Identification of the human sphingolipid C4-hydroxylase, hDES2, and its up-regulation during keratinocyte differentiation

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Abstract The C4-hydroxylation of dihydrosphingosine or dihydroceramide is a key reaction in the biosynthesis of phytosphingolipids, both in yeasts and in mammalian cells. Mouse DES2 (mDES2) was recently cloned and shown to work as a Δ4-desaturase/C4-hydroxylase, when expressed in yeast cells. Here, we cloned a human homologue of mDES2, hDES2, by homology search utilizing a BLAST program. When expressed in HEK 293 cells, hDES2 exhibited hydroxylase activity for dihydroceramide. Northern blot analyses of hDES2 revealed high expression in skin, intestines, and kidney, sites reportedly possessing high levels of phytosphingolipids. Furthermore, up-regulation of hDES2 mRNA expression and subsequent phytoceramide production were observed during vitamin C/serum-induced differentiation of human keratinocytes. These results suggest that the newly cloned hDES2 plays an essential role in phytosphingolipid synthesis in human skin and other phytosphingolipid-containing tissues.

Key words: DES2; Hydroxylase; Phytosphingosine; Phytoceramide; Keratinocyte; Differentiation

1. Introduction

Sphingolipids are essential components of the plasma membranes of eukaryotic cells, especially within lipid microdomains or rafts [1]. Additionally, sphingolipid metabolites, including ceramide, sphingosine, and sphingosine 1-phosphate, play important roles in diverse biological phenomena, such as cell growth, differentiation, apoptosis, and stress responses, in both mammalian and yeast cells [2–4].

Sphingolipids in the yeast *Saccharomyces cerevisiae* contain mainly phytosphingosine as a sphingoid base, such as that synthesized from dihydrosphingosine by C4-hydroxylase, a product of the *SUR2* gene [5,6]. In contrast, the major sphingoid bases of mammalian cells are sphingosine and dihydrosphingosine. However, some mammalian cells, specifically those in skin, intestines, and kidney, have been shown to contain a considerable amount of phytosphingosine-based sphingolipids [7–9]. Until recently, it had been unclear whether these are derived from food or from de novo synthesis. However, the mouse DES2 (mDES2) gene was recently cloned and expressed in Δ*sur2* yeast mutants, and its product was identified as sphingolipid Δ4-desaturase/C4-hydroxylase [10]. Since then, mDES2 expressed in COS7 cells has also been found to exhibit C4-hydroxylase activity [11].

In this study, we have cloned the human homologue of DES2 (hDES2), using the BLAST program with the GenBank™ human EST data base, and have demonstrated that hDES2 expressed in HEK 293 cells exhibits C4-hydroxylation activity. Northern blotting analyses of hDES2 revealed high expression in skin, intestines, and kidney, sites previously reported as having high levels of phytosphingolipids. We have also examined roles for hDES2 during vitamin C/serum-induced differentiation of human primary keratinocytes.

2. Materials and methods

2.1. Cell culture and transfection

HEK 293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, in a humidified atmosphere of 5% CO_2 at 37°C. Cells were transfected with the indicated cDNA using LipofectAMINE Plus (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's manual. Lysates of transiently transfected cells were prepared 24 h after transfection.

Normal human epidermal keratinocytes, isolated from neonatal skin, were obtained from Cambrex (MD, USA). Primary cultures were maintained in a serum-free keratinocyte growth medium (Gibco, Grand Island, NY, USA), containing 0.07 mM calcium, and grown to 90–100% confluence. Keratinocyte differentiation was performed as described previously [12] using differentiation medium, a mixture of Dulbecco's modified Eagle's medium and Ham F-12 medium (2:1, v/v), supplemented with 1.3 mM calcium, 10% fetal bovine serum, 10 μg/ml insulin, 0.4 μg/ml hydrocortisone, and 50 μg/ml vitamin C.

