Neutral Ceramidase Encoded by the Asah2 Gene Is Essential for the Intestinal Degradation of Sphingolipids*

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Complex sphingolipids are abundant as eukaryotic cell membrane components, whereas their metabolites, in particular ceramide, sphingosine, and sphingosine 1-phosphate, are involved in diverse cell signaling processes. In mammals, degradation of ceramide by ceramidase yields sphingosine, which is phosphorylated by the action of sphingosine kinase to generate sphingosine 1-phosphate. Therefore, ceramidases are key enzymes in the regulation of the cellular levels of ceramide, sphingosine, and sphingosine 1-phosphate. To explore the physiological functions of a neutral ceramidase with diverse cellular locations, we disrupted the Asah2 gene in mice. Asah2 null mice have a normal life span and do not show obvious abnormalities or major alterations in total cellular levels in tissues. The Asah2-encoded neutral ceramidase is highly expressed in the small intestine along the brush border, suggesting that the neutral ceramidase may be involved in a pathway for the digestion of dietary sphingolipids. Indeed, Asah2 null mice were deficient in the intestinal degradation of ceramide. Thus, the results indicate that the Asah2-encoded neutral ceramidase is a key enzyme for the catabolism of dietary sphingolipids and regulates the levels of bioactive sphingolipid metabolites in the intestinal tract.

Sphingolipids have diverse functional roles (1, 2). Complex sphingolipids such as sphingomyelin and glycosphingolipids are abundant plasma membrane constituents in plant and animal cells. Here they function as organizers of membrane structure, regulators of cell signaling, and mediators of cell-cell interactions. Simple sphingolipid metabolites that can be derived by the degradation of the complex sphingo- lipids, including ceramide, sphingosine, and sphingosine 1-phosphate, fulfill a different role as signaling molecules. These simple sphingolipids utilize both intracellular and extracellular targets and are potent regulators of cell growth and survival.

In the sphingolipid degradative pathway, ceramidases (EC 3.5.1.23) catalyze the hydrolysis of the N-acyl group of ceramide to yield sphingosine and fatty acids. Sphingosine can then be phosphorylated by sphingosine kinase to yield sphingosine 1-phosphate. Thus, cerami- dases are key regulators of sphingolipid signaling metabolites. Cerami- dases have traditionally been classified according to the pH range (acid, neutral, or alkaline) that supports their optimal activity. Recently, the mammalian ceramidases have been defined more precisely by genetic approaches to establish their enzymatic activity. Indeed, the action of sphingosine kinase to generate sphingosine 1-phosphate is critical for the metabolism of dietary sphingolipids and, thus, the regulation of bioactive ceramide and sphingosine levels in the gastrointestinal tract.

EXPERIMENTAL PROCEDURES

Preparation of Polyclonal Antibody—A rabbit anti-mouse neutral ceramidase antibody (Ab174) was raised against the peptide corresponding to amino acids 741–756 of mouse neutral ceramidase (CILAFEGISSPFEVTT). The peptide was conjugated to keyhole limpet hemocyanin as a carrier and injected into rabbits. The specific antibody was isolated by affinity purification from immune serum using the peptide antigen.

Generation of Asah2 Null Mice—To construct the Asah2 targeting vector, a 4.8-kbp cassette containing the neomycin-resistance gene was inserted between a BamHI and a MluI sites in the same orientation as the Asah2 gene (Fig. 1). Gene targeting in TC1 embryonic stem (ES) cells and generation of chimeric and heterozygous mice were performed as described previously (26). One targeted ES clone was used to establish chimeric mice, which were crossed with C57BL/6 mice to obtain Asah2 heterozygous mice. The heterozygous mice were viable and appear without severe defects. However, the Asah2 null mice are impaired in the intestinal degradation of sphingolipids, a process that has been reported to alter susceptibility to intestinal tumorigenesis (19–24).

