DES2 protein is responsible for phytoceramide biosynthesis in the mouse small intestine

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The C-4 hydroxylation of sphinganine and dihydroceramide is a rate-limiting reaction in the biosynthesis of phytosphingolipids. Mouse DES1 (MDES1) cDNA homologous to the *Drosophila melanogaster* degenerative spermatocyte gene-1 (*des-1*) cDNA leads to sphingosine Δ^4 -desaturase activity, and another mouse homologue, MDES2, has bifunctional activity, producing C-4 hydroxysphinganine and Δ^4 -sphingenine in yeast [Ternes, Franke, Zahringer, Sperling and Heinz (2002) J. Biol. Chem. 277, 25512–25518]. Here, we report the characterization of mouse DES2 (MDES2) using an *in vitro* assay with a homogenate of COS-7 cells transfected with MDES2 cDNA and *N*-octanoyl-sphinganine and sphinganine as substrates. MDES2 protein prefers dihydroceramide as a substrate to sphinganine, and exhibits

dihydroceramide Δ^4 -desaturase and C-4 hydroxylase activities. MDES2 mRNA content was high in the small intestine and abundant in the kidney. *In situ* hybridization detected signals of MDES2 mRNA in the crypt cells. Immunohistochemistry using an anti-MDES2 peptide antibody stained the crypt cells and the adjacent epithelial cells. These results suggest that MDES2 is the dihydroceramide C-4 hydroxylase responsible for the biosynthesis of enriched phytosphingoglycolipids in the microvillous membranes of intestinal epithelial cells.

Key words: C-4 hydroxylase, ceramide C-4 hydroxylase, DES2, desaturase, phytoceramide.

INTRODUCTION

Cell membranes are composed of phospholipids, cholesterol, glycosphingolipids and proteins. Complex lipids are the membrane constituents that support membrane protein function by providing a suitable membrane environment, called the microdomain, and determine the physical properties of membranes [1–3].

The microvillous membranes of epithelial cells in the mammalian small intestine are very rich in glycosphingolipids containing phytosphingosine and α -hydroxy fatty acid, and this characteristic feature is conserved in various mammals [4–7]. Phytosphingosine has an additional hydroxyl group at the C-4 position of sphingosine (Figure 1). It was first identified in glycolipids isolated from plants [8], and subsequently in those from yeast [9], protozoans [10,11], sea urchins [12], fish [13], amoebae [14] and mammals [4,15]. Mammalian phytosphingosine biosynthesis was first reported *in vivo* by Crossman and Hirschberg [16], who detected radiolabelled phytosphingosine in a glycolipid-rich fraction prepared from the small intestine of a rat after intraperitoneal injection of radiolabelled sphinganine.

However, the biosynthetic pathways and biological functions of phytosphingosine-containing sphingolipids long remained unknown. Recently, several important findings have been reported. In yeast, the gene *Sur2/Syr2* was cloned and found to encode a hydroxylase that acts at the C-4 position of sphinganine [17,18]. Plant genes homologous to the yeast *Sur2/Syr2* gene were subsequently cloned, and their sphinganine C-4 hydroxylase activities were confirmed using a yeast expression system [19]. After the *Drosophila melanogaster* degenerative spermatocyte (*des-1*) gene and its mouse homologue (MDES1) were identified [20,21], another mouse homologue (MDES2) was shown to have both sphingolipid C-4 hydroxylase and sphingolipid Δ^4 -desaturase activities, suggesting that MDES2 is a major and bifunctional enzyme responsible for sphingolipid C-4 hydroxylation [22]. This report by Ternes et al. [22] was the first to characterize a mammalian sphingolipid C-4 hydroxylase.

Here, we characterized MDES2 protein expressed in COS-7 cells as a bifunctional enzyme with Δ^4 -desaturase and C-4 hydroxylase activities using dihydroceramide as the substrate. We also confirmed the physiological involvement of the MDES2 gene by analysing the tissue distribution of MDES2 mRNA, and the localization of MDES2 mRNA and protein in the mouse small intestine.