2.2. Plasmid construction

The hDES2 cDNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) using human epidermal keratinocyte total RNA and the primers 5′-ACCATGGGCAACAGCGCGAGCCGC-3′ and 5′-GCTCACAGACCATCTTTTGCCAGC-3′. The amplified DNA fragment was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) to generate pGEM-hDES2. The obtained nucleotide sequence has been deposited with GenBank™ with the accession number AY541700.

pCE-puro-3×FLAG, a derivative of pCE-puro [13], was constructed to create a C-terminally triple FLAG (3×FLAG)-tagged gene. *Bam*HI sites were created at both the 5′- and 3′-termini of hDES2 by PCR amplification using primers 5′-GGGATCCACCATGGGCAACAGCGCGAGCCGC-3′ and 5′-GGGATCCGACAGACCATCTTTTGCCAGCC-3′ from the pGEM-hDES2. The amplified 0.98 kb fragment was digested with *Bam*HI and cloned into the *Bam*HI site of the pCE-puro-3×FLAG to generate pCE-puro-hDES2-3×FLAG. pcDNA3-hDES2 was constructed by cloning 1.0 kb *Eco*RI fragments from pGEM-hDES2 into the *Eco*RI sites of pcDNA3 (Invitrogen).

2.3. In vitro dihydroceramide hydroxylase assay

In vitro dihydroceramide hydroxylase assays were performed essen-

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tially as described previously [14], with minor modifications. Briefly, cells suspended in lysis buffer (50 mM HEPES–NaOH (pH 7.5) containing a 1×protease inhibitor mixture (Complete™, Roche Molecular Biochemicals)) were lysed by sonication. Total cell lysates (0.5 mg protein) were mixed with 3.0 nmol *N*-octanoyl-D-*erythro*-dihydrosphingosine (Biomol, Plymouth Meeting, PA, USA) and 0.5 μCi [4,5-^{3}H]*N*-octanoyl-D-*erythro*-dihydrosphingosine (40 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO, USA), diluted with 50 mM HEPES buffer (pH 7.5) to a volume of 300 μl, and treated with octyl-β-glucoside (final concentration 0.3%). After a preincubation of 5 min at 37°C, the reaction was started by the addition of NADPH (final concentration 5 mM), followed by incubation for 1 h at 37°C. Lipids were then extracted as described previously [14] and separated by thin layer chromatography (TLC) on silica gel 60 plates (Merck), coated with sodium borate, using chloroform/methanol/water (80:10:1, v/v).

2.4. Immunoblotting

Immunoblotting was performed as described previously [15] using the anti-FLAG antibody, M2 (Sigma, St. Louis, MO, USA).

2.5. Methanolysis

The reagent for lipid methanolysis was prepared by diluting 0.86 ml of concentrated HCl and 0.94 ml of water to methanol (final volume 10 ml) [16]. After lipids were reacted with 0.5 ml reagent at 70°C for 20 h, samples were mixed with 0.25 ml of 2 N NaOH and 0.5 ml chloroform. After centrifuging the sample at 1500×*g* for 5 min the lower phase was collected. The extraction step was repeated twice. The combined organic phase was then washed with distilled water to remove NaOH. Once dried, the lipids were suspended in 20 μl chloroform/methanol (1:2, v/v) and resolved by TLC on Silica Gel 60 high performance TLC plates (HPTLC, Merck) with chloroform/methanol/4.2 N ammonia (15:3.8:0.8, v/v).

2.6. [^{3}H]Dihydrosphingosine labeling assay

Cells were metabolically labeled with 2.0 μCi [4,5-^{3}H]D-*erythro*-dihydrosphingosine (50 Ci/mmol, American Radiolabeled Chemicals). After incubating at 37°C for 24 h, the cells were washed with phosphate-buffered saline, followed by lipid extraction as described previously [17]. The labeled lipids were separated on HPTLC plates using chloroform/methanol/acetic acid (190:9:1, v/v). After drying, the HPTLC plates were developed again with the same solvent system.

2.7. Northern blot analysis

Analysis was performed on total RNA blots containing 20 μg of RNA from adult human tissues (RNWAY Laboratories, Korea). The hDES2 probe was prepared from a DNA fragment, corresponding to the N-terminal 500 bp of the hDES2 ORF, which was amplified from pGEM-hDES2 using the primers 5′-CCATGGGCAACAGCGCGAGCCGC-3′ and 5′-GCGGGTGTGCAGAAGAACCAGC-3′. The amplified fragment was then labeled with [^{32}P]dCTP using a Random Primer DNA labeling Kit (Takara, Shiga, Japan). Hybridization was carried out in ExpressHyb buffer (Clontech, Palo Alto, CA, USA) for 5 h at 68°C.

