Differentiation-associated expression of ceramidase isoforms in cultured keratinocytes and epidermis

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Abstract Ceramides (Cers) accumulate within the interstices of the outermost epidermal layers, or stratum corneum (SC), where they represent critical components of the epidermal permeability barrier. Although the SC contains substantial sphingol, indicative of ceramidase (CDase) activity, which CDase isoforms are expressed in epidermis remains unresolved. We hypothesized here that CDase isoforms are expressed within specific epidermal compartments in relation to functions that localize to these layers. Keratinocytes/epidermis express all five known CDase isoforms, of which acidic and alkaline CDase activities increase significantly with differentiation, persisting into the SC. Conversely, neutral and phytoalkaline CDase activities predominate in proliferating keratinocytes. These differentiation-associated changes in isoform activity/protein are attributed to corresponding, differentiation-associated changes in mRNA levels (by quantitative RT-PCR). Although four of the five known CDase isoforms are widely expressed in cutaneous and extracutaneous tissues, alkaline CDase-1 occurs almost exclusively in epidermis. These results demonstrate large, differentiation-associated, and tissue-specific variations in the expression and activities of all five CDase isoforms. Because alkaline CDase-1 and acidic CDase are selectively upregulated in the differentiated epidermal compartment, they could regulate functions that localize to the distal epidermis, such as permeability barrier homeostasis and antimicrobial defense.— Houben, E., W. M. Holleran, T. Yaginuma, C. Mao, L. M. Obeid, V. Rogiers, Y. Takagi, P. M. Elias, and Y. Uchida. **Differentiation-associated expression of ceramidase isoforms in cultured keratinocytes and epidermis. *J. Lipid Res.* 2006.** 47: **1063–1070.**

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Ceramides (Cers) are bulk components of the epidermal permeability barrier, a critical function of the epidermis that is required for survival in a terrestrial environment (1). To fulfill barrier requirements, Cer synthesis increases during epidermal differentiation, accelerating still further in response to all forms of barrier abrogation (2). Ultimately, these Cers are deposited within the extracellular matrix of the stratum corneum (SC), along with cholesterol and FFAs, forming distinctive lamellar membranes (1). Importantly, newly synthesized Cers must first be converted to either glucosylceramide or sphingomyelin within the suprabasal, nucleated cell layers of the epidermis; it is only after secretion at the stratum granulosum (SG)-SC interface that these polar sphingolipid precursors are hydrolyzed again into Cers (3).

Excess cellular Cers can trigger antimitogenic signals and induce apoptosis in all cell types, including keratinocytes (reviewed in 4, 5), whereas distal Cer metabolites, sphingosine and sphingosine-1-phosphate, also are important bioregulatory molecules (reviewed in 6). Cer hydrolysis (catabolism) occurs in the nucleated cell layers, where this pathway regulates keratinocyte proliferation and apoptosis in response to external stress, such as irradiation and other oxidative stress (7). Yet, Cer hydrolysis apparently also operates in the enucleated layers of the SC, because substantial free sphingoid base (or "sphingol") is also present in the SC interstices (8), where it appears to function as a potent, endogenous antimicrobial agent (8, 9).

The role of Cer catabolites in other epidermal functions remains unresolved. We hypothesize here that the function of Cer catabolites could be linked to the expression of specific ceramidase (CDase) isoforms within the layer(s)

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Abbreviations: aCDase, acidic ceramidase; alkCDase, alkaline ceramidase; CDase, ceramidase; Cer, ceramide; CHK, cultured human keratinocyte; DIG, digoxigenin; nCDase, neutral ceramidase; phytoCDase, phytoalkaline ceramidase; SC, stratum corneum; SG, stratum granulosum.

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that subserve(s) these functions. Of the five major CDase isoforms of varying pH and substrate specificity that exist in various mammalian tissues, activities that could be ascribed to the acidic ceramidase (aCDase), neutral ceramidase (nCDase), and alkaline ceramidase (alkCDase; subtype undetermined) isoforms have been tentatively identified in epidermis (8, 10, 11). Whether phytoalkaline ceramidase (phytoCDase) is present is not known; nor is it known whether the putative alkCDase activity is attributable to one or both alkCDase isoforms. Finally, nothing is known about the localization of CDase isoforms in epidermis. Four of these isoforms, aCDase (12), nCDase (13, 14), alkCDase-1 (15, 16), and alkCDase-2 (accession number AY312516), display distinctive pH optima, which could restrict their activities to selective epidermal strata. The activity of phytoCDase, if present, would be further limited by its substrate preference for phytosphingosine-containing Cers (15), which are known to be plentiful in epidermis (17, 18). Moreover, the differing subcellular localization of the various isoforms could further restrict certain CDase isoforms to specific layers. For example, aCDase localizes preferentially within the lysosomal compartment in extracutaneous tissues (19), whereas alkCDase-1/-2, where present, occur in Golgi and endoplasmic reticulum fractions, respectively (15, 16), and nCDase is present within both mitochondria and plasma membrane "raft" microdomains (14, 20, 21). Tissue-specific differences in CDase localization could further affect function (e.g., in gastrointestinal epithelium, nCDase is postulated to hydrolyze dietary sphingolipids) (22). Finally, the unique requirements of the epidermis could dictate isoform expression within specific cell layers or subcellular fractions.