EXPERIMENTAL

Materials

N-Octanoyl-D-erythro-[4,5-³H]C₁₈-sphinganine and D-erythro-[4,5-³H]C₁₈-sphinganine were purchased from American Radiolabelled Chemicals; D-erythro-C₁₈-sphinganine, D-erythro-C₁₈-sphingenine and D-ribo-1,3,4-trihydroxy-2-amino-octadecane (C₁₈-phytosphingosine) were from Sigma; *N*-octanoyl-D-erythro-C₁₈-sphinganine and *N*-octanoyl-D-erythro-C₁₈-sphingenine were from Matreya, and HPTLC plates were from Merck. *N*-Octanoyl-C₁₈-phytosphingosine (*N*-octanoyl-D-ribo-1,3,4-trihydroxy-2-amino-octadecane) was prepared from C₁₈-phytosphingosine and octanoic anhydride [23]. *N*-[8-³H]Octanoyl-D-erythro-C₁₈-sphingenine purchased from American Radiolabelled Chemicals was hydrogenated with NaBH₄ and PdCl₂ according to the method of Schwartzmann [24], and *N*-[8-³H]octanoyl-D-erythro-C₁₈-sphinganine was purified by HPLC with an ODS column and 90 % methanol as the elution solvent. Figure 1 lists the chemical structures and names of sphingolipids used in this study.

PCR cloning of MDES-1 and -2 cDNAs and the yeast C-4 hydroxylase gene

MDES1 and MDES2 cDNAs were cloned by PCR from mouse brain cDNA (Clontech), using the following oligonucleotide primers: for MDES1 (accession number AF466376), 5'-CCTCG-AGGGCCATGGGTAGCCGCGTGTCC-3' as the forward primer

Abbreviations used: MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight.

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Figure 1 Chemical structures and nomenclature of sphingolipids

and 5'-GATATTTACTCCAGAATCTCGTTCCC-3' as the reverse primer; for MDES2 (accession number AF466377), 5'-CCTCG-AGGCCATGGGTAATAGCGCGGCC-3' as the forward primer and 5'-GGCTCACAGGTGGTCCCTCGCCAGCTTA-3' as the reverse primer. The yeast C-4 hydroxylase gene (accession number U10427) was cloned by PCR from *Saccharomyces cerevisiae* genomic DNA (Promega), using the oligonucleotide primer 5'-CCTCGAGATACGATGAACGTAACATCGAAT-3' as the forward primer and 5'-GGCGGCCGCTTATTCTCTT-TCTTACTTCAT-3' as the reverse primer. The resulting PCR products were cloned into the pCRII-TOPO vector (Invitrogen) and the DNA sequences were confirmed using an ABI PRISM 3100 genetic analyser.

Expression of MDES cDNA in COS-7 cells

MDES1 and MDES2 cDNAs and a yeast C-4 hydroxylase DNA fragment were cloned into a mammalian expression vector, pME18S, which carries a strong chimaeric promoter of simian virus 40 and retrovirus, SR α [25], to construct pME-MDES1, pME-MDES2 and pME-yC4H, respectively. COS-7 cells were routinely maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 i.u./ml penicillin and 50 μ g/ml streptomycin. Transfection was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Then, 72 h after transfection, the cells were harvested, washed twice with PBS and suspended in 20 mM Tris/HCl, pH 7.5, and 250 mM sucrose. Cells were disrupted using a Physcotron Polytron-type microhomogenizer (NITI-ON). The protein concentrations were determined using a Bio-Rad Protein Assay Kit with BSA as the standard.

