Rat intestinal ceramidase: purification, properties, and physiological relevance

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Submitted 7 April 2004; accepted in final form 28 May 2004

Olsson, Maria, Rui-Dong Duan, Lena Ohlsson, and Åke Nilsson. Rat intestinal ceramidase: purification, properties, and physiological relevance. Am J Physiol Gastrointest Liver Physiol 287: G929–G937, 2004. First published June 24, 2004; 10.1152/ajpgi.00155.2004.—Neutral ceramidase activity has previously been identified in the intestinal mucosa and gut lumen and postulated to be important in the digestion of sphingolipids. It is found throughout the intestine but has never been fully characterized. We have purified rat intestinal neutral ceramidase from an eluate obtained by perfusing the intestinal lumen with 0.9% NaCl and 3 mM sodium taurodeoxycholate. Using a combination of acetone precipitation and ion-exchange, hydrophobic-interaction, and gel chromatographies, we obtained a homogenous enzyme protein with a molecular mass of ∼116 kDa. The enzyme acts on both [14C]octanoyl- and [14C]palmitoyl-sphingosine in the presence of glycolycolic and taurocholic acid and the bile salt analog 3-{[3-cholamidopropyl]dimethylammonio}-1-propanesulfonate but is inhibited by 2 mM or more of other bile salts. It is a glycosylated protein stable to trypsin and chymotrypsin exposure, is not influenced by Ca2+, Mg2+, or Mn2+, and is inhibited by Zn2+ and Cu2+. Mass fragmentographic analysis identified 12 fragments covering 17.5% of the sequence for neutral/alkaline ceramidase 2 purified (Mitsutake S, Tani M, Okino N, Mori K, Ichinose S, Omori A, Iida H, Nakamura T, and Ito M. J Biol Chem 276: 26249–262459, 2001) from rat kidney and located in apical membrane of renal tubular cells. Intestinal and kidney ceramidases also have similar molecular mass and ion dependence. Intestinal ceramidase thus is a neutral ceramidase 2 released by bile salts and resistant to pancreatic proteases. It is well suited to metabolize ceramide formed from dietary and brush border sphingolipids to generate other bioactive sphingolipids.

bile salt; intestine; mass spectrometry; octanoyl-sphingosine; palmitoyl-sphingosine

SPHINGOLIPIDS OCCUR IN DIETARY meat, milk, and fish products and in some vegetable products, such as soybeans. The daily intake was calculated to be 0.3–0.4 g/day (52). Sucking babies ingest 80–200 mg milk sphingomyelin (SM)/day and some glycosphingolipids (36). The dietary sphingolipids are mixed in the gut with bile SM and with SM and glycosphingolipids from sloughed mucosal cells. Sphingolipids in food are sequentially hydrolyzed to ceramide, sphingosine, and fatty acids (35) but not transported intact via the chyle (34). In the case of SM the initial step is catalyzed by alkaline sphingomyelinase (Alk-SMase) (35, 39) and in the case of glycosyl ceramides by lactase-phlorizin hydrodase (21, 23). The amide bond of the ceramide formed is hydrolyzed in the lumen and at the brush border mainly by intestinal ceramidase. The question as to whether ceramide also permeates intact into mucosal cells is unresolved. Free sphingosine formed is rapidly absorbed, phosphorylated in the mucosal cells to sphingosine 1-phosphate (S-1-P), and converted to chylomicron palmitic acid or incorporated into mucosal ceramide and sphingolipids (34, 47). Dietary SM is hydrolyzed mainly in the middle and lower small intestine (35, 39) where Alk-SMase is most abundant (12). Digestion of glycosylceramide follows a similar course (33). Because of the extended course of sphingolipid digestion, the whole small intestine and colon are exposed to the metabolites formed (34, 39).

Ceramide, sphingosine, and S-1-P are signaling substances that influence numerous cell functions, including growth, differentiation, and apoptosis (8, 22). Ceramide and sphingosine commonly favor cell differentiation or growth inhibition, and S-1-P often has antagonistic effects (42). Dietary sphingolipids were shown to counteract chemically induced colon carcinogenesis and tumor development in a chemical carcinogenesis model and in multiple intestinal neoplasia (MIN) mice with an inactivating mutation in the familial adenomatous polyposis (FAP) gene (46, 48). In cultures of colon carcinoma HT29 cells, sphingosine induced apoptosis and inhibited proliferation by decreasing the level of free β-catenin (48). Purified intestinal Alk-SMase (34) from the brush border of the gut inhibited proliferation of colon carcinoma HT29 cells (18). Dietary sphingolipids may also interact with bile acids to counteract their carcinogenic action in colon (30) and with sterols to cause inhibition of cholesterol absorption and delayed SM digestion (37). To understand the sequence of events involved in the mediation of these effects, knowledge about the participating enzymes is crucial.