As noted above, neither the spectrum of CDase isoforms present in epidermis nor their expression/localization in relation to keratinocyte differentiation is known. Yet, this type of information is a prerequisite to understanding the role of each isoform in relation to epidermal structure and function. Hence, we assessed the expression and activities of the five major CDase isoforms in human and murine epidermis, as well as in cultured human keratinocytes (CHKs), subjected to graded levels of differentiation. We report here that all five isoforms are expressed constitutively in human keratinocytes; furthermore, significant alterations in the expression of four of these CDase isoforms occur in relation to differentiation. Moreover, epidermis demonstrates highly selective and very high expression of the alkCDase-1 (but not alkCDase-2) isoform compared with extracutaneous tissues.

MATERIALS AND METHODS

Materials

Cers were purchased from Sigma (St. Louis, MO) and Matreya (Pleasant Gap, PA). Radiolabeled chemicals were from American Radiolabeled Chemicals, Inc. (Arlington Heights, IL). High performance thin-layer chromatography plates (Silica Gel 60) were purchased from Merck (Darmstadt, Germany). Hairless male mice (Skh:hr-1) were purchased from Simonsen Laboratories (Gilroy, CA). Human total RNAs were from Ambion (Austin, TX).

Cell culture

Normal human keratinocytes were isolated from human neonatal foreskins by a modification of the method of Pittelkow and Scott (23, 24) under an Institutional Review Board-approval protocol (University of California, San Francisco). Cells were grown in keratinocyte growth medium supplemented with bovine epidermal growth factor, bovine pituitary extract, insulin, hydrocortisone, and 0.07 mM calcium chloride (Cascade Biologics, Portland, OR). CHKs at three subsequent stages of differentiation were used for these studies. To generate undifferentiated keratinocytes, cells were grown in a low-Ca^{2+}-containing (0.07 mM) and serum-free medium and harvested at 80–90% confluence. For early-stage differentiated CHKs, the medium was switched to 1.2 mM Ca^{2+} and serum-free medium at 90% confluence, and cells were harvested 2 days after the Ca^{2+} switch. Advanced-stage differentiated CHKs, which show both lamellar bodies and the full spectrum of epidermal Cer species (25), were obtained by growth in Dulbecco's and Ham's F-12 medium (2:1, v/v) containing ≥1.2 mM calcium, supplemented with 10% FBS, 10 μg/ml insulin, 0.4 μg/ml hydrocortisone (26), and 50 μg/ml ascorbic acid, as described previously (25). The cultures each were maintained at 37°C under 5% CO_2 in air, with medium changes performed three times weekly.

Human epidermis

Full-thickness human skin samples were obtained from neonatal foreskins under a human research protocol approved by the local Ethics Commission (Vrije Universiteit Brussels). The epidermis was isolated from the dermis after overnight incubation with dispase solution at 4°C (24).

Enzyme assays

Either CHK cell lysates or epidermal homogenates (≤500 μg of protein) were used as the source for enzyme activity assays. Activities of each CDase isoform were measured using ^{3}H-labeled palmitoyl-D-erythro-sphingosine as a substrate in the presence of detergent (a mixture of 16 mg/ml sodium cholate, 10 mg/ml Triton X-100, and 4 mg/ml Tween 80). Alternatively, phytoCDase activity was assayed fluorometrically using N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]lauroyl]-phytosphingosine (Molecular Probes, Eugene, OR) as substrate, as described previously (15).

Western immunoblot analysis

Western blot analysis was performed using a previously described procedure (27). Briefly, cell lysates (prepared as described above) were suspended in sample buffer (60 mM Tris-HCl, pH 6.8 containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.005% bromophenol blue) and resolved by electrophoresis on 10% SDS-polyacrylamide gels. The resultant bands were electrophoretically transferred to polyvinylidine fluoride membranes (Invitrogen, Carlsbad, CA), probed with anti-human aCDase (BD Sciences, Franklin Lakes, NJ), and detected using an enhanced chemiluminescence system (Invitrogen). The same membranes were reprobed with β-actin antibody to normalize for protein loading.