Neutral ceramidase in mice is encoded by the Asah2 gene (9–11). Orthologous genes have been identified in human (12), rat (13), zebrafish (14), Drosophila (15), and bacteria (16). The neutral cerami- dase has been identified as a type II integral membrane protein that can be cleaved to yield a soluble secreted protein (17). Interestingly, a short- ened human neutral ceramidase truncated at the amino terminus was shown to localize to mitochondria (12). The enzyme has also been demon- strated in the apical membranes of proximal and distal tubules, collect- ing ducts of kidney, endosome-like organelles of hepatocytes (13), and in the epithelia of the jejunum and ileum (11, 18), suggesting pos- sible diverse physiologic functions. In zebrafish, the expression of the enzyme was found to be critical for early development (14). To identify the physiologic functions of this neutral ceramidase in mice, we inactiv- ated the Asah2 gene by homologous recombination. We found that Asah2 null mice are viable and appear without severe defects. However, the Asah2 null mice are impaired in the intestinal degradation of sphingolipids, a process that has been reported to alter susceptibility to intestinal tumorigenesis (19–24). Our findings indicate that an essential physiologic function of the neutral ceramidase is for the metabolism of dietary sphingolipids and, thus, the regulation of bioactive ceramide and sphingosine levels in the gastrointestinal tract.

An acid ceramidase is encoded by the Asah1 gene (3–5). Mutations in the corresponding human gene cause Farber disease, a storage disorder produced by the lysosomal accumulation of ceramide (6). An alkaline ceramidase is specified by the Asah3 gene that is highly expressed in the skin (7). A second related but distinct alkaline ceramidase has been identified that has a substrate preference for phytoceramide (8).

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‡ The abbreviations used are: ES, embryonic stem; NBD, [7-nitro-2-1,3-benzoxadiazol-4-yl]amino; PBS, phosphate-buffered saline; IS, internal standard; WT, wild type; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol.

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mice, and mice back-crossed to the fifth generation were used for experiments. The Asah2 genotypes were determined by Southern blot analysis and PCR. For PCR genotyping, three primers (Fig. 1) were used: 5’-ACAGGACACCCAATCTCCATT3’ (primer 1), 5’-GAGTGCCTGGAGAGATGGAAGA3’ (primer 2), and 5’-ATCGCCCTCTATCGCTTCTTGA3’ (primer 3). Primers 1 and 2 detected the wild-type Asah2 allele and amplified an ~280-bp fragment. Primers 2 and 3 detected the Asah2 neo allele and amplified an ~390-bp fragment. Forty cycles of 94 °C (30 s), 55 °C (30 s), and 72 °C (1 min) were used.

**Northern Analysis**—Total RNA was prepared from mouse liver and kidney using TRIZol® reagent (Invitrogen). Total RNA (50 µg) was fractionated on denaturing formaldehyde-gel agarose gels (1%) and then transferred to positively charged nylon membranes (GeneScreen Plus; PerkinElmer Life Sciences and Analytical Science). The membranes were prehybridized in PerfectHyb Plus (Sigma-Aldrich) with 0.1 mg/ml denatured salmon sperm DNA at 68 °C for 1 h. Hybridization was performed at 68 °C overnight with 11P-labeled DNA probe prepared by the random priming method. Asah2 cDNA was used as a probe. The membranes were washed in 2X SSC (sodium chloride/sodium citrate buffer), 0.1% SDS at 25 °C for 5 min and in 0.2X SSC, 0.1% SDS at 50 °C for 5 min, 55 °C for 5 min, 60 °C for 5 min, and 65 °C for 5 min. Radiolabeled bands were detected using a BAS-2500 radioimage analyzer (Fujifilm).

**Real-time PCR**—Regional dissection of the intestinal tract was accomplished as described (27). cDNA was synthesized from 1 µg of total RNA. Real-time PCR was performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems) as described previously (28). Assay-on-Demand™ gene expression products for Asah2, Sphk1, Sphk2, Sggpl, Sgpl1, Eubp5, and TagMan Universal PCR Master Mix (Applied Biosystems) were used for real-time PCR. For quantitation, Gapdh was amplified simultaneously.