Enzymic activity assay

To assay the Δ^4 -desaturase and C-4 hydroxylase activities, 1 μ Ci of *N*-octanoyl-D-erythro-[4,5- 3 H]C₁₈-sphinganine (22.9 pmol) or *N*-[8- 3 H]octanoyl-D-erythro-C₁₈-sphinganine (94.8 pmol) and 22.2 nmol of *N*-octanoyl-D-erythro-C₁₈-sphinganine in methanol were dried and then resuspended in 40 μ l of 1.5% octylglucoside by sonication with a bath-type sonicator. Then 10 μ l of 1 M Tris/HCl, pH 7.5, and 1 mg of the protein from COS-7 cell homogenates were added, and the total volume was adjusted to 190 μ l with distilled water. The reaction was initiated by adding 10 μ l of 100 mM NADPH or NADH (in 10 mM Tris/HCl, pH 7.5, and 1 mM EDTA) and incubated for 2 h at 37 °C. We compared the effects of five detergents, octylglucoside, CHAPS, Nonidet P-40, deoxycholic acid and digitonin, at 0.03, 0.1 and 0.3% final concentrations, and 0.3% octylglucoside was the best for the hydroxylase activity. NADPH was used for the assays, unless noted otherwise. The reaction was stopped by adding 380 μ l of methanol and 190 μ l of chloroform. The mixture was vortex mixed thoroughly and spun for 30 s to separate it into two phases. The lower chloroform phase was transferred to a fresh tube, while the remaining aqueous phase was washed with 200 μ l of chloroform. The two chloroform phases were both washed with 200 μ l of a solution containing 135 mM NaCl, 10 mM Tris/HCl, pH 7.5, and 15 mM EDTA, and dried under nitrogen. After suspending the dried materials in 20 μ l of chloroform/methanol (1:1, v/v), 10 μ l aliquots were applied to a borate-impregnated silica gel HPTLC plate. Before applying the sample, the plates were dipped in a solution of 70 mM sodium borate in methanol for 30 min, and dried at room temperature for 2 h [26]. Chloroform/methanol/water (80:10:1, by vol.) was used as the developing solvent [26]. The ceramides were visualized and quantified by autoradiography using a Fujix Bas 2000 Bioimaging

analyser. To identify spots detected by autoradiography, plates on which the reaction products were separated together with non-labelled *N*-octanoyl-sphinganine, *N*-octanoyl-sphingenine and *N*-octanoyl-phytosphingosine were analysed after spraying them with 3% (w/v) cupric acetate in an 8% (v/v) phosphoric acid solution and heating them at 180 °C for 10 min.

To assay the sphinganine C-4 hydroxylase activity, 1 μ Ci (29.1 pmol) of D-erythro-[4,5-³H]C₁₈-sphinganine and 28.2 nmol of D-erythro-C₁₈-sphinganine in methanol were dried and resuspended in 40 μ l of 1.5% octylglucoside after sonication with a bath-type sonicator. The other assay conditions were the same as those using *N*-octanoyl-sphinganine as the substrate. The reaction products were separated by TLC with silica gel plates, using a chloroform/methanol/NH₄OH (15:3.8:0.8, by vol.) solution as the developing solvent.

The amounts of the reaction products in the assays with *N*-octanoyl-D-erythro-[4,5-³H]C₁₈-sphinganine were calculated from the activity ratios, 2.29 for the desaturase and 1.46 for the hydroxylase, obtained in actual experiments ($n=3$) with *N*-octanoyl-D-erythro-[4,5-³H]C₁₈-sphinganine and *N*-[8-³H]-octanoyl-D-erythro-C₁₈-sphinganine in the presence of the same amounts of homogenate proteins. The theoretical values for the correction were 2 for the desaturase and 1.33 for the hydroxylase, since *N*-octanoyl-D-erythro-[4,5-³H]C₁₈-sphinganine was synthesized with *N*-octanoyl-D-erythro-C₁₈-sphingenine and NaB³H₄ in the presence of catalysts and the enzymes are stereospecific [27].

Product identification

A large-scale incubation mixture consisting of 10 mg of protein from the homogenates obtained from MDES2-transfected COS-7 cells, 564 nmol of *N*-octanoyl-sphinganine, and other factors, in a final volume of 2 ml was incubated for 2 h. The lipids were extracted using the method described above. Lower-phase lipids in chloroform solution were applied to an Iatrobeads column (0.5 cm $\times$ 5 cm, 500 mg). After the column was washed with 5 ml of chloroform, the ceramide fraction was recovered with 5 ml of chloroform/methanol (95:5, v/v) [28]. This ceramide fraction was separated by HPLC with an ODS column (0.3 cm $\times$ 15 cm; Nomura Chemical Co.) using 85% methanol as the elution solvent at a flow rate of 1 ml/min. The peaks were monitored using a UV detector at 210 nm. To further characterize the material, peaks exhibiting the same retention times as *N*-octanoyl-phytosphingosine, *N*-octanoyl-sphinganine and *N*-octanoyl-sphingenine were recovered and subjected to MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS using an AXIMA-CFR mass spectrometer (Shimadzu Corp.) and 2, 5-dihydroxybenzoic acid as the matrix.