RT-PCR analysis

RT-PCR or quantitative RT-PCR was performed using cDNA prepared from total RNA as described previously (27, 28). RT-

PCR was performed using *Taq* DNA polymerase (Invitrogen) with specific primer sets (15, 16). For quantitative RT-PCR, 10 ng of cDNA was mixed with sets of primer pairs (final concentration, 200 pM) and SYBR Green PCR mix containing *Taq* DNA polymerase and SYBR Green I dye (Applied Biosystems, Foster City, CA). The following primer sets were used for quantitative RT-PCR: for human aCDase, 5′-TCAACAAGCTGACCGTATACACAA-3′ and 5′-CCCGCAGGTAAGTTTCGAATT-3′ (68 bp); for human nCDase, 5′-GCTAACCCGAAGAATTCAGTACAAA-3′ and 5′-CCACGATGTTGAAGTAGCCTCAT-3′ (84 bp); for human alkCDase-1, 5′-GGTGGATGCCAACTATGAGATG-3′ and 5′-CAACTGTCCCAGGCCAGTA-3′ (61 bp); for human alkCDase-2, 5′-GAGTTCTACAACACCGATCAGCAATG-3′ and 5′-TGACGAAACAAGCACATGCA-3′ (70 bp); for human phytoCDase, 5′-CAGGGCTAGTCGGAGGATCAT-3′ and 5′-TGGCCATAGACAGACACCATAAA-3′ (92 bp); for murine aCDase, 5′-TGGATCAAAAGCTGCCTGGTAT-3′ and 5′-TGCAATTCCCCTCATTTCCT-3′ (68 bp); for murine nCDase, 5′-ACGAGGCAGCATCTACAATCTATG-3′ and 5′-TGGCTACTGTGTCCGTAGCAA-3′ (92 bp); for murine alkCDase-1, 5′-GCATCTGATTGCGGTTTCTGT-3′ and 5′-CAAAGTACACGGTCACTGATCCA-3′ (98 bp); for murine phytoCDase, 5′-TGGTGCATTTCAAGGCATTAGA-3′ and 5′-CCATTCCTACCACTGTGAGTGCTA-3′ (79 bp). The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min, repeated 40 times on ABI Prism 7700 (Applied Biosystems). The products were analyzed using 1.5% or 3% agarose gels with ethidium bromide.

In situ hybridization

The sequences of aCDase and alkCDase-1 cDNAs (coding regions, 600 and 520 bases, respectively) were prepared by PCR using platinum *Taq* DNA polymerase (Invitrogen) with CHK cDNA as a template; primers were aCDase sense (5′-TCGTGCCAAGTGGAAAAGTTA-3′) and antisense (5′-GGTTGCCTCCCAGGATAAAGT-3′) and alkCDase-1 sense (5′-GGCCTGTTCTCCATGTATTTCC-3′) and antisense (5′-AGTAGCGGACTTTGAGGGTTTC-3′). The PCR products were inserted into pCR II-TOPO vectors using TOPO TA Cloning (Invitrogen), with which One Shot TOP10 competent cells were transformed. Plasmid DNAs purified with the Qiagen Plasmid Mini Kit (Operon Biotechnologies GmbH, Cologne, Germany) were linearized with the restriction enzymes *Spe*I and *Not*I for aCDase and *Spe*I and *Eco*RV for alkCDase-1. Digoxigenin (DIG)-labeled RNA probes to detect human aCDase and alkCDase mRNA were made from these linearized cDNA sequences as templates using the DIG RNA Labeling Kit (SP6/T7) supplied by Roche Diagnostics (Indianapolis, IN).

Sections from human newborn foreskins were fixed in 4% paraformaldehyde and embedded in paraffin. The sections were hybridized at 50°C, and hybridization of DIG-labeled probes to the endogenous mRNA was detected by GenPoint (DakoCytomation) with modification. Briefly, after an initial binding of rabbit polyclonal anti-DIG antibody-HRP conjugate to the DIG probes, peroxidase oxidation of biotinyl tyramide deposits additional biotin at the hybridization site, allowing additional streptavidin-HRP capture and enhanced signal generation. The signal was finally detected using the Vector VIP peroxidase substrate kit (Vector Laboratories, Burlingame, CA), and sections were counterstained with hematoxylin. The sense control probes resulted in no signal (data not shown), indicating the specificity of hybridization with the antisense probes.

Statistical analysis

Statistical analyses were performed using an unpaired Student's *t*-test.