**Assay of Ceramidase Activity**—The neutral ceramidase activity was measured as described previously using NBD C12-ceramide (Avanti Polar Lipids Inc.) and palmitoyl-ethyl-[erythro]-9,10-2Hsphingosine (American Radiolabeled Chemicals, Inc.) as the substrates with slight modification (29). Small intestine, brain, liver, and kidney were harvested after mice were fasted for 18 h. Regional dissection of the intestinal tract was accomplished as described (27). Mucosa was obtained by cutting the entire small intestine longitudinally and scraping the mucosa off with a glass slide. Mucosa, brain, liver, and kidney were homogenized in buffer A (1 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.32 M sucrose, and protease inhibitor mixture (Sigma-Aldrich)) and centrifuged at 10,000 × g at 4 °C for 30 min. The pellet was resuspended in buffer A, and 50 µg of homogenate protein was incubated in 20 µl of reaction buffer (25 mEq Tris-HCl, pH 7.5, 1% sodium cholate, and 50 µM NBD C12-ceramide or 50 µM palmitoyl-ethyl-[erythro]-9,10-2Hsphingosine (1 µCi of palmitoyl-ethyl-[erythro]-9,10-2H-sphingosine) at 37 °C for 10 min. The reaction was terminated by the addition of 100 µl of chloroform/methanol (2/1, v/v). This solution was dried in a SpeedVac concentrator, dissolved in 30 µl of chloroform/methanol (2/1, v/v), and applied onto TLC plates (silica gel 60, Merck). The TLC was developed with chloroform/methanol/2.5 N ammonia (60/35/8, v/v). NBD C12-ceramide and NBD C12 fatty acid, the enzyme reaction products, were separated from NBD C12-sphingomyelin by TLC, and band intensity was measured using a luminescent image analyzer (LAS-1000 plus, Fujifilm). The amount of NBD C12 fatty acid, NBD C12-ceramide, and NBD C12-sphingomyelin were quantitated using a standard curve.

**Ceramide Digestion In Vitro**—The capacity of intestinal segments to degrade NBD ceramide was accomplished by a method similar to that described by Schmelz et al. (23). Small intestine was harvested after mice were fasted for 18 h. Regional dissection of the intestinal tract was accomplished as described (27), and 2-cm segments were cut off from the middle of the respective intestinal regions. Each segment was opened longitudinally, rinsed with cold PBS, and incubated in 200 µl of reaction buffer (PBS, 0.017% sodium cholate, and 20 µM NBD C12-ceramide) at 37 °C. After various incubation times, the reaction mixture was transferred to a glass tube that was placed on ice. Residual intestinal segments were washed with 200 µl of PBS, and the buffer was combined with the reaction mixture. Another 200 µl of PBS was added to intestinal segments. One ml of chloroform/methanol/hydrochloric acid (100:100:1, v/v) and 200 µl of 1 M sodium chloride were added to both fractions of the reaction mixture and the intestinal segment, mixed vigorously, and centrifuged at 2,000 × g for 10 min. The organic phase of the solution was collected, evaporated under nitrogen gas, and dissolved in 100 µl of chloroform/methanol (1:1, v/v), and 10 µl was applied onto TLC plates (silica gel 60, Merck). TLC was developed with chloroform/methanol/2.5 N ammonia (90/20/0.5, v/v) and visualized using a luminescent image analyzer (LAS-1000 plus, Fujifilm).

**Liquid Chromatography/Tandem Mass Spectrometry Analysis of Sphingolipids**—Sphingolipids were determined in extracts of major tissues (brain, lung, heart, thymus, spleen, kidney, liver, and skeletal muscle and in the gastrointestinal tract, excluding luminal contents) and in the serum of Asah2 null and wild-type mice. Gastrointestinal tract tissues were harvested after fasting the mice for 18 h. Regional dissection of the intestinal tract was accomplished as described (27). Each intestinal segment was opened longitudinally and rinsed with cold PBS.

