β-Glucocerebrosidase activity in murine epidermis: characterization and localization in relation to differentiation

Walter M. Holleran,[1] Yutaka Takagi, Genji Imokawa, Simon Jackson, Jane M. Lee, and Peter M. Elias

Department of Dermatology, University of California School of Medicine, and Dermatology Service, Veterans Administration Medical Center, San Francisco, CA, and Biological Science Laboratories, Kao Corporation, Tochigi, Japan

Abstract The intercellular lipids of the stratum corneum, which are highly enriched in ceramides, are critical for the mammalian epidermal permeability barrier. During the terminal stages of epidermal differentiation, the glucosylceramide content is dramatically reduced, while the content of free ceramides increases. To investigate whether β-glucocerebrosidase (β-GlcCer'ase) could be responsible for this change in lipid content, we characterized its activity in murine epidermis, compared enzyme activity to other murine tissues, and localized β-GlcCer'ase activity within the epidermis. Epidermal extracts demonstrated linear 4-methylumbelliferyl-β-D-glucose hydrolysis (to 3 h) with protein concentrations between 1 and 250 μg/ml. Whole epidermis contained comparable β-glucosidase activity (9.1 ± 0.4 nmol/min per mg DNA) to murine brain and liver, and 5-fold higher activity than spleen. Epidermal β-glucosidase activity was stimulated >15-fold by sodium taurocholate at pH 5.6, and inhibited at acidic pH (3.5–4.0). Bromoconduritol B epoxide (≥1.0 μM), inhibited epidermal enzyme activity by >75%, while activity in brain, liver, and spleen was only inhibited by 6, 17, and 14%, respectively. Moreover, β-GlcCer'ase mRNA expression in murine epidermis exceeded levels in liver, brain, and spleen. Finally, β-GlcCer'ase activity was highest in the outer, more differentiated epidermal cell layers including the stratum corneum. In summary, mammalian epidermis contains an usually high percentage (~75%) of β-glucocerebrosidase activity, and the concentration of activity in the more differentiated cell layers may account for the replacement of glucosylceramide by ceramides in the outer epidermis. —**Holleran, W. M., Y. Takagi, G. Imokawa, S. Jackson, J. M. Lee, and P. Elias.** β-Glucocerebrosidase activity in murine epidermis: characterization and localization in relation to differentiation. *J. Lipid Res.* 1992. **33:** 1201–1209.

Supplementary key words β-glucosidase • glucosylceramide • ceramide • bromoconduritol B epoxide • permeability barrier

Lipids are concentrated in the interstitial domains of the stratum corneum of terrestrial mammals, where they mediate the barrier to water loss (1). During the transition from the basal proliferating epidermal layers to the outer, anucleate stratum corneum, the usual phospholipid–neutral lipid mixture is replaced by a relatively nonpolar mixture of ceramides, cholesterol, and free fatty acids (2–5). Glucosylceramide levels, which peak in the stratum granulosum, decline rapidly in the lower stratum corneum coincident with an increase in ceramide levels, which ultimately account for 30–40% of stratum corneum lipid content by weight. Since stratum corneum ceramides display sphingoid base and N-acyl compositional profiles similar to those of glucosylceramides present in lower layers (6, 7), hydrolysis of the glucose moiety could account for these changes in lipid content.

Although at least two enzymes can catalyze the hydrolysis of glucose from 4-methylumbelliferyl-β-D-glucopyranoside (4MUG), only one, β-glucocerebrosidase (β-GlcCer'ase; EC 3.2.1.45), is capable of specifically hydrolyzing glucosylceramides. Much is known about human β-GlcCer'ase due to its deficiency in Gaucher's disease (8, 9), a condition associated with tissue accumulations of glucocerebrosides (reviewed in references 10 and 11). A second, relatively nonspecific β-glucosidase has been distinguished from β-GlcCer'ase by a number of criteria. First, sodium taurocholate stimulates the in vitro activity of β-glucocerebrosidase, while simultaneously inhibiting nonspecific β-glucosidase activity (12). Second, conduritol B epoxide (CBE), a 1,2-anhydro-myo-inositol derivative,

Abbreviations: PBS, phosphate-buffered saline; SB, stratum basale; SS, stratum spinosum; SG, stratum granulosum; SC, stratum corneum; DTT, dithiothreitol; ME, mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; BrCBE, bromoconduritol B epoxide (1,2-anhydro-6-bromo-6-deoxy-myo-inositol); Cer, ceramide; GlcCer, glucosylceramide; 4MU, 4-methylumbelliferone; 4MUG, 4-methylumbelliferyl-β-D-glucoside; β-GlcCer'ase, β-D-glucocerebrosidase; PMSF, phenylmethylsulfonylfluoride; RNA-H_2O, RNase-free water.

[1]To whom correspondence should be addressed at: Dermatology Service (190), Veterans Administration Medical Center, 4150 Clement Street, San Francisco, CA 94121.

specifically inhibits β-GlcCer'ase, without affecting nonspecific β-glucosidase activity (13, 14). Third, the pH optimum for β-GlcCer'ase ranges from 5.0 to 6.0 (12, 13), while nonspecific β-glucosidase reportedly displays a lower (12, 15), similar (13, 14), or neutral (16, 17) pH optimum. Taken together, these three criteria allow delineation of the relative amounts of β-GlcCer'ase and nonspecific β-glucosidase activity in specific tissues.

Although a number of reports have described β-glucosidase activity in whole skin as well as in isolated epidermis (18–25), these studies did not distinguish between nonspecific β-glucosidase and β-GlcCer'ase activity. Hence, neither the absolute quantities of β-GlcCer'ase nor the proportion of β-glucosidase activity present as β-GlcCer'ase in the epidermis, the major site of glycosphingolipid metabolism in the skin (vide supra), are known. In addition, neither the localization of β-GlcCer'ase activity within the epidermis nor its relationship to keratinocyte differentiation have been addressed previously. We report high β-glucosidase activity, with an unusually large proportion of β-GlcCer'ase, and correspondingly high levels of β-GlcCer'ase mRNA expression in murine epidermis. Moreover, a significant gradient of increasing β-GlcCer'ase activity appears in relation to differentiation and formation of the cornified cell layers of the epidermis. These results support a link between epidermal β-GlcCer'ase activity and the changes in sphingolipid distribution that occur during epidermal differentiation and development of the mammalian permeability barrier.

MATERIALS AND METHODS

Materials

4-