2-Hydroxy-ceramide synthesis by ceramide synthase family: enzymatic basis for the preference of FA chain length

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Abstract **Ceramide is unusually abundant in epidermal stratum corneum and is important for permeability barrier function. Ceramides in epidermis also comprise an unusual variety, including 2-hydroxy (α-hydroxy)-ceramide. Six mammalian ceramide synthase/longevity assurance homologue (CerS/LASS) family members have been identified as synthases responsible for ceramide (CER) production. We reveal here that of the six, *CerS3/LASS3* mRNA is the most predominantly expressed in keratinocytes. Moreover, its expression is increased upon differentiation. CerS family members have known substrate specificities for fatty acyl-CoA chain length and saturation, yet their abilities to produce 2-hydroxy-CER have not been examined. In the present study, we demonstrate that all CerS members can produce 2-hydroxy-CER when overproduced in HEK 293T cells. Each produced a 2-hydroxy-CER with a chain length similar to that of the respective nonhydroxy-CER produced. In HeLa cells overproducing the FA 2-hydroxylase FA2H, knockdown of *CerS2* resulted in a reduction in total long-chain 2-hydroxy-CERs, confirming enzyme substrate specificity for chain length. In vitro CerS assays confirmed the ability of CerS1 to utilize 2-hydroxy-stearoyl-CoA as a substrate. These results suggest that all CerS members can synthesize 2-hydroxy-CER with specificity for 2-hydroxy-fatty acyl-CoA chain length and that CerS3 may be important in CER and 2-hydroxy-CER synthesis in epidermis.—**Mizutani, Y., A. Kihara, H. Chiba, H. Tojo, and Y. Igarashi. **2-Hydroxy-ceramide synthesis by ceramide synthase family: enzymatic basis for the preference of FA chain length. *J. Lipid Res.* 2008.** 49: **2356–2364.**

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Sphingolipids are essential lipid components of the eukaryotic plasma membrane. The hydrophobic backbone of sphingolipids, ceramide (CER), functions in cellular signaling during such processes as apoptosis, cell differentiation, and cell cycle arrest (1, 2). In epidermal stratum corneum, CER is a major lipid component and is important for epidermal permeability barrier formation (3, 4).

CER consists of an FA and an amide-linked long-chain base (LCB). In mammals, FA chain length typically ranges from 14 to 26 carbons (C14 to C26), with the C16 (C16:0) and C24 (C24:0 and C24:1) species being predominant in most tissues. FAs in CER are typically nonhydroxylated, although in certain tissues, including skin and brain, α-hydroxy (2-hydroxy) FAs are abundant (5, 6). In these tissues, 2-hydroxy-FA is converted from FFA by the hydroxylase FA2H (7, 8), which is also known to be induced during keratinocyte differentiation (9). In addition, skin contains ω-hydroxy FA, with a chain length of 30–36, linked to a linoleic acid (C18:2) (10–13).

The major LCB in mammals is sphingosine (Sph), which carries a *trans* double bond between the C-4 and C-5 positions, although dihydrosphingosine (dihydro-Sph), which lacks the double bond, also exists. In addition, phytosphingosine (phyto-Sph), containing a hydroxyl group at the C-4 position, is detectable in certain tissues, including skin, small intestine, and kidney (14–16); skin also contains a unique LCB, 6-hydroxy-sphingosine (6-hydroxy-Sph) (17). Thus, due to the diverse species of LCBs and FAs available, skin contains unusually varied CER types. In fact, in the human stratum corneum of epidermis, 10 subclasses of ceramide synthase (CerS) have been identified, which differ in their LCB (Sph, phyto-Sph, and 6-hydroxy-Sph) and

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Abbreviations: CER, ceramide; CerS, ceramide synthase; FB_1, fumonisin B_1; LASS, longevity assurance homologue; LCB, long-chain base; MS/MS, tandem mass spectrometry; Sph, sphingosine.

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FA (nonhydroxy, 2-hydroxy, ω-hydroxy linked to a linoleic acid) species (4, 10–13).

In de novo sphingolipid biosynthesis, dihydro-CER is synthesized from dihydro-Sph and fatty acyl-CoA by CerS. The dihydro-Sph portion of dihydro-CER is then desaturated by the Δ4-desaturase DES1, creating CER with a Sph moiety (18). Alternatively, in certain tissues, including skin, the C4-hydroxylase DES2 produces CER with a phyto-Sph (phyto-CER) (19–21). CERs can also be produced by the salvage pathway, in which Sph generated by the deacylation of CER is reacylated by CerS.

There are six mammalian CerSs: CerS1/longevity assurance homolog 1 (LASS1), CerS2/LASS2, CerS3/LASS3, CerS4/LASS4, CerS5/LASS5, and CerS6/LASS6 (22). These enzymes share high sequence similarity and together constitute the CerS family. Each CerS member exhibits a characteristic fatty acyl-CoA preference (CerS1, C18; CerS2, C22 and C24; CerS4, C20, C22 and C24; and CerS5 and CerS6, C16), with the exception of CerS3, which exhibits broad substrate specificity toward medium- to long-chain fatty acyl-CoAs (short-chain having ≤C18; medium-chain, C18–C22; and long-chain, ≥C22) (23–28).

CerS members also exhibit substrate preference with regard to the saturation of the fatty acyl-CoA. For example, CerS1, CerS4, and CerS6 can use C18:0-CoA but not C18:1-CoA as a substrate (26). However, the substrate preference of CerS members regarding 2-hydroxy-fatty acyl-CoA has not yet been examined. Therefore, in the present study, we tested the ability of each CerS member to produce 2-hydroxy-CER (CER containing 2-hydroxy-FA). In in vivo metabolic labeling experiments and in vitro CerS assays, all CerS members produced 2-hydroxy-CER with efficiency similar to that observed for their synthesis of nonhydroxy-CER. Furthermore, the expression of CerS3 and CerS4 was found to increase during keratinocyte differentiation, whereas the expression levels of other CerS family members remained mostly unchanged. These data suggest that differing expression patterns for CerS family members play important roles in the production of unique epidermal CERs, including 2-hydroxy-CER.