Electrospray ionization/tandem mass spectrometry analysis of endogenous sphingosine, sphingoid base-1-phosphates and ceramide species were performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer, operating in a multiple reaction monitoring (MRM) positive ionization mode, using a modified version (30) of a published protocol (31). Briefly, the biological materials were fortified with internal standards (C17 base-d-erythro-sphingo-1-phosphate, C17 sphingosine-1-phosphate, N-palmitoyl-d-erythro-C17-sphingosine, and heptadecanoyl-d-erythro-sphingosine and C6-phytoceramide), then extracted with ethyl acetate/isopropanol/water (60/30/10, v/v) solvent system. After evaporation and reconstitution in 150 µl of methanol, samples were injected on the Surveyor/TSQ 7000 LC/MS system and gradient-eluted from the BDS Hypersil C8, 150 × 3 mm, 3-µm particle size column, with a 1.0 µl methanolic ammonium formate/2 mM aqueous ammonium formate mobile phase system. Peaks corresponding to the target analytes and internal standards (ISs) were collected and processed using the Xcalibur software system. Quantitative analysis was based on the calibration curves generated by spiking an artificial matrix with the known amounts of the target analyte synthetic standards and an equal amount of the ISs. The target analyte/IS peak areas ratios were plotted against analyte concentration. The target analyte/IS peak area...
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ratios from the samples were similarly normalized to their respective ISs and compared with the calibration curves, using a linear regression model. Introduction of the internal standards to the samples prior to extraction yielded results already "recovery-corrected", therefore, no further data manipulations were necessary.

Western Blot Analysis—Stomach, duodenum, jejunum, ileum, cecum, and colon were homogenized in buffer A and centrifuged at 10,000 × g at 4 °C for 30 min. The pellet was resuspended in buffer A. Ten μg of protein was incubated in NuPAGE sample buffer with reducing agent (Invitrogen) at 70 °C for 10 min, loaded onto a NuPAGE 4–12% bis-Tris gel (Invitrogen), and blotted onto nylon membrane (Invitrogen). The nylon blot was blocked by immersion in Odyssey blocking buffer (LI-COR Bioscience) at room temperature for 1 h. After washing with 0.1% Tween 20 containing PBS for four times (5 min per wash), the membrane was incubated with Alexa Fluor 680 goat anti-mouse IgG (H+L) (0.4 μg/ml, Molecular Probes) and IRDye 800-conjugated affinity-purified goat anti-rabbit IgG Fc (1:5000 dilution, Rockland Immunochemicals for Research) at room temperature for 1 h. After washing with 0.1% Tween 20 containing PBS for four times (5 min per wash), and with PBS once (5 min per wash), immunoreactivity was detected using an Odyssey infrared system (LI-COR Bioscience).

Histological Analysis—Small intestine and cecum were harvested after fasting mice for 18 h. Small intestine was rolled tightly on No. 3MM chromatography paper (Whatman) soaked in PFA (4% paraformaldehyde in 100 mM phosphate buffer). The rolled small intestine and cecum, kept separately in histology cassettes with foam pads, were soaked in PFA at 4 °C overnight. The cassettes were transferred to 30% sucrose in PBS and kept at 4 °C overnight. The small intestine and cecum were removed from cassettes and embedded in OCT compound (Sakura Finetechical Co., Ltd., Tokyo). Serial sections (5-μm thick) were stained with hematoxylin and eosin. For immunostaining, the specimens were fixed in PFA at 4 °C for 15 min, and antigen retrieval was accomplished by a 30-min incubation at 95 °C in Target retrieval solution (DAKO Corp.). Endogenous peroxidase activity was quenched by incubation with 0.3% hydrogen peroxide in methanol for 5 min. After rinsing with TBST (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% Tween 20), blocking was performed by a 20-min incubation in CAS-BLOCK (Zymed Laboratories Inc.). The specimens were incubated with anti-neutral ceramidase antibody (Ab174; 5 μg/ml) at room temperature for 1 h followed by a 20-min incubation in horseradish peroxidase-conjugated goat anti-rabbit IgG (1 μg/ml; Upstate) after rinsing with TBST. The neutral ceramidase expression was visualized using a peroxidase substrate kit (Vector Laboratories Inc.).