Whereas Alk-SMase was recently purified and cloned (5, 9, 10), the intestinal ceramidases have not been purified and fully characterized in the purified form. Early studies demonstrated the presence of ceramidase with neutral pH optimum in the mucosa, in brush-border preparations, and in intestinal content (35). Later, purified bile salt-stimulated lipase (BSSL) in pancreas and human milk was shown to have lipoamidase (19) and ceramidase activity (38). Most of the intestinal neutral ceramidase activity cannot, however, be attributed to BSSL. The longitudinal distribution of ceramidase activity in the rat intestine was different (25), and neutral ceramidase of rat and human intestinal content was separated from and differed in properties from BSSL (11). Furthermore, mucosal neutral ceramidase activity was present in BSSSL(−/−) mice (20). In this study, we further examined the identity of the enzyme accounting for the major digestive ceramidase activity in the gut.
The first possibility was that it is a member of the highly conserved neutral/alkaline ceramidase family. Members of this family have been purified and/or cloned from a number of tissues such as kidney (29), rat brain (13), mouse liver (40, 50), human liver (14), and endothelial cells (44) and from bacteria (41), yeast (26, 27), and *Drosophila* (53). During the progress of this work, Choi et al. (6) cloned mouse neutral ceramidase and reported a high expression of neutral ceramidase in mouse intestine and kidney. The second possibility was that the intestinal ceramidase activity is accounted for by a brush-border enzyme related to another enzyme family, e.g., peptidases or lipases. This possibility was not considered unlikely, since we recently found that Alk-SMase is not homologous to any other known SMase but to a new tissue-specific ectoenzyme related to the alkaline nucleotidase/phosphodiesterase (NPP) family (9). We have purified neutral ceramidase from a luminal bile salt eluate of rat small intestine and found that the enzyme is identical or closely related to the neutral ceramidase 2 from rat kidney (29) and highly homologous to the mouse neutral ceramidase recently shown to be expressed also in the intestine (6). Characterization of the purified enzyme indicates important physiological roles of the enzyme in ceramide digestion.

**MATERIALS AND METHODS**

**Materials**

\[1^{14}C\]octanoic acid was purchased from American Radiolabeled Chemicals (St. Louis, MO). \(\beta\)-Erythro-sphingosine was obtained from Matreya (Pleasant Gap, PA). Taurocholate (TC), glycocholate (GC), taurodeoxycholate (TD), glycocoxycholate (GDC), taurochenodeoxycholate (TCDC), glycocychocholate (GCCD), phenylmethanesulfonyl fluoride (PMSF), 3-[\(\beta\)-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), trypsin, chymotrypsin, and octanoyl chloride were from Sigma (St. Louis, MO). PD-10 columns, anion exchange HQ cartridges were purchased from Amersham Bioscience (Uppsala, Sweden). Protein determination was performed with Bio-Rad Detergent-Compatible Protein assay. Anion exchange HQ cartridges were purchased from Bio-Rad (Stockholm, Sweden). \(1^{14}C\)palmitoyl-labeled ceramide was provided by Dr. Peter Ström (AstraZeneca; Lund, Sweden) and was prepared by conversion of \(1^{14}C\)palmitic acid to the \(1^{14}C\)palmitoyl chloride. \(1^{14}C\)octanoyl-sphingosine was taken to dryness under nitrogen and then sonicated on ice for 2 min in assay buffer, i.e., 50 mM K$_2$CO$_3$/K$_2$BO$_3$, pH 10 (1). After mixing, the tubes were centrifuged at 10,000 rpm for 10 s. An aliquot of 200 \(\mu\)l of upper phase was taken for liquid scintillation counting. The total disintegrations per minute in the upper phase were calculated. The distribution of released fatty acids in the upper phase under these condition was 90\%, which was considered in calculation of enzyme activity (25). After the detergent effects on the activity of the purified enzyme had been considered, the assay condition was changed to 50 mM Tris•HCl buffer, pH 8.0, with 8 mM CHAPS, because of the lower cost of CHAPS than of TC.