2.8. RT-PCR

Total RNA was isolated from human epidermal keratinocytes using Trizol reagent (Invitrogen). RT-PCR was performed using a Super-Script One-Step RT-PCR with Platinum Taq kit (Invitrogen). Primers used were as follows: hDES1, 5′- CCATGGGGAGCCGCGTCTCGCG-3′ and 5′-TGAAAGCGGTACAGAAGAACCAGC-3′; hDES2, 5′-ACACCCGCCCGCAAGCTGCTCTG-3′ and 5′-TGCCAGCCTGTACACCCGCTTC-3′; keratin 1, 5′-GGTGGACGTGGTAGTGGCTTTG-3′ and 5′-TTCAGTTCCGAATCCAACCGAG-3′; and β-actin, 5′-TGATGATATCGCCGCGCTCGTCGTC-3′ and 5′-GCATACCCCTCGTAGATGGGCACAG-3′.

3. Results

3.1. Identification of hDES2

A previous study identified mDES1, mDES2, and hDES1 as mammalian DES family members and characterized their sphingolipid Δ4-desaturase/C4-hydroxylase activities by expression in *S. cerevisiae* [10]. However, cloning and characterization of hDES2 have not been reported. To identify the hDES2 cDNA, the GenBank™ human EST data base was searched using the BLAST program, for sequences similar

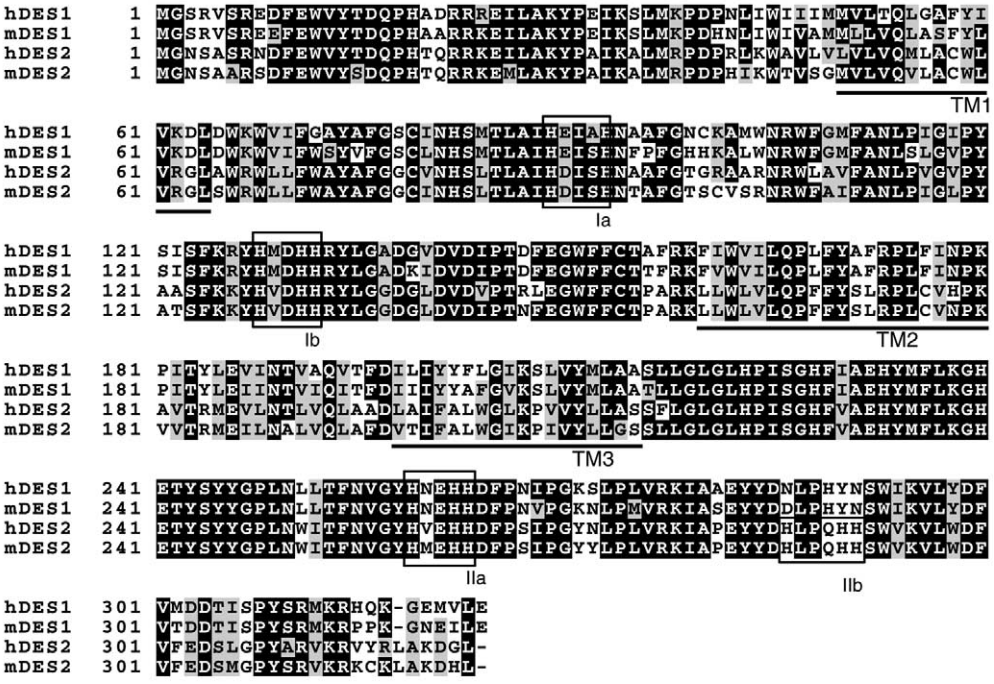

Fig. 1. Comparison of the amino acid sequences of human and mouse DES1 and DES2. An alignment of amino acid sequences from hDES1 (GenBank™ accession number AF466375), mDES1 (AF466376), hDES2 (AY541700), and mDES2 (AF466376) was generated using ClustalW [32] and BOXSHADE (Institute for Animal Health, Surrey, UK) programs. The black boxes indicate identical residues, and the gray boxes show amino acid similarity. Putative transmembrane segments (TM1–TM3) predicted by the SOSUI program (http://sosui.proteome.bio.tuat.ac.jp) are underlined. The histidine box motifs are enclosed by boxes.

