β-Glucocerebrosidase activity in mammalian stratum corneum

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Abstract Although previous studies have demonstrated a crucial role for the enzyme β-glucocerebrosidase (GlcCer'ase) in the final steps of membrane structural maturation in mammalian stratum cornuem (SC) and epidermal homeostasis, the precise in vivo localization of GlcCer'ase activity and protein is not known. Here, we developed a fluorogenic in situ assay on histologic sections (zymography) to elucidate the in vivo distribution of GlcCer'ase activity, and further characterized and localized the SC GlcCer'ase activity in vitro. The zymographic technique revealed higher GlcCer'ase activity in upper stratum granulosum and SC, both in murine and human SC; activity that was both inhibited by conduritol B epoxide, a specific GlcCer'ase inhibitor, and pH-dependent; i.e., present at pH 5.2, and absent or significantly reduced at neutral pH (7.4), consistent with the known pH optimum for epidermal GlcCer'ase in vitro. Immunohistochemical staining for GlcCer'ase protein showed enhanced fluorescent signal in the outer layers of human epidermis, concentrated at the apex and margins of stratum granulosum and lower SC. Moreover, in extracts from individual epidermal layers, GlcCer'ase activity was present throughout murine epidermis, with the highest activity in the SC, peaking in the lower-to-mid-SC. The SC activity was stimulated >10-fold by sodium taurocholate, and inhibited by bromoconduritol B epoxide. Finally, isolated membrane couplets, prepared from SC sheets, also demonstrated significant GlcCer'ase activity. These data localize GlcCer'ase activity to the outer epidermis by three different techniques, and support the role of this enzyme in extracellular processing of glucosylceramides to ceramides, required for permeability barrier maturation and function.—Takagi, Y., E. Kriehuber, G. Imokawa, P. M. Elias, and W. M. Holleran. **β-Glucocerebrosidase activity in mammalian stratum corneum. *J. Lipid Res.* 1999.** 40: **861–869.**

Supplementary key words β-glucocerebrosidase • β-glucosidase • epidermis • stratum corneum • Gaucher disease • glucosylceramide • ceramide • sphingolipid • permeability barrier • cutaneous

Lipids of the stratum corneum (SC) of skin comprise a distinctive mixture that is enriched in ceramides, cholesterol, and free fatty acids. These lipids appear to provide the barrier against excess water loss and to limit the ingress of xenobiotics. The ceramide–cholesterol–free fatty acid mixture replaces a phospholipid–neutral lipid mixture during the transition of keratinocytes from the stratum granulosum to the SC (1–4). A number of lipid catabolic enzymes, including sphingomyelinase (5), phospholipase A (6), triacylglycerol hydrolase (5, 7), and steroid sulfatase (8) have been localized in sites where these transformations occur. Moreover, many of these enzymes also have been localized within epidermal lamellar bodies (9–11), suggesting that, although alternative delivery pathways may exist, most of these lipid hydrolytic activities in the SC result from the secretion of this organelle's contents.

A high concentration of glucosylceramides exists in the stratum granulosum (SG), but in the inner SC, glucosylceramides are eliminated while the ceramide content is markedly increased. This distribution pattern for glucosylceramide, along with other more direct evidence discussed below, suggests that the conversion of glucosylceramide to ceramide may be important for the maintenance of skin barrier function. For example, mucosal epithelia (12, 13) and cetacean epidermis (9) do not display a fully competent barrier, and the ratio of glucosylceramides to ceramides in these tissues remains high (14). Although a number of prior studies have reported epidermal β-glucosidase activity in vitro (12, 15–20), we found this activity is due specifically to β-glucocerebrosidase (GlcCer'ase) (21), which only hydrolyzes glucosylceramides to ceramides. Moreover, several studies indicate an important role for this specific enzyme in the epidermal permeability barrier: *1*) inhibition of GlcCer'ase diminishes epidermal permeability barrier function in association with altered lamellar membrane formation (22, 23); and *2*) depletion of GlcCer'ase in Gaucher disease and in transgenic, knock-

Abbreviations: GlcCer'ase, β-glucocerebrosidase; BrCBE, bromoconduritol-B epoxide; 4-MUG, 4-methylumbelliferyl-β-d-glucoside; PBS, Dulbecco's calcium- and magnesium-free phosphate-buffered saline; SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale.

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out mice, results in abnormal bilayer structures and compromised barrier function (25, 26).

In order to further address the hypothesis that the hydrolysis of glucosylceramides to ceramides in the *intercellular* domains of the SC is critical for epidermal homeostasis and permeability barrier function, we have examined the localization of GlcCer'ase within murine and human SC. We report here that: *a*) high levels of GlcCer'ase are found in the SC, both by classical in vitro methods, and by novel in situ zymology techniques; *b*) the inner SC shows higher levels of GlcCer'ase than are found at the surface; and *c*) GlcCer'ase activity is localized to intercellular membrane domains within the SC. These results demonstrate that conversion of glucosylceramide to ceramide within the outer epidermis can be attributed to GlcCer'ase activity within the membrane/intercellular domains of the SC.

MATERIALS AND METHODS

Materials

4-Methylumbelliferone (4-MU), 4-methylumbelliferyl-β-d-glucoside (4-MUG) were obtained from Sigma Chemical Co. (St. Louis, MO). Bromoconduritol B epoxide (BrCBE) was a gift from Dr. G. Legler (Köln University, FRG) (27), while conduritol B epoxide (CBE) was obtained from Toronto Research Chemicals (Ontario, Canada). Staphylococcal exfoliative toxin was purchased from Toxin Technology (Sarasota, FL). The Bio-Rad Protein Assay Kit and bovine serum albumin (BSA) were obtained from Bio-Rad (Richmond, CA). All solvents were of reagent or HPLC grade.