**Purification of Ceramidase**

**Collection of intestinal protein.** Thirty rats fasted overnight were anesthetized (im) with Ketalar-Rompun (2:1) (Ketalar, 50 mg/ml, Parke Davis, Detroit, MI; Rompun Vet, 20 mg/ml). A segment of small intestine from 5–10 cm below pylorus to 5–10 cm above the cecum was cannulated. The segment was flushed twice with 20 ml 0.9\% NaCl containing 1 mM benzamidine, 1 mM PMSF, and 3 mM TDC, which serves to dissociate both Alk-SMase and ceramidase from the intestinal brush border. The eluted solution was centrifuged at 5,000 g (3,000 g at C), and the supernatant was concentrated by ultrafiltration through a YM-30 membrane. The proteins were precipitated by addition of acetone to 50\% (vol/vol) at temperatures below \(-10^\circ\)C. After centrifugation at 8,000 rpm (7,600 g) for 15 min at 0\°C, the protein pellets were dissolved in 50 ml of 20 mM Tris•HCl buffer, pH 8.2, and 0.075 M NaCl containing 1 mM benzamidine. The nondissolved proteins were removed by centrifugation at 15,000 rpm (27,000 g) for 15 min at 0\°C. The supernatant was used for purification. All chromatographic procedures were performed at 4°C with the use of a Bio-Logic HR chromatography system. The elution of protein was monitored by ultraviolet light when applicable.

**Anion-exchange chromatography.** The acetone precipitate dissolved in 20 mM Tris•HCl, pH 8.2, containing 1 mM benzamidine was loaded on an anion-exchange HQ column preequilibrated with the same buffer. To remove unbound proteins, the column was washed with 15 ml of equilibrating buffer. The column was first eluted with a NaCl gradient from 0 to 0.25 M, which elutes Alk-SMase. The salt concentration was then increased to 0.5 M to elute remaining proteins, including ceramidase. The ceramidase fractions were pooled and applied to a PD-10 column equilibrated with 20 mM Tris•HCl buffer, pH 7.5, containing 1 mM PMSF, 1 mM benzamidine, and 0.005\% (wt/vol) Triton X-100. After buffer change, the sample was concentrated by ultrafiltration using Amicon PM-10 Ultrafiltration Discs. The sample containing \(-32 mg\) proteins was loaded on a HiTrap Q XL column. The column was equilibrated with the same buffer as the PD-10 column. To elute unbound proteins, the column was washed with 15 ml of equilibrating buffer. The bound proteins were eluted with a NaCl gradient from 0 to 0.4 M at 2 ml/min. The fractions with ceramidase activity were pooled and concentrated by ultrafiltration.

**Hydrophobic-interaction chromatography.** The ceramidase sample now containing \(-1 mg\) of protein was applied to a HiTrap Butyl FF column. The column was equilibrated with 25 ml of 20 mM Tris•HCl buffer, pH 7.5, containing 1 mM PMSF, 1 mM benzamidine, 0.005\% (wt/vol) Triton X-100, and 1 M ammonium sulfate. After loading of the sample, the column was washed with equilibration buffer. The bound proteins were eluted from the column by a gradient of decreasing ammonium sulfate concentration, from 1 M to 0. The proteins were collected in 1-ml fractions. The fractions with ceramidase activity were pooled and concentrated for the next purification step.

**Gel-filtration chromatography.** An aliquot of 200 \(\mu\)l of the pooled fractions from the hydrophobic-interaction chromatography (HiCa) was applied to a Superdex 200 HR 10/30 column equilibrated with 20 mM Tris•HCl buffer, pH 7.5, containing 1 mM PMSF, 1 mM benzamidine, 0.005\% (wt/vol) Triton X-100, and 0.1 M NaCl. The
proteins were eluted with 30 ml of equilibration buffer and collected in 0.3-ml fractions.

