for *in vitro* translation in a 96-well microplate and incubated at 25 °C for 20 h. Twelve microliters from the reaction mixture was withdrawn and then subjected to 10% SDS-PAGE, and proteins were visualized using an anti-myc antibody.

**Preparation and Injection of AMOs—**AMOs were designed with sequences complementary to cDNA encoding znCD around the initiating start codon based on the manufacturer's recommendations (Fig. 7A). Three AMOs and a four-base mismatched AMO were synthesized: AMO1, 5'-GGGGTTCTCAGCTCAGTGATGATGATGATG-3'; AMO2, 5'-GGGGTTCTCAGCTCAGTGATGATGATGATGATG-3'; AMO3, 5'-GGGAGCAGACGCAGATG-3'; AMO4, 5'-GGGAGCAGACGCAGATG-3'. An inert standard oligonucleotide, which has no sequence similarity to zebrafish genes, was used as a control. Morpholinol oligonucleotides were solubilized in sterile water at a concentration of 10 μg/μl. The stock solution was diluted to working concentration in water before injection into one- to four-cell stage embryos. Injected embryos were cultured in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4) at 28 °C for specific periods. The injection of AMO and a control oligonucleotide was performed according to the method of Nasevicius and Ekker (25).

**Acridine Orange Staining—**Embryos were stained with the vital dye acridine orange (Sigma) to detect the hematopoietic cells. Embryos were placed in the dye (2 μg/ml) in E3 medium. After treatment with the dye for 30 min, embryos were washed with E3 medium. The visualization and photography was performed using an MZFLIII (Leica, Germany) fluorescence microscope and the Cool Pix 990 (Nikon, Japan) camera system.

**Preparation of Recombinant Cer Kinase—**CHOP cells were transformed with human Cer kinase cDNA, pcR/cERK, using LipofectAMINE™ Plus (26). After 2 days of culture, cells were scraped off and collected in a 1.5-ml tube by centrifugation. The cell pellet was rinsed with PBS, suspended in lysis buffer (20 mM Mops, pH 7, containing 1 mM dithiothreitol, 10% glycerol, and proteinase inhibitors), lyzed by sonication, and subjected to centrifugation (100,000 g/11003 H9262). The precipitated membrane fraction was resuspended in lysis buffer (20 mM Mops buffer, 1 M KCl in 20% glycerol, and proteinase inhibitors), incubated on a thin layer chromatography plate that was developed with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1; v/v). Using an imaging analyzer FLA5000 (Fuji Film, Japan), radioactive bands were visualized and quantified.

**Rescue of znCD Activity by znCD mRNA Injection—**For the preparation of mRNA, a znCD construct was generated by PCR using pcDNA/znCD and cloned into a pCG2+ vector. Capped mRNA was synthesized from the znCD construct using mMESSAGE mMACHINE (Ambion) according to the manufacturer's instructions. The synthesized mRNA and AMO3 were simultaneously injected into the yolk of embryos. For the control experiment, the same amount of AMO3, but without the mRNA, was injected into embryos. The sequence of znCD mRNA did not overlap with that of AMO3.

**RESULTS**

**cDNA Cloning of Zebrafish Neutral CDase—**To obtain a DNA fragment encoding znCD, PCR amplification was performed using primers designed from a mouse neutral CDase sequence (AB037111) and the cDNA library from an adult zebrafish as a template as described under "Experimental Procedures." Then, 5'- and 3'-RACE PCR were performed to obtain the putative ORF of znCD. Finally, a CDase clone encoding the znCD ORF was subcloned into the pcDNA3.1/myc-His(+)-vector (Invitrogen) to give pcDNA/znCD. The ORF of the znCD gene was 2,229-bp long and encoded 743 amino acid residues (Fig. 1A). The znCD exhibits sequence identity to other neutral CDases, i.e. 32.7% for *Pseudomonas aeruginosa*, 40.6% for Dictyostelium discoideum, 40.2% for *Drosophila melanogaster*, 57.2% for mouse, 57.2% for rat, and 59.8% for human, respectively. The predicted molecular weight and pI of the enzyme were 82,065 and 6.04, respectively, judging from the deduced amino acid sequence. The ORF contained eight potential N-glycosylation sites (Fig. 1A, underlined sequences). A hydrophobicity analysis revealed the presence of a prominent hydrophobic segment in the N-terminal region of a putative signal anchor sequence (Fig. 2B). Like neutral CDases from mammals, znCD has a Ser/Thr/Pro-rich domain (mucin-like domain) just downstream of the N-terminal hydrophobic region in which four potential O-glycosylation sites were found (Fig. 1A, circles). A alignment of the mucin-like domain of the znCD with that of other neutral CDases is shown in Fig. 1C. Interestingly, this specific domain is present in neutral CDases from vertebrates but not in enzymes from invertebrates such as bacteria, fruit fly, and slime mold.