to the mDES2 sequence. We found several EST clones, including CA488047, BQ129385 and CA314430, which exhibited high sequence similarity to mDES2, although they contain only partial but overlapping sequences. By assembling these sequences, we were able to predict an entire open reading frame (ORF), subsequently named hDES2. Based on this nucleotide sequence, we performed an RT-PCR using total RNA from human epidermal keratinocytes and obtained cDNA for the hDES2 ORF. The ORF of hDES2 encodes 323 amino acids with a predicted molecular mass of 37.2 kDa (Fig. 1). Since hDES2 is highly homologous to mDES2 throughout the entire sequence (86.4% identity and 91.9% similarity), it is most likely a counterpart of mDES2.

The DES family members illustrated in Fig. 1 belong to the desaturase/hydroxylase superfamily, which is characterized by the existence of three conserved histidine boxes, $HX_{3-4}H$, $HX_{2-3}HH$, and $HX_{2-3}HH$, designated Ia, Ib, and IIa, respectively [18]. However, although DES1 members (mDES1 and hDES1) and DES2 members (mDES2 and hDES2) exhibit similarity overall (in amino acid sequence, polypeptide length, and hydropathy profile), certain amino acids are conserved only in the DES2 members. One of the most striking differences between DES1 and DES2 members is a variation in the second amino acid residue of each histidine box. Whereas DES1 members contain Glu, Met and Asn at that position in Ia, Ib, and IIa, respectively, DES2 members have Asp, Val, and Val/Met. Another characteristic feature of DES2 members is the existence of an additional $HX_{2-3}HH$ motif designated IIb.

3.2. hDES2 exhibits dihydroceramide hydroxylase activity

HEK 293 cells were transiently transfected with pCE-puro-hDES2-3×FLAG encoding the C-terminally 3×FLAG-tagged hDES2 (hDES2-FLAG). Immunoblots using an anti-FLAG antibody detected hDES2-3×FLAG as a 35 kDa band (Fig. 2A). This mobility was slightly faster than the predicted molecular mass of 41.2 kDa.

To investigate the dihydroceramide hydroxylase activity of hDES2, an in vitro assay was performed using [^{3}H]dihydroceramide (*N*-octanoyl dihydrosphingosine) as a substrate. Cell lysates prepared from HEK 293 cells transfected with a control vector plasmid effectively converted dihydroceramide to ceramide (corresponding to *N*-nonhydroxyacyl sphingosine (Cer-NS)) (Fig. 2B). On the other hand, only a very low amount of phytoceramide (corresponding to *N*-nonhydroxyacyl phytosphingosine (Cer-NP)) was detected. These results indicate that HEK 293 cells possess high dihydroceramide desaturase activity, whereas the hydroxylase activity is very weak.

Overexpression of hDES2-3×FLAG in HEK 293 cells had no effect on the amount of ceramide produced but resulted in a marked increase in phytoceramide production (Fig. 2B). Methanolysis of the ceramide and phytoceramide generated sphingosine and phytosphingosine, respectively (Fig. 2C), which co-migrated with cold sphingosine and phytosphingosine standards (data not shown).

We next investigated the hydroxylase activity of hDES2 in cultured cells by [^{3}H]dihydrosphingosine labeling assay. HEK 293 cells transfected with control vector plasmid produced two bands corresponding to long-chain ceramide/dihydroceramide and short-chain ceramide/dihydroceramide, but no phytoceramide (Fig. 3). On the other hand, HEK 293 cells