Real-time quantitative reverse transcriptase-PCR

Total RNA prepared from mouse small intestinal epithelial cells was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA from mouse small intestine was obtained from UNITECH, and total RNA from mouse kidney, testis, brain, liver, heart, lung, uterus and stomach were obtained from Clontech. MDES1 and MDES2 mRNA were quantified using the ABI PRISM 7900HT Sequence Detection System (TaqMan). The oligonucleotide primers and fluorescently labelled TaqMan probes used are listed in Table 1. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as an endogenous control to normalize the amount of total RNA in each sample.

Table 1 Sequences of TaqMan primers and probes

TaqMan primers and probes	Sequence
MDES1	
Forward primer	5'-TGGTGAGGAAGATCGCAAGTG-3'
Reverse primer	5'-CGTCACAAAGTCATAGACACCTT-3'
Probe	5'-TGACCTCCCGCACTACAACCTCTGGA-3'
MDES2	
Forward primer	5'-GCATCAACCACTCGCTGACA-3'
Reverse primer	5'-CGGTTTCGGGAGACACAAC-3'
Probe	5'-TCCATGACATCTCGACAATACTGCCTT-3'

In situ hybridization

The small intestine of a mouse was fixed in 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.4, at 4 °C for 12 h. The fixed tissues were immersed with 30% sucrose in the same buffer, frozen in OCT compound at –80 °C, and cut into 8 μ m-thick sections using a cryostat. The sections were incubated with riboprobes of digoxigenin-labelled antisense and sense RNA prepared from the MDES2 cDNA sequence, from nucleotides 473 to 972, using a Digoxigenin RNA Labelling kit (Roche) according to the manufacturer's instructions. *In situ* hybridization was performed at 42 °C for 16 h. Hybridized riboprobes were detected with an alkaline phosphate-conjugated anti-digoxigenin antibody (Roche), and visualized with nitroretrozolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche).

Immunohistochemistry with a polyclonal antibody against a synthetic MDES2 peptide

A peptide (CSMGYPYSRVKRRKRLAKDHL) corresponding to the C-terminal sequence of MDES2 in which Cys-13 was replaced with Arg was synthesized on an automatic peptide synthesizer. It was conjugated with keyhole limpet haemocyanin via the N-terminal Cys residue. A polyclonal anti-MDES2 peptide antibody was raised in a rabbit using complete Freund's adjuvant.

For Western blotting, 25 μ g of protein from the homogenate of COS-7 cells transfected with pME-MDES2 was separated by SDS/PAGE under reducing conditions and transferred to PVDF membranes. The membranes were incubated with a 1:2000 dilution of the anti-MDES2 peptide antibody or pre-immune serum. Antibody binding was detected using a chemiluminescence detector, LAS 1000 plus (Fuji Photo Film), after incubation with a 1:4000 dilution of horseradish peroxidase-conjugated Protein A (Bio-Rad) using an ECL detection kit (Amersham Biosciences).

The frozen sections prepared as described previously were incubated with 10% goat serum in PBS at room temperature for 1 h, with rabbit anti-MDES2 peptide antibody or the pre-immune serum in a 1:100 dilution at 4 °C for 12 h, and finally with goat anti-rabbit IgG antibody coupled to Alexa Fluor-488 (Molecular Probes) at room temperature for 1 h. For an inhibition experiment, a frozen section was incubated with the rabbit antibody in the presence of 300 μ M MDES2 peptide conjugated BSA. The sections were examined under a Zeiss LSM 510 laser confocal microscope.

