Endoglycoceramidase (EGCase; EC 3.2.1.123) is an enzyme capable of cleaving the glycosidic linkage between oligosaccharides and ceramides of various glycosphingolipids. We detected strong EGCase activity in animals belonging to Cnidaria, Mollusca, and Annelida and cloned the enzyme from a hydra, Hydra magnipapillata. The hydra EGCase, consisting of 517 amino acid residues, showed 19.2% and 50.2% identity to the Rhodococcus and jellyfish EGCases, respectively. The recombinant hydra enzyme, expressed in CHOP (Chinese hamster ovary cells expressing polyoma LT antigen) cells, hydrolyzed [14C]GM1a to produce [14C]ceramide with a pH optimum at 3.0–3.5. Whole mount in situ hybridization and immunocytochemical analysis revealed that EGCase was widely expressed in the endodermal layer, especially in digestive cells. GM1a injected into the gastric cavity was incorporated and then directly catabolized by EGCase to produce GM1a-oligosaccharide and ceramide, which were further degraded by exoglycosidases and ceramidase, respectively. However, hydra exoglycosidases did not hydrolyze GM1a directly. These results indicate that the EGCase is indispensable for the catabolic processing of dietary glycosphingolipids in hydra, demonstrating the unique catabolic pathway for glycosphingolipids in the animal.

Glycosphingolipids (GSLs), amipathic compounds consisting of oligosaccharides and ceramides, are characteristic

Unique Catabolic Pathway of Glycosphingolipids in a Hydrozoan, *Hydra magnipapillata*, Involving Endoglycoceramidase*

Yasuhiro Horibata‡, Keishi Sakaguchi‡, Nozomu Okino‡, Hiroshi Iida‡, Masanori Inagaki‡, Toshitaka Fujisawa**, Yoichiro Hama**, and Makoto Ito‡ ‡‡

From the Department of Biotechnology, Graduate School of Bioscience and Biotechnology, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, and the Department of Applied Genetics and Pest Management, Graduate School of Bioscience and Biotechnology, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, the Faculty of Pharmaceutical Sciences, Graduate School Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, and the Department of Developmental Genetics, National Institute of Genetics, 1,111 Yata, Mishima, Shizuoka 411-8540, and the **Department of Applied Biological Sciences, Faculty of Agriculture, Saga University, 1 Honjo, Saga 840-8502, Japan

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mmer was further converted to fatty acid and fatty acid-derived metabolites, whereas the latter was converted to shorter chain oligosaccharides, possibly GM2- and GM3-oligosaccharides. It was, however, noted that GM1a per se was resistant to hydrolysis by hydra exoglycosidases. These results strongly suggest that EGCase is involved in the catabolic processing of dietary GSLs in hydra. This catabolic pathway is completely different from that of mammals, which are likely to be missing the EGCase.

**EXPERIMENTAL PROCEDURES**

**Materials**—GM1a was prepared from crude bovine brain ganglioside using the sialidase-producing bacterium, *Pseudomonas sp.* FY-2 as previously described (12). ^14C(GM1a, labeled at the fatty acyl chain in ceramide (14C)stearic acid, d18:1 sphinganine), or sialic acid/GalNAc residues in sugar chain was prepared as described in a previous study (13). Glabridin was purchased from Dafron Lab. Inc. (Tokyo, Japan). GTbH, GD1a, GD1b, GD3, GM3, asialo-GM1, and lactosylceramide (LacCer) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Gl4- (Galβ1,6Galβ1,4Glcβ1,1′Cer) was donated from Dr. M. Hoshi, Keio University, Japan (14). t-5-(GalNAcβ1,4GalNAcβ1,4GlcNAcβ1,3Manβ1,4Glcβ1,1′Cer) from larvae of the green bottle fly, *Lucilia coarctata* (15), and Lipid IV (Glcα4Meβ1,4GalNAcα1,3Fucα1,4GlcNAcβ1,3Manβ1,4Glcβ1,1′Cer) from spermatocytes of a freshwater bivalve, *Hyriopsis schlegelii* (16) were kindly donated by Dr. M. Sugita, Shiga University, Japan. Neogalactosylceramide (TGG; Galβ1,6Galβ1,4Glcβ1,1′Cer) was isolated from a turban shell, *Turbo cornutus*, by a method described previously (17). Triton X-100 and Lubrol PX were obtained from Sigma and Nacalai Tesque Inc. (Kyoto, Japan), respectively. A precasted Silica Gel 60 TLC plate was purchased from Merck (Darmstadt, Germany). All other reagents were of the highest purity available.