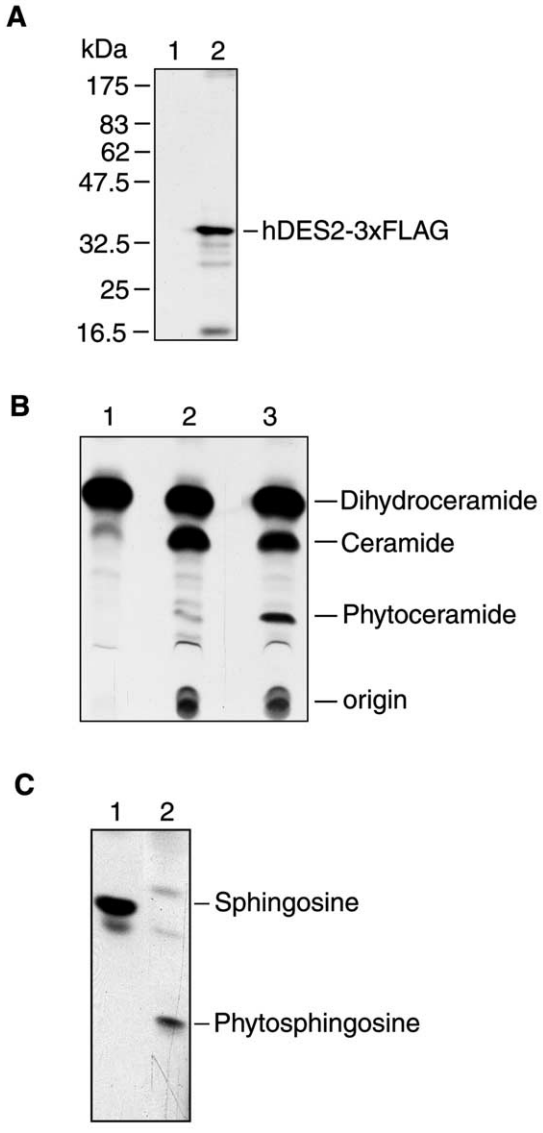

Fig. 2. hDES2 is a dihydroceramide hydroxylase. A: HEK 293 cells were transfected with pCE-puro-3×FLAG (vector, lane 1) and pCE-puro-hDES2-3×FLAG (lane 2). Total cell lysates (25 μg protein) were subjected to immunoblotting using an anti-FLAG antibody. B: Total lysates (0.5 mg protein) prepared from HEK 293 cells transfected with pCE-puro-3×FLAG (lane 2) or with pCE-puro-hDES2-3×FLAG (lane 3) were incubated with [^{3}H]*N*-octanoyl dihydrosphingosine at 37°C for 60 min. Lipids were extracted and separated by TLC on borate-coated silica plates. Lane 1, [^{3}H]*N*-octanoyl dihydrosphingosine substrate. C: TLC lipid spots were recovered from B, and ceramide (lane 1) and phytoceramide (lane 2) were subjected to methanol-HCl hydrolysis and resolved by HPTLC.

overexpressing hDES2 did produce phytoceramide (Fig. 3). These results provide evidence that hDES2 functions as a dihydroceramide hydroxylase.

3.3. Tissue distribution of hDES2 mRNA expression

We examined the tissue distribution pattern of hDES2 by Northern blotting. Hybridization of the hDES2 probe to total RNA from 12 different tissues detected a 1.8 kb band in brain, lung, kidney, large intestine, small intestine, and skin (Fig. 4). The expression level was the highest in skin. We could not detect transcription in heart, liver, spleen, skeletal muscle,

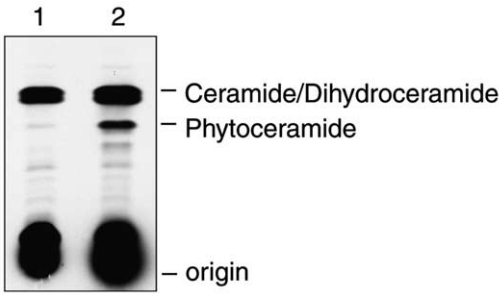

Fig. 3. Overexpression of hDES2 increases phytoceramide in cultured cells. HEK 293 cells transfected with pcDNA3 (vector, lane 1) or pcDNA3-hDES2 (lane 2) were labeled with [^{3}H]dihydrosphingosine for 24 h. Lipids were extracted and separated by HPTLC. Note that ceramide and dihydroceramide could not be separated by the solvent system used.

thymus or placenta. Thus, in contrast to hDES1 mRNA, which is expressed ubiquitously [19], distribution of hDES2 mRNA appears to be highly tissue-specific.