Detection of Ceramide in Feces—Asah2 null and wild-type mice were maintained on a AIN-76A diet supplemented with 0.1% (w/w) milk sphenogmyelin (Avanti Polar Lipids, catalog No. 860065) or on a AIN-76A diet alone. After 1 week of feeding, feces were collected during a 1-h period and dried. Total lipids were extracted from 25 mg of feces (dry weight) with 2 ml of chloroform/methanol (1:1, v/v). Organic and aqueous phases were separated by adding 1 ml of water and centrifugation at 2,000 × g for 10 min. An aliquot of organic phase was transferred to a glass tube, dried under nitrogen gas, and dissolved as micelles by brief sonication in 40 μl of 10 mM imidazole (pH 6.6), 7.5% octyl-β-D-glucopyranoside, 5 mM cardiolipin, and 0.2 mM diethylenetriamine-pentaacetic acid (Sigma). 140 μl of reaction buffer (100 mM imidazole, pH 6.6, 100 mM NaCl, 25 mM MgCl2, 2 mM EGTA, 10 mM dithiothreitol, and 10 milliunits of Escherichia coli diacylglycerol kinase) was added to lipid micelles, and reaction was initiated by adding 20 μl of [γ-32P]ATP (10 μCi; 10 mM ATP, 200 mM MgCl2). After incubation at room temperature for 1 h, lipids were fractionated in the organic phase by adding 0.5 ml of chloroform/methanol/hydrochloric acid (100:100:1, v/v) and 100 μl of 1 M sodium chloride followed by centrifugation. An aliquot of organic phase was applied onto TLC plates (silica gel 60, Merck). The TLC was developed with the solvent chloroform/methanol/acetic acid/water (10:3:4:2.1, v/v) and analyzed with a BAS-2500 radioimage analyzer (Fujiﬁlm).

RESULTS

Generation of Asah2 Mutant Mice—The mouse Asah2 gene spans more than 70 kbp and consists of 27 exons and 26 introns (10, 11). An Asah2 targeting vector was constructed to produce a deletion of a portion of exon 17 and the entire exon 18 (Fig. 1A). Southern blot analysis of KpnI-digested genomic DNA from properly targeted ES cells yielded a >20-kbp band, corresponding to the Asah2 wild-type allele, and a 8-kbp band corresponding to the recombined Asah2 allele (Fig. 1, A and B). A targeted ES clone was used to produce chimeras and subsequently heterozygous mice. Interbreeding of the Asah2 heterozygous mice yielded wild-type, heterozygous, and homozygous mutant (null) offspring (Fig. 1C) at approximately Mendelian ratios (wild type:heterozygote:homozygote mutant = 151:319:153), demonstrating the absence of significant embryonic lethality in the mutant mice. Intercrossing of the Asah2 homozygous mutant mice produced Asah2 homozygous mutant offspring, indicating that fertility of males and females was largely unaffected. The Asah2 null mice grew normally, did not exhibit obvious abnormalities, and were viable for up to 1 year. A histological evaluation of major organs and tissues did not reveal unusual pathology.

Asah2 Expression—To confirm the absence of Asah2 transcripts in Asah2 null mice, Northern blot analysis was performed using total RNA from liver and kidney (Fig. 1D). In Asah2 null tissues, the Asah2 6.0-kb transcript was not detected, whereas wild-type and heterozygous mice expressed the Asah2 transcript in a gene dosage-dependent manner (Fig. 1D). In agreement with a previous report (11), in normal mice the Asah2 gene was found to be highly expressed in small intestine when compared with expression levels in the brain, liver, and other portions of the gastrointestinal tract, including stomach, cecum, and colon (Fig. 2A). Correspondingly, in normal (wild-type) mice, neutral ceramidase activity was highly elevated in small intestinal mucosa compared with extracts of brain, liver, and kidney (Fig. 2B). Neutral ceramidase activity was not detectable in Asah2 null tissues, confirming the functional disruption of the Asah2 gene (Fig. 2B). Similar results were obtained using 3H-C16-ceramide as a substrate (data not shown; specific activities in wild-type small intestinal mucosa: NBD C12-ceramide, 50.7 ± 11.4 nmol/h/mg protein; 3H-C16-ceramide, 12.6 ± 0.17 nmol/h/mg protein).