**SDS-PAGE.** The progress of the purification was followed by monitoring the protein composition of the peaks containing ceramidase on 10% SDS-PAGE. The protein bands were visualized with silver staining. The migration of the purified ceramidase was compared with standards of known molecular mass. To examine the effect of deglycosylation on migration, ceramidase was deglycosylated with the use of a glycoprotein deglycosylation kit from Calbiochem-Merck (Darmstadt, Germany) according to the provided protocol. The kit contained N-glycosidase F (peptide N\(^{-}\)-acyetyl-\(\beta\)-glucosaminyl) asparagine amidase cloned from *Flavobacterium meningosepticum* and expressed in *Escherichium coli*, endo-\(\alpha\)-N-acetyl-galactosaminidase, neuraminidase, \(\beta\)-1,4-galactosidase, and \(\beta\)-\(N\)-acetyl-glucosaminidase.

**Characterization of Ceramidase**

**Effects of bile salts.** Varying concentrations of TC, GC, TDC, GDC, TCDC, GCDC, CHAPS, and Triton X-100 in 0.05 M Tris-HCl buffer, pH 8.0, were incubated with 2.5 ng ceramidase and \([^{14}C]octanoyl- and \([^{14}C]palmitoyl-sphingosine as substrates. Optimal pH was determined by incubations in 0.1 M acetate buffer at pH 4–6.5, in 0.1 M phosphate buffer at pH 7.0–8.0, in 0.05 M Tris-HCl buffer at pH 8.5, and in 0.1 M glycine-NaOH buffer at pH 9–10. The effects of 0.1–4.0 mM concentrations of Li\(^{+}\), Ca\(^{2+}\), Cu\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\) on the ceramidase activity were examined. All buffers contained 8 mM CHAPS.

**Effects of octanoyl- and palmitoyl-sphingosine.** The rates of reaction with \([^{14}C]octanoyl- and \([^{14}C]palmitoyl-sphingosine as substrates were compared in assays as above with different concentrations of unlabeled ceramide present. \(V_{\text{max}}\) and \(K_{m}\) were calculated from Lineweaver-Burk plots.

**Effects of trypsin and chymotrypsin.** Purified ceramidase was incubated with 0.05 M Tris-HCl buffer, pH 8.0, containing 8 mM CHAPS and 0, 0.05, 0.5, and 1 mM trypsin and chymotrypsin for 30 min at 37°C. After preincubation the ceramidase activity was measured. The activity of trypsin was verified with the use of benzoyl-L-arginine-p-nitroanilide as substrate (17).

**Mass spectroscopic analysis.** The purified enzyme band from the SDS-PAGE was eluted and subjected to trypsin digestion. The fragments were then subjected to mass fragmentographic (MALDI-TOF) analysis. These analyses were performed by methods established at the Swegene Proteomics Unit (Lund, Sweden). Briefly, the gel was stained with GelCode blue and the ceramidase band was cut and subjected to a procedure involving destaining and dehydration/rehydration, reduction/alkylation, trypsin digestion, and extraction of peptides.

**RESULTS**

**Purification of Ceramidase**

The results from the different chromatographic steps are summarized in Table 1. The protein peak that was not retained on the HQ column contained Alk-SMase and was collected and used for purification of this enzyme (5). The ceramidase was retained on the column, and the enzyme was eluted with 0.5 M NaCl. The fractions with ceramidase activity were pooled and desalted on PD-10 columns. On the subsequent HiTrap Q XL column, the ceramidase was retained to the column and was eluted with a NaCl gradient as a broad peak between 0.02 M and 0.3 M NaCl, with maximal ceramidase activity at 0.1 M NaCl (Fig. 1A). During elution thereafter with equilibration buffer containing 0.5% (wt/vol) Triton X-100, a second ceramidase peak was detected that was not further investigated in this paper. The ion-exchange step gave a 13-fold increase of the specific activity with a recovery of 20% (Table 1).

In the subsequent HIC step, the pooled fractions with ceramidase activity were applied to a HiTrap Butyl FF column. Ceramidase was retained when applied in 1 M ammonium sulfate. When the salt concentration was reduced gradually, a distinct peak containing ceramidase activity was eluted between 0.38 and 0 M ammonium sulfate. The maximum ceramidase activity was detected at 0.15 M ammonium sulfate (Fig. 1B). This purification step yields 244-fold purification. The recovery was 12% of the ceramidase activity obtained from the initial HQ chromatography (Table 1).