RESULTS

Δ^4 -Desaturase and C-4 hydroxylase assay conditions

Homogenates prepared from COS-7 cells transfected with pME-MDES2 (MDES2 COS-7) were used as an enzyme source

Figure 2 Δ^4 -Desaturase and C-4 hydroxylase activities in the homogenates of COS-7 cells transfected with pME-MDES1, pME-MDES2 and pME-yC4H

(A) Homogenates prepared from COS-7 cells transfected with pME18S (mock transfection), pME-yC4H, pME-MDES1 or pME-MDES2 were assayed for Δ^4 -desaturase and C-4 hydroxylase activities using *N*-octanoyl-[4,5- 3 H]sphinganine as a substrate. A borate-impregnated silica gel TLC plate was developed with chloroform/methanol/water (80:10:1, by vol.) and the spots were visualized and quantified by autoradiography using a bioimaging analyser. (B) C-4 hydroxylase activity in the homogenates of COS-7 cells transfected with pME-yC4H and pME-MDES2; these were assayed with [4,5- 3 H]sphinganine as a substrate. A silica gel TLC plate was developed with chloroform/methanol/ NH_4OH (15:3.8:0.8, by vol.) and spots were quantified as with (A). (C, D) The product yields were calculated from the results of (A) and (B), respectively, as described in the Experimental section. The Δ^4 -desaturase and C-4 hydroxylase activities are expressed as the means $\pm$ S.E.M. ($n = 4$ for pME-yC4H and pME-MDES1, $n = 10$ for pME18S and pME-MDES2). * indicates that the difference in Δ^4 -desaturase activity between homogenates prepared from COS-7 cells transfected with pME18S (mock transfection) and pME-MDES2 is statistically significant ($P < 0.05$, Mann-Whitney test).

and incubated with radiolabelled *N*-octanoyl-sphinganine as the substrate. The reaction products analysed by TLC autoradiography are shown in Figure 2(A). We established the standard assay conditions by determining the optimal pH, a suitable detergent, detergent concentrations, homogenate-protein amount, incubation time and substrate concentration. When *N*-octanoyl-sphinganine was used as the substrate, the MDES2 COS-7 homogenates had the maximal Δ^4 -desaturase and C-4 hydroxylase activities between pH 7.0 and 8.0, with 0.3% octylglucoside. Both reactions were linear up to 1 mg of protein and a 4 h incubation. The desaturase and hydroxylase activities required either NADPH or NADH. MDES2 COS-7 homogenates exhibited no additive effect of NADPH and NADH and no significant

preference of NADPH and NADH for both Δ^4 -desaturase and C-4 hydroxylase activities.

Product identification

TLC with borate-impregnated plates yielded good separation of the products and substrate, as shown in Figure 2(A) (unlabelled compounds are not shown). Two radiolabelled products co-migrated with the unlabelled authentic compounds, *N*-octanoyl-sphingenine and *N*-octanoyl-phytosphingosine, on TLC. As shown in Figure 3 (left-hand panel), the HPLC retention times of peaks a and b (the products) obtained with the large-scale

Figure 3 Identification of the products of Δ^4 -desaturase and C-4 hydroxylase activity in pME-MDES2-transfected COS-7 cells

Left-hand panel: the products (a, b and c) were separated using HPLC with a C_{18} reversed-phase column and 15% water in methanol as the elution solvent. Peaks were detected at 210 nm using a UV detector. Peaks a, b, and c have the same retention times as *N*-octanoyl-phytosphingosine, *N*-octanoyl-sphingene and *N*-octanoyl-sphinganine, respectively; the peaks were recovered and subjected to MALDI-TOF MS. Right-hand panels: mass spectra of peaks a, b and c. The peaks between a and b, and after c were not ceramides, but were unidentified lipids detected also in the ceramide fraction obtained from an incubation mixture containing pME-MDES2-transfected COS-7 cell homogenates without the substrate.

incubation were identical with those of the authentic compounds *N*-octanoyl-phytosphingosine and *N*-octanoyl-sphingene respectively. As shown in Figure 3 (right-hand panels), peak a has

ions at m/z 466.4 for $[M + Na]^+$ and m/z 482.3 for $[M + K]^+$; consequently the obtained molecular mass, 443, is the same as that of *N*-octanoyl- C_{18} -phytosphingosine. Peak b has ions at

Figure 4 Substrate-saturation experiment of Δ^4 -desaturase and C-4 hydroxylase in the homogenates of COS-7 cells transfected with pME-MDES2

Radioautography of TLC for the reaction products with MDES2 homogenates and radiolabelled *N*-octanoyl-dihydrosphinganine (22.9 pmol) in the presence of various amounts of unlabelled *N*-octanoyl-dihydrosphinganine.