Human skin

Human skin samples were obtained as surgical margins from anonymous donors under an approved human research protocol.

Animals

Hairless male mice (hr/hr) and germ-free nude mice (nu/nu) were purchased from Simonsen Laboratories (Gilory, CA). All animals were 8–12 weeks old at the time of study, and all studies were performed under an approved animal research protocol.

In situ zymography

To first estimate the relative activity of GlcCer'ase activity throughout the epidermis in vivo, we utilized a zymographic method we recently developed using tissue sections to assess enzyme activity in situ, followed by in situ zymology on intact samples (described below). Although similar activity patterns are seen in both mouse and human skin sections, localization is more readily visualized in human samples due to the increased number of epidermal and SC cell layers. Briefly, either freshly isolated murine skin or human skin from surgical margins were immediately placed into keratinocyte growth medium, and then snap frozen in OCT (10.24% polyvinyl alcohol/4.26% polyethylene glycol; Miles Lab., Elkhart, IN) within 30 min of excision, sectioned (20 μm), and mounted onto poly-l-lysine-coated slides. Sections were fixed briefly (10 min; 22–25°C) with 10% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) (aldehyde fixation eliminated enzyme activity in this system), overlayered with substrate solution (0.5 mm 4MUG in McIlvaine citrate/phosphate buffer, pH 5.6) (28) for 30 min, covered with glass cover slips, and sealed. Additional sections were overlayered with substrate solutions containing 10 mm CBE (in citrate/phosphate buffer) for 30 min to inactivate GlcCer'ase. Sections then were incubated for 16 h at 4°C (less signal diffusion occurs at 4°C), and viewed on an inverted Zeiss (Thornwood, NY) laser scanning confocal microscope (excitation wavelength, 360 nm; emission wavelength, 450 nm; objective 40×; aperture 1.2; brightness set to maximal and contrast adjusted to 329 arbitrary units). Scans were subjected to signal analysis (representing relative rates of 4-MU release) using Zeiss imaging software. Controls included both inhibitor-treated samples (i.e., 10 mm CBE), and substrate-excluded samples. The pH of the buffer solutions was measured at the beginning and end of each incubation to insure that the optimal pH for epidermal GlcCer'ase (i.e., approx. 5.6) (21) was maintained.

In situ zymology

As an additional verification of the in situ zymography technique described above, we developed a zymology assay for use with unfixed, intact skin samples. Highly differentiated, human skin equivalent cultures (MatTek®; Ashland, MA) were placed in assay medium and incubated for 4 h (37°C; 5% CO_2 in air). The 4-MUG assay solution (2 mm ± 10 mm CBE in citrate-phosphate buffer; pH 5.6) was placed on top of the culture(s) for 30 min. The culture medium was then changed to keratinocyte growth medium (KGM; 0.07 mm Ca^{2+}; Clonetics, San Diego, CA), and samples were incubated for an additional 16 h (37°C; 5% CO_2 in air). For analysis, 6 mm punch biopsies were obtained, placed on a glass slide, covered, and immediately viewed by confocal microscopy, as described above.

Immunofluorescence

Human tissue (surgical margins, as above), were kept in PBS (4°C) for less than 30 min after excision, then frozen in OCT, and stored at −80°C until use. Cryosections (10 μm) were obtained, briefly fixed in acetone–methanol 1:1 (7 min at 4°C), and allowed to air dry for 2 h (22–25°C). Sections were rehydrated for 1 h in PBS, blocked for 1 h, and rabbit anti-human GlcCer'ase (kindly provided by Drs. Ed Ginns and Ellen Sidransky, NIH) was applied (diluted 1:5000 in blocking solution) for 16 h (4°C). Sections then were washed three times with blocking solution, incubated 45 min (22–25°C) with secondary antibody (goat anti-rabbit-FITC; diluted 1:100 in blocking solution). Sections were washed extensively and mounted with vector shield. Fluorescence was visualized with a Bio-Rad/Nikon confocal microscope. Edge-enhancement was used for data analysis, and exposure settings remained unchanged between control and treated samples.

Preparation of whole epidermal samples

Skin samples were excised from killed mice and subcutaneous tissues were removed by scraping with scalpel blades, as described previously (21, 29). Epidermal sheets were obtained from skin samples by exposure to dry heat (60°C for 60 sec) and by gentle scraping with a scalpel blade. GlcCer'ase is relatively heat stable as 100% of enzyme activity remains after incubation at 50°C for 60 min (22).

Preparation of individual epidermal cell layers

Staphylococcal exfoliative toxin separation was used to prepare individual cell layers as described previously (2, 21, 22). Mice were injected intradermally with 50 μg staphylococcal exfoliative toxin, which had been dissolved in 100 μl PBS. After 2 h, mice were killed by cervical dislocation. The upper epidermis, consisting of SC, SG, and some residual stratum spinosum (SS), was removed gently with tweezers. The remaining, subjacent skin, comprising the lower epidermis (i.e., stratum basale [SB] and some SS), was excised and immediately floated on a solution of 10 mm EDTA in PBS-CMF at 37°C for 30 min. The lower epidermis was then removed from the dermis by gentle scraping

with a blade. The dermis was patted dry with a paper towel, weighed, and its surface area was determined by planimetry.

In a second method (21), after skin excision and removal of subcutaneous fat (as described above), samples were floated dermal-side down onto a 10 mm dithiothreitol solution in PBS at 37°C for 1.5 h. The upper epidermis (i.e., SC and SG) then was gently removed with tweezers, followed by gentle scraping of the remaining epidermal cell layers (i.e., SB and SS) from the dermis with a blade. The dermis again was dried, weighed, and its area was determined by planimetry.