RESULTS

aCDase and alkCDase activities increase with keratinocyte differentiation

We first investigated the activities of the aCDase, nCDase, alkCDase, and phytoCDase isoforms in both CHKs and human epidermis in relation to differentiation. AlkCDase activity represented the total activities of both alkCDase-1 and alkCDase-2. Three different stages of keratinocyte differentiation were assessed: undifferentiated CHKs (proliferative stage) and two stages of progressively increased differentiation in CHKs, induced initially by increasing the Ca^{2+} concentration of the medium from 0.07 to 1.2 mM, and then boosted further by adding 10% serum and 50 μM vitamin C, with a still higher Ca^{2+} concentration (≥1.2 mM) (25). We designate CHKs grown in low Ca^{2+} here as undifferentiated CHKs. Cellular proliferation declines and differentiation initiates by 2 days after an increase in medium Ca^{2+} to 1.2 mM (29); these CHKs are designated early-stage differentiated CHKs. However, expression of late differentiation markers, such as loricrin (30), and the full spectrum of Cer species, characteristic of SC, occurs only when CHKs are cultured for prolonged periods not only in high Ca^{2+} (≥1.2 mM) but also in vitamin C (50 μM) and 10% serum (31, 32). These cells are designated advanced-stage differentiated CHKs. The enzyme activities of both aCDase and total alkCDase increased significantly in advanced-stage differentiated over undifferentiated CHKs ($P < 0.01$) as well as in whole epidermis compared with early-stage differentiated CHKs ($P < 0.01$) (**Fig. 1**). In contrast, both nCDase and phytoCDase activities declined significantly in homogenates of whole epidermis and in differentiated (both levels, $P < 0.01$) compared with undifferentiated CHKs (Fig. 1). Together, these results suggest that various types of CDase activities are regulated divergently in relation to keratinocyte/epidermal differentiation.

Differentiation-associated alterations in CDase activities are attributable to prior alterations in mRNA expression

We next investigated whether the differentiation-linked changes in CDase activities could be attributed to corresponding changes in mRNA expression. First, we examined mRNA expression for the five CDase isofoms (aCDase, nCDase, alkCDase-1, alkCDase-2, and phytoCDase) in CHKs at the three different stages of CHK differentiation as well as in whole human epidermis by RT-PCR. mRNA was detected for all five CDase isoforms in CHK epidermis at all stages of differentiation and in human epidermis using RT-PCR (data not shown).

To further assess alterations of the five CDase isofoms during differentiation, we next quantitated mRNA levels of these enzymes by quantitative RT-PCR. We used 18S rRNA as both a control and to normalize CDase expression, because the expression of this housekeeping gene does not change with differentiation (data not shown). Whereas both undifferentiated CHKs and early-stage differentiated CHKs showed the same mRNA levels for aCDase, mRNA levels for this CDase isoform were 2- and 3-fold higher, respectively, in both advanced-stage differen-