Western blot analysis using an anti-neutral ceramidase antibody (Ab174) demonstrated the expression of an 115 kDa major band and an ~100 kDa minor band in duodenum, jejunum, and ileum of wild-type mice, with the strongest signal in the jejunum (Fig. 3A). No immunoreactive bands were detected in tissues from the Asah2 null mice. Immunostaining localized the neutral ceramidase to the epithelial mucosa in jejunum and ileum in wild-type mice (Fig. 3B; ileum data not shown). The Asah2 null tissues showed no expression of the neutral ceramidase (Fig. 3B).

Sphingolipid Levels—Sphingolipid levels, including total and individual ceramide species, sphingosine, and sphingosine 1-phosphate, were determined in major tissues (brain, lung, heart, thymus, spleen, kidney, liver, skeletal muscle, and serum) and in gastrointestinal tract tissues of Asah2 null and wild-type mice by mass spectrometry analysis. With the
exception of the intestinal tract tissues, sphingolipid levels were similar in wild-type and Asah2 null mice (Fig. 4, data not shown). In jejunum, the level of C16:0 ceramide (the major species of ceramide in the gastrointestinal tract tissues) was significantly increased in Asah2 null mice when compared with wild type (Fig. 4A). The level of total ceramide (Fig. 4B), however, was not significantly different in wild-type versus Asah2 null mice in intestinal tissues. Phytoceramide levels were not elevated in tissues of the gastrointestinal tract (data not shown).

Levels of sphingosine, a product of the ceramidase reaction, were significantly decreased in jejunum and colon of Asah2 null mice when compared with those of wild-type mice (Fig. 4C). Phytosphingosine in Asah2 null jejunum, ileum, and cecum was also depressed compared with wild-type tissues (jejunum: wild type (WT), 8.20 ± 3.26 versus Asah2 null, 6.75 ± 1.35, p = 0.0087; ileum: WT, 14.67 ± 4.40 versus Asah2 null, 4.47 ± 0.24, p = 0.0814; cecum: WT, 3.97 ± 0.20 versus Asah2 null, 2.63 ± 0.18, p = 0.0077). Sphingosine 1-phosphate levels were not significantly different in the gastrointestinal tract tissues or in other tissues in wild-type versus Asah2 null mice (data not shown).

Intestinal Metabolism of Sphingolipids—The ability of wild-type and Asah2 null intestinal mucosa to degrade sphingomyelin carrying the NBD label on the fatty acid was assessed (Fig. 5). Wild-type mucosa produced a time-dependent decrease in the level of the sphingomyelin substrate accompanied by a transient appearance of ceramide. As the incubation period continued, both sphingomyelin and ceramide