Preparation of stratum corneum samples

Prior to obtaining individual SC preparations, animal flanks were gently swabbed with ethanol to remove surface bacterial contaminants. The following three different techniques were then used to prepare SC samples, and each provided histologically homogeneous sheets of anucleate, cornified cells.

Whole SC also were prepared by cyanoacrylate glue-stripping (30), as follows. One to 2 drops of cyanoacrylate resin were spread on a sterile glass slide, and immediately pressed against the surface of alcohol-wiped adult murine skin. After 1.0 min, the glass slide(s) were gently removed. This procedure results in near-complete removal of SC, although small focal areas with residual SC may remain associated with the underlying epidermis (30). The SC sheets (including dried cyanoacrylate glue) were trimmed to 1.0 cm^2, and removed from the plates with a sterile razor blade.

Epidermal sheets were obtained from adult hairless mice by floating whole skin samples in 10 mm ethylenediaminetetraacetic acid in PBS for 1.0 h at 37°C, followed by gentle scraping with a scalpel blade. Epidermal sheets were then floated in a 0.5% trypsin solution in PBS and incubated at 37°C. After 1.0 h, the SC samples were transferred to fresh trypsin solution and incubated for an additional 30 min. Tissue samples then were sonicated gently for 10 min to completely remove any residual nucleated cells. As GlcCer'ase activity is not significantly altered by direct exposure to trypsin (0.1%), even in tissue homogenates (data not shown), it is unlikely that significant activity is lost during this preparation.

Finally, sequential tape strippings were used to estimate relative GlcCer'ase activity in the outer versus inner SC. Tape strips (Tesa Tape; Mesa, CO) were evenly pressed against alcohol-wiped skin, removed, with repeated strippings representing successively deeper layers of the SC. Samples then were trimmed to 1.0 cm^2 squares, and used directly in the enzyme assay (see below). Fresh tape, used as control, showed no effect on in vitro enzyme activity (i.e., no background fluorescence).

Preparation of tissue homogenates

All procedures were performed at 4°C. Epidermal and SC preparations, excluding cyanoacrylate or tape stripping samples, were minced into small pieces (1 mm^3) with a scalpel blade and transferred to small plastic tubes. Fifteen volumes (by weight) of PBS (containing 0.1 mM PMSF) were added, and tissue samples were homogenized (15 sec ×3) using a Polytron PCU2 Tissue Homogenizer (Kinematica, GmbH, Lucerne, Switzerland), followed by sonication (35%, 10 sec ×2) using a Fisher Sonic Dismembranator Model 300 (Artec Corp, Farmingdale, NY). Protein concentrations were measured using a Bio-Rad Protein Assay Kit, using BSA as a standard (31).

Stratum corneum membrane couplet preparation

SC sheets were first prepared using trypsin, as described above. After removal of excess water with a cotton cloth, flattened SC sheets were covered with three layers of aluminum foil, frozen with liquid nitrogen, and pulverized by pounding with a hammer. The SC powder was then suspended in PBS, homogenized with a Stansted Cell Disrupter twice (15,000 lbs/in^2), as described previously (32), and then trypsinized (0.005%) for 1 h at room temperature. After centrifugation for 15 min at 25,000 *g*, membrane couplets were isolated as previously described (32).

In vitro β-glucocerebrosidase assay

Enzyme assays were performed as described previously (21, 23). Briefly, the assay buffer consisted of citrate–phosphate buffer (pH 5.6, and 0.54% sodium taurocholate, unless otherwise indicated). The enzyme solution was preheated to 37°C in the assay buffer (50 μl); reactions were initiated by addition of 50 μl substrate solution (0.5 mm 4-MUG in assay buffer); incubated for 60 min (37°C); and terminated with 1.25 ml 200 mm carbonate–bicarbonate buffer, pH 10.5. Enzyme activity was determined at various pHs (pH 3.2–7.0) as the production of fluorescent 4-MU from the β-d-glucoside substrate (4-MUG). Fluorescence was measured (Ex = 360 nm, Em = 450 nm) with a Perkin-Elmer spectrofluorimeter. A standard 4-MU solution (0–300 nm) in carbonate–bicarbonate buffer was used for calibration.

Statistical analysis

Statistical significance was determined using a two-tailed Student's *t* test, as appropriate. Results are expressed as mean ± SEM unless otherwise indicated.

RESULTS

Characterization of GlcCer'ase versus β-glucosidase in whole stratum corneum

We previously reported that in vitro GlcCer'ase activity is present in the homogenates from whole epidermis and crude SC preparations (21, 33). Here, we further characterized this enzyme in whole SC sheets, prepared by cyanoacrylate stripping, to be certain that only SC activity was being assessed. Consistent with prior results, hydrolysis of 4-MUG by SC enzyme is maximal near pH 5.2 in the presence of 5 mm sodium taurodeoxycholate; i.e., approximately 10-fold higher than non-taurocholate controls (**Fig. 1**). That this activity is clearly pH-dependent, is shown by the paradoxical inhibition of enzyme activity (i.e., approximately 50%) by taurocholate at still lower pHs (pH 3.6), which characteristically are associated with nonspecific β-glucosidase activity (14). We also used BrCBE, a specific inhibitor for GlcCer'ase (27), to further distinguish between β-GlcCer'ase and other types of nonspecific β-glucosidase activity. SC β-glucosidase activity is highly sensitive to BrCBE in the presence of sodium taurodeoxycholate at pH 5.6 (**Table 1**); i.e., 0.1 and 100 μm BrCBE inhibit activity by 50.0 ± 3.1% and >99%, respectively. These data demonstrate that GlcCer'ase accounts for the majority of β-glucosidase activity in whole stratum corneum, consistent with the findings in whole epidermis (21).