MATERIALS AND METHODS

Cell culture and transfection

Normal human epidermal keratinocytes isolated from neonatal skin were obtained from Cambrex (Walkersville, MD), and were grown in a serum-free keratinocyte growth medium (Invitrogen, Carlsbad, CA) containing 0.07 mM calcium. Keratinocyte differentiation was performed as described previously (21) using differentiation medium [DMEM and Ham's F-12 medium (2:1, v/v), supplemented with 1.3 mM calcium, 10% FBS, 10 μg/ml insulin, 0.4 μg/ml hydrocortisone, and 50 μg/ml vitamin C].

Human embryonic kidney (HEK) 293T cells and HeLa cells were grown in DMEM containing 10% FBS and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. HEK 293T cells were grown in culture dishes coated with 0.3% collagen. Transfections were performed using Lipofectamine Plus™ reagent (Invitrogen), according to the manufacturer's manual. Lysates of transiently transfected cells were prepared 24 h after transfection.

Plasmids

Human *FA2H* cDNA was amplified by RT-PCR using human epidermal keratinocyte total RNA and the primers 5′-GAGATCT-ATGGCCCCCGCTCCGCCCCCCGC-3′ and 5′-TCAGGTGGGG-TTTCTCTGGAGTGAGG-3′. The amplified DNA fragment was cloned into pGEM-T Easy vector (Promega, Madison, WI) to generate pGEM FA2H (*Bgl*II). The mammalian expression vector pCE-puro 3xFLAG-1, a derivative of pCE-puro, was constructed to create an N-terminally triple FLAG (3xFLAG)-tagged gene (29). The 1.1 kb *Bgl*II-*Not*I fragment of pGEM-FA2H (*Bgl*II) was cloned into the *Bam*HI-*Not*I site of the pCE-puro 3xFLAG-1 to generate pCE-puro 3xFLAG-FA2H.

The pcDNA3 HA-CerSx plasmids (with x representing the CerS numbers) encode N-terminally HA-tagged mouse CerS family members, and the pcDNA3 DES2 plasmid encodes untagged human DES2, as described previously (21, 26, 27).

RT-PCR

Total RNA was isolated from cultured cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's manual. RT-PCR was performed on total RNA using a SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen). PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. Primers for actin were designed and used as an endogenous control. The following primer sets were used for this assay: *FA2H*, 5′-CAAG-GACCTGGTGGACTGGC-3′ and 5′-GCTGCATGCACAAGTA-GAAGAC-3′; *Keratin 1*, 5′-GGTGGACGTGGTAGTGGCTTTG-3′ and 5′-TTCAGTTCCGAATCCAACCGAG-3′; *CerS1*, 5′-ATGCC-GAGCTACGCGCAGCTAGTGC-3′ and 5′-TTGCGCCAGGTGTC-CATGTATAGC-3′; *CerS2*, 5′-CCGTCATTGTGGATAAACCCTG-3′ and 5′-GAGGTAGGCCCAGAAGATATG-3′; *CerS3*, 5′-TCAAA-CATTCCACAAGGCAACC-3′ and 5′-AGCAGACTCCAG-CCAAATG-3′; *CerS4*, 5′-ATGCTGTCCAGTTTCAACGAG-3′ and 5′-AGAGTCTGGTTTGGGTACCTG-3′; *CerS5*, 5′-CAGGGACCCC-TAAGCTTGCTG-3′ and 5′-CAGCACTGTCGGATGTCCCAG-3′; *CerS6*, 5′-GTGGAATACGAGGCATTGCTG-3′ and 5′-ATCATCCTT-GGACACCTTGC-3′; and *actin*, 5′-TGATGATATCGCCGCGCTC-GTCGTC-3′ and 5′-GCATACCCCTCGTAGATGGGCACAG-3′.

Real-time quantitative PCR

Total RNA was isolated from cultured cells using an RNeasy Mini Kit (Qiagen) and converted to cDNA using Affinity Script QPCR cDNA (Stratagene, San Diego, CA) according to the manufacturer's protocol. Real-time quantitative PCR was performed using a standard TaqMan PCR kit protocol on an Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The 20 μl PCR reaction mixture included 2 μl (20 ng) cDNA, 10 μl 2× TaqMan Universal Master Mix (Applied Biosystems), and 1 μl 20× TaqMan gene expression assay mix, which includes primers and a fluorescent probe for each target gene (Applied Biosystems; *CerS1*, Hs00242151; *CerS2*, Hs00604577; *CerS3*, Hs00698859; *CerS4*, Hs00226114; *CerS5*, Hs00332291; *CerS6*, Hs00826756; and *18S rRNA*, Hs99999901). Primers for *18S rRNA* were used as an endogenous control for data normalization. The reaction mixture was incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The amount of template was quantified using the standard curve of each target as outlined in the manufacturer's technical bulletin. All reactions were run in triplicate.

[^{3}H]dihydro-Sph labeling assays

[^{3}H]dihydro-Sph labeling assays were performed as described previously (26).

In vitro CER synthase assays

An in vitro CerS assay was performed as described previously (26) with some modification. HEK 293T cells transfected with the CerS members expression plasmids were suspended in buffer A (50 mM HEPES-NaOH, pH 7.5), 1× protease inhibitor mixture (Complete™ EDTA free; Roche Diagnostics, Indianapolis, IN), and 0.5 mM dithiothreitol and lysed by sonication. The resulting total cell lysates (20 μg protein) were incubated with 5 μM dihydro-Sph (Biomol, Plymouth Meeting, PA), 0.2 μCi [4,5-^{3}H]dihydro-Sph (50 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO), and 25 μM fatty acyl-CoA (nonhydroxy C14:0-CoA, nonhydroxy C16:0-CoA, nonhydroxy C18:0-CoA, nonhydroxy C20:0-CoA; all from Sigma, St. Louis, MO) or 2-hydroxy C18:0-CoA (Avanti Polar Lipids, Alabaster, AL) in buffer B (50 mM HEPES-NaOH, pH 7.5), 0.5 mM dithiothreitol, 1 mM $MgCl_2$, and 0.1% digitonin in a final volume of 100 μl. After a 15 min incubation at 37°C, lipids were extracted by successive addition and mixing of 3.75 vol chloroform-methanol (1:2, v/v), 1.25 vol chloroform, and 1.25 vol 1% KCl. Phases were separated by centrifugation, and the organic phase was recovered, dried, and suspended in chloroform-methanol (2:1, v/v). The lipids were separated on Silica Gel 60 high performance TLC plates (Merck, Whitestation, NJ), using a solvent system of chloroform-methanol-acetic acid (190:9:1, v/v/v). The plates were sprayed with the fluorographic reagent EN^{3}HANCE™ (PerkinElmer Life Sciences, Ontario, Canada), dried, and exposed to X-ray films at −80°C.