**Hydra Culture**—Strain 105, the standard wild-type strain of *H. magnipapillata*, was used in this study. Animals were cultured in hydra medium (1 mM Tris-HCl buffer, pH 7.6, 1 mM NaCl, 1 mM CaCl2, 0.1 mM KCl, and 0.1 mM MgSO4) at 18 °C using the standard method (18). Freshly hatched brine shrimp *Artemia salina* were fed to hydra three times a week, and the medium was changed daily. Before the experiment, animals were starved for 3 days.

**EGCase Assay**—Ten nanomoles of GM1a (containing 100 pmol of ^14C) in ethanolic GM1a, 5 nCi) was incubated at 37 °C for a specific period with an appropriate amount of the enzyme in 20 μl of 25 mM sodium acetate buffer, pH 3.0, containing 0.2% (v/v) Triton X-100. The reaction was stopped by heating in a boiling water bath for 5 min. After drying, the sample was dissolved in 20 μl of 50% methanol and applied to TLC plates, which were then developed with chloroform/methanol/0.02% CaCl2, 5/4/1, v/v). Radioactive GM1a and ceramide separated by TLC were analyzed with an imaging analyzer (Cryoscan, FujiFilm, Tokyo, Japan) and quantified with Image Gauge version 3.0 (FujiFilm). One unit of the EGCase was defined as the amount of enzyme that catalyzes the release of 1 μmol of GM1a/min under the conditions described above. A value of 10^−10, 10^−9, and 10^−8 units of enzyme was expressed as 1 milliunit, 1 microunit, and 1 nanounit, respectively.

For substrate specificity, non-radioactive GSLs (10 nmol each) were employed. GSLs and oligosaccharides were visualized by spraying the TLC plates with orcinol-H2SO4 reagent and scanning them by using a Shimadzu CS-9300 chromatoscanner (Kyoto, Japan) with the reflection mode.

**Function of Animal Endoglycoceramidase**

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**Ceramidase Assay**—The activity of ceramidase was measured using C12-NBD-ceramide (C12-NBD-Cer, d18:1) as a substrate (19). 100 pmol of C12-NBD-ceramide was incubated with the enzyme in 20 μl of the indicated buffer containing 0.4% Triton X-100 at 37 °C for 16 h. After drying, the sample was dissolved in 15 μl of chloroform/methanol (2/1, v/v) and applied to a TLC plate, which was then developed with chloroform/methanol/acetone/ammonia (90/10/5/1, v/v). C12-NBD-fatty acid was separated from C12-NBD-ceramide by TLC and then analyzed and quantified with a Shimadzu CS-9300 chromatoscanner (excitation 475 nm, emission 525 nm).

**Purification of Hydra EGCase**—Hydra EGCase—Ceramide assay—Hydra EGCase was purified using a DIG BNA Labeling Mix (Roche Diagnostics). In situ hybridization of whole mounts was carried out as described previously (20). Briefly, hydra were fixed with 4% paraformaldehyde after relaxation of the polyps with 2% urethane. Specimens were subsequently treated with ethanol and proteinase K. To stabilize the digested tissues, specimens were re-fixed with 4% paraformaldehyde for 15 min and then prehybridized in prehybridization solution (50 mM NaCl, 5 × SSC, 1 × Denhardt’s solution, 200 μg/ml total RNA, 0.1% Tween 20, 0.1% CHAPS, and 100 mg/ml heparin) to block non-specific hybridization sites. This was followed by hybridization for 16 h with the DIG-labeled RNA probe and subsequent washing in hybridization solution and then in 1 × SSC. The specimens were further washed in MBA (100 mM maleic acid, 150 mM NaCl, pH 7.5) and pre-blocked for 2 h in MBA with 2% blocking reagent (Roche Diagnostics). This was followed by a 16-h incubation at 4 °C in the same solution with anti-DIG antibody (Roche Diagnostics). The specimens were washed eight times with MBA and then briefly in alkaline phosphatase buffer (100 mM Tris-HCl buffer, pH 9.5, containing 50 mM MgCl2, 100 mM NaCl, and 0.1% Tween 20). Sections were then stained with BM-purple (Roche Diagnostics).