m/z 448.4 for $[M + Na]^+$ and at m/z 464.3 for $[M + K]^+$, so the obtained molecular mass, 425, is the same as that of *N*-octanoyl- C_{18} -sphinganine. Combined with the results of Ternes et al. [22] for the product identification with MDES2 cDNA transformation in yeast, this supports the conclusion that the homogenates of pME-MDES2-transfected COS-7 cells produced *N*-octanoyl- C_{18} - Δ^4 -sphingene (peak b) and *N*-octanoyl- C_{18} -4-hydroxy- C_{18} -sphinganine (peak a). Peak c has ions at m/z 450.4 for $[M + Na]^+$ and at m/z 466.4 for $[M + K]^+$, and was identified as the *N*-octanoyl- C_{18} -sphinganine used as the substrate.

Characterization of MDES1 and MDES2 proteins

The result of a substrate-saturation experiment using *N*-octanoyl-sphinganine and the MDES2 COS-7 homogenates is shown in Figure 4. Substrate concentrations above 44.4 nmol/200 μ l incubation volume were not tested due to substrate insolubility. The MDES2 COS-7 cell homogenates had specific Δ^4 -desaturase and C-4 hydroxylase activities of 15.33 ± 0.37 and 6.36 ± 0.17 pmol/mg per min (mean $\pm$ S.E.M., $n = 10$), respectively (Figure 2C). We measured the Δ^4 -desaturase and C-4 hydroxylase activities in the homogenates of COS-7 cells transfected with pME-MDES1 (MDES1 COS-7) under the same conditions as for MDES2 COS-7. The MDES1 COS-7 cell homogenates showed specific Δ^4 -desaturase and C-4 hydroxylase activities of 71.34 ± 6.18 and 1.36 ± 0.23 pmol/mg per min ($n = 4$), respectively (Figure 2C). The homogenates of pME18S-transfected COS-7 cells were analysed similarly; the specific activity of Δ^4 -desaturase and C-4 hydroxylase was 13.95 ± 0.31 and 0.64 ± 0.02 pmol/mg per min ($n = 10$), respectively (Figures 2A and 2C). We consider these activities representative of the endogenous activities of host COS-7 cells.

Based on these measurements, we conclude that (i) MDES2 COS-7 homogenates contain dihydroceramide C-4 hydroxylase activity with a low but significant dihydroceramide Δ^4 -desaturase activity ($P < 0.05$, Mann-Whitney test), and (ii) MDES1 COS-7 homogenates contain dihydroceramide Δ^4 -desaturase activity with a negligible amount of C-4 hydroxylase activity.

The homogenates of COS-7 cells transfected with pME-yC4H showed sphinganine C-4 hydroxylase activity (20.7 ± 1.2 pmol/

Figure 5 Tissue distribution of MDES1 and MDES2 mRNA

Real-time quantitative reverse transcriptase-PCR was performed with 50 ng of total RNA. The amounts of MDES1 and MDES2 mRNA were determined using plasmid DNA containing MDES1 and MDES2 genes as standards and normalized with the amount of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA. The amounts of MDES1 and MDES2 mRNA are expressed as pg/50 ng of total RNA (means $\pm$ S.E.M.; $n = 3$).

mg per min, $n = 3$). Phytosphingosine was formed (Figures 2B and 2D), but no *N*-octanoyl-sphinganine C-4 hydroxylase activity was detected (Figures 2A and 2C). Grilley et al. [17] reported that yeast C-4 hydroxylase expressed in yeast exhibited both dihydroceramide C-4 hydroxylase and sphinganine C-4 hydroxylase activity, but did not mention the specific activity of the former. We were unable to confirm their detection of dihydroceramide C-4 hydroxylase activity using *N*-octanoyl-sphinganine. The MDES2 COS-7 homogenates exhibited much lower sphinganine C-4 hydroxylase activity (0.26 ± 0.03 pmol/mg per min, $n = 3$; Figures 2B and 2D) than *N*-octanoyl-sphinganine C-4 hydroxylase activity (6.36 ± 0.17 pmol/mg per min, $n = 10$; Figures 2A and 2C), indicating that MDES2 COS-7 homogenates prefer *N*-octanoyl-sphinganine to sphinganine as a substrate.