3.4. *hDES2 mRNA expression during keratinocyte differentiation*

Correlating with the high expression of hDES2 mRNA, skin contains a large amount of phytosphingosine-based sphingolipids. Previous studies have demonstrated that, upon differentiation, keratinocytes produce heterogeneous ceramides, including phytoceramides (Cer-NP and *N*-α-hydroxyacyl phytosphingosine (Cer-AP)) [12,20]. We examined whether expression of hDES2 mRNA was regulated during in vitro keratinocyte differentiation, using RT-PCR. Differentiation of normal human keratinocytes was induced by vitamin C and serum. Normal progression of the differentiation was verified by monitoring the synthesis of ceramides characteristic to epidermis, including Cer-NP and Cer-AP (Fig. 5A) [12], as well as by the mRNA expression of keratin 1, a marker for terminal differentiation of keratinocytes [21] (Fig. 5B). hDES2 mRNA was not expressed at day 0 or 3 days after differentiation (Fig. 5B). At day 6, DES2 transcription was detected, increasing by day 9 (Fig. 5B). On the other hand, the expression levels of hDES1 and β-actin mRNAs did not change throughout the keratinocyte differentiation (Fig. 5B).

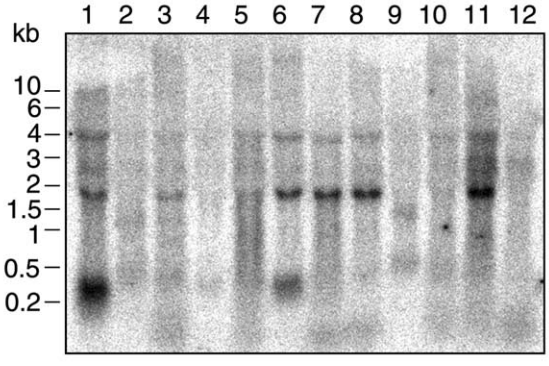

Fig. 4. Tissue-specific expression of hDES2 mRNA. A ^{32}P-labeled hDES2 probe was hybridized to total RNA from 12 different human tissues (20 μg/lane). Lane 1, brain; lane 2, heart; lane 3, lung; lane 4, liver; lane 5, spleen; lane 6, kidney; lane 7, large intestine; lane 8, small intestine; lane 9, skeletal muscle; lane 10, thymus; lane 11, skin; lane 12, placenta.

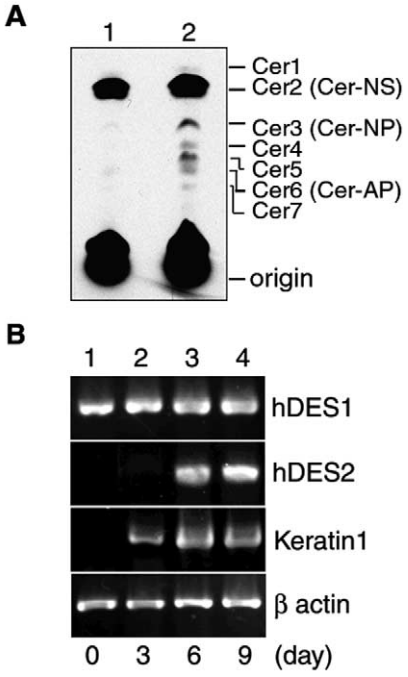

Fig. 5. Expression of hDES2 mRNA is induced in keratinocyte differentiation. A: Undifferentiated keratinocytes (lane 1) or those differentiated for 9 days (lane 2) were labeled with [^{3}H]dihydrosphingosine for 24 h at 37°C. Lipids were extracted and separated by HPTLC. Cer 1, *N*-(*O*-linoleoyl) ω-hydroxyacyl sphingosine; Cer 2, Cer-NS; Cer 3, Cer-NP; Cer 4, *N*-(*O*-linoleoyl) ω-hydroxyacyl 6-hydroxysphingosine; Cer 5, *N*-α-hydroxyacyl sphingosine; Cer 6, Cer-AP; Cer 7, *N*-α-hydroxyacyl 6-hydroxysphingosine. B: Keratinocytes were differentiated for 0, 3, 6, and 9 days. mRNAs prepared at each time point were subjected to RT-PCR using primers for hDES1, hDES2, keratin 1, and β-actin.