In the last step, an aliquot of 200 μl of the concentrated ceramidase peak from the preceding step was applied to a Superdex 200 HR 10/13 column with a total volume of 25 ml. Ceramidase was eluted as a distinct peak at an elution volume of 13.5 ml (Fig. 1C). After this step, 5% of the original ceramidase activity remained and the increase in specific activity was 307-fold, compared with the ceramidase containing protein fraction from the initial HQ column (Table 1).

In initial experiments, most of the enzyme activity in hydrophobic interaction and gel filtration steps was lost when no detergent was present. Addition of 0.005% Triton X-100 was found to be sufficient to avoid this problem and did not interfere with the ceramidase assay. This detergent concentration also gave a favorable elution pattern.

The protein composition of the ceramidase-containing peaks was monitored by SDS-PAGE (Fig. 2). The purified ceramidase obtained after Superdex 200 HR appeared as one single band visible with silver staining. The band corresponds to a molecule mass of ~116 kDa (Fig. 2, left). After incubation of the native enzyme with glycoprotein deglycosylation kit, a major protein band with a mass of ~97 kDa was observed (Fig. 2, right).

**Characterization of Ceramidase**

**Mass spectroscopic analysis.** The MALDI-TOF analysis identified 12 peptide fragments with sequences identical to parts of the sequence for the ceramidase purified from rat kidney by Mitsutake et al. (29). The sequence coverage was 17.5%. Of the sequence between amino acid 107 and 251, 88 out of 144 amino acids, i.e., sequence coverage of 61%, were obtained. At the COOH-terminal end between amino acid 617 and 761, 68 amino acids, i.e., 47%, were covered (Fig. 3, A and B). The sequences exhibit 92% homology with the sequence for mouse ceramidase clones from liver (50), neonatal adipose tissue, central nervous system, adrenal gland, intestine, and kidney (6) and 78.7% homology with human mitochondrial neutral ceramidase (14). Clones from *Drosophila melan-*
gaster (53), Arabidopsis thaliana, and Mycobacterium tuberculosis also exhibited significant homology. Unigene search of the mouse ceramidase 2 reveals that a full-length cDNA Riken clone accounts for 0.07% of the adult mouse small intestine cDNA [Mn-104900, db-expressed sequence tag (EST) library ID no. 7218]. The search also indicates that Unigene Cluster Rn-81031, i.e., N-acylsphingosine hydrolase 2 from rat, has homologous EST sequences consisting of up to 400 bases, with 73–100% homology. These are derived from libraries from fetal colon, a combined library, dorsal root ganglion, prostate tissue, and hypothalamus.

Effects of pH, ions, and bile salts. Effects of pH. Intestinal ceramidase has a broad pH optimum. The enzyme has optimal activity between pH 6 and 8 and is active at a pH as high as 9.5. At pH < 5.0 the ceramidase has little activity (Fig. 4).

Effects of cations. The ceramidase activity of the pure enzyme was strongly inhibited by Zn$^{2+}$. As low a concentration as 0.2 mM Zn$^{2+}$ caused >90% inhibition. Also, Cu$^{2+}$ was distinctly inhibitory, causing >80% inhibition at 2 mM or higher concentrations. Li$^{+}$ had a slight inhibitory effect, whereas Mn$^{2+}$, Mg$^{2+}$, and Ca$^{2+}$ had no effect on ceramidase activity (Fig. 5). The inhibition caused by Zn$^{2+}$ and Cu$^{2+}$ was reversible by EDTA (data not shown).

Effects of bile salts. When the enzyme was incubated with [14C]octanoyl-sphingosine in the presence of varying concentrations of TC, GC, TDC, GDC, TCDC, and GCDC, all bile salts were found to stimulate the ceramidase activity to some extent. TC and GC dose dependently stimulated the action of the enzyme, with maximal effect at a concentration of 8 mM, i.e., around their critical micellar concentration. With GCDC and TCDC, stimulation occurred at a concentration of 2–2.5 mM, and GDC and TDC were stimulatory only at concentrations of 1 mM or lower. All bile salts were inhibitory at high concentrations (Fig. 6A). The effects of CHAPS on ceramidase activity were similar to those of TC and GC (Fig. 6B). CHAPS was as effective as TC and GC and had maximal stimulatory effect at a concentration of 8 mM. Higher concentrations of CHAPS tended to inhibit the enzyme. Triton X-100 had an inhibitory effect on ceramidase even at low concentration (Fig. 6C).
The presence of 0.005% Triton X-100 in the chromatographic steps did not, however, interfere with the monitoring of the purification with the aliquot sizes used.