FIGURE 1. Targeted disruption of Asah2. A, a schematic representation of the Asah2 targeting strategy. The structure of the wild-type mouse Asah2 locus is shown at the top, the structure of the Asah2 targeting vector is in the middle, and the predicted structure of the recombined locus is at the bottom. Numbers indicate exons; Neo, neomycin-resistance gene. B, Southern blot analysis of KpnI-digested genomic DNA from a wild-type ES cell clone (WT) and from an ES cell clone that had undergone targeted recombination (Rec) at the Asah2 locus. The probe used for Southern blot analysis is shown in A. The >20-kbp band corresponds to the wild-type Asah2 allele and the 8-kbp band to the targeted Asah2 allele. C, genotyping of mouse offspring using tail DNA by PCR with primers 1, 2, and 3, shown in A. Asah2 WT allele yielded a 280-bp band, and the targeted Asah2 allele yielded a 390-bp band. M, DNA markers; HT, heterozygous; C, control without template DNA. D, Northern blot of liver and kidney RNA from Asah2 WT, heterozygous, and null mice was performed using Asah2 cDNA as a probe. The ethidium bromide-stained gel is shown at the bottom as the loading control.
decreased to low levels and were replaced by the free fatty acid product of the ceramidase reaction (Fig. 5A). In contrast, degradation of sphingomyelin substrate by Asah2 null mucosa yielded only ceramide, without further degradation (Fig. 5B). The results demonstrate that the absence of the neutral ceramidase in intestinal mucosa from Asah2 null mice blocked the degradation pathway of sphingomyelin after the generation of ceramide. Localization of ceramide digestion in intestine was studied by incubation of NBD-labeled ceramide with intestinal tissue segments as enzyme sources (Fig. 6). Wild-type small intestine segments, especially duodenum and jejunum (proximal and distal) segments, substantially degraded NBD-labeled ceramide to the NBD-labeled fatty acid. In contrast, only minimal degradation of NBD-labeled ceramide was observed with Asah2 null intestinal segments. The ceramide content of feces was determined after feeding wild-type and Asah2 null mice a diet supplemented with 0.1% milk sphingomyelin (Fig. 7). Asah2 null feces contained 725 ± 79.2 pmol of ceramide in 100 g of feces, whereas wild-type feces contained 13.6 ± 3.10 pmol of ceramide/100 g of feces (Asah2 null versus wild-type, p = 0.0008). Collectively, these results indicate the Asah2 null mice are defective in the intestinal digestion of dietary ceramide.
Sphingolipid Digestion by Neutral Ceramidase

Ceramide exists at a critical branch point in sphingolipid metabolism, utilized both as a backbone for the synthesis of complex sphingolipids and via degradation, for the generation of bioactive lipids including sphingosine and sphingosine 1-phosphate. Ceramide itself is a signaling molecule in a variety of cellular contexts, most notably in stress and apoptosis responses. The regulation of cellular ceramide amounts is therefore carefully controlled and occurs at multiple levels, through de novo synthesis, as a precursor for complex sphingolipid synthesis, and via the breakdown by ceramidas. Here we have established Asah2 null mice lacking a neutral ceramidase to probe the function of this enzyme in mammalian physiology.

In contrast to the essential nature of the neutral ceramidase in development of zebrafish (14), Asah2 null mice showed no signs of embryonic lethality, indicating that this enzyme is not essential for mammalian development. It is notable that Asah1 null mice lacking the acid ceramidase are blocked in embryonic development (33) and that Faber disease patients, with mutations in the ASAH1 gene, accumulate ceramide in their cells and tissues (6). As adults, the Asah2 null mice appeared healthy and were fertile. No pathology of major organ systems was apparent. In major tissues of the Asah2 null mice the total ceramide levels were not significantly different from those in wild-type mice, although the level of one subspecies, C16:0-ceramide, was elevated in the Asah2 null jejunum. These results indicate that the neutral ceramidase is not necessary to regulate bulk ceramide levels in most tissues, which is consistent with a lack of apoptosis-related pathology in the Asah2 null mice. Presumably, other ceramidas, such as the acid and alkaline forms, function to control metabolic levels of ceramide in cells.

Although the neutral ceramidase was detected in several tissues including heart, brain, liver and kidney, the highest level of Asah2 expression was found to be within the small intestine, with peak levels in the jejunum. The ceramidase protein was localized to the brush border membrane of the small intestinal epithelia. These results are consistent with earlier reports of the detection of high levels of neutral ceramidase activity in rat intestine (18) and a high level of neutral ceramidase expression in the mouse intestinal mucosa (11). Western blot analysis revealed an ~115 kDa major ceramidase protein form along with a minor ~100 kDa form. As defined by Tani et al. (17), the larger band may represent the membrane-bound enzyme, whereas the smaller band may correspond to a soluble form with the membrane anchor clipped. NBD-labeled ceramide was degraded by the incubation with wild-type intestinal segments but not with Asah2 null intestinal segments as an enzyme source (Fig. 6), indicating that the active site of neutral ceramidase is exposed to the lumen of the small intestine at the brush border.

The activity of the intestinal alkaline sphingomyelinase would directly precede that of the neutral ceramidase for the degradation of sphingomyelin and is believed to have a similar topology (34–36), indicating that the sequential degradation of sphingomyelin and ceramide occurs within the lumen of the intestine.