Localization of β-GlcCer'ase activity in human epidermis by in situ zymography

Based on the prior in vitro biochemical localization of GlcCer'ase activity in SC preparations reported above and previously (21), we next developed an in situ method for a more direct visualization of enzyme activity in *intact epidermis.* Confocal microscopy demonstrates cytoplasmic fluo-

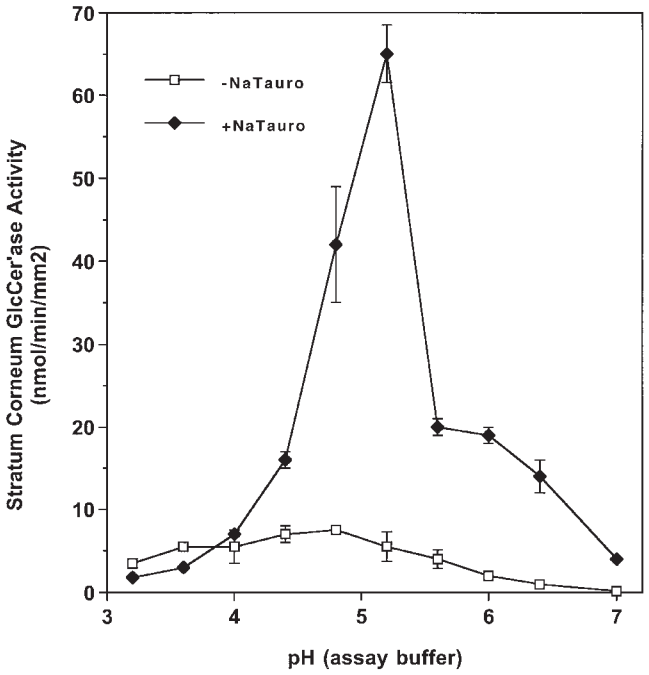

Fig. 1. The effect of pH and taurodeoxycholate on GlcCer'ase activity in the stratum corneum. Full-thickness stratum corneum homogenates were prepared from cyanoacrylate strippings (see Methods). Mean GlcCer'ase activity was determined as the pH in the assay buffer was varied from 3.2 to 7.0 (±range, n = 2). Closed squares represent values obtained without added sodium taurocholate (as control), while open squares represent values with 5 mm sodium taurodeoxycholate. Optimal activity was obtained at pH 5.2.

rescence (i.e., GlcCer'ase activity) throughout the epidermis (**Fig. 2A**); fluorescent signal is excluded from regions corresponding to nuclei. In addition, the fluorescent signal increases from the mid-spinous layer toward the upper SG, while maximal (red) signal intensity (i.e., approximately 2-fold increase over signal in nucleated cell layers) is evident in the lower-to-mid SC; with signal decreasing toward the outer SC. Epidermal appendages (i.e., hair follicles, sebaceous glands) in the dermis also show an increased signal intensity as compared to surrounding dermis (not shown). The GlcCer'ase inhibitor, CBE (10 mm), completely suppresses the fluorescent signal (Fig. 2B), demonstrating the specificity of this signal. Generation of fluorescent signal in the epidermis also was significantly reduced (i.e., >10-fold) at neutral pH (pH 7.6; not shown) (34). Similar to intact human skin samples, murine skin also displayed increased fluorescence intensity in the outer epidermis, with maximal signal evident in the SG and lower SC regions (Fig. 2C). Moreover, fluorescent signal again was diminished in CBE-treated murine samples (Fig. 2D). These results demonstrate the specificity and confirm that the localization of GlcCer'ase activity is concentrated in the outer epidermal cell layers in both human and mouse skin.

TABLE 1. Inhibition of stratum corneum GlcCer'ase activity by bromoconduritol B epoxide (BrCBE)

BrCBE	Specific Activity[a]	Ratio
	nmol/min/mm² surface area	*%*
0 (control)	0.069 ± 0.001	100
0.1 μm	0.034[b] ± 0.002	50.0
0.1 mm	0.003[c] ± 0.0004	0.4

[a] Activity determined using SC homogenate; reported as means ± SEM; n ≤ 5 for each.
[b] $P < 0.005$ vs. control.
[c] $P < 0.001$ vs. control.

In situ zymology on unfixed epidermal samples

To further corroborate and validate that the activity described above for fixed epidermis is representative of that found in unfixed, viable epidermis, the fluorogenic substrate was applied topically to intact, highly differentiated human epidermal skin equivalents (MatTek® cultures). After 16 h incubation, unfixed punch biopsies revealed a sharp increase in fluorescence (i.e., enzyme activity) in the SG, with elevated signal extending into the lower-to-mid SC (**Fig. 3A** and **B**). As a control for specificity, CBE again completely suppressed the generation of the fluorescent signal generation due to 4-MUG hydrolysis (Fig. 3C). This in situ fluorescent pattern is equivalent to that described with the fixed tissue sections above (c.f., Fig. 2), demonstrating that this localization technique closely approximates the in vivo GlcCer'ase activity.

Localization of GlcCer'ase protein in epidermis by immunofluorescence

To obtain further information about the localization of GlcCer'ase enzyme protein in vivo, we next performed immunolocalization studies. Immunofluorescence staining of human skin samples shows fluorescence signal in the mid-to-upper SG (**Fig. 4A**). Moreover, cells in the outer SG display a punctate, apical fluorescence pattern, with a perinuclear distribution reminiscent of lysosomal association. Fluorescence is significantly diminished in the basal layer, except for areas of focal staining (Fig. 4B). Normal serum controls, without the primary antibody against GlcCer'ase, do not generate a measurable fluorescent signal (Fig. 4C). Although the level of nonspecific protein reactivity remains to be determined, these results are consistent with localization of epidermal GlcCer'ase protein to the mid-to-outer epidermis, with a punctate, apical, cytosolic distribution within these cell layers.