**Expression and Characterization of the znCD in CHOP and BRF41 Cells—**To verify whether pcDNA/znCD encodes the neutral CDase, CHOP and BRF41 cells were transfected with pcDNA/znCD, and the CDase activity in cell lysates and culture supernatants was then measured using C12-NBD-Cer as a substrate at pH 7.5. The activity of the CDase in cell lysates of the transfected CHOP (Fig. 2A) and BRF41 (Fig. 2B) cells increased markedly compared with that of mock transfected (mock) or untransfected cells (data not shown). A part of the activity, possibly from the enzyme detached from the cells by processing of the N-terminal signal anchor sequence, was detected in the culture supernatants of the transfected CHOP and BRF41 cells (data not shown). It is noteworthy that the recombinant CDase from CHOP cells exhibited a neutral pH optimum (Fig. 2C). These results indicate that the cloned cDNA (pcDNA/znCD) certainly encodes the neutral CDase.

**Subcellular Localization of the znCD in CHOP and BRF41 Cells—**To examine the subcellular localization of znCD, myc-tagged znCD, tagged at the C-terminal end of the enzyme, was expressed in CHOP and BRF41 cells, and then myc signals were observed under a confocal fluorescence microscope. The myc signals were observed in endoplasmic reticulum/Golgi compartments as well as on plasma membranes when cells were permeabilized with Triton X-100 (Fig. 3, A and C). Endoplasmic reticulum/Golgi localization of the znCD was confirmed by simultaneous staining with specific markers, namely
FIG. 1. Nucleotide and deduced amino acid sequences (A), hydrophobicity plot of znCD (B), and alignment of N-terminal sequences of neutral CDases (C). A, the deduced amino acid sequence of znCD is shown in one-letter symbols below the nucleotide sequence. Amino acid residues are numbered beginning with the first Met, and the translation termination codon is denoted by an asterisk. Eight potential \( \text{N} \)-linked glycosylation sites and four potential \( \text{O} \)-linked glycosylation sites are underlined and surrounded with circles, respectively. Numbers correspond to amino acids (lower) and nucleotides (upper). B, a hydrophobicity plot of znCD was analyzed by the method of Kyte and Doolittle (33). C, the N-terminal sequence of znCD was compared with that of other neutral CDases based on deduced amino acid sequences using the CLUSTAL algorithm (34). The mucin-like domain is a Ser/Thr/Pro-rich domain.
Functional Analysis of Zebrafish Neutral Ceramidase

Fig. 2. Expression of znCD in CHOP (A) and BRF41 (B) cells and pH-dependence of the recombinant znCD (C). A and B, cDNA transfection was carried out using LipofectAMINE Plus for CHOP cells (A) and LipofectAMINE 2000 for BRF41 cells (B), respectively. pcDNA/znCD and mock indicate the transfectants bearing the whole ORF of znCD and the pcDNA3.1/Myc-His (+) vector without znCD ORF, respectively. The znCD activity of cell lysates was measured at pH 7.5 using 3 \( \mu \)g of protein and 400 pmol of C12-NBD-Cer as a substrate. C, pH-dependence of the recombinant znCD was measured using a lysate of CHOP cells transformed with pcDNA/znCD. The enzyme activity was measured at different pH using 3 \( \mu \)g of protein of the lysate and 400 pmol of C12-NBD-Cer as a substrate. In this assay, 150 mM GTA buffer (50 mM 3,3-dimethyl-glutaric acid, 50 mM Tris(hydroxymethyl)aminoethane, and 50 mM 2-amino-2-methyl-1,3-propanediol) with different pH values was used instead of 50 mM Tris-HCl buffer, pH 7.5. Details are shown under "Experimental Procedures."