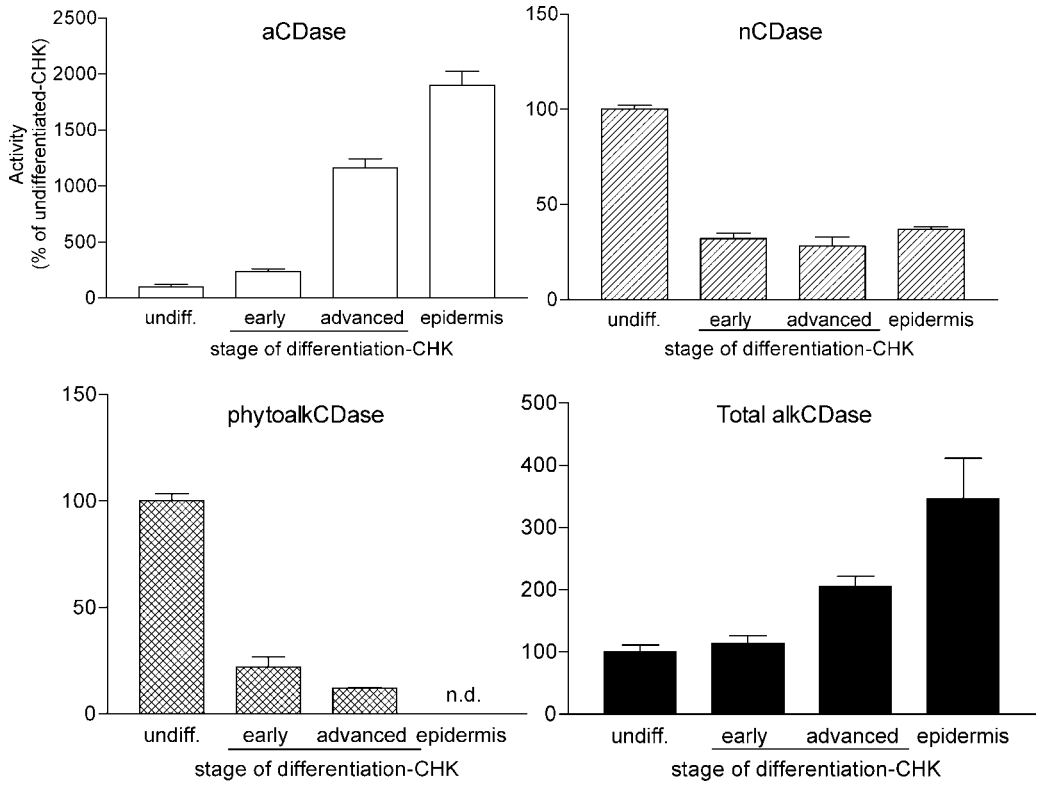

Fig. 1. Alterations of ceramidase (CDase) isoform activities during keratinocyte differentiation. Activities for acidic ceramidase (aCDase), neutral ceramidase (nCDase), total alkaline ceramidase (alkCDase), and phytoalkaline ceramidase (phytoCDase) were determined in homogenates prepared from cultured human keratinocytes (CHKs) or epidermis, as detailed in Materials and Methods. Values represent means ± SEM for three determinations each. n.d., undetectable or insufficient for quantification. * $P < 0.01$ versus undifferentiated CHKs; ** $P < 0.01$ versus early-stage differentiated CHKs. The activities of aCDase, nCDase, total alkCDase, and phytoCDase in undifferentiated CHKs are 51.0 ± 12.2, 34.5 ± 1.2, 1,628 ± 450, and 24.1 ± 2.8 pmol/mg/h. The activities were assayed using [^{3}H]palmitoylsphingosine for aCDase, nCDase, and total alkCDase and NBD-lauroylphytoshingosine for phytoCDase.

tiated CHKs and in whole epidermis versus undifferentiated CHKs (**Table 1**). Moreover, mRNA expression of alkCDase-1 again was significantly higher in advanced-stage differentiated CHKs (79-fold; $P < 0.01$) and in epidermis (369-fold; $P < 0.01$) than in undifferentiated and early-stage differentiated CHKs, whereas mRNA levels of alkCDase-2 did not change significantly in early-stage versus advanced-stage differentiated CHKs (Table 1). In contrast, the mRNA levels for both nCDase and phytoCDase declined in both advanced-stage differentiated CHKs and epidermis compared with undifferentiated and early-stage differentiated CHKs (Table 1). Together, these results suggest, first, that alterations in mRNA expression of the various CDase isoforms largely account for the observed changes in enzyme activities that occur during keratinocyte differentiation, and second, that expression of the various CDase isoforms is linked to keratinocyte differentiation. Finally, alkCDase-1 expression is markedly upregulated compared with the other isoforms during differentiation, including aCDase.

TABLE 1. Quantitative RT-PCR for CDase mRNA levels during keratinocyte differentiation (mRNA expression relative to undifferentiated CHKs)

Sample	aCDase	nCDase	alkCDase-1	alkCDase-2	phytoCDase
Undifferentiated	1.00 ± 0.21	1.00 ± 0.15	1.00 ± 0.19	1.00 ± 0.14	1.00 ± 0.21
Early-stage differentiated	0.90 ± 0.22	1.04 ± 0.51	0.89 ± 0.16	1.59 ± 0.24	0.94 ± 0.21
Advanced-stage differentiated	1.99 ± 0.49	0.35 ± 0.12	79.0 ± 30.4	1.34 ± 0.23	0.20 ± 0.10
Human whole epidermis	2.98 ± 0.68	0.14 ± 0.10	369 ± 74.8	0.65 ± 0.19	0.28 ± 0.08

aCDase, acidic ceramidase; alkCDase, alkaline ceramidase; CDase, ceramidase; CHK, cultured human keratinocyte; nCDase, neutral ceramidase; phytoCDase, phytoalkaline ceramidase. CDase mRNA expression was normalized to levels of 18S rRNA expression, with expression in undifferentiated keratinocytes to 18S rRNA set to 1.00. Further details of quantitative RT-PCR methods are provided in Materials and Methods. Values represent means ± SD.

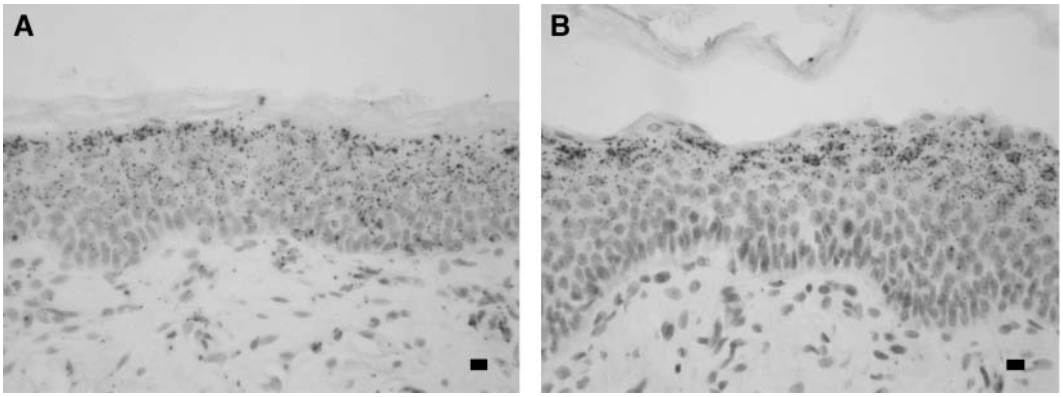

Fig. 2. aCDase mRNA (A) is expressed across the epidermis, whereas alkCDase mRNA (B) peaks in the outermost nucleated layers of human epidermis. mRNAs were detected with digoxigenin-labeled antisense riboprobes (black stain), as detailed in Materials and Methods. Bars = 10 μm.