Enzyme localization in epidermis in relation to differentiation

The in vitro specific activity of GlcCer'ase was previously shown to be almost 1.5-times higher in murine SC homogenates than in the whole epidermal samples (21). Therefore, we next determined the activity of GlcCer'ase within different layers of the epidermis and the SC. The possibility that SC enzyme activity (i.e., especially at the surface of the SC) derives from bacteria first was eliminated by comparing the ratio of activities obtained both in cyanoacrylate strips and in whole epidermis, with or without prior wiping with 70% ethanol (see Methods).

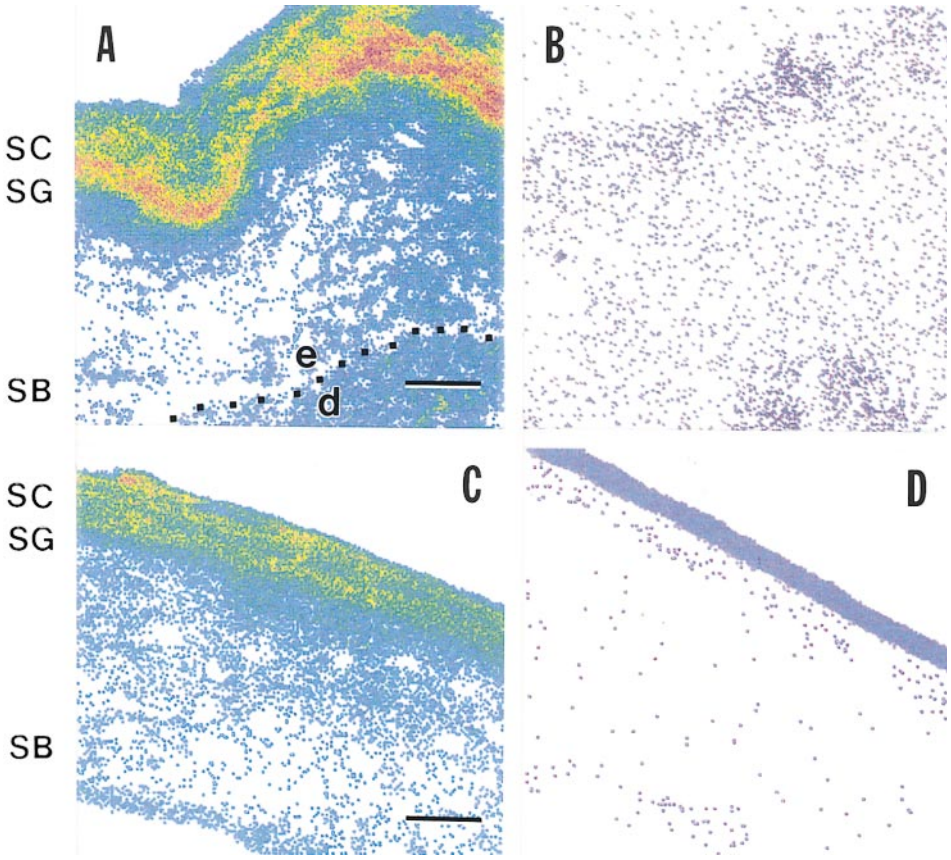

Fig. 2. Localization of epidermal GlcCer'ase activity by in situ fluorescence zymography in human and murine epidermal samples. Serial sections were obtained from OCT-embedded frozen human skin (panels A and B), and murine skin (panels C and D). Buffered fluorophore substrate was incubated on top of section to optimize penetration / activity throughout the section. GlcCer'ase activity (i.e., fluorescence) is represented by pseudocolor scale (see scale Fig. 2), with red representing the brightest and purple the weakest fluorescent signal. The color shift from red-to-purple represents a >10-fold decrease in fluorescence intensity. Note paucity of signal in lower epidermal layers, and increased signal in SG-to-SC layers. Co-incubation with the GlcCer'ase inhibitor CBE significantly diminishes fluorescent signal (panels B and D), demonstrating the specificity of the fluorescent signal. Magnification: panels A–D, ×400; size bar = 25 μm.

No significant difference in GlcCer'ase activity were evident in the SC samples from either ethanol-wiped mice or germ-free nude mice when compared with the activity in the SC of normal mice (**Table 2**). These results further confirm that the observed SC activity is due to endogenously produced GlcCer'ase enzyme. Finally, nearly 50% of the total in vitro epidermal GlcCer'ase activity is localized to the upper epidermal layers, including SC and SG, with the SC alone accounting for approximately 25% of total epidermal activity (**Table 3**). These studies demonstrate that GlcCer'ase activity at the surface of the stratum corneum is not derived from bacterial contamina-