Activity against octanoyl-sphingosine and palmitoyl-sphingosine. Two substrates were examined. [14C]octanoyl-sphingosine was used for the assay during the purification and characterization of the enzyme. The reason is that this substrate has higher micellar solubility in presence of bile salt and provides a sensitive and reliable assay. [14C]palmitoyl-sphingosine was used to document that the enzyme is active toward a major physiological ceramide species. The results obtained with different concentrations of the two substrates are presented as a Lineweaver-Burk plot that gives a $V_{\text{max}}$ of 160 $\mu$mol·min$^{-1}·mg^{-1}$ and a $K_{m}$ of 71.4 $\mu$M for octanoyl-sphingosine (Fig. 7A) and a $V_{\text{max}}$ of 16 $\mu$mol·min$^{-1}·mg^{-1}$ and a $K_{m}$ of 66 $\mu$M for palmitoyl-sphingosine (Fig. 7B). The enzyme was thus active against both substrates.

Effects of proteolytic enzymes. Because trypsin and chymotrypsin are present in the intestine and ceramidase is exposed to these enzymes at the brush border and in the lumen, we examined whether ceramidase is rapidly inactivated by or resistant to these proteases. The ceramidase was preincubated with various concentrations of trypsin and chymotrypsin followed by a measurement of ceramidase activity. Trypsin and chymotrypsin in concentrations up to 1 mg/ml had no effect on ceramidase activity during preincubation at 37°C for 30 min, indicating that the enzyme in its native form is resistant to the major pancreatic proteases. Trypsin efficiently hydrolyzed benzoyl-DL-$p$-nitroanilide under the conditions used (data not shown).

**DISCUSSION**

This paper reports the first purification and characterization of rat intestinal neutral ceramidase. The enzyme can be released from the mucosa by bile salts at a concentration that is presented as a Lineweaver-Burk plot that gives a $V_{\text{max}}$ of 160 $\mu$mol·min$^{-1}·mg^{-1}$ and a $K_{m}$ of 71.4 $\mu$M for octanoyl-sphingosine and 16 $\mu$mol·min$^{-1}·mg^{-1}$ and a $K_{m}$ of 66 $\mu$M for palmitoyl-sphingosine.
not tissue damaging. Together with earlier data showing that the enzyme is present in rat (25) and human (11) intestinal content and enriched in brush-border preparations (35), this indicates that it is a releasable ectoenzyme from the brush border. The luminal bile salt eluate provided a favorable starting material that is enriched in the enzyme. A similar procedure was used in our earlier purification procedure for Alk-SMase, which is also located at the brush-border surface and released into the lumen (10). By a series of chromatographic steps, a protein appearing as a single protein band (SDS-PAGE) with a molecular mass corresponding to 116 kDa was obtained. A crucial step was the HIC in the presence of a low concentration of detergent (0.005% Triton X-100) that gave effective purification.

The MALDI-TOF analysis identified the purified enzyme as a member of the neutral/alkaline ceramidase family. Twelve fragments containing 8–18 amino acids each and a total of 160 amino acids are identical to sequences in the segment containing amino acid 107 to 251 and 617 to 736 in the neutral ceramidase 2 earlier purified and cloned from rat kidney and shown to be located in the apical membrane of the renal tubular cells (Fig. 3A) (29). The sequence of rat kidney neutral ceramidase 2 also exhibits 92% homology with the mouse neutral/alkaline ceramidase 2 (Fig. 3B), which is expressed at mRNA level in several tissues including the small intestine (Mn-104900, dbEST library ID no. 7218, Riken full-length cDNA). Thus the intestinal ceramidase purified in this study is very likely a product of the neutral ceramidase 2 gene. We have not excluded that it is an isoform that may be differently spliced than the kidney enzyme, nor have we excluded that other neutral ceramidases may be expressed in the intestine. During

Fig. 6. Effects of bile salts and detergents on ceramidase activity. A and B: effects of different bile salts and 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), respectively, at various concentrations (mM). Incubations with [14C]octanoyl-sphingosine and purified ceramidase for 60 min were performed under the conditions described in MATERIALS AND METHODS. C: effect of different amounts of Triton X-100, as percentage. GDC, glycodeoxycholate; TC, taurocholate; GC, glycocholate; TCDC, taurochenodeoxycholate; GCDC, glycochenodeoxycholate; TDC, taurodeoxycholate; Δ, ceramide hydrolysis without bile salt. All values are means ± SD of 3 observations and are expressed as %dpm in upper phase of total dpm added.