Localization of aCDase and alkCDase-1 mRNA within human epidermis

To delineate variations in CDase mRNA expression that might indicate specific functions in the outer epidermis, we next localized the mRNA for the two differentiation-associated isoforms in human epidermis, aCDase and alkCDase-1, by in situ hybridization (**Fig. 2**). Although aCDase mRNA was evident throughout all of the nucleated layers of the epidermis, expression increased in the outer nucleated (SG) layers (Fig. 2A). Whereas alkCDase-1 mRNA also increased significantly in the outer epidermis (i.e., upper stratum spinosum and SG), mRNA for this isoform was not detected in the basal cell layer (Fig. 2B).

aCDase and alkCDase activities persist in SC

Because the activities and gene expression of both aCDase and alkCDases are increased in advanced-stage differentiated CHKs, we next investigated whether one or both activities remain(s) active in the enucleate layers of the SC. Consistent with prior studies (8), we detected both aCDase (127 ± 63 pmol/mg protein/h) and (total) alkCDase (42 ± 16 pmol/mg protein/h) activities in SC. However, we could not compare activities in SC with nucleated cell layers, because the highly cross-linked cornified proteins and hydrophobic extracellular lipid structures decrease the extraction efficiencies of CDase from SC.

aCDase activator proteins, such as saposin D, which are present both at the SG-SC junction and within the SC (33), also could influence in vitro activity for these fractions. Hence, we next ascertained whether increased aCDase activity in advanced-stage differentiated CHKs reflects alterations in protein levels for this isoform. Western immunoblot analysis revealed a significant increase in the α-subunit of aCDase (13 kDa), the mature catalytic form of the enzyme, in advanced-stage differentiated CHKs (1.69- and 1.29-fold vs. undifferentiated and early-stage differentiated CHKs, respectively), whereas protein levels of a control protein, β-actin, did not change (**Fig. 3**, lanes 1–3). These results suggest that an increase in aCDase protein levels accounts for the increased enzyme activity in advanced-stage differentiated CHKs. Finally, we also demonstrated the expression of aCDase protein in the SC (Fig. 3, lanes 4, 5).

Unique expression patterns of CDase isoforms in epidermis correlate with unique functions of this tissue

The differentiation-associated variations in mRNA expression and localization of the five CDase isoforms in mammalian epidermis suggest unique roles for each of these enzymes in the divergent functions of this tissue. Hence, we next compared epidermal expression of the five CDase isoforms with expression levels of these isoforms in several extracutaneous human and murine tissues by quantitative RT-PCR. Specifically, we quantitated CDase isoform mRNA expression in undifferentiated CHKs, whole human epidermis, and several other extracutaneous

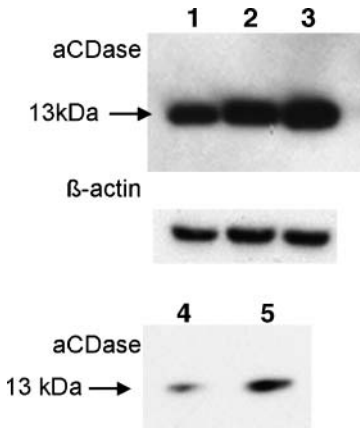

Fig. 3. aCDase protein levels increase in advanced-stage differentiated CHKs. Cell lysates (10 μg of protein) or protein extracts (10 and 35 μg) from stratum corneum (SC) were resolved by electrophoresis on 10% SDS-polyacrylamide gels and probed with monoclonal anti-aCDase antibody; representative blots are shown. Lane 1, undifferentiated CHKs; lane 2, early-stage differentiated CHKs; lane 3, advanced-stage differentiated CHKs; lanes 4, 5, SC extracts (10 and 35 μg, respectively). Lanes 1–3, upper panel, aCDase; lower panel, β-actin. A mature aCDase comprises the α-subunit (13 kDa) and the β-subunit (40 kDa). Monoclonal anti-aCDase antibody recognizes an epitope of the α-subunit of aCDase.