The intestinal mucosa preparations from the Asah2 null mice liberated ceramide, presumably through the alkaline sphingomyelinase, but were unable to hydrolyze ceramide further. Segments of small intestine from Asah2 null were highly deficient in the ability to degrade ceramide. Finally, substantially higher amounts of ceramide were found in the feces of Asah2 null mice compared with the levels of ceramide in the feces of wild-type mice. These experiments indicate a critical role of the neutral ceramidase in the intestinal metabolism of ceramide.

**DISCUSSION**

**Gene Expression of Intestinal Enzymes Involved in Metabolism of Sphingolipids**—We determined the relative gene expression from regions of the intestinal tract of other enzymes potentially involved in the degradation of sphingolipids (Fig. 8). Of the enzymes tested, alkaline sphingomyelinase (Enpp5) (32), sphingosine kinase 1 (Sphk1), and sphingosine 1-phosphate lyase (Sgpl1) showed an expression gradient similar to Asah2, with expression peaking in the distal jejunum.
ently, the bile salt-stimulated lipase, reported to exhibit neutral ceramidase activity within the proximal small intestine, was unable to fully substitute for the neutral ceramidase (37).

Animal food products (such as meat, milk, and fish) and some plant food products (such as soybeans) contain substantial amounts of sphingolipids (38). Dietary sphingolipids are hydrolyzed to sphingosine and fatty acids in the middle and lower intestinal tract (39–41). A pathway for the intestinal digestion of complex sphingolipids is shown in Fig. 9. Ceramide is liberated from sphingomyelin by the action of the highly expressed alkaline sphingomyelinase within the lumen of the small intestine. We have shown here that the neutral ceramidase encoded by Asah2 is essential for the further degradation of ceramide. As an ectoenzyme, with the active site of the neutral ceramidase oriented extracellularly, the ceramidase reaction would also occur in the intestinal lumen. Further degradation, based on the results of Schmelz et al. (41) and the predominantly intracellular location of the sphingosine kinases (42, 43), would take place after sphingosine is transported within the epithelial cells, where it is phosphorylated, yielding sphingosine 1-phosphate. Based on its expression pattern in the intestine (Fig. 8), sphingosine kinase 1 may participate. Sphingosine-1-phosphate lyase, which is also coordinately expressed with alkaline sphingomyelinase, neutral ceramidase, and sphingosine kinase 1 in the small intestine, could then degrade the sphingosine 1-phosphate to hexadecenal and phosphoethanolamine. Synthesis back to ceramide and complex sphingolipids is also believed to occur via a salvage pathway that is initiated by the action of phosphatase on sphingosine 1-phosphate (41). Proapoptotic ceramide can be derived in substantial amounts from dietary sources of sphingolipids. This ceramide may be largely excluded from the interior of cells, where its molecular targets exist, because of its efficient degradation in the lumen (Fig. 9). Instead, sphingosine liberated by the extracellular action of neutral ceramidase would be transported into cells and enter the intracellular metabolic pathway. Interestingly, sphingoid bases derived from diet have been proposed to suppress intestinal tumorigenesis (19). Asah2 null mice would be a useful model to test this hypothesis.

In summary, we have shown that neutral ceramidase, the product of the Asah2 gene, controls an essential step in the digestion of dietary sphingolipids. As such, the enzyme regulates the levels of ceramide and

**FIGURE 8.** Gene expression of enzymes of sphingolipid metabolism in intestinal tract. Real-time PCR was used to determine mRNA expression of Enpp5, Asah2, Sphk1, Sphk2, Sgpl1, and Sgpp1 in portions of the gastrointestinal tract from wild-type mice. The expression levels relative to the same gene in brain are shown. The data represent the means ± S.E., n = 3.

**FIGURE 9.** Scheme for intestinal digestion of sphingomyelin. The model is based on the work of Schmelz et al. (41) and on the findings presented here. PE, phosphoethanolamine.
sphingosine derived from food, which may have important effects on intestinal biology and disease.

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