Additional samples (100 μg protein) of cell lysates were mixed with 5 μM dihydro-Sph and 25 μM 2-hydroxy C18:0-CoA in buffer B in a final volume of 1 ml and incubated for 15 min at 37°C. The lipids were then extracted for further analysis by normal-phase HPLC/ion-trap mass spectrometry as described below.

Mass spectrometric analyses

To assess the molecular compositions of CER, glucosyl CER, and galactosyl CER in the in vitro CerS reaction products or HeLa cells cotransfected with pCE-puro 3xFLAG-FA2H and siRNA specific for CerS members, we used normal-phase HPLC/ion-trap mass spectrometry as reported elsewhere (19) with some modifications. Lipid extracts prepared from the synthase reaction mixtures above and from cell pellets were mixed with appropriate amounts of myristoyl-CER and glucosyl lauroyl-CER as internal standards. The lipids were evaporated under N_2 and dissolved in hexane-2-propanol (3:2). To remove the majority of ester-containing lipids, aliquots were hydrolyzed for 60 min at 60°C in a mild alkaline pH, followed by a Bligh and Dyer extraction (30). The samples were evaporated and dissolved again in the hexane-propanol solvent. An aliquot (1–2.5 μl) of each extract was directly subjected to HPLC/mass spectrometry. Sphingolipids were separated into their classes and subclasses in the order of lower to higher polarity on a trap, and separation silica columns were connected in series (Fortispack 1 × 20 mm and 1 × 100 mm; OmniSeparo-TJ, Inc., Hyogo, Japan). Effluents were monitored with an LCQdeca-XP Plus mass spectrometer interfacing with an xyz stage equipped with a fluoropolymer-coated electrospray tip (FortisTip; OmniSeparo-TJ) of 150 μm outer diameter/20 μm inner diameter. Lipids were identified by the order of elution from the HPLC column, the mass-to-charge ratio (m/z) values, and data-dependent first- or second-stage tandem mass spectrometry (MS/MS or MS^3), and quantified based on a comparison of the peak areas on chromatograms of target and internal standard ions of the same class. Peak area values were corrected for the contributions from the presence of ^{13}C isotope of the natural abundance, including the difference between the carbon numbers of a given molecular species and an internal standard, and an overlap with the second isotope peak of molecular species ions with one double bond more than the species under consideration (31).

RNA interference

Commercially available siRNAs for human *CerS2* (Hs-LASS2-2, -4, and -5), *CerS5* (Hs-LASS5-1 and -3), and control siRNA were all purchased from Qiagen. Three days prior to experiments, HeLa cells were transfected with the appropriate siRNA (final concentration 2 nM) using Lipofectamine™ RNAiMAX reagent (Invitrogen) following the manufacturer's recommendations. Knock-down of target gene expression was confirmed by RT-PCR.

RESULTS

Expression of FA2H and CerS family members during keratinocyte differentiation

The mammalian epidermis contains unique CERs, including those bearing an acyl chain with 2- or ω-hydroxylation and/or an LCB of phyto-Sph or 6-hydroxy-Sph (4). We previously demonstrated that human primary keratinocytes produce such unique CERs and, when differentiated in vitro to an advanced stage, express mRNA for the phyto-Sph, producing C4-hydroxylase *DES2* (21). Furthermore, under these same differentiation conditions, expression of the FA 2-hydroxylase *FA2H* mRNA is also induced (**Fig. 1A**) (9). *Keratin 1*, a marker for terminal differentiation of keratinocytes, is also detectable at day 3 and increases by day 6 (Fig. 1A), confirming differentiation.

To investigate whether CerS family members might also have a role in the production of these unique CERs, we examined the expression of CerS family members during keratinocyte differentiation using real-time quantitative PCR. For all *CerS* family members except *CerS1*, mRNA expression was detected in undifferentiated keratinocytes (day 0 in Fig. 1B). Upon differentiation, however, *CerS3* mRNA expression significantly increased and remained high throughout differentiation (Fig. 1B). In contrast, only a slight increase in *CerS4* mRNA was observed at the late stage of differentiation, and the expression of *CerS2*, *CerS5*, and *CerS6* mRNAs remained nearly unchanged (Fig. 1B). These results suggest that differences in the expression patterns of CerS family members may play an important role in the production of the CER/2-hydroxy CER compositions of different chain lengths observed in different cell types and even in the altered production that occurring during keratinocyte differentiation.