TABLE 2. mRNA expression (quantitative RT-PCR) for CDase isoforms in various human tissues (mRNA expression relative to epidermis)

Tissue	aCDase	nCDase	alkCDase-1	alkCDase-2	phytoCDase
Whole epidermis	1.00 ± 0.29	1.00 ± 0.14	1.00 ± 0.27	1.00 ± 0.39	1.00 ± 0.13
Undifferentiated CHKs	0.52 ± 0.16	1.36 ± 0.19	0.01 ± 0.01	1.53 ± 0.44	5.25 ± 0.91
Brain	3.41 ± 0.38	0.32 ± 0.18	n.d.	0.27 ± 0.09	0.29 ± 0.99
Thymus	0.05 ± 0.00	0.20 ± 0.04	<0.01	0.58 ± 0.17	0.69 ± 0.47
Heart	38.3 ± 1.76	0.17 ± 0.19	n.d.	0.40 ± 0.12	0.55 ± 1.17
Lung	6.16 ± 2.19	0.35 ± 0.13	n.d.	0.32 ± 0.09	4.48 ± 1.82
Trachea	1.50 ± 0.40	0.21 ± 0.10	0.04 ± 0.01	0.68 ± 0.20	0.60 ± 0.19
Liver	0.83 ± 0.16	0.44 ± 0.10	n.d.	0.13 ± 0.04	0.23 ± 0.09
Kidney	21.7 ± 2.71	2.23 ± 1.01	0.14 ± 0.01	1.07 ± 0.28	5.08 ± 0.53
Skeletal muscle	2.50 ± 0.44	0.70 ± 0.25	n.d.	0.10 ± 0.03	0.49 ± 0.06

mRNA expression was normalized to levels of 18S rRNA expression. Further details of quantitative RT-PCR methods are provided in Materials and Methods. Values represent means ± SD. n.d., not quantitatively detected.

human tissues by quantitative RT-PCR. Although the relative mRNA expression levels for aCDase, nCDase, alkCDase-2, and phytoCDase differed between tissues, these four isoforms were all detected in the tissues examined (**Table 2**). Whereas aCDase mRNA expression levels in heart and kidney were substantially higher than in other tissues, nCDase mRNA expression levels were comparable in kidney, epidermis, and undifferentiated CHKs (Table 2). PhytoCDase mRNA expression levels were higher in undifferentiated CHKs, lung, and kidney than in other tissues. Although alkCDase-1 mRNA was also expressed in a few extracutaneous epithelia (e.g., trachea and kidney), its expression levels were 8- to 250-fold lower than in whole epidermis (Table 2). In contrast, mRNA for alkCDase-2 was expressed in all tissues examined, with the highest levels in kidney, epidermis, and undifferentiated CHKs (Table 2).

As in human tissues, aCDase, nCDase, and phytoCDase were detected in all murine tissues examined (**Table 3**; cf. Table 2). In addition, higher expression of aCDase and phytoCDase was evident in brain, heart, lung, and kidney than in epidermis, thymus, liver, and small intestine (Table 3). However, whereas nCDase mRNA levels were significantly higher in kidney than in other tissues, epidermis and heart also showed high expression of this isoform (Table 3). In contrast to these three CDase isoforms, mRNA for alkCDase-1 was detected only in thymus, small intestine, and epidermis, and its expression in epidermis again greatly exceeded that in both thymus and small intestine (Table 3). These results reveal a comparable expression pattern for the various CDase isoforms in human and mouse tissues, with relatively selective, high levels of alkCDase-1 expression in epidermis.

TABLE 3. mRNA expression (quantitative RT-PCR) for CDase isoforms in various murine tissues (mRNA expression relative to epidermis)

Tissue	aCDase	nCDase	alkCDase-1	phytoCDase
Whole epidermis	1.00 ± 0.04	1.00 ± 0.07	1.00 ± 0.14	1.00 ± 0.05
Brain	2.38 ± 0.77	0.41 ± 0.14	n.d.	6.88 ± 2.41
Thymus	0.81 ± 0.25	0.10 ± 0.03	0.24 ± 0.03	0.87 ± 0.34
Heart	3.36 ± 0.03	0.95 ± 0.08	n.d.	1.74 ± 0.23
Lung	4.21 ± 0.50	0.47 ± 0.14	n.d.	3.18 ± 0.40
Liver	0.82 ± 0.05	0.51 ± 0.09	n.d.	0.69 ± 0.07
Kidney	11.8 ± 1.20	3.02 ± 0.32	n.d.	2.49 ± 0.29
Small intestine	1.13 ± 0.17	0.63 ± 0.08	0.04 ± 0.01	0.60 ± 0.21

CDase mRNA expression was normalized to levels of 18S rRNA expression, with expression in whole epidermis to 18S rRNA set to 1.00. Further details of quantitative RT-PCR methods are provided in Materials and Methods. Values represent means ± SD. n.d., not quantitatively detected.