**Expression of Hydra EGCase in CHO Cells**—A cDNA fragment encoding the hydra EGCase was prepared by PCR using a 5′ primer containing a Kozak sequence (21) and EcoRI site (5′-GAATTCGACCATGTGAAGGCTGACATTTAT-3′), a 3′ primer containing an XhoI site (5′-CCGTCGATTGATAGCTATTATCAACCA-3′), and pSHKGC Ecoli DNA polymerase (Takara, Shiga, Japan). The PCR product was digested with EcoRI and XhoI and introduced into these sites in the vector of pCDNA3.1/Myc-His^®^/A (Invitrogen, Carlsbad, CA). CHO cells, Chinese hamster ovary cells expressing polyoma lymphomavirus TK to support the replication of eukaryotic expression vectors (22), were grown in α-minimal essential medium supplemented with 10% fetal bovine serum, 100 μg/ml of kanamycin, and 100 units/ml penicillin in a humidified incubator containing 5% CO2. Cells were seeded at 10^4 cells/dish. Transfection with vector alone or vector containing hydra EGCase was performed using LipofectAMINE Plus (Invitrogen) according to the instructions of the manu-
facturer. After 4 h of incubation with the mixture, the cells were grown in fresh medium. After 24 h, cells were harvested and suspended in 100 μl of 20 mM sodium acetate buffer, pH 6.0, containing 0.1% Triton X-100. The enzyme activity in the cell lysate was measured as described above.

Release of EGCase—One-hundred-starved hydra were fed brine shrimp, washed, and then cultured in fresh hydra medium (1 ml/well on 6-well plates). After a period of time, 100 μl of the medium was withdrawn and then centrifuged at 17,400 × g for 5 min. The EGCase activity of the supernatant was assayed at 37 °C for 2 h using [14C]stearyl-GM1a as a substrate.

In Vivo Digestion of [14C]GM1a in Hydra—50 pmol of [14C]GM1a (2.5 nCi), dissolved in hydra medium (–1 ml), was injected through the mouth into the gastric cavity of the hydra using a micropipette. Just before the injection, the hydra were fed brine shrimp. After the animals were cultured for a period of 18 °C, total lipids were extracted from 10 animals by homogenization with 400 μl of chloroform/methanol (1/2, v/v). The extract was evaporated dry, redissolved in 20 μl of the same solvent, and analyzed by TLC, using a solvent system of chloroform/methanol/water (60/30/5, v/v). Radioactive lipids were analyzed by the method described above.

Extraction of Glycolipids from Brine Shrimp and Fast Atom Bombardment Mass Spectrometry—Total lipid was extracted from 13 g of freshly hatched brine shrimp by homogenization with 15 ml of chloroform/methanol (2/1, v/v). The extraction was repeated twice. Combined extracts were divided into upper and lower phases with Folch's partition (23). After being evaporated to dryness, the lower phase was incubated in 0.1 M methanolic KOH at 37 °C for 1 h to eliminate glycolipids. After the removal of salt using Folch's partition, the lipid was dissolved in chloroform/methanol (98/2, v/v) and applied to a column of Sep-Pak plus silica (Waters Co., Milford, MA). GSLs were eluted from the column with methanol. Each fraction was spotted onto a TLC plate for separation. The plates were developed in a solvent system of chloroform/methanol/0.2% CaCl2 (5/4/1, v/v), and visualized by spraying with orcinol-H2SO4 reagent. For fast atom bombardment mass spectrometry, GSLs were purified by TLC. The portions corresponding to the orcinol-H2SO4-positive bands were scraped from the unstained duplicate TLC plate and then extracted with chloroform/methanol (1/1, v/v). After removal of the silica by centrifugation, the supernatant was dried under N2 gas. Approximately 10 μg of GSL was mixed with triethyleneglycol as a matrix and subjected to FAB mass spectrometric analysis, which was performed in the negative ion mode.

Fatty Acid and Sugar Composition Analysis—The fatty acid and sugar compositions of hydra GSLs were analyzed by the methods described previously (24, 26). Briefly, methyl esters of fatty acids and methyl glycosides were obtained by methanolysis of GSLs with 1.0 M anhydrous methanolic HCl at 100 °C for 3 h. Fatty acid methyl esters were extracted with hexane, and the methyl glycosides remaining in the methanolic solution were concentrated, N-acetylated, and trimethylsilylated. The fatty acid and sugar compositions were determined separately by GLC on a Shimadzu GC-14A equipped with a fused silica capillary column (DB-1, 0.25 mm × 30 m, J & W Scientific).

Preparation of Anti-hydra EGCase Antibody—To obtain the recombinant hydra EGCase, the ORF was subcloned into the bacterial expression vector pET32b (+Novagen, Madison, WI), and introduced into AD494(DE3)pLysS, an E. coli strain, for protein expression. The cells were cultured at 37 °C in 1.5 liters of LB medium containing 50 μg/ml ampicillin, 15 μg/ml kanamycin, and 35 μg/ml chloramphenicol. When the absorbance at 600 nm reached 0.4, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM, and the mixture was incubated for 5 h at 37 °C. Cells were harvested by centrifugation at 7,000 × g for 10 min at 4 °C, and the pellet was lysed with 20 mM Tris-HCl buffer, pH 7.5, containing 1% Triton X-100. After centrifugation at 15,000 × g for 10 min, the pellet (inclusion bodies) was lyzed by sonication in 50 mM Tris-HCl buffer, pH 7.5, containing 8 mM urea. The recombinant protein was purified using a Hi-Trap chelating column (Ni2+–, Amersham Biosciences) according to the manufacturer's instructions. The purified protein was then subjected to 10% SDS-PAGE, and the protein band was extracted from the gel using 20 mM Tris-HCl buffer, pH 7.5, containing 1% SDS. Antiserum against recombinant hydra EGCase was obtained from a rabbit immunized with the purified protein.