Tissue distributions of MDES1 and MDES2 mRNA

Real-time quantitative reverse transcriptase-PCR demonstrated that MDES1 mRNA was expressed ubiquitously in the tissues tested (Figure 5). By contrast, MDES2 mRNA expression showed tissue specificity. MDES2 mRNA was abundant in small-intestinal epithelial cells, intermediate in the small intestine and kidney, and quite sparse in testis, brain, liver, uterus, heart and lung.

In situ hybridization

In situ hybridization of the small intestine with the antisense probe for MDES2 mRNA produced clear signals in crypt and goblet cells and weak signals in epithelial cells (Figure 6). No signals were observed with the sense probe (results not shown). These results indicate that the crypt cells actively produce MDES2 mRNA, the epithelial cells produce it at very low levels, and goblet cells maintain a high level of production.

Figure 6 *In situ* hybridization of mouse small intestine for MDES2 mRNA

Magnification, $\times 100$.

Immunohistochemistry of MDES2 protein

The anti-MDES2 peptide antibody detected a band from homogenates of MDES2 COS-7 cells with a molecular mass of 32 kDa on Western blots (Figure 7A), which showed that the anti-MDES2 peptide antibody reacts with MDES2 protein.

With the same antibody, the crypt cells and the adjacent epithelial cells stained strongly, while the other epithelial cells stained weakly (Figure 7B). Preimmune sera did not give signals, and the positive staining with the antibody was eliminated by the antibody incubation in the presence of the peptide-conjugated BSA. These results together with the *in situ* results indicate that MDES2 is transcribed in the crypt cells and the amount of mRNA produced in the epithelial cells decreases. MDES2 protein is produced in the crypt cells and the adjacent epithelial cells, and is stored in the more-differentiated epithelial cells at low concentrations.

DISCUSSION

This study confirmed that MDES2 protein has both dihydroceramide C-4 hydroxylase and dihydroceramide Δ^4 -desaturase activity. By contrast, MDES1 protein has dihydroceramide Δ^4 -desaturase activity. MDES2 is transcribed in crypt cells and epithelial cells near the crypts, and these cells produce DES2 protein. Consequently, MDES2 contributes to the very high concentration of phytosphingoglycolipids in the epithelial cells of mouse small intestine.

In our expression experiments with COS-7 cells, yeast C-4 hydroxylase exhibited sphinganine C-4 hydroxylase activity and converted trace amounts of *N*-octanoyl-sphinganine into *N*-octanoyl-phytosphingosine. Grilley et al. [17] previously reported that yeast C-4 hydroxylase hydroxylates both dihydrosphingosine and dihydroceramide. However, our result indicates that yeast C-4 hydroxylase expressed in COS-7 cells prefers dihydrosphingosine to *N*-octanoyl-sphinganine. Ternes et al. [22] reported that MDES2 and yeast C-4 hydroxylases belong to different protein families and their sphingolipid C-4 hydroxylase activities evolved independently. Our data on MDES2 and yeast C-4 hydroxylase kinetics support this assertion.

MDES1 mRNA was expressed in all of the mouse tissues examined, with the greatest expression in the lung. Dihydro-

Figure 7 Western blotting and immunohistochemistry of MDES2 with anti-MDES2 peptide antibody

(A) Homogenates (25 μ g of protein) of COS-7 cells transfected with pME-MDES2 were analysed by Western blotting with anti-MDES2 peptide antibody and peroxidase-conjugated Protein A. (B) Immunohistochemistry of the mouse small intestine using anti-MDES2 peptide antibody and goat anti-rabbit IgG coupled to Alexa Fluor-488. Magnification, $\times 100$.

ceramide Δ^4 -desaturase activity has also been detected in various rat tissues [29]. MDES2 mRNA was expressed in small-intestinal epithelial cells, small intestine and kidney, in decreasing quantities, but not in the other mouse tissues that we tested. These results agree with observations that phytosphingoglycolipids are considerably enriched in the microvillous membranes of intestinal epithelial cells. These lines of evidence suggest that DES2 C-4 hydroxylase activity is the rate-limiting step in phytosphingoglycolipid biosynthesis. Unfortunately, we were unable to detect *N*-octanoyl-sphinganine C-4 hydroxylase activity in the homogenates or microsomal fractions of small intestine and intestinal mucosa, because of the very high ceramidase activity.