Fig. 7. Kinetics for the hydrolysis of [14C]octanoyl-sphingosine (A) and [14C]palmitoyl-sphingosine (B) presented in Lineweaver-Burk plots. Purified ceramidase was incubated with increasing concentrations of the substrate under the conditions described in detail in MATERIALS AND METHODS. Values are means of triplicates. V, reaction rate; S, substrate concentration.
the progress of this work, Choi et al. (6) reported that the highly homologous (92%) mouse neutral ceramidase 2 is strongly expressed in duodenum, jejunum, and ileum, with highest levels in the jejunum. This is in agreement with our studies (25) of the longitudinal distribution of neutral ceramidase in the rat intestine.

The enzymatic properties of the enzyme purified in the present study are also identical to those of the neutral ceramidase from kidney (29). The molecular mass, the broad pH optimum between 6 and 8, and the effects of metal ions are all similar. Thus both enzymes were inhibited by \( \text{Zn}^{2+} \) and \( \text{Cu}^{2+} \) ions, the effect of the \( \text{Zn}^{2+} \) ions being most pronounced, and were not influenced by \( \text{Ca}^{2+}, \text{Mg}^{2+}, \text{or Mn}^{2+} \) ions. This contrasts with the brain ceramidase that was stimulated by \( \text{Mn}^{2+} \) ions (13) and the bacterial (41) and hepatic enzymes (50) that were stimulated by \( \text{Ca}^{2+} \).

An important feature of the intestinal ceramidase is its resistance to trypsin and chymotrypsin in its native form. The reason is unknown but may be related to extensive glycosylation. The amino acid sequence of the rat kidney enzyme indicates the presence of nine potential glycosylation sites, and the estimated molecular mass (113 kDa) exceeds the one predicted from the deduced sequence of 761 amino acids (83.5 kDa). This indicates a high degree of glycosylation, and it has been suggested that mannose glycosylation is essential for its association as a type II transmembrane protein with the tubular membrane in the kidney (49). The change in molecular mass observed after deglycosylation in our study (Fig. 2, right) indicates a high degree of glycosylation of the intestinal ceramidase also.

The longitudinal distribution and the resistance to pancreatic proteases make the enzyme suited for a key role in the digestion of ceramide. In our earlier studies, neutral ceramidase was found throughout the small intestine (25). In BSSL gene knockout mice the total ceramidase was decreased only in the most proximal part, where BSSL is normally abundant, but not in the remaining small intestine (20). The enzyme is thus expected to be most active in the part of the small intestine where most of the lipid digestion has been finished but where the bile salt concentration is still high. In the present study, the activity of the enzyme was found to be influenced by bile salts. The trihydroxy bile salts TC and GC stimulated over a relatively broad range and may have a stimulatory effect in vivo. The dihydroxy bile salts, which have a lower critical micellar concentration, had their optimal effect at lower concentrations and were strongly inhibitory at higher concentrations. To evaluate the role of bile salts for the kinetics of ceramide hydrolysis in vivo is therefore difficult on the basis of the present data.

Protease resistance, inhibition by \( \text{Zn}^{2+} \) ions, and ability to act at neutral/alkaline pH are properties that the intestinal ceramidase shares with Alk-SMase. Being active under similar conditions, the enzymes may act in a concerted manner during SM digestion. The choline, the sphingoid base, and the fatty acid parts of SM can thereby be utilized without the need for powerful pancreatic sphingolipidases that might threaten the integrity of the brush border. As a consequence of the moderate capacity of the enzymes, the digestion of SM and ceramide is extended and incomplete, and the colon is exposed to sphingolipid metabolites (34, 39). Because of the protease resistance of both enzymes, significant amounts of both Alk-SMase and ceramidase are also transferred with the luminal content into colon where they may continue to generate sphingolipid metabolites. The factors that regulate the sphingolipid hydrolysis and colon exposure in vivo need further study.