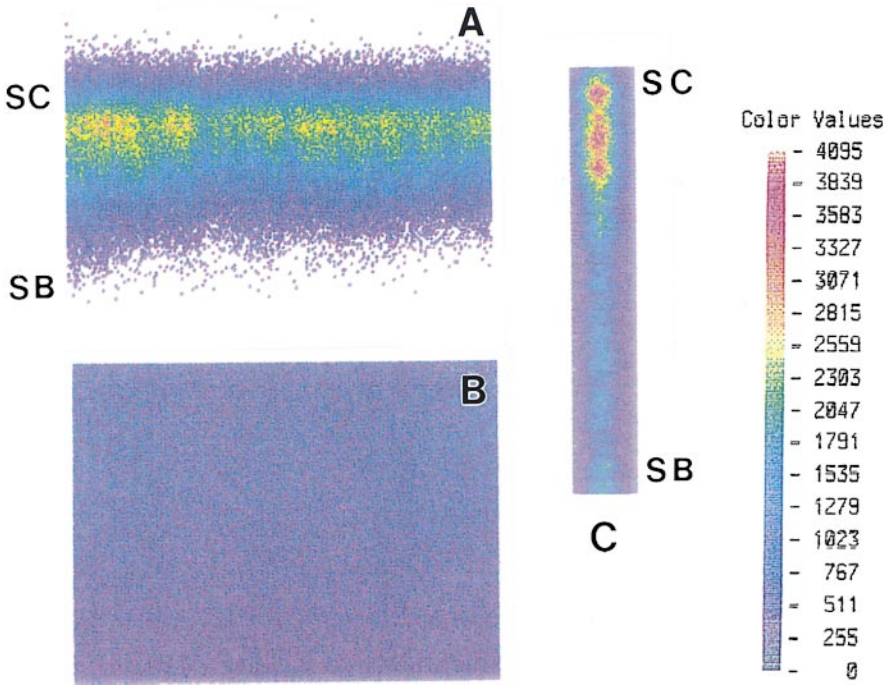

Fig. 3. Localization of epidermal GlcCer'ase by in situ fluorescence zymology of unfixed epidermal samples. Epidermal skin equivalents were used to demonstrate in situ GlcCer'ase activity without prior fixation or sectioning. Planar cross-section (i.e., digital) (panel A) and a cumulative section (panel C) display elevated fluorescent signal in the outer vs. the lower epidermal layers. Co-incubation with CBE again eliminates the generation of fluorescent signal (panel B). These results demonstrate that incubation with the 4-MUG fluorophore yields fluorescent signal (4-MU) in intact epidermal samples, indicating that GlcCer'ase activity is present in vivo. Assigned pseudocolor values for fluorescent intensity are shown and are equivalent for both Figs. 2 and 3.

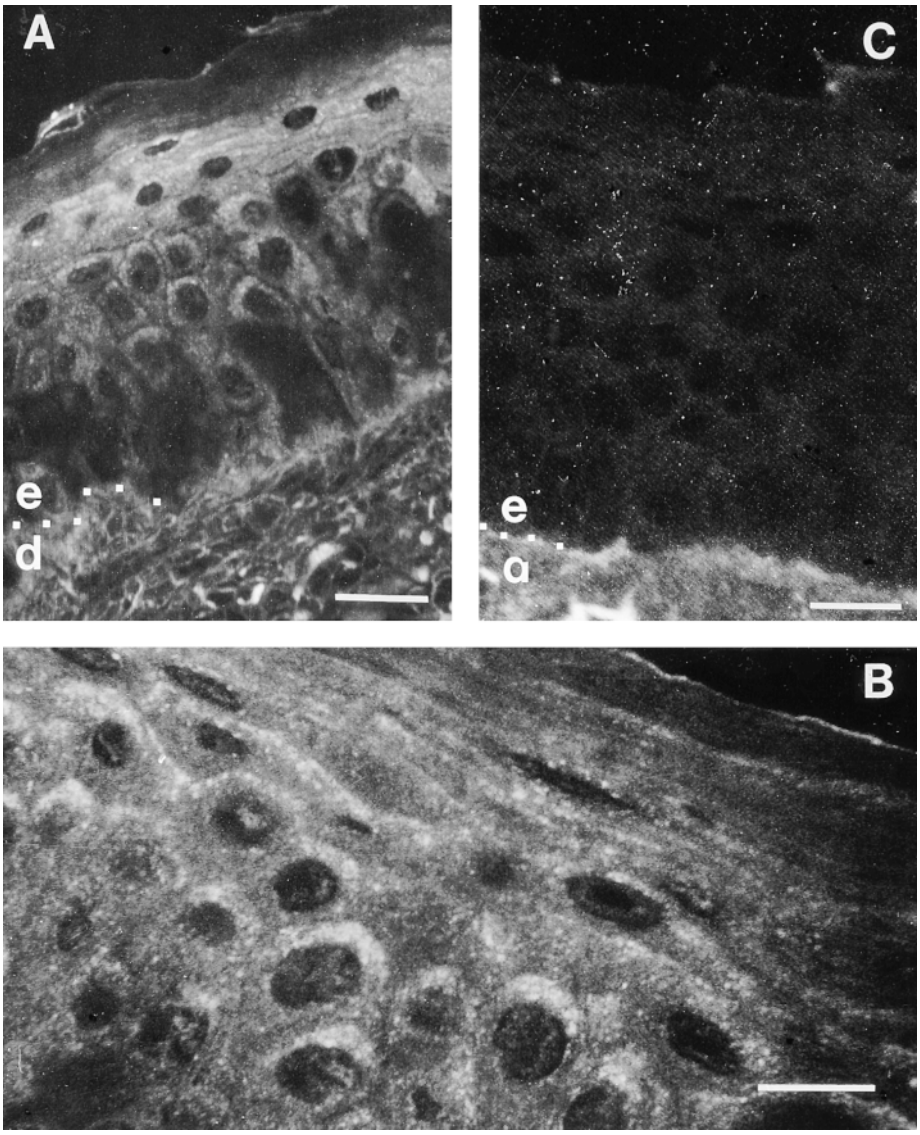

Fig. 4. GlcCer'ase protein is localized to upper epidermis and SC by immunofluorescence. Confocal images demonstrate increased fluorescent signal due to GlcCer'ase immunofluorescence in outer layers of human epidermis (panel A; Mag. ×600). Signal is concentrated in the apical margins of mid-to-upper epidermal cells (panel B; Mag. ×1500). Lack of primary antibody eliminated epidermal signal (panel C; Mag. ×600). Demarcation between dermis (d) and epidermis (e) is indicated as a dotted line; size bars = 20 μm (panels A and C); 10 μm (panel B).

tion, and that significant GlcCer'ase activity is retained in the SC.