Fig. 3. Subcellular localization of znCD expressed in CHOP and BRF41 cells. CHOP and BRF41 cells were transfected with a pcDNA/znCD vector. After cultivation for 24 h, cells were fixed and then stained with anti-myc antibody before and after permeabilization with 0.1% Triton X-100 as described under "Experimental Procedures." Arrows and arrowheads indicate the expression of znCD in plasma membranes and endoplasmic reticulum/Golgi compartments, respectively. A, CHOP cells after permeabilization with Triton X-100. B, CHOP cells before treatment with Triton X-100. C, BRF41 cells after permeabilization with Triton X-100. D, BRF41 cells before treatment with Triton X-100.

calnexin for the endoplasmic reticulum and Rab6 for the Golgi apparatus (data not shown). In the absence of Triton X-100, the signal at the C-terminal end of znCD was only observed on plasma membranes (Fig. 3, B and D). These results suggest that the subcellular localization of the znCD is almost the same as that of mouse and rat neutral CDases and that znCD is likely to present itself as a type II integral protein with its N-terminal end anchored to the plasma membrane and C-terminal end exposed to the extracellular space (10, 19).

Distribution and Glycosylation of znCD in Zebrafish Intestine—To investigate the localization of the znCD in zebrafish tissues, immunostaining was performed with frozen sections of adult zebrafish intestine, which was simply a useful organ to evaluate. Actually, the enzyme activity was detected widely in tissues such as brain, fin, muscle, small intestine, etc. Using anti-neutral CDase antiserum and Cy3-labeled anti-rabbit IgG antibody as a secondary antibody, a strong signal for znCD was observed at the luminal surface of the villi (Fig. 4A) and microvilli (Fig. 4D) of absorptive epithelial cells in zebrafish intestine. Counterstaining the actin filaments with phallolidin-fluorescein isothiocyanate (green) (Fig. 4, B and E) revealed that znCD was localized to upper part of the microvilli in the epithelial cells (Fig. 4, C and F). Collectively, in the zebrafish intestine the neutral CDase seems to concentrate at the apical surface of the epithelial cells.

Eight possible N-glycosylation and four possible O-glycosylation sites were detected in the deduced amino acid sequence of znCD (Fig. 1A). To verify whether znCD is actually glycosylated, the enzyme was immunoprecipitated from the membrane fraction of zebrafish small intestine using an anti-neutral CDase antibody as described under "Experimental Procedures" and then analyzed using glycopeptidase F and endoglycosidase H. These enzymes cleave the N,N'-chitobiose structure in Asn-linked glycoproteins but clearly differ in specificity, i.e., the former cleaves both high mannose and complex/hybrid-type N-glycans, whereas the latter acts specifically on high mannose type N-glycans. As shown in Fig. 5, the immunoprecipitated znCD showed a 110-kDa band on SDS-PAGE after visualization with an anti-neutral CDase antibody (Fig. 5A, lane 1). The 110-kDa protein band was reduced to 82.5 kDa after treatment with glycopeptidase F (lane 2), whereas the band was not affected by endoglycosidase H (lane 3), indicating that the znCD possesses complex/hybrid type N-glycans. Next, to verify whether the znCD is glycosylated with O-glycans, the immunoprecipitated enzyme was subjected to lectin blotting using peanut agglutinin, which specifically binds to the GalB1,3GalNAc sequence in O-glycans (28). As shown in Fig. 5B, znCD was stained with HRP-labeled peanut agglutinin lectin (lane 1) as in the mouse kidney CDase (lane 2). In conclusion, znCD from the zebrafish small intestine was glyco-
sylated with N- as well as O-glycans.

Change of znCD Activity during Zebrafish Embryogenesis—To address the biological roles of neutral CDase in the zebrafish embryogenesis, the activity of znCD was first examined at pH 7.5 using C12-NBD-Cer as a substrate during the course of embryogenesis. It was found that the activity of the enzyme decreased transiently at 9 h post-fertilization (hpf) (90%-epiboly stage) and then gradually increased until at least 60 hpf, when it was 4-fold higher than that of 1-cell stage embryos (Fig. 6). The drastic change in the activity during the course of embryogenesis may indicate the significance of znCD in the early development of zebrafish embryos.