An intriguing question is whether gut Alk-SMase and ceramidase are only digestive enzymes or whether their action also influences mucosal functions. A number of observations indicate that sphingolipid hydrolysis does indeed influence mucosal function and cell growth. Field et al. (15) showed that exposure of Caco-2 cells to SMase decreased cholesterol absorption (15) and lipoprotein and apolipoprotein B secretion (28), and Kirby et al. (20) that hydrolysis of ceramide by BSSL influenced the size and composition of intestinal lipoproteins. BSSL(-/-) mice lacking BSSL had less total ceramidase activity in the upper gut and produced predominantly smaller lipoproteins than the BSSL(+/-) mice. In polarized Caco-2 cells, SMase induced an inhibition of secretion of large lipoproteins that could be reversed by BSSL hydrolyzing ceramide formed (20). Thus SM and ceramide hydrolysis in/at the brush border may influence sterol absorption and lipoprotein secretion. Studies with free sphingoid bases (48) and with purified Alk-SMase (18) indicate that ceramide and sphingosine generated by the combined action of Alk-SMase and ceramidase may influence growth of colon carcinoma cell lines.

Choi et al. (6) recently reported that in the mouse, feeding of C-2 ceramide (N-acetyl-sphingosine) induced epithelial apoptosis in the gut, particularly when the ceramidase inhibitor (1S,2R)-n-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol (n-erythro-MAPP) was fed simultaneously. Choi et al. (6) proposed that neutral ceramidase in the gut is an essential component of a detoxifying mechanism to prevent ceramide-induced apoptosis. An important consideration is, however, that long-chain ceramides formed from natural dietary and brush-border sphingolipids are expected to permeate much less efficiently into the mucosal cells in intact form than the C-2 ceramide because of their molecular structure and low solubility in bile salt micelles. The question as to whether long-chain ceramides from dietary and brush-border sphingolipids permeate into the mucosal cells has not been solved. Radioactive ceramide is found to be associated with the mucosal tissue after a feeding of radioactive SM (39), but the intracellular location has not been confirmed, and very little dietary ceramide appears intact in chyle (34). There may be alternative mechanisms by which ceramide reaches intracellular signaling targets. Recent data indicated that SM hydrolysis in the outer layer of erythrocyte ghosts and model membranes caused a transmembrane movement of lipids due to transient formation of nonlamellar structural intermediates and loss of asymmetry in the lipid bilayer with a transbilayer movement of ceramide together with other lipids (7). If similar events occur in the brush border, ceramide generated in the outer layer could reach intracellular signaling targets. If so, the mucosal ceramidase must be important to balance signaling effects of ceramide. Interestingly, gut mucosa and isolated crypt and villus cells from intestinal mucosa contain significant amounts of ceramide located in several subcellular fractions (4).

Ceramidase generates free sphingosine, which is absorbed much more efficiently than long-chain ceramides (34). Because of the high levels of sphingosine-1-phosphokinase in the mucosa (16), sphingosine is also rapidly converted to S-1-P,
which could act as a signal to favor cell growth and survival in the mucosa. Sphingosine may also be rapidly reacylated to ceramide formation from glycosphingolipids (21, 43) and intestinal neutral ceramidase in the mouse (6) and the Alk-SMase in the rat (24) are promptly expressed during late gestation soon after delivery. All three enzymes are thus developmentally regulated and appear with the differentiated absorptive epithelium just in time to participate in digestion of milk sphingolipids. Recent studies (31) on newborn rats receiving artificial milk containing SM or equivalent amounts of phosphatidylcholine suggested that SM may influence maturation of the small intestine as judged by differences in lactase levels and morphological criteria. The relation of the gut ceramidase to neonatal nutrition and biological effects of suckling is thus an interesting area for further study.

In summary, the neutral ceramidas found in the gut brush border, the apical membrane of the renal tubular cells (29), and the caveolae of endothelial cells (44, 45) are likely to be products of the same gene. The neutral ceramidase 2 is thus an important ectoceramidase, the multiple roles of which deserve further study. Possible functional aspects are related to digestion, to detoxifying and scavenging functions, and to distinct signaling functions.

ACKNOWLEDGMENTS

The MALDI-TOF analysis was performed by Liselotte Andersson, Swe-gene Proteomics Unit, Lund, Sweden.

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