Localization of GlcCer'ase within murine stratum corneum

We next determined the specific localization of GlcCer'ase *within* the SC by measuring enzyme activity in successive layers of murine SC. Prior studies have shown that each tape stripping removes between 1 and 3 layers of SC. Using consecutive tape strippings, preparations from the surface-to-deeper layers within the SC were obtained. As tape stripping does not inhibit GlcCer'ase enzyme activity (data not shown), we used measured surface areas of the tape containing layers of the SC as the enzyme source. Tape/SC samples were placed in assay buffer, and the in vitro enzyme assays were performed as above; enzyme activities were sufficiently high to allow measurements on individual strips. GlcCer'ase enzyme activity increased with each incremental tape strip, peaking 6–8 layers (i.e., 3 strippings) from the skin surface (**Fig. 5**). These findings demonstrate that in vitro levels of GlcCer'ase are highest in the lower SC, and decline toward the surface of the epidermis/SC.

Localization of GlcCer'ase in corneocytes

Finally, to examine the localization of GlcCer'ase in the intercellular membrane versus corneocyte domains of the SC, whole SC sheets (prepared with trypsin) were homogenized with a cell disrupter, further trypsinized and centrifuged to prepare membrane couplets (i.e., fragments of

TABLE 2. GlcCer'ase activity in germ-free murine stratum corneum

	Activity[a]		
	Epidermis[c]	Stratum Corneum[d]	% vs. Whole[b] Epidermis
	nmol/min/mm²		
Normal (hr−/hr−)	1.07 ± 0.19	0.066 ± 0.006	6.2
Ethanol-wiped[e] (hr−/hr−)	1.13 ± 0.07	0.061 ± 0.002	5.4
Aseptic (nude; th−/th−)	1.99 ± 0.31	0.109 ± 0.010	5.5

[a] Specific activities were determined using homogenates normalized for mm² dermis, and reported as mean ± SEM; n ⩾ 5 for each value.
[b] Ratio as stratum corneum/epidermis ×100.
[c] Separated by heat treatment.
[d] Separated with cyanoacrylate glue stripping.
[e] Skin was wiped with 70% ethanol before tissue preparation.

opposing cornified envelopes of two corneocytes with sandwiched intercellular domains) as previously described (32). As noted above, GlcCer'ase is stable to trypsin treatment and incubation of SC homogenates with 0.005% trypsin for 1 h at room temperature does not decrease enzyme activity (not shown). The resultant membrane couplets display an almost 2-fold increase in GlcCer'ase specific activity versus SC homogenates (i.e., 1.73 ± 0.005 v. 0.89 ± 0.001 for membrane couplets and whole SC homogenates, respectively; $P < 0.005$; n = 4). These results suggest that a significant amount of the SC GlcCer'ase activity is localized within membrane domains between adjacent corneocytes, consistent with a role for this enzyme in *intercellar* lipid processing.

DISCUSSION

During the transition from the basal proliferating epidermal layer to the outer cornified layer, the phospholipid–neutral lipid mixture is replaced by ceramides, cholesterol, and free fatty acids (1–4), and the total amount of lipids increases. In the SC, these lipids are localized to the intercellular spaces, where they form lamellar membrane structures, which are essential for epidermal permeability barrier function. Whereas glucosylceramide levels are highest in the SC, ceramide concentrations rapidly increase at the level of the inner SC. One pathway to produce ceramides is by hydrolysis of glucosylceramides by GlcCer'ase. We previously reported that GlcCer'ase, rather than nonspecific β-glucosidase activity, is present in whole epidermis, and that in vitro GlcCer'ase activity is highest in isolates from the outer epidermis (21). As inhibition of GlcCer'ase activity diminishes permeability barrier formation (22–24), the hydrolysis of glucosylceramide to ceramide represents a key step in the processing of ceramides for barrier function. In addition, since both glucosylceramide (9, 10) and β-glucosidase activity (11) appear to be co-localized within lamellar bodies, it seems likely that hydrolysis occurs in intercellular domains of the SC, after secretion of lamellar body contents. Hence, in this study, we ascertained whether the localization of GlcCer'ase activity

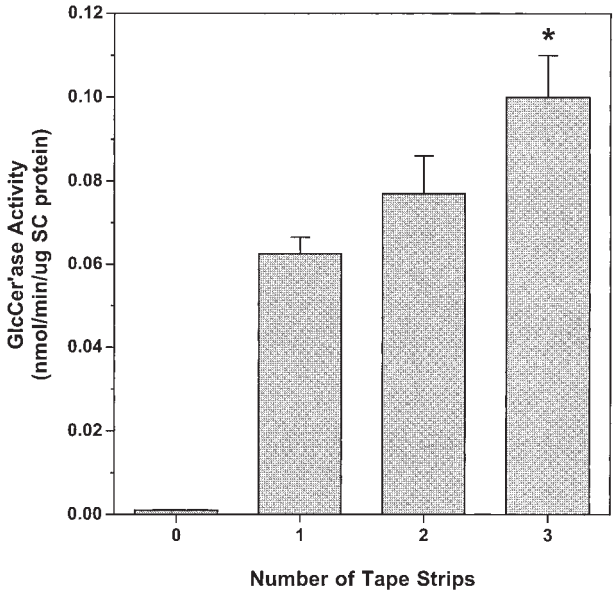

Fig. 5. Localization of GlcCer'ase within the stratum corneum. Stratum corneum layers were prepared by the sequential tape stripping. The activity of GlcCer'ase in each layer of the stratum corneum was measured directly on the tape. Activities were calculated per protein, and data are reported as nmol/min/μg SC protein (±SEM; n ⩾ 6 for each). Control (i.e., 0 strips) represents background fluorescence generated in the in vitro assay by the tape alone; (*$P \leqslant 0.05$ vs. strip #1; and $P < 0.001$ for each tape-stripped sample vs. control).