Construction of AMOs and Inhibition of znCD Translation in Vitro by the AMOs—To elucidate the functions of znCD in zebrafish embryogenesis, we performed gene knockdown of the enzyme using three different antisense morpholino oligonucleotides (AMOs 1, 2, and 3) that were designed based on sequences at different sites of the 5′-untranslated region of the znCD mRNA (Fig. 7A). To evaluate the potency and specificity of AMO, we examined whether AMO inhibits the in vitro translation of the znCD in a wheat germ cell-free protein synthesis system. Although both AMO1 and a four-base mismatched morpholino oligonucleotide inhibited the protein translation of znCD in a dose-dependent manner, the four-base mismatched oligonucleotide was much less effective than AMO1 (Fig. 7B). On the other hand, no inhibitory effects were observed even at 50 μM by the control oligonucleotide, which does not show sequence similarity in znCD (Fig. 7B).

The Activity of znCD in AMO-injected Embryos—The injection of AMO1 into 1-cell-stage embryos at a concentration of 2 mg/ml (about 3 ng/embryo) led to a decrease in the znCD activity of embryos at 36 hpf by 25% compared with that of the control oligonucleotide-injected embryos. On the other hand, the injection of AMO2 and AMO3 at the same concentration resulted in a decrease of the activity by 20 and 40%, respectively. In summary, the order of potency for decreasing the znCD activity is AMO3 > AMO1 > AMO2. It should be noted that the inhibition of znCD activity by AMOs continued until at least 36 hpf. The four-base mismatched oligonucleotide slightly decreased the enzyme activity (<10%), and the control oligonucleotide did not affect the activity at all (Fig. 8A). To examine the specificity of AMO, the acid sphingomyelinase activity of embryos was measured after injection with AMO1. Notably, the AMO1 showed no effects on acid sphingomyelinase activity of embryos at 36 hpf, indicating that the AMO is specific to znCD (Fig. 8B).

The Phenotype of Zebrafish Embryos after Injection with AMOs—Interestingly, the knockdown of the znCD with AMOs led to an increase in the number of embryos with severe morphological and cellular abnormalities. A phenotype with pericardial edema and little (or the complete lack of) blood cell circulation at 36 hpf was characterized as "weak" in this study.
(Fig. 9B), whereas a phenotype was “strong” when head and tail morphogenesis was abnormal in addition to so-called weak characteristics (Fig. 9C). In a phenotype lacking the circulation of blood cells, a heartbeat was observed, but it seemed to be weaker than normal. Among ~200 embryos tested, <10 and 20% were abnormal when the control oligonucleotide and the four-base mismatched oligonucleotide were injected into one-cell stage embryos, respectively. In contrast, >90, 70, and 95% of embryos were abnormal when AMO1, 2, and 3 were injected, respectively, and these abnormal embryos were classified into weak and strong phenotypes as shown in Fig. 9D. The degree of abnormality was closely correlated to that of the decrease in znCD activity (Fig. 8A), that is, the abnormality became more severe in the following order: AMO3 > AMO1 > AMO2 (Fig. 9D). With the injection of AMO, abnormality was also detected at the cellular level when embryos were stained with acridine orange, which was used to stain apoptotic but not necrotic cells in Drosophila (29). Dead cells, possibly apoptotic cells, were detected in the head, dorsal, and tail regions of embryos at 24 and 36 hpf when 3 ng of AMO1 was injected into a one-cell stage embryo (Fig. 10, B–E). On the other hand, few dead cells were detected in control oligonucleotide-injected embryos at the same developmental stage (Fig. 10, A and C). Whole mount in situ hybridization using the znCD gene suggests that the expression of znCD mRNA corresponded roughly to the region where the dead cells were detected (data not shown).