SDS-PAGE and Western Blotting—SDS-PAGE was carried out according to the method of Laemmli (26). The transfer of protein to polyvinylidene difluoride membrane was performed using Trane-Blot SD (Bio-Rad) as described previously (27). After treatment with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (T-TBS) for 1 h, the membrane was incubated with anti-hydra EGCase antiserum for 1 h at room temperature. After a wash with T-TBS, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit antibody for 1 h. After another wash with T-TBS, the ECL reaction was performed for 2–3 min as recommended by the manufacturer, and chemiluminescent signals were visualized with an ECL Mini-camera (Amersham Biosciences).

Immunohistochemistry and Fluorescence Microscopy—Hydra were fixed with 4% paraformaldehyde for 2 h after relaxation of the polyps with 2% urethane. The samples were rinsed with PBS and then infiltrated with 20% sucrose in PBS overnight at 4 °C. They were embedded in Tissue-Tek OCT compound (Sakura Finetek Co., Tokyo, Japan), rapidly frozen using liquid nitrogen, and stored at −80 °C. The frozen materials were cut into 9- to 10-μm thick sections using a cryostat (Leica CM1850, Wetzlar, Germany) and mounted on poly-l-lysine-coated glass slides. After treatment with 5% skim milk in PBS (blocking buffer) for 20 min at room temperature, the samples were incubated with the anti-hydra EGCase antiserum diluted 1:500 with a blocking buffer for 2 h at room temperature followed by Cy3-labeled anti-rabbit IgG (Amersham Biosciences) at room temperature for 1 h. For controls, pre-immune serum was used as the primary antibody. For nuclear staining, SYTOX Green (Invitrogen) was used. To identify more precisely the EGCase-expressing cells, macerated samples were used. Hydra were placed in maceration fluid, acetic acid/glycerol/water (1/1/13, v/v), for 10 min, and then shaken to disperse the cells (28). The suspended cells were fixed using 4% paraformaldehyde and spread on a gelatin-coated glass slide. Immunostained samples were observed under a confocal laser-scanning microscope (Digital Eclipse C1, Nikon, Tokyo, Japan).

RESULTS

Distribution of EGCase in Animals—The EGCase activity in various invertebrates was examined using [14C]GM1a ([14C]stearyl acid, d18:1 sphingenine) as the substrate (Table I). The activity was found to be widely distributed in animals of the phyla Cnidaria, Mollusca, and Annelida. In addition to the animals listed in Table I, we detected EGCase activity in sea sponges, baby octopus, ear shells, etc., but not in mammalian or...
insect cell lines such as HEK293 cells (human embryonic kidney cells), COS-1 cells (African green monkey cells), and Schneider 2 cells (Drosophila melanogaster) cells (data not shown). Among the animals tested, we found a relatively high specific activity of EGCase in cnidarians and thus selected H. magnipapillata as a model to study the biological roles of animal EGCase.

General Properties of the Hydra EGCase—First, EGCase was purified from extracts of H. magnipapillata by conventional column chromatography as described under “Experimental Procedures.” The optimum pH of the purified enzyme was found to reside in an extremely acidic range, pH 3.0–4.0, when [14C]GM1a was used as a substrate (Fig. 1A). The enzyme required detergents for the hydrolysis of GSLs. The optimal concentrations of Triton X-100 and Lubrol PX were 0.2% (w/v) and 0.4% (w/v) at pH 3.0, respectively, although taurodeoxycholate at 0.1–1% (w/v) strongly inhibited the enzyme activity. Cu2+, Zn2+, Mn2+, Ca2+, Mg2+, and EDTA had no significant effects on the activity at 5 mM. Fig. 1B shows the specificity of the purified enzyme for various GSLs. The extent of hydrolysis of GSLs by the enzyme was expressed under different conditions; one expressed the relative initial reaction velocity (30 min, open bar) and the other the degree of hydrolysis after exhaustive digestion (16 h, solid bar). Of all the substrates tested, GSLs having a gangliotetraose structure appeared to be the best substrates for the enzyme regardless of the presence or absence of sialic acid residues. GT1b was the most favored substrate under both conditions. Hydra EGCase hydrolyzed globoside very slowly compared with ganglio-series GSLs, in-
the hydrophobic motif was also clearly indicated by a hydrophobicity plot (Fig. 2B). The deduced amino acid sequence of the hydra EGCase contained four potential N-glycosylation sites, consistent with the notion that the hydra EGCase is glycosylated by N-glycans (Fig. 1C). The ORF showed 19.2% and 50.2% identity to that of the Rhodococcus M-777 and jellyfish EGCases, respectively, at the amino acid level (Fig. 3). In addition to the NEP sequence, seven amino acid residues, which are essential for the catalytic activity of GH5 endo-β-1,4-glucanases (32), were all conserved in these three EGCases; Glu230 and Glu349 are thought to be an acid/base catalyst and a nucleophile, respectively, and His125 is thought to be important for interaction with glucose (Fig. 3).