Production of 2-hydroxy-CER by overproduced CerS family members

We next investigated the roles of FA2H and CerS family members in 2-hydroxy-CER production using enzymes overproduced in HEK 293T cells. HEK 293T cells transfected with a control vector or with an *FA2H*-encoding plasmid were metabolically labeled with [^{3}H]dihydro-Sph. Control cells produced only nonhydroxy-CER, which appeared as two bands corresponding to long-chain and short-chain CERs (**Fig. 2A**, upper and lower bands, respectively). Our previous study using electrospray ionization-mass spectrometry demonstrated that the upper band

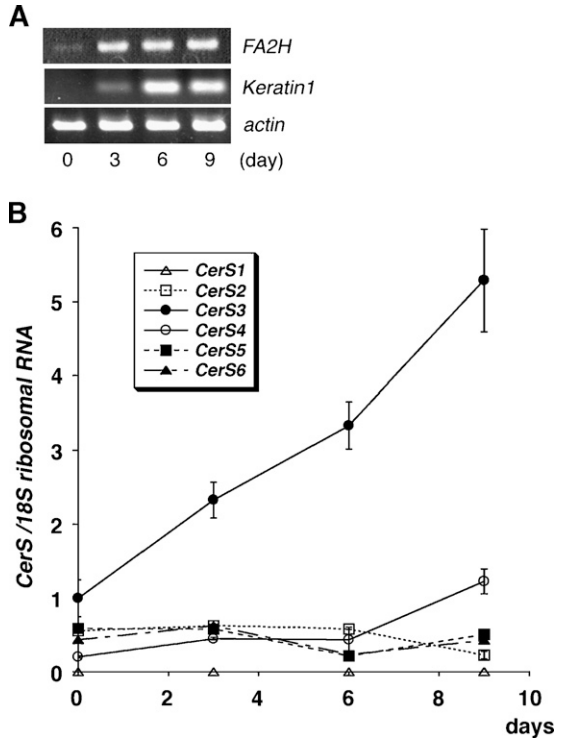

Fig. 1. Expression of ceramide synthase 3 (CerS3) mRNA is induced during differentiation of human keratinocytes. A: Cultured human keratinocytes were differentiated for 0, 3, 6, or 9 days, and total RNA was prepared at each time point. RT-PCR was performed using primers specific for FA2H, keratin 1 (a terminal differentiation marker), and actin (a loading control). PCR products were separated by 1.5% agarose gels and visualized. B: Keratinocyte cDNA was prepared at each time point during differentiation. Real-time quantitative PCR was performed by the TaqMan method using primers and probes specific for CerS1, CerS2, CerS3, CerS4, CerS5, and CerS6, with 18S rRNA as a loading control. Values represent the mean ± SD of the amount of each CerS mRNA relative to that of the 18S rRNA from three independent experiments.

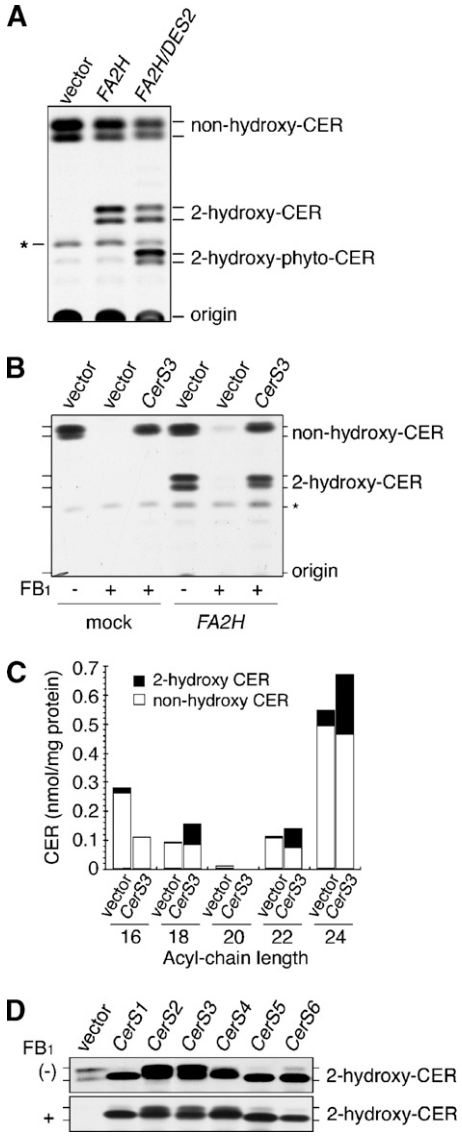

Fig. 2. CerS family members produce 2-hydroxy-ceramide (2-hydroxy CER). A: HEK 293T cells were transfected with pcDNA3 HA-1 (vector), pCE-puro 3xFLAG-FA2H (FA2H), or pCE-puro 3xFLAG-FA2H/pcDNA3 DES2 (FA2H/DES2), and labeled with 1 μCi [3H]dihydro-Sph for 3 h. Lipids were extracted, separated by TLC, and detected by X-ray film. B: HEK 293T cells were transfected with pCE-puro 3xFLAG-FA2H (FA2H), together with pcDNA3 HA-1 (vector), or pcDNA3 HA-CerS3 (CerS3). Cells were untreated or treated with 20 μM fumonisin B_1 (FB_1) (a CerS inhibitor) then labeled and visualized as in (A). The asterisk in A and B indicates an unidentified band. C: HEK 293T cells were cotransfected with FA2HpCE-puro 3xFLAG-FA2H and pcDNA3 HA-1 (vector) or pcDNA3 HA-CerS3 (CerS3). Cells were harvested 3 days after transfection. Total lipids were extracted and hydrolyzed under mild alkaline conditions, and the remaining sphingolipids were extracted by the Bligh and Dyer method. The FA compositions of the total CER, including 2-hydroxy- and nonhydroxy FAs, were determined by HPLC/ion-trap mass spectrometry as described in Materials and Methods. Each column is shaded to illustrate the total amounts of nonhydroxy CER (white) and 2-hydroxyl CER (black), relative to zero. D: HEK 293T cells were cotransfected with pCE-puro 3xFLAG-FA2H and a CerS-encoding plasmid, pcDNA3 HA-CerS1 (CerS1), pcDNA3 HA-CerS2 (CerS2), pcDNA3 HA-CerS3 (CerS3), pcDNA3 HA-CerS4 (CerS4), pcDNA3 HA-CerS5 (CerS5), or pcDNA3 HA-CerS6 (CerS6). Cells were untreated or treated with 20 μM FB_1 (a CER synthase inhibitor) then labeled and visualized as in (A).

contained CERs with C22:0, C24:0, and C24:1 acyl chains, whereas the lower band contained C16:0- and C18:0-CERs (27). In contrast, overproduction of FA2H resulted in the generation of two bands of 2-hydroxy-CER, and co-overproduction of both FA2H and DES2 created 2-hydroxy-CER with phyto-Sph (Fig. 2A). Thus, when FA2H and DES2 are expressed, HEK 293T cells are able to produce 2-hydroxy-FA and phyto-Sph, i.e., CERs found in epidermis.