Accumulation of Cer in Embryos with AMO Injection—To examine the change in the metabolism of Cer in zebrafish embryogenesis, we compared the amount of Cer between AMO- and control oligonucleotide-injected embryos. Interestingly, the Cer content of embryos injected with AMO1 and AMO3 was significantly high compared with that of control oligonucleotide-injected embryos (Fig. 11). Furthermore, the accumulation of Cer paralleled the degree of znCD inhibition by each AMO, i.e. the order in terms of Cer content in embryos was AMO3 > AMO1 > AMO2. These results strongly suggest that znCD is essential for Cer metabolism in zebrafish embryogenesis.
Rescue of znCD Activity and Abnormal Phenotype by znCD mRNA—The decrease of znCD activity caused by AMOs resulted in an increase in the incidence of abnormal phenotypes in zebrafish embryogenesis as described above. To address whether these phenotypes were actually due to an inhibition of znCD translation by the AMO, we examined whether exogenously supplied znCD mRNA rescues znCD activity and abnormal phenotypes. In this experiment, the synthesized znCD mRNA was injected into one-cell stage embryos simultaneously with AMO3, which showed the strongest enzyme inhibition and phenotype (Fig. 9). The sequence of znCD mRNA did not overlap with that of AMO3. Expectedly, znCD activity recovered to a normal level when 1.2 ng of znCD mRNA was simultaneously administrated with 3 ng of AMO (Fig. 12). The rescue of the phenotype was evaluated at 36 hpf using the index for blood cell circulation in the dorsal aorta and the posterior cardinal vein. As shown in Table I, 44% of embryos showed the phenotype of no circulation of blood cells at 36 hpf when 3 ng of AMO3 was injected, whereas 94% of control oligonucleotide-injected embryos displayed a normal vasculature (data not shown). Interestingly, the phenotype of no circulation of blood cells was greatly, but not completely, rescued when 1.2 ng of znCD mRNA was injected into one-cell stage embryos simultaneously with 3 ng of AMO3 (Table I and the accompanying movie available as supplemental material in the on-line version of this article). At the same time, the proportion of strong phenotypes was significantly decreased from 93.8 to 48.1% by the simultaneous injection of the znCD mRNA (data not shown). These results strongly suggested that the phenotype, especially the abnormality in circulation of blood cells, was triggered by a dysfunction of znCD. In conclusion, the znCD is essential for the metabolism of Cer and the early development of zebrafish.
DISCUSSION

The mammalian neutral CDases have a Ser/Thr-rich domain (mucin box) downstream of the N-terminal hydrophobic region, whereas bacterial and invertebrate enzymes do not (20). The mucin box of the mammalian enzymes is highly glycosylated with O-glycans and retains the enzyme at the plasma membrane as a type II integral membrane protein, because a mucin box-deleted mutant CDase and an Ala-replacement mutant enzyme were found to be secreted into the culture medium when expressed in human embryonic kidney 293 cells (20). Furthermore, it was clarified that bacterial and invertebrate neutral CDases, lacking a mucin box, were released into the extracellular milieu (9, 18). The present study revealed that znCD possesses a mucin box in which four possible O-glycosylation sites are present (Fig. 1A), and znCD from intestine, which localized at the apical membrane of the epithelial cells (Fig. 4), was actually glycosylated with O-glycans like the mammalian enzymes (Fig. 5B). These results clearly indicate that the mucin box is conserved in neutral CDases, but not in acidic or alkaline enzymes and not only from mammals but also from teleosts, and retains the enzyme at the plasma membrane, possibly as a type II integral membrane protein. Interestingly, the mucin-like domain seems to have increased in length during evolution, because it has become longer in this order: zebrafish/H11021 mouse/H11005 rat/H11021 human (Fig. 1C). However, it should be stressed that vertebrate CDases, including znCD, could be detached from cells by processing the N-terminal signal/anchor sequence, although these CDases are mainly localized at plasma membranes as type II integral proteins. Actually, znCD was detected in the culture medium when expressed in CHOP and BRF41 cells in this study, and the mouse enzyme was found in the serum (20).

The apparent decrease of znCD activity in embryos caused by AMOs seems to be lower than that expected (Fig. 8A). This may indicate the presence of other CDase species in zebrafish embryos that could not be inhibited by the AMOs used. We
found that acid and alkaline CDase genes are present in the zebrafish gene data base (www.ncbi.nlm.nih.gov/genome/seq/DrBlast.html), and we detected CDase activity in not only in neutral but also in acidic and alkaline ranges (data not shown). Thus, the CDase activity measured at pH 7.5 in this study seems to be overestimated because of contamination from acid and alkaline enzymes that could not be inhibited by the AMOs used, leading to an underestimation of the decrease in znCD activity caused by the AMOs.