To verify whether the putative ORF actually encodes the EGCase, CHOP cells were transfected with an expression vector containing the ORF. EGCase activity was detected in the
lysate of the CHOP cells transfected with the ORF-containing plasmid but not in that of the mock transfectant (Fig. 4A). The activity of hydra EGCase was not detected in the bacterial expression system using *E. coli* as a host cell (data not shown), suggesting that N-glycosylation is necessary for the enzyme activity. This speculation was supported by the finding that the hydra EGCase showed no activity when expressed in CHOP cells in the presence of tunicamycin, a specific inhibitor for N-glycosylation (data not shown).

The pH dependence of the recombinant hydra EGCase was quite similar to that of the purified native enzyme (Fig. 1A), the optimum pH being 3.0–4.0 (Fig. 4B). This result indicates that the enzyme is a typical acid EGCase like the jellyfish enzyme.

**Whole Mount in Situ Hybridization and Immunocytochemical Analysis of the Hydra EGCase**—The expression of the EGCase mRNA in hydra was analyzed with whole mount *in situ* hybridization. The transcripts were detected specifically in the endodermal layer throughout the body. It was noticeable that signals were strong in the tentacles and peduncles (Fig. 5A, right panel). No signals were detected with the sense RNA probe (Fig. 5A, left panel). The expression of EGCase protein was also examined using anti-hydra EGCase antibody under a...
flourescent microscope. As shown in Fig. 5B, strong signals for EGCase were detected in endodermal cells. This result indicates that the site of expression of the EGCase protein is well consistent with that of the mRNA. Endodermal cells are made up of digestive cells and gland cells. To identify which cells express the EGCase, macerated cells were subjected to immunostaining with anti-hydra EGCase antibody. As shown in Fig. 5C, the enzyme was specifically detected in digestive cells but not gland cells. To investigate the distribution of EGCase activity in the hydra, the animal was cut into three parts (tentacles, gastric cavity, and peduncle regions), from each of which the enzyme was extracted and the activity was measured. The specific activity in the three parts was almost the same (tentacles, 0.25 milliunit/mg; gastric cavity, 0.21 milliunit/mg; and peduncle regions, 0.23 milliunit/mg), suggesting that EGCase was expressed in the endoderm throughout the whole body.

Release of the Hydra EGCase in the Gastric Cavity—It is reported that digestive enzymes such as proteases are secreted into the gastric cavity when hydra ingest food (33). To clarify the possibility that hydra EGCase functions as an extracellular digestive enzyme, its release into the medium during the feeding process was examined. Interestingly, EGCase activity was released into the surrounding medium when brine shrimp was fed to the hydra (Fig. 6A). Notably, however, no EGCase activity was detected in the medium without prey (brine shrimp). It was confirmed that the EGCase activity was not derived from the prey, because the extract of brine shrimp contained no EGCase activity (data not shown). Next, we examined the total EGCase activity in the whole body of the hydra before and after release of the enzyme. EGCase activity in body was transiently reduced when the prey was given (Fig. 6B). This timing coincides with the increase of the enzyme activity in the medium. EGCase activity levels were restored after 24 h (Fig. 6B). These results strongly suggest that the hydra EGCase in the digestive cells is released during the digestive process into the gastric cavity and eventually into the surrounding medium. The enzyme seemed to be released from digestive cells but not from gland cells, because EGCase was exclusively detected in the digestive cells but not gland cells (Fig. 5C).