A possible function of phytosphingoglycolipids in the microvillous membrane is to support the functions of membrane proteins, such as maltase-isomaltase [30], glucose transporters

[31], amino acid transporters [32], fatty acid transporters [33] and bile acid transporters [34], by providing specific microdomains that differ from those in other biological membranes composed of glycosphingolipids carrying ceramide. This is an interesting hypothesis that should be tested by manipulating the functions of the molecules listed above in cultured cells after introducing MDES2 cDNA.

In mouse small intestine, MDES2 mRNA is abundant and MDES1 mRNA is also present. Therefore, this organ has the capacity to produce ceramide via the Δ^4 -desaturase activity of the two proteins. However, the major glycolipids (glucosylceramide and gangliosylceramide) are composed of phytosphingosine, which makes up more than 90 % of the total sphingosines [7]. This discrepancy suggests that there must be a molecular mechanism controlling their enzymic activities besides the regulation of MDES1 and MDES2 protein production. We are in the process of searching for such factors by establishing a reconstitution system using sub-purified recombinant MDES2 protein and electron-transfer components, because our preliminary results suggest the involvement of cytochrome *b₅* in the hydroxylation reaction.

The hydropathy of the amino acid sequences of MDES1 and MDES2 was analysed with the algorithm of SOSUI program (<http://sosui.proteome.bio.tuat.ac.jp/welcomeE.html>), HMMTOP (<http://www.enzim.hu/hmmtop/>), PSORT (<http://psort.nibb.ac.jp/>) and TMPred (http://www.ch.embnet.org/software/TMPRED_form.html). The SOSUI program provided a model which meets the following criteria: the simplest membrane-spanning structure and three His domains locating at the cytoplasmic side. The model predicts three putative membrane-spanning domains with cytoplasmic localization of three His domains, which form the catalytic domain to transfer electrons from NADPH or NADH. The cytoplasmic localization of three His domains fits the concept that ceramide is synthesized on the cytosolic side of the endoplasmic reticulum, then flip-flopped to the endoplasmic reticulum lumen, and elongated to glucosylceramide and higher glycosphingolipids [35]. Cadena et al. [36] reported that human DES1 is located in the endoplasmic reticulum of cultured HeLa cells and suggested that the human DES1 protein has four membrane-spanning domains and three catalytic His domains localized on the cytoplasmic side. However, the fine analysis of the topology of MDES1 and MDES2 remains for the future and is an important research subject; determining the domain structures responsible for the formation of the active sites of desaturation and hydroxylation is also of interest.

The *in situ* hybridization and immunohistochemistry studies demonstrated that crypt cells express much more MDES2 mRNA and protein than epithelial cells. Immunohistochemistry with anti-gangliosylceramide antibody demonstrated that glycolipids containing phytoceramide are distributed at the microvillous membrane of the epithelial cells of mouse small intestine [37]. These results suggest that crypt cells and the adjacent epithelial cells actively transcribe and translate MDES2 and produce phytosphingoglycolipids, while there is a low level of MDES2 protein expression in further differentiated epithelial cells. The epithelial cells are detached from the top of the villi 48–72 h after crypt cell proliferation. This rapid turnover of epithelial cells also supports the notion that the majority of MDES2 mRNA and protein is produced in the crypt cells and adjacent epithelial cells.

In addition to the small intestine, the kidney and skin are reported to contain glycosphingolipids carrying phytoceramide [38,39]. The concentrations of phytosphingoglycolipids in these tissues are much lower than in the small intestine, and the functions of these molecules are unknown. In the kidney, phytosphingoglycolipids are distributed in tubular epithelial cells,

suggesting that they have similar functions to those of the small intestinal epithelial cells; they may be involved in the active absorption of molecules from glomerular urine. In the skin, phytoceramides and acyl-glucosylphytoceramides are reported to be components of the water barrier lipids formed by differentiated keratinocytes or corneum cells. Therefore, a study of the functions of phytoceramides in water barriers is another subject of interest.

We are currently designing functional studies of phytosphingoglycolipids that involve manipulating the MDES2 gene to modulate its enzyme activity in cultured cells and mice; the results will clarify the physiological importance of phytosphingoglycolipids in mammals.

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