Each CerS family member exhibits a unique substrate preference, not only for the chain length but also for the saturation in the fatty acyl-CoA (26). To date, however, the substrate preference of CerS members toward hydroxy-fatty acyl-CoA has not been examined. To investigate this, we first examined the ability of CerS3, the most abundant CerS in keratinocytes, to produce 2-hydroxy-CER. HEK 293T cells overproducing FA2H and/or CerS3 were metabolically labeled with [^{3}H]dihydro-Sph in the presence or absence of the CerS inhibitor fumonisin B_1 (FB_1). Consistent with previous reports that FB_1 inhibits endogenous CerS activity but not the activity of overproduced CerS members (23, 25–27), cells transfected with a *CerS3*-encoding plasmid produced nonhydroxy-CER in the presence of FB_1,

whereas those transfected with the control vector did not (Fig. 2B). Co-overproduction of CerS3 and FA2H resulted in the production of 2-hydroxy-CER in the presence of FB_1. Moreover, both medium- and long-chain 2-hydroxy-CERs were produced by CerS3 (Fig. 2B). We carefully examined the FA composition of 2-hydroxy-CERs in HEK 293T cells overproducing CerS3 and/or FA2H in the absence of FB_1 using HPLC/mass spectrometry (Fig. 2C). In FA2H-overproducing HEK 293T cells, HPLC/mass spectrometry analysis demonstrated that the upper band contained mainly 2-hydroxy-CERs with a C24:0 acyl chain, whereas the lower band contained mainly C16:0 (Fig. 2C). The ratio of the 2-hydroxy-CERs carrying these two chain lengths was similar to that observed for nonhydroxy-CER, as we previously reported (27). In cells overproducing both CerS3 and FA2H, no 2-hydroxy-C16:0-CER was detected, but both medium- and long-chain 2-hydroxy-CERs (C18, 0.07 nmol/mg protein; C22, 0.06 nmol/mg protein; C24, 0.21 nmol/mg protein) were clearly detected by HPLC/mass spectrometry (Fig. 2C). We previously reported that CerS3 exhibits relatively broad substrate specificity for the chain length of nonhydroxy-fatty acyl-CoA (27). These results suggest, then, that CerS3 may use both medium- and long-chain 2-hydroxy-fatty acyl-CoAs as substrates.

We also investigated the ability of other CerS members to produce 2-hydroxy-CER by overproducing each of them together with FA2H in the absence or presence of FB_1. All CerS members produced 2-hydroxy-CER, although the chain length of the resulting CERs varied (Fig. 2D). CerS2, CerS3, and CerS4 each produced two bands, whereas CerS5 and CerS6 synthesized only one band. The lower band produced by CerS2, CerS3, and CerS4 migrated slightly faster than the single band observed with CerS5 and CerS6, suggesting that this band may correspond to medium-chain CERs. Therefore, the chain length preference of CerS members toward 2-hydroxy-CoAs may be similar to that toward nonhydroxy-fatty acyl-CoAs (CerS1, C18; CerS2, C22 and C24; CerS4, C20, C22, and C24; CerS5 and CerS6, C14 and C16) (23–27).

Chain length-specific production of 2-hydroxy-stearoyl-CER by CerS1 in vitro

We next performed an in vitro CER synthesis assay using commercially available 2-hydroxy-stearoyl (C18:0)-CoA. Total cell lysates were prepared from HEK 293T cells overproducing CerS1, the CerS that exhibits the highest activity toward C18:0-CoA, and CerS6, which exhibits the highest activity toward C16:0-CoA. CerS1 lysate, when incubated with the corresponding nonhydroxy fatty acyl-CoA and [^{3}H]dihydro-Sph, produced a strong C18:0-dihydro-CER band and weak C14:0- and C16:0-dihydro-CER bands (**Fig. 3A**), in agreement with previous reports (23, 26). Furthermore, when 2-hydroxy-C18:0-CoA was used instead of a nonhydroxy fatty acyl-CoA, 2-hydroxy-C18:0-dihydro-CER was synthesized (Fig. 3A). In contrast, CerS6 lysate produced nonhydroxy-dihydro-CERs that included strong bands for C14:0- and C16:0-dihydro-CER, but a weak band for C18:0-dihydro-CER (Fig. 3A), also consistent with our previous report (26). When CerS6 lysate was incubated with 2-hydroxy-C18:0-CoA, very little 2-hydroxy-C18:0-dihydro-CER production was detected. Control cell lysates produced neither of the two dihydro-CER species. These results indicate that CerS members have 2-hydroxy-CER synthesis activities with chain length-dependent substrate preference for 2-hydroxy-fatty acyl-CoAs.

We also examined 2-hydroxy-CER synthesis in vitro using HPLC/mass spectrometry. To confirm the structure of the reaction products, cell extracts were incubated as above with 2-hydroxy-C18:0-CoA and dihydro-Sph, then total lipids were extracted from the mixtures and subjected to analysis. Figure 3B shows simultaneously acquired positive- and negative-ion chromatograms on a single run. Chromatograms of the $(M + H)^+$ ions of *m/z* 584.7 and $(M + HCOO)^-$ ions of *m/z* 628.8, corresponding to *N*-(2-hydroxy-stearoyl)-dihydro-Sph, reveal two peaks, but the retention time of the peak eluted later corresponds to 2-hydroxy-dihydro-CER, consistent with the results of the TLC (Fig. 3A). Analysis of control extracts did not indicate any peak at the retention time of 2-hydroxy-dihydro-CER, but endogenous nonhydroxy-CERs of C24:0 and C24:1 were similarly detected in both vector and CerS1-overproducing cell extracts. On-line data-dependent MS/MS of the *m/z* 584.7 ions in the positive-ion mode did show the presence of diagnostic ions, i.e., protonated hydroxy-stearoylamide ions (OH-C18:0-NH_3^+, *m/z* 300.2) derived from the acyl moiety of CER, and a series of ions (Sa′ of *m/z* 284.3 and Sa″ of *m/z* 266.4) formed from protonated dihydro-Sph (Sa of *m/z* 302.4) by successive dehydration. More-definitive peaks of these important ions were obtained by the successive MS/MS scan of the *m/z* 566.4 ions arising from the *m/z* 584.7 ions by dehydration on the first MS/MS scan (Fig. 3C). The MS/MS spectrum of the ions eluted earlier on the *m/z* 584.7 positive-ion chromatogram was the same as that of the 2-hydroxy-dihydro-CER ions eluted later, suggesting that the substrate contains a structural isomer of 2-hydroxy-C18:0-CoA. These results, together with those above, confirm that 2-hydroxy-fatty acyl-CoAs are good substrates for CerS members.

Synthesis of 2-hydroxy-CER by CerS members is confirmed in knock-down studies

In addition to the experiments using overproduced enzymes described above (Fig. 2B–D), we also examined the involvement of CerS members in 2-hydroxy-CER synthesis using a knock-down method in an FA2H-overproducing system. For this purpose, we chose HeLa cells, which express only *CerS2*, *CerS4*, and *CerS5* mRNAs, as confirmed by RT-PCR (**Fig. 4A**), rather than HEK 293T cells, which express *CerS2*, *CerS4*, *CerS5*, and *CerS6* mRNAs (data not shown). Furthermore, in HeLa cells, the expression level of *CerS2* is eight times greater than that of *CerS4*, as determined by real-time quantitative PCR (data not shown). Therefore, using these cells, we introduced RNA interference and examined the long-chain preference of *CerS2* and the short-chain preference of *CerS5* in the synthesis of 2-hydroxy/nonhydroxy-CER. We confirmed that in the presence of siRNA for *CerS2* or *CerS5*, each corresponding mRNA was specifically reduced in the HeLa cells (Fig. 4B).

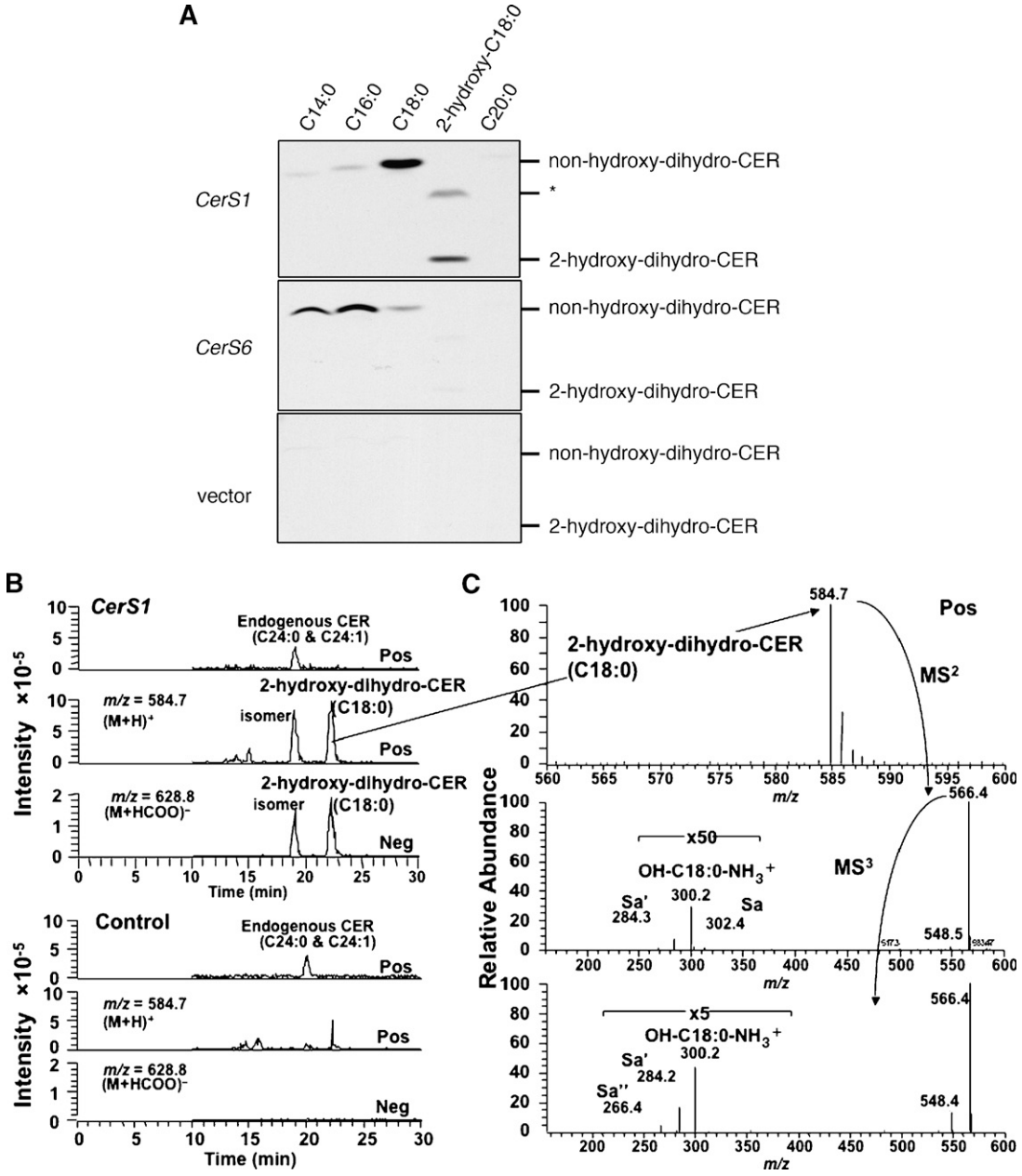

Fig. 3. CerS1 exhibits 2-hydroxy-C18:0-CER synthesis activity in vitro. A: Total cell lysates (20 μg protein) prepared from HEK 293T cells transfected with pcDNA3 HA-1 (vector), pcDNA3 HA-CerS1 (CerS1), or pcDNA3 HA-CerS6 (CerS6) were incubated at 37°C for 15 min with 0.2 μCi [3H]dihydro-Sph, 5 μM dihydro-Sph, and either a nonhydroxy-CoA (C14:0, C16:0, C18:0, or C20:0) or 2-hydroxy-18:0-CoA (each at 25 μM). Lipids were extracted, separated by TLC, and detected by X-ray film. An asterisk indicates an unidentified band. B: Total cell lysates (100 μg protein) prepared from HEK 293T cells transfected with pcDNA3 HA-1 (control) or pcDNA3 HA-CerS1 (CerS1) were incubated at 37°C for 15 min with 5 μM dihydro-Sph and 2-hydroxy-18:0-CoA. Lipids were extracted, directly separated on a normal-phase HPLC, and then monitored on-line with an LCQdeca-XP mass spectrometer via an electrospray ion source. A fluoropolymer-coated electrospray ionization (ESI) tip (FortisTip, 20 μm ID and 150 μm OD) (35) was fit onto an xyz stage. Spectra in the positive and negative modes were alternately acquired by switching polarity at the ESI source on a single chromatographic run. C: A mass spectrum near a peak at the retention time of 22.5 min on a chromatogram of *m/z* 584.7 ions (upper panel), a data-dependent tandem mass spectrometry (MS/MS) spectrum of *m/z* 584.7 ions (middle panel), and a successive MS/MS spectra of the *m/z* 566.4 ions formed by collision-induced dissociation (lower panel).

When FA2H was overproduced in the control siRNA-treated cells, strong synthesis was observed for long-chain nonhydroxy-CERs but weak synthesis for short-chain nonhydroxy-CERs. Strong synthesis of long-chain 2-hydroxy-CER was also apparent in these cells, but no short-chain 2-hydroxy-CER band was detected (Fig. 4C). These results suggest that *CerS2* and *CerS4*, which prefer long-chain fatty acyl-CoAs, are dominant over *CerS5*, which prefers short-chain fatty acyl-CoAs, in HeLa cells.

Because the distribution pattern of CER molecular species was more clearly exhibited in hexosyl CERs, including galactosyl and glucosyl CERs, than in CERs, we also examined the FA composition of nonhydroxy- and 2-hydroxy-hexosyl CERs in FA2H-overproducing HeLa cells using

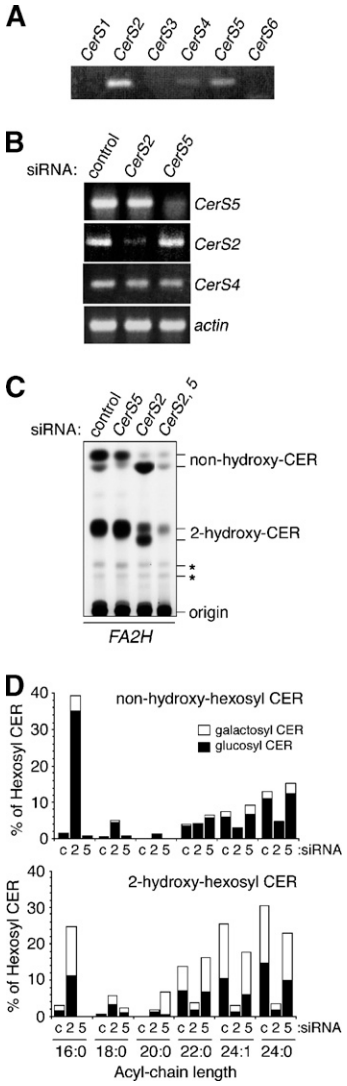

Fig. 4. CerS2 produces long-chain 2-hydroxy-CER and CerS5 short-chain 2-hydroxy-CER in HeLa cells overproducing FA2H. A: Total RNA was prepared from HeLa cells and subjected to RT-PCR using primers specific for each CerS family member. PCR products were separated on 1.5% agarose gels and visualized. B: HeLa cells were transfected with control siRNA, or siRNA specific for CerS2 or CerS5. Three days after transfection, total RNA was prepared from each culture and subjected to RT-PCR using primers specific for CerS2 or CerS5. CerS4 and actin were used as loading controls. PCR products were separated on 1.5% agarose gels and visualized. C: HeLa cells were cotransfected with pCE-puro 3xFLAG-FA2H (FA2H) and control siRNA (control) or siRNA specific for CerS2 (CerS2), CerS5 (CerS5), or CerS2 and 5 (CerS2, 5). Three days after transfection, cells were labeled with 1 μCi [3H]dihydro-Sph for 3 h. Lipids were extracted, separated by TLC, and detected by autoradiography. The asterisk indicates an unidentified band. D: HeLa cells were cotransfected with pCE-puro 3xFLAG-FA2H and control siRNA (c) or siRNA specific for CerS2, or CerS5. Cells were harvested three days after transfection, and total lipids were extracted then hydrolyzed under mild alkaline conditions, and the remaining sphingolipids were extracted by the Bligh and Dyer method. The FA compositions of hexosyl CERs, including glucosyl and galactosyl CERs, were determined by HPLC/ion-trap mass spectrometry as described in Materials and Methods. For each cell treatment, the column is shaded to illustrate the percentages of glucosyl CER (black) and galactosyl CER (white) present in either the nonhydroxy-hexosyl CER (upper panel) or 2-hydroxy-hexosyl CER (lower panel) lipid portion.

HPLC/mass spectrometry (Fig. 4D). In control siRNA-treated HeLa cells, nonhydroxy- and 2-hydroxy-hexosyl CERs carrying C22:0-, C24:1-, and C24:0-FAs were the major components, and 2-hydroxy-C16:0-hexosyl CER was a minor one. Treatment with siRNA specific for *CerS2* resulted in a decrease in both long-chain CERs (nonhydroxy- and 2-hydroxy-CERs), and in the appearance of respective short-chain CERs (Fig. 4C). HPLC/mass spectrometry revealed that long-chain nonhydroxy- and 2-hydroxy-hexosyl CERs (C22:0, C24:1, and C24:0) were significantly decreased and that the short-chain nonhydroxy- and 2-hydroxy-CER bands observed by TLC corresponded mainly to the C16:0 acyl-chain species (Fig. 4D). These data suggest that CerS2 exhibits a substrate specificity for long-chain FAs. Additionally, in CerS2 siRNA-treated HeLa cells, we detected CerS5 mRNA expression by RT-PCR (Fig. 4B) but no CerS6 mRNA expression (data not shown). These data support the conclusion that the predominance of CerS5 regulates the appearance of short-chain CERs in CerS2 siRNA-treated cells. In contrast, interference by *CerS5*-specific siRNA reduced the level of the short-chain nonhydroxy CER band (Fig. 4C), and no signal for 2-hydroxy-C16:0-hexosyl CER was detected by HPLC/mass spectrometry (Fig. 4D). Furthermore, cotreatment with *CerS2*- and *CerS5*-specific siRNAs resulted in decreases in both long- and short-chain CERs (nonhydroxy- and 2-hydroxy-CERs) (Fig. 4C). The *CerS2*-specific siRNA-dependent short-chain band (mainly C16:0) was reduced by *CerS5*-specific siRNA. These results demonstrate that CerS5 exhibits C16:0 specific activity for nonhydroxy and 2-hydroxy CERs. Thus, this knock-down approach provided further evidence that each CerS member exhibits a specific chain length preference for 2-hydroxy-fatty acyl-CoAs as well as for nonhydroxy-fatty acyl-CoAs.

DISCUSSION

CERs are unusually abundant in epidermal stratum corneum, where they have pivotal roles in maintaining epidermal permeability barrier function (3, 4). Epidermal CERs are unique in the composition of their FAs and LCBs. The FAs found in epidermis include 2-hydroxy and ω-hydroxy types in addition to the more common nonhydroxy type, and the LCBs include Sph, phyto-Sph, and 6-hydroxy-Sph (10, 17, 32). However, the molecular basis for the generation of this variety of CERs remains largely unknown. Recent identification of members of the CerS family (22–28), of the sphingolipid C4-hydroxylase DES2 (18), and of the FA 2-hydroxylase FA2H (7) have enabled us to investigate their roles in the production of epidermal CERs. We previously reported that the expression of *DES2* mRNA is induced upon keratinocyte differentiation (21). In the present study, we have demonstrated that *FA2H* and *CerS3* mRNAs are also significantly increased in the differentiation of these cells (Fig. 1A, B). Of the CerS members, *CerS3* exhibited the highest mRNA expression throughout differentiation (Fig. 1B). Reportedly, the ratios of medium- and long-chain 2-hydroxy-FAs (C18–C26 chain length) were increased in the CER/glucosyl CER of differentiated

keratinocytes (9). CerS3 can utilize both medium- and long-chain fatty acyl-CoAs as substrates regardless of the hydroxylation status at the C-2 position (Fig. 2B, C) (27). Recently, studies in testis suggested that CerS3 might display a significant affinity for polyunsaturated acyl-CoA with 28 or more carbon atoms (33). Furthermore, CerS3 exhibits similar activity toward Sph and phyto-Sph (unpublished observations). Therefore, the increase in CerS3 levels observed during keratinocyte differentiation (Fig. 1B) may be responsible for the increased production of certain epidermal CERs, including long-chain 2-hydroxy CER, also observed during the differentiation (9).

Although some CerS members are inactive toward unsaturated fatty acyl-CoA (26), we revealed here that all CerS members can utilize 2-hydroxy-fatty acyl-CoA, regardless of their chain length preference. Overproduction of each CerS member resulted in the production of a 2-hydroxy-CER with a specific chain length (Fig. 2B–D). Mass spectrometry indicated that CerS3 produced medium- and long-chain 2-hydroxy-CERs (C18, C22, and C24 chain length) in HEK 293T cells (Fig. 2C). Furthermore, the knock-down of *CerS2* gene expression caused reduced production of long-chain 2-hydroxy-CERs (Fig. 4C, D), and the knock-down of *CerS5* gene expression caused a reduction in the amount of short-chain 2-hydroxy-CERs, especially C16:0-CERs (Fig. 4C, D). Above all, utilization of 2-hydroxy-fatty acyl-CoA was directly confirmed by an in vitro assay (Fig. 3).

The presence of 2-hydroxy-CER is limited to specific tissues, such as brain, skin, and kidney (6, 16, 32). This distribution pattern coincides with the tissue-specific expression of *FA2H* mRNA (7). The hydroxyl group of 2-hydroxy-CER may function in interactions with other 2-hydroxy-CER molecules or with other lipid molecules via hydrogen bonds. Sphingolipids, together with cholesterol, form lipid microdomains, which are known to serve as platforms for effective signal transduction in the eukaryotic plasma membrane (34). We speculate that such hydrogen bonds strengthen lipid-lipid interactions, affecting microdomains in size, mobility, and stability. In epidermis, another hydroxyl group exists in CER, at the C4 position of the LCB (phyto-Sph). Therefore, it is possible that this hydroxyl group, together with that in the 2-hydroxy-FA moiety of CER, produces rigid interactions among different CERs via hydrogen bonds, providing the barrier function of the epidermal stratum corneum.

Although we demonstrated here the cooperation of DES2, FA2H, and CerS members in the production of some epidermal CERs, including phyto-CER, 2-hydroxy-CER, and 2-hydroxy-phyto-CER, the molecular mechanism behind the generation of other epidermal CERs, such as those containing ω-hydroxy-FA and 6-hydroxy-Sph, is completely unknown. To understand the entirety of epidermal CER biogenesis, the identification of genes encoding an FA ω-hydroxylase and sphingolipid C-6-hydroxylase will be necessary.

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