To avoid misleading data from the nonspecific inhibition by AMOs, we synthesized three AMOs based on different sequences that correspond to three different parts of the znCD mRNA around the initiating start codon (Fig. 7A). Three AMOs were found to reduce the znCD activity when injected at the one-cell stage, but the potency for the inhibition of znCD activity was somewhat different (Fig. 8A). significantly, however, the degree of Cer accumulation (Fig. 11) and phenotype (Fig. 9) clearly correlated with the potency of the AMOs to inhibit znCD activity, whereas a control oligonucleotide that has no sequence similarity and a four-base mismatched oligonucleotide did not affect that of sphingomyelinase in zebrafish embryos (Fig. 8A).

Furthermore, the recovery of znCD activity with the simultaneous injection of AMO3 with znCD mRNA partially rescued the phenotype of a defect in the blood cell circulation (Table I). These results strongly suggest that a decrease of znCD activity by AMOs caused Cer accumulation, which was strongly related to abnormal phenotypes in zebrafish embryogenesis. We conclude that znCD is integral for the metabolism of Cer and normal embryogenesis of zebrafish.

This study suggests that a dysfunction of znCD causes the accumulation of Cer, which may trigger the abnormal phenotype in zebrafish embryogenesis. In this context, the report of Acharya et al. is of interest (16). They found that in arachidonic acid and phospholipase C mutants of Droso phila, Cer was significantly accumulated, possibly causing retinal degeneration. The decrease of Cer to the normal level by targeted overexpression of neutral CDase in the retina rescued the retinal degeneration. These findings suggest that neutral CDase functions to keep the Cer content at a normal level and that accumulation of Cer leads to abnormal phenotypes in invertebrates (the fruit fly; Refs. 16 and 17) and vertebrates (zebrafish; this study). However, the molecular mechanism underlying these phenomena, such as how Cer content is regulated by the neutral CDase or why Cer accumulation causes dysfunctions of certain cellular processes, remains to be clarified.

It should be elucidated whether the decrease of znCD activity could also influence the amount of Sph and S1P in zebrafish embryos, because Sph is thought to be solely produced from Cer through hydrolysis by CDases (6). It is widely accepted that S1P, which is produced through the phosphorylation of Sph by Sph kinases, promotes the proliferation and migration of endothelial cells through the activation of G protein-coupled receptors, i.e. Edgs (S1Pvs) (30). In zebrafish, abnormal organogenesis of the heart could be caused by the mutation of the gene encoding Edg-5 (S1P2) (31). It is possible that the phenotype of defective blood cell circulation (Fig. 9 and the accompanying movie available as supplemental material in the on-line version of this article) may stem from the decrease of S1P caused by the knockdown of znCD. We thus analyzed the amounts of Sph and S1P in embryos at 36 hpf by high performance liquid chromatography after extraction and derivatization of samples with o-phthalaldehyde, but no significant change in the quantity of these metabolites was observed between AMO- and control oligonucleotide-injected embryos (data not shown). However, the possibility that temporal and/or local decreases of Sph and S1P could occur cannot be ruled out.

Accumulating evidence suggests that neutral CDase regulates the intracellular contents of Cer by which sphingolipid-mediated signaling could be modulated (1, 14, 16, 32). However, almost all observations of neutral CDase functions were carried out at the cellular level and not at an individual level. This paper is the first report to reveal the functions of neutral CDase at an individual level and to show the usefulness of gene knockdown technology with AMOs in zebrafish embryogenesis for analyzing the functions of sphingolipid-signaling enzymes.