Digestion of Brine Shrimp GSLs by the Hydra EGCase—The results described above suggest that EGCase hydrolyzes diet-derived GSLs. We thus investigated whether brine shrimp possesses GSLs, which are actually degraded by hydra EGCase. We detected several alkaline-resistant GSLs in brine shrimp (Fig. 7A, lane 1). These GSLs were not stained with either resorcinol or Dittmer reagent, indicating that these compounds contain no sialic acids or phosphate groups (data not shown). Brine shrimp GSLs were degraded by Rhodococcus EGCase II to generate oligosaccharides (Fig. 7A, lane 4) and ceramide (data not shown). As previously reported (5), the microbial EGCase did not hydrolyze glucosylceramide (GlcCer), which then remained intact after the enzyme treatment. On the other hand, the treatment of brine shrimp GSL with the
partially purified hydra EGCase diminished levels of GlcCer and other GSLs with the simultaneous production of glucose and GSL-derived oligosaccharides (Fig. 7A, lane 2). It is of note that the \( R_p \) of GSL-derived oligosaccharides by either EGCase was the same. The addition of Conduiritol B epoxide, a specific inhibitor for \( \beta \)-glucosylceramidase (34), suppressed the production of glucose and decrease of GlcCer, suggesting that the hydra EGCase preparation contained a \( \beta \)-glucosylceramidase. In fact, the hydra extract showed that the hydra EGCase preparation contained a \( \beta \)-glucosidase activity using CM-5PW chromatography, indicating that the major \( \beta \)-glucocerebroside activity in the hydra is not due to EGCase (data not shown). However, the possibility that the hydra EGCase per se hydrolyzed GlcCer can not be ruled out at present, because the leech ceramide glycanase (EGCase) hydrolyzes GlcCer very slowly (7). In any case, the dietary GSLs could be degraded by the combination actions of EGCase and \( \beta \)-glucocerebroside in the hydra.

The major EGCase-digestible GSL of brine shrimp (GSL-X) was subjected to negative ion FAB-MS analysis. The parental molecular ion [M-H] was observed at \( m/z \) 1265. Fragment ions of GSL-X indicated the sequential elimination of N-acetylgalactosamine, deoxyhexose, hexose, and hexose being detected at \( m/z \) 1062, 916, 754, and 592, respectively (Fig. 7B). The sugar composition of GSL-X, determined by GLC with trimethylsilyl derivatives, was GlcNAc, Fuc, Man, and Glc in a molar ratio of about 1:1:1:1. GSL-X seems to be composed of two bands on TLC, thus, each was extracted from the TLC and analyzed separately for sugar and fatty acid composition. The major fatty acids of the upper and lower bands were C22:0 and 2-hydroxy-C22:0, respectively, whereas the sugar compositions of both were the same. Conclusively, brine shrimp, a prey of the hydra, possesses an EGCase-digestible neutral GSL, although the precise structure of the GSL remains to be determined.

**Digestion of \( ^{14}C \)GSLs by EGCase in Vivo**—To examine whether hydra EGCase can hydrolyze exogenously added GSLs in vivo, \( ^{14}C \)GM1a labeled at the fatty acyl chain or sugar chain was injected into the gastric cavity of the hydra just after the animal had captured brine shrimp, and then the catabolism of GM1a was monitored. After a period, radioactive metabolites of GM1a was fed to hydra, \( ^{14}C \)ceramide first appeared after 0.5 h as a digestion product, after which \( ^{14}C \)stearic acid and its putative metabolites were observed at 1–2 h (Fig. 8A). This
result indicated that the EGCase hydrolyzed the exogenously added GM1a to generate the ceramide relatively early during the digestive process. To clarify why $^{14}$C-stearic acid was produced just after the generation of $^{14}$C-ceramide, the hydra was examined for ceramidase activity using a fluorescent ceramide as the substrate at different pHs. Ceramidase is an enzyme capable of hydrolyzing the $\text{N}$-acyl linkage of ceramide to generate fatty acid and sphingosine (EC 3.5.1.23). As shown in Fig. 8B, an acid ceramidase having a pH optimum at 5.0 was actually found in the hydra. The pH dependence of the ceramidase activity of hydra was assayed at different pHs using C12-NBD-ceramide as the substrate as described under “Experimental Procedures.”

Hydra were labeled with $^{14}$C-stearyl GM1a (2.5 nCi) or $^{14}$C-stearic acid (2.5 nCi) for 24 h, and then radioactive metabolites were extracted and analyzed as described under “Experimental Procedures.” Lane 1, metabolites from $^{14}$C-stearic acid-labeled hydra; lane 2, metabolites from $^{14}$C-stearyl GM1a-labeled hydra. D, TLC shown in A was quantified with Image Gauge version 3.0. $^{14}$C-ceramide, $^{14}$C-stearic acid, $^{14}$C-GlCer, $^{14}$C-GM1a, $^{14}$C-GM3, $^{14}$C-GM2, and $^{14}$C-GM1a-labeled oligosaccharides were extracted and analyzed as described under “Experimental Procedures.”