TABLE 3. Localization of GlcCer'ase activity within different layers of murine epidermis

	Specific Activity[a,b]	Total Activity[c]	% vs. Whole Epidermis[d]
	nmol/min/mg protein	*nmol/min/mg derm*	
Epidermis	2.60 ± 0.07	10.6 ± 3.51	100
Stratum basal/spinosum[e]	1.43 ± 0.05	5.19 ± 1.05	55
Stratum granulosum/corneum[e]	3.17 ± 0.21	4.86 ± 1.81	46
Stratum corneum[f]	4.09 ± 0.02[g]	2.89 ± 1.00	27

[a] Specific activity determined using tissue homogenates; results presented as mean ± SEM.
[b] Previously reported in Holleran et al. (22).
[c] Total activity (i.e., specific activity × total homogenate protein) per dermal weight; n ⩾ 9 determinations.
[d] Percent of total activity.
[e] Separated with staphylococcal epidermolytic toxin (see Methods).
[f] Isolated by trypsinization (see Methods).
[g] $P \leqslant 0.005$ vs. whole epidermis.

in the SC is consistent with its purported key role in barrier formation.

Using primarily in vitro techniques, a number of lipid catabolic enzyme activities, including acid sphingomyelinase, phospholipase A_2, acidic and neutral triacylglycerol hydrolase, and steroid sulfatase have been found either in lamellar bodies and/or the SC interstices (5, 6, 8, 9, 35) Thus, these enzymes are present at the sites where critical membrane transformations occur. Lamellar bodies are not enriched in ceramides and free fatty acids but rather in glycosphingolipids and phospholipids (9, 35), and their contents are secreted between the SC and SG by fusion with the outermost SG cell. Enzymes that are co-packaged with their lipid substrates in the lamellar bodies are thought to be delivered to membrane domains of the SC, although this process has yet to be well delineated. Despite the localization data of these catabolic enzymes, including the recent description of in vitro β-glucosidase activity in lamellar body-enriched preparation (11), their activity in intact epidermis and their functional relationship to epidermal barrier homeostasis also is incompletely understood. In the present study, the pattern of immunofluorescence in the epidermis is consistent with a lamellar body localization for GlcCer'ase. Furthermore, the in situ activity studies, both with epidermal sections and intact skin samples, further suggest that GlcCer'ase is present in lamellar bodies, and with their fusion to the plasma membrane at the apical aspect of the outermost SG cells, the enzyme is delivered, along with its lipid substrate, GlcCer, to the lower SC intercellular domains. Moreover, the observation that a high percentage of the SC GlcCer'ase activity is present in membrane couplets (i.e., in the lipid-rich membrane domains between adjacent corneocytes) is consistent with GlcCer'ase being a membrane-bound enzyme in extracutaneous systems. However, in the unique case of the epidermis, enzyme activity occurs in an intercellular milieu, with the subsequent conversion of glucosylceramides to their corresponding ceramides being required for lamellar membrane organization and subsequent permeability barrier homeostasis (21, 22, 24).

In the present study, we also used a number of criteria to further distinguish SC GlcCer'ase from nonspecific β-glucosidase. First, optimal enzyme activity in SC preparations was observed at acidic pH (i.e., pH 5.2–5.6), consistent with GlcCer'ase (i.e., pH 5.0–6.0) (22). Second, sodium taurocholate stimulates GlcCer'ase, but is inhibitory to β-glucosidase activity (14). In this study, 4-MUG hydrolysis was activated >10-fold by 5 mm sodium taurodeoxycholate at pH 5.2, further suggesting the involvement of GlcCer'ase. Third, BrCBE, and other related conduritol epoxides, specifically inhibit GlcCer'ase, without affecting nonspecific β-glucosidase (21, 27). Enzymatic activity in the SC was inhibited >99% in the presence of BrCBE. Furthermore, as GlcCer'ase activity was comparable in both germ-free and normal hairless mouse SC, the reported activity is of epidermal rather than bacterial origin. These results, in conjunction both with prior studies (21, 22, 24) and with the in situ and immunolocalization studies presented above, demonstrate that the GlcCer'ase is the enzyme responsible for the hydrolysis of GlcCer to Cer in mammalian SC, rather than a nonspecific β-glucosidase.

In summary, the results of this study demonstrate that the conversion of glucosylceramide to ceramide within the outer epidermis can be attributed to GlcCer'ase activity, localized primarily, albeit indirectly, to the intercellular spaces of the inner stratum corneum. Although GlcCer'ase activity is also present throughout the epidermis and concentrated in the outer SG, the activity in the SC is most likely responsible for the conversion of GlcCer-to-Cer within the SC interstices. This is the first report showing such localization of this enzyme which is critical to the formation of the lamellar membrane structures which is in turn essential for epidermal barrier function. The recent finding that prosaposin-deficient mice, which lack the activator protein for GlcCer'ase (i.e., SAP C), both accumulate GlcCer species in the SC and have abnormal SC lamellar membrane structures (36), further substantiates a key role for this enzyme in epidermal homeostasis.

This work was supported by National Institutes of Health Grants AR 39448 and 19098. Kao Corporation (Japan) supported Y. Takagi as a visiting research fellow. The authors thank Ms. Sue Allen for her capable assistance with manuscript preparation.

Manuscript received 23 October 1998 and in revised form 14 January 1999.

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