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**REFERENCES**

1. Hannun, Y. A., Obeid, L. M., and Wolff, R. A. (1993) Adv. Lipid Res. 25, 43–64
2. Mathias, S., and Kolesnick, R. (1993) Adv. Lipid Res. 25, 65–90
3. Van Brocklyn, J. R., Lee, M., Menzeleev, R., Oliveira, A., Edsall, L., Cuvillier, O., Thomas, M. D., Cooperr, J. P. P., Thangada, S., Liu, H. C., Hla, T., and Spiegel, S. (1998) J. Cell Biol. 142, 229–240
4. English, D., Garcia, J. G. N., and Brindley, D. N. (2001) Cardiovasc. Res. 49, 588–599
5. Klink, J. M., and Hla, T. (2002) Biochim. Biophys. Acta 1582, 72–80
6. Michel, C., van Echten-Deckert, G., Rothen, J., Sandhoff, K., Wang, E., and Merrill, A. H., Jr. (1997) J. Biol. Chem. 272, 22432–22437
7. Tani, M., Okino, N., Mori, K., Tanigawa, T., Ito, H., and Ito, M. (2000) J. Biol. Chem. 275, 11229–11234
8. Okino, N., Ichinose, S., Omori, A., Imayama, S., Nakamura, T., and Ito, M.
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(1999) J. Biol. Chem. 274, 36616–36622
9. Yoshimura, Y., Okino, N., Tani, M., and Ito, M. (2002) J. Biochem. 132, 229–236
10. Mitsutake, S., Tani, M., Okino, N., Mori, K., Ichinose, S., Omori, A., Iida, H., Nakamura, T., and Ito, M. (2001) J. Biol. Chem. 276, 26249–26259
11. Bawab, S. E., Robby, P., Qian, T., Blieawksa, A., Lemasters, J. J., and Hannun, Y. H. (2000) J. Biol. Chem. 275, 21508–21513
12. Monjashe, H., Okino, N., Tani, M., Maeda, M., Yoshida, M., and Ito, M. (2003) Biochem. J. 376, 473–479
13. Nikolva-Karakashian, M., Morgan, E. T., Alexander, C., Liotta, D. C., and Merrill, A. H., Jr. (1997) J. Biol. Chem. 272, 18718–18724
14. Franzen, R., Pautz, A., Brautigam, L., Geisslinger, G., Pleilschifter, J., and Huwiler, A. (2001) J. Biol. Chem. 276, 35382–35389
15. Coronesos, E., Martinez, M., McNeelley, S., and Kester, M. (1995) J. Biol. Chem. 270, 23005–23009
16. Acharya, U., Patel, S., Koundakjian, E., Nagashima, K., Han, X., and Acharya, J. K. (2003) Science 299, 1740–1743
17. Acharya, U., Moven, B. M., Nagashima, K., and Acharya, J. K. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 1922–1926
18. Okino, N., Tani, M., Imayama, S., and Ito, M. (1998) J. Biol. Chem. 273, 14368–14373
19. Tani, M., Okino, N., Mitsutake, S., Tanigawa, T., Izu, H., and Ito, M. (2000) J. Biol. Chem. 275, 3462–3468
20. Tani, M., Iida, H., and Ito, M. (2003) J. Biol. Chem. 278, 10523–10530
21. Tani, M., Kita, K., Komori, H., Nakagawa, T., and Ito, M. (1998) Anal. Biochem. 263, 183–188
22. Hefferman, M., and Dennis, W. J. (1991) Nucleic Acids Res. 19, 85–92
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
25. Naservic, A., and Ekker, S. C. (2000) Nat. Genet. 26, 216–220
26. Sugura, M., Kono, K., Shimizu-gawa, T., Minekura, H., Spiegel, S., and Ko- hama, T. (2002) J. Biol. Chem. 277, 23294–23300
27. Bektas, M., Jolly, S. P., Mälstein, S., and Spiegel, S. (2003) Anal. Biochem. 320, 259–265
28. Lotan, R., Skutelsky, E., Danon, D., and Sharon, N. (1975) J. Biol. Chem. 250, 8518–8523
29. Abrams, J. M., White, K., Fessler, L. I., and Steller, H. (1993) Development 117, 29–43
30. Lee, M.-J., Thangada, S., Claffey, K. P., Ancellin, N., Liu, H. C., Kluk, M., Volpi, M., Sha’afi, I. R., and Hla, T. (1999) Cell 99, 301–312
31. Kupperman, E., An, S., Osborne, N., Waldron, S., and Stainier, Y. R. D. (2000) Nature 406, 192–195
32. Franzen, R., Fabbrro, D., Aschrafi, A., Pleilschifter, J., and Huwiler, A. (2002) J. Biol. Chem. 277, 46184–46190
33. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
34. Thompson, J. D., Huggins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680