DISCUSSION

These studies demonstrate that all five known CDase isoforms, aCDase, nCDase, alkCDase-1, alkCDase-2, and phytoCDase, are expressed in both CHKs and whole epidermis, greatly extending prior studies (8, 10, 11). We further show that the expression of four of the five CDase isoforms (except alkCDase-2) changes markedly in relation to keratinocyte differentiation, but only aCDase and alkCDase-1 gene expression are upregulated during keratinocyte differentiation, likely accounting for parallel, differentiation-associated increases in enzyme activities. In contrast, both the expression and activities of nCDase and phytoCDase peak in undifferentiated keratinocytes (i.e., basal, proliferating layer) and decline with differentiation, whereas alkCDase-2 (but not alkCDase-1) did not change significantly during differentiation. Although alkCDase activity in the SC could derive, in part, from skin-colonizing pathogenic bacteria (34), a prior study demonstrated that alkCDase activity does not increase in the SC of atopic dermatitis, despite high pathogen colonization (34). Moreover, microbial origin certainly cannot explain our observation of high levels of alkCDase in advanced-stage differentiated CHKs. The high expression of alkCDase-1 mRNA in epidermis versus extracutaneous tissues suggests an endogenous, epidermal origin of most of this activity. Nevertheless, the possibility exists that some alkCDase activity in the SC could originate from bacterial sources.

Whereas both mRNA and enzyme activities for aCDase also increase in parallel with differentiation, the relative increase in enzyme activity in normal SC far exceeds the more modest changes in mRNA level. In fact, aCDase is synthesized as a 55 kDa precursor protein, which is then processed into mature, α (13 kDa) and β (40 kDa) subunits (35). Thus, posttranscriptional modification(s) of the aCDase into mature subunits could alter/amplify enzyme

activity during normal terminal differentiation. Whether posttranscriptional modification of one or more of the other CDase isoforms also occurs remains unknown.

We recently demonstrated that both aCDase and nCDase accompanied by downstream conversion of sphingosine to sphingosine-1-phosphate restrict increases in intracellular Cer levels in CHKs, thereby protecting cells from oxidative stress-mediated Cer-induced apoptosis (36). The role of CDase in protecting against Cer-induced apoptosis has been explored in a variety type of cells (reviewed in 5). For example, blockade of CDase activity using specific inhibitors sensitizes cancer cells to chemotherapeutic agents (reviewed in 37, 38), which operate by inducing an increase in Cer (39, 40). In addition, Cer-induced apoptosis mediated by tumor necrosis factor-α can be attenuated by the overexpression of aCDase (41). A related recent study showed that nCDase activity is expressed in duodenum, jejunum, and ileum cells, where it is thought to detoxify dietary Cer (42). Both nCDase and alkCDase are deployed in cellular compartments, such as plasma membranes (21, 43), mitochondria (13), endoplasmic reticulum, and Golgi apparatus (15, 16), where Cer generation occurs, suggesting that these CDase isoforms attenuate the potentially deleterious effects of Cer in cells. Because metabolites of Cer, sphingosine, and sphingosine-1-phosphate also regulate myriad cellular functions (reviewed in 44), CDase could also generate other functionally important bioregulatory molecules. Although the role of aCDase and nCDase in protecting against oxidative stress-induced apoptosis seems clear, whether phytoCDase, alkCDase-1, or alkCDase-2 protects against apoptosis remains unknown.

Because both aCDase and alkCDase-1 mRNA expression and activities are strongly linked to differentiation, and both of their activities further persist into the SC, it seems likely that they have separate or additional functions related to the distal compartment of the epidermis. Although sphingosine appears to be an important endogenous antimicrobial species, Cers, rather than sphingosine, are essential components of the epidermal permeability barrier (1). Thus, unrestricted Cer hydrolysis could threaten lamellar membrane structures that mediate permeability function in the SC. To minimize excess Cer hydrolysis under basal conditions, CDase activity is likely restricted by ambient pH and/or its localization to specific microdomains within the SC. For example, the extracellular spaces in the SC are more acidic than the cytosol (45), and the outer SC is more acidic than the lower SC (45). Hence, aCDase activity likely is restricted to specific sites within the SC interstices.

What is the basis, then, of further abundant alkCDase activity present in the SC? Although the low pH of the SC (46) may restrict alkCDase activity to ensure the retention of sufficient bulk Cer for barrier function under basal conditions, barrier abrogation, which is accompanied by both a threat of pathogen invasion and an increased pH, would activate alkCDase, thereby generating additional antimicrobial sphingosine. Yet, like aCDase, alkCDase activity could also be active within specific microdomains where the pH is higher (e.g., corneocyte cytosol vs. lower SC).

In summary, this study reveals the presence of five CDase isoforms within human and murine epidermis as well as significant gradients of these isoforms in relation to epidermal differentiation. This pattern strongly suggests specific function(s) for individual CDase isoforms in the organization of normal epidermal structure and function, such as protection against Cer-induced apoptosis, generation of proliferation and prodifferentiation signaling molecules, and maintenance of the balance between requirements for a competent permeability barrier and the generation of antimicrobial Cer metabolites.

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