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 2xTaqMan Universal Master Mix (Applied Biosystems), and 1 μl 20x 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 ribosomal RNA*, Hs99999901). Primers for *18S ribosomal RNA* 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 sec 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* CER synthase 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), 1X 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

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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 (non-hydroxy C14:0-CoA, non-hydroxy C16:0-CoA, non-hydroxy C18:0-CoA, non-hydroxy 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, 1mM $MgCl_2$, 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 volumes of chloroform/methanol (1:2, v/v), 1.25 volumes of chloroform, and 1.25 volumes of 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). 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

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To assess the molecular compositions of CER, glucosyl CER, and galactosyl CER in the *in vitro* ceramide synthase reaction products or HeLa cells co-transfected 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 connected in series (Fortispack 1x20 mm and 1x100 mm, OmniSeparo-TJ, Inc. Hyogo, Japan). Effluents were monitored with an LCQdeca-XP Plus mass spectrometer interfacing with a xyz stage equipped with a fluoropolymer-coated electrospray tip (FortisTip; OmniSeparo-TJ, Inc. Hyogo, Japan) 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 species in 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 (Cambridge, MA). 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.

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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 a long-chain base 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 fatty acid 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 a *FA2H*-encoding plasmid were metabolically labeled with [^{3}H]dihydro-Sph. Control cells produced only non-hydroxy-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 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 CER synthase in keratinocytes, to produce 2-hydroxy-CER. HEK 293T cells

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overproducing FA2H and/or CerS3 were metabolically labeled with [^{3}H]dihydro-Sph in the presence or absence of the CER synthase inhibitor fumonisin B_1 (FB_1). Consistent with previous reports that FB_1 inhibits endogenous CER synthase activity but not the activity of overproduced CerS members (23, 25-27), cells transfected with a *CerS3*-encoding plasmid produced non-hydroxy-CER in the presence of FB_1, while 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 fatty acid 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 mainly contained 2-hydroxy-CERs with a C24:0 acyl-chain, whereas the lower band mainly contained C16:0 (Fig. 2C). The ratio of the 2-hydroxy-CERs carrying these two chain lengths was similar to that observed for non-hydroxy-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 length 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 non-hydroxy-fatty acyl-CoA (27). These results suggest, then, that CerS3 may use both medium- and long-chain 2-hydroxy-fatty acyl-CoA as substrates.

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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 non-hydroxy-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 non-hydroxy 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), agreeing with previous reports (23, 26). Furthermore, when 2-hydroxy-C18:0-CoA was used instead of a non-hydroxy fatty acyl-CoA, 2-hydroxy-C18:0-dihydro-CER was synthesized (Fig. 3A). In contrast,

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CerS6 lysate produced non-hydroxy-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. Fig. 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-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-CER, but endogenous non-hydroxy-CERs of C24:0 and C24:1 were similarly detected in both vector and CerS1-overproducing cell extracts. On-line data-dependent tandem mass spectrometry (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

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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-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/non-hydroxy-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). When FA2H was overproduced in the

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control siRNA-treated cells, strong synthesis was observed for long-chain non-hydroxy-CERs but weak synthesis for short-chain non-hydroxy-CERs. Strong synthesis of long-chain 2-hydroxy-CER was also apparent in these cells, but no short-chain 2-hydroxy-CER band was not detected (Fig. 4C). These results suggest that *CerS2* and *CerS4*, which prefer long-chain fatty acyl-CoAs, are dominant over *CerS5*, which prefer short-chain fatty acyl-CoAs, in HeLa cells.

Since the distribution pattern of ceramide molecular species was more clearly exhibited in hexosyl CERs, including galactosyl- and glucosyl-CERs, than in CERs, we also examined the fatty acid composition of non-hydroxy- and 2-hydroxy-hexosyl CERs, in FA2H-overproducing HeLa cells, using HPLC/mass spectrometry (Fig. 4D). In control siRNA-treated HeLa cells, non-hydroxy- and 2-hydroxy-hexosyl CERs carrying C22:0-, C24:1-, and C24:0-fatty acids 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 (non-hydroxy- and 2-hydroxy-CERs), and in the appearance of respective short-chain CERs (Fig. 4C). HPLC/mass spectrometry revealed that long-chain non-hydroxy- and 2-hydroxy-hexosyl CERs (C22:0, C24:1, and C24:0) were significantly decreased and that the short-chain non-hydroxy- 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 fatty acids. 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 non-hydroxy CER band (Fig. 4C), and no signal for 2-hydroxy-C16:0-hexosyl CER was detected by HPLC/mass spectrometry (Fig. 4D). Furthermore, co-treatment with *CerS2*- and *CerS5*-specific siRNAs resulted in decreases in both long- and short-chain CERs (non-hydroxy- 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 non-hydroxy 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 non-hydroxy-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 non-hydroxy type, and the LCBs include Sph, phyto-Sph, and 6-hydroxy-Sph (10, 17, 32). However, the molecular basis behind the generation of this variety of CERs remains largely unknown. Recent identification of members of the CER synthase CerS family (22-28), of the sphingolipid C4-hydroxylase DES2 (18), and of the fatty acid

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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 (Figs. 1A and 1B). Of the CerS members, *CerS3* exhibited the highest mRNA expression throughout differentiation (Fig. 1B). Reportedly, the ratios of medium- and long-chain 2-hydroxy-fatty acids (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 (Figs. 2B and 2C) (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 (our unpublished data). 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 (Figs. 4C and 4D), and the knock-down of *CerS5* gene expression caused a reduction in the amount of short-chain 2-hydroxy-CERs, especially C16:0-CERs (Figs. 4C and 4D). 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, proving 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

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biogenesis, the identification of genes encoding a FA ω–hydroxylase and sphingolipid C-6-hydroxylase will be necessary.

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