$^{14}$C-GM1a was injected into the gastric cavity of 10 hydra. After incubation at 18°C for the periods indicated, radioactive lipids and oligosaccharides were extracted and analyzed as described under “Experimental Procedures.”

Fig. 8. In vivo catabolism of exogenously added $^{14}$C-GM1a in hydra. A, time course for catabolism of $^{14}$C-stearyl GM1a in hydra. A 50 pmol of $^{14}$C-GM1a (2.5 nCi) was injected into the gastric cavity of 10 hydra. After incubation at 18°C for the periods indicated, radioactive lipids were extracted and analyzed as described under “Experimental Procedures.” STD shows the standard $^{14}$C-ceramide and $^{14}$C-stearic acid. B, the pH dependence of the ceramidase activity of hydra was assayed at different pHs using C12-NBD-ceramide as the substrate as described under “Experimental Procedures.” $\bullet$, glycine-HCl buffer, pH 2.0–3.0; $\blacktriangle$, sodium acetate buffer, pH 3.0–6.0; $\square$, sodium phosphate buffer, pH 6.0–7.0; $\bigcirc$, Tris-HCl buffer, pH 7.5–9.0. C, comparison of the catabolic pattern obtained with $^{14}$C-stearyl GM1a and $^{14}$C-stearic acid as a starting precursor. Hydra were labeled with $^{14}$C-stearyl GM1a (2.5 nCi) or $^{14}$C-stearic acid (2.5 nCi) for 24 h, and then radioactive metabolites were extracted and analyzed as described under “Experimental Procedures.” Lane 1, metabolites from $^{14}$C-stearic acid-labeled hydra; lane 2, metabolites from $^{14}$C-stearyl GM1a-labeled hydra. D, TLC shown in A was quantified with Image Gauge version 3.0. $\blacksquare$, $^{14}$C-GM1a; $\bm{\square}$, a + b + c + d; $\bullet$, $^{14}$C-ceramide; $\bigcirc$, $^{14}$C-stearic acid. E, time course for catabolism of $^{14}$C-GM1a labeled at GalNAc/sialic acid residue in hydra. 50 pmol of $^{14}$C-GM1a (2.5 nCi) was injected into the gastric cavity of 10 hydra. After incubation at 18°C for the periods indicated, radioactive lipids and oligosaccharides were extracted and analyzed as described under “Experimental Procedures.”

Metabolites a–d were suggested to be triacylglycerol (TAG), phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine, respectively, judging from the $RF$ values for the TLC and alkaline sensitivity. It is noteworthy that metabolites e and f were also detected by metabolic labeling of hydra with $^{14}$C-choline (data not shown). Metabolite e showed the same $RF$ value of the standard GlcCer, and f was hydrolyzed by bacterial sphingomyelinase to generate ceramide, suggesting that alkaline-resistant e and f were cerebroside and sphingomyelin, respectively. However, alkaline-resistant EGCase-sensitive GSLs were not found in hydra in this study (data not shown). Fig. 8D shows the quantification of Fig. 8A, i.e. the time course for metabolism of $^{14}$C-fatty acyl-GM1a in the hydra, indicating that the amount of GM1a gradually
decreased during the course of the experiment, whereas levels of ceramide, stearic acid, and other metabolites \((a + b + c + d)\) increased. These results suggested that the dietary GSLs were degraded by EGCase and ceramidase to produce fatty acids, which were further metabolized to various glycerophospholipids and triacylglycerol.

The \([14\text{C}]\)sugar-GM1a was also fed to hydra, and its catabolism was monitored. As shown in Fig. 8E, GM1a-oligosaccharide was first observed after 0.5–1 h by the action of the EGCase. The oligosaccharide was gradually converted to the sugar-GM1c, whereas sialidase was not detectable in hydra (data not shown). Interestingly, \([14\text{C}]\)GM1a was directly converted by EGCase to GM1a-oligosaccharide while hydra exoglycosidases did not attack GM1a directly under the conditions used because no GM2 or GM3 was found after prolonged incubation (Fig. 8E). In summary, catabolism of GSLs in the hydra is initiated by the removal of sugar chains from GSLs by EGCase; subsequently, sugar chains and ceramides are degraded by exoglycosidases and ceramidase, respectively. Both enzymes were actually detected in hydra, whereas sialidase was not detectable in hydra (data not shown).

The catalytic pathway for GSLs involving EGCase that is likely to be missing in mammals.

**DISCUSSION**

The catabolism of GSLs has been studied extensively for several decades mainly in mammals. In mammals, endogenous GSLs are finally decomposed by various exoglycosidases in lysosomes to generate monosaccharides and ceramide. The latter is further degraded by ceramidase to fatty acid and sphingosine. On the other hand, there have been few reports on the catabolism of dietary GSLs. Nilsson (35) reported that exoglycosidase activity of GSLs is conserved in several decades mainly in mammals. In mammals, endogenous glucosylceramide was catabolized by the actions of glucosylceramidase (EC 3.2.1.45) and ceramidase to generate sphingosine and fatty acid, the latter of which was further converted to TAG and PC in the intestinal tract of rats. It should be emphasized that EGCase is not likely to be present in mammals. Thus, the new catalytic pathway for GSLs involving EGCase in hydra is completely different from that in mammals. This catalytic pathway seems to be unique but quite ubiquitous in invertebrates except insects, because EGCase activity is widely distributed in Cnidaria, Mollusca, Annelida, Porifera, Echinodermata, and others.

Hydra are the most primitive group of animals with a defined body plan and widely used for the study of pattern formation, cell differentiation, and morphogenesis (36). The animal has a simple tube-shaped body, column and foot, and is made up of two multicellular layers separated by an extracellular matrix, named the mesoglea. The outer layer (ectodermal cell layer) consists of epithelialmuscular cells and cells in the interstitial cell lineage (nematocytes, nerve, and germ cells), whereas the inner layer (endodermal cell layer) is made up of digestive cells and gland cells. During the feeding process, the prey are captured and paralyzed by the action of nematocysts and organelle in nematocytes, most of which are located on the tentacle ectodermal epithelial cells called battery cells. The captured prey are ingested into the gastric cavity and degraded into small particles by digestive enzymes secreted from gland cells. The digestive cells phagocytize the particles into phagosomes/lysosomes (food vacuoles) where the particles are further digested into organic compounds to be used as nutrients (33). Hydra EGCase seems to participate in the catabolic processing of GSLs in two different stages. First, EGCase functions as an extracellular digestive enzyme in the gastric cavity. This conclusion is supported by the finding that EGCase is transiently released into the cavity during feeding (Fig. 6, A and B) and actually hydrolyzes the diet-derived GSLs (Fig. 7A). However, the enzyme seems to be released from the digestive cells but not from gland cells, because the enzyme is exclusively found in the former cells in the endoderm (Fig. 5, B and C). Second, the enzyme could be involved in the catabolism of GSLs in the phagosomes/lysosomes of digestive cells. This was suggested by the extremely acidic pH optimum of the hydra EGCase (Figs. 1A and 4B).

The question of whether the hydra EGCase participates in the catabolism of endogenous GSLs is still unanswered. The metabolic labeling of hydra using \([14\text{C}]\)stearic acid, \([14\text{C}]\)serine, and \([14\text{C}]\)galactose indicated de novo synthesis of several alkaline-resistant lipids, among which sphingomyelinase-sensitive lipid and cerebroside are included. However, we were not able to detect alkaline-resistant EGCase-digestible GSLs in the hydra in the present study.

We isolated a full-length cDNA of EGCase from jellyfish (10) and hydra (this work) and showed that the NEP sequence, the active site of GH5 endo-\(\beta\)-1,4-glucanases (cellulase) (32) and Rhodococcus EGCase II (30, 31), was conserved in both eukaryotic and microbial EGCases. Furthermore, seven integral amino acid residues, which form the active site region of GH5 endo-\(\beta\)-1,4-glucanases (32), were completely conserved in animal and microbial EGCases. These results strongly suggest that EGCases and endo-\(\beta\)-1,4-glucanases form a similar catalytic domain and hydrolyze internal \(\beta\)-glucosyl linkages via the same mechanism. However, in substrate specificities, EGCase differs completely from endo-\(\beta\)-1,4-glucanases, that is, EGCase did not hydrolyze cellulose, whereas endo-\(\beta\)-1,4-glucanases did not hydrolyze GSLs. Homology modeling of Rhodococcus EGCase II using Clostridium endo-\(\beta\)-1,4-glucanases as a template indicated EGCase II to be a \(\beta\)Ct barrel, a so-called triosephosphate isomerase-barrel structure. This model also suggested that the different substrate specificities of the two enzymes are derived from the shape of the substrate-binding cleft (3). Because active site residues are thought to be conserved during evolution, EGCases and GH5 endo-\(\beta\)-1,4-glucanases may be derived from the same ancestral gene.

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Unique Catabolic Pathway of Glycosphingolipids in a Hydrozoan, *Hydra magnipapillata*, Involving Endoglycoceramidase

Yasuhiro Horibata, Keishi Sakaguchi, Nozomu Okino, Hiroshi Iida, Masanori Inagaki, Toshitaka Fujisawa, Yoichiro Hama and Makoto Ito

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