4. Discussion

Mammalian cells, specifically those in skin, intestines, and kidney, have long been known to contain phytosphingosine-based sphingolipids [7–9], although it was unclear whether these were derived from food or synthesized locally. In recent studies, forced expression of the mouse DES2 protein in the Δ*sur2* yeast mutants restored the C4-hydroxylase activity [10]. Furthermore, results presented here (Fig. 2B) and recent studies using mouse DES2 [11] have demonstrated that mammalian DES2 expressed in cultured cells exhibits in vitro dihydroceramide hydroxylase activity. We also revealed that overexpression of hDES2 results in the production of phytoceramide in cultured cells (Fig. 3). Thus, mammals have the potential to synthesize phytosphingosine-based sphingolipids.

The distribution of phyto-type sphingolipids correlates well with the expression pattern of DES2. For example, tissue-specific expression of DES2 was observed in tissues containing phyto-type sphingolipids. Recently, high levels of mDES2 mRNA were observed in small intestine and kidney using an RT-PCR method, although skin was not examined [11]. Here, we demonstrated by Northern blot analysis that hDES2 is highly expressed in skin, small and large intestines, and kidney. Additionally, synthesis of phyto-type sphingolipids correlated with the appearance of hDES2 mRNA during keratinocyte differentiation. Finally, a decrease in mDES2 mRNA in cultured keratinocytes from mice lacking ARNT (transcription factor of the Per/AHR/ARNT/Sim family)

was accompanied by a decrease in phytoceramide level [22]. Such correlation supports the hypothesis that phyto-type sphingolipids are synthesized in mammals by DES2, especially in light of an in vivo study in rats which detected radiolabeled phytosphingosine in the intestine and kidneys following an intravenous injection of [^{3}H]dihydrosphingosine [23].

When expressed in yeast, mDES2 exhibits both desaturase and hydroxylase activity. However, no desaturase activity was detected following expression of hDES2 in cultured cells (Fig. 2B). It is not clear whether, in mammalian cells, DES2 possesses only hydroxylase activity or whether the desaturase activity is masked by a high background activity. In contrast, DES1 exhibits only desaturase activity, despite sharing high sequence similarity with DES2 [10,11].

Substitutions of four key residues in the *Arabidopsis thaliana* oleate desaturase or the *Lesquerella fendleri* hydroxylase/desaturase, members of the same desaturase/hydroxylase family as DES1 and DES2, influenced the partitioning of the catalysis between desaturation and hydroxylation [24,25]. However, the equivalent residues in DES1 and DES2 are identical. This suggests that these residues are not important in the determination of catalytic partitioning in the DES family members. In contrast, some differences were found between DES1 and DES2 members, such as a disparity in the second residue of each histidine box motif (Ia, Ib, and IIa). Moreover, only DES2 members contain an additional histidine box motif, termed IIb. The yeast Scs7p, which exhibits sphingolipid α-hydroxylase but not desaturase activity [18], also possesses this additional motif, suggesting that the motif is important for the hydroxylase activity.

The function of phyto-type sphingolipids in skin remains unclear. However, the levels of these lipids are altered in several skin diseases displaying epidermal barrier abnormalities. Psoriasis is a hyperproliferative disease of the epidermis characterized by a defective skin barrier function. In psoriatic skin, the phytosphingosine-carrying ceramides (Cer-NP and Cer-AP) are significantly decreased [26]. Additionally, levels of *N*-(*O*-linoleoyl) ω-hydroxyacyl dihydrosphingosine, as well as those of Cer-NP, were low in patients with atopic dermatitis [27]. Moreover, the quantity of Cer-NP can be significantly correlated with transepidermal water loss [28]. Thus, it is possible that phyto-type sphingolipids play an important barrier function in skin.

In addition to its role as a component of certain sphingolipids, phytosphingosine can function as a bioactive lipid molecule via its conversion to phytosphingosine 1-phosphate. Edg6/S1P$_4$, which is specifically expressed in lymphoid tissue [29], shows a high affinity to phytosphingosine 1-phosphate [30]. When overproduced in CHO cells, Edg6/S1P$_4$ stimulated motility via activation of Cdc42 [31]. Recently, we detected kinase activity against phytosphingosine in certain tissues (including small intestine), which differed from that of already known sphingosine kinases (SPHK1 and SPHK2) [15]. Future studies of DES2 may provide clues towards elucidating the physiological functions of both phyto-type sphingolipids and phytosphingosine 1-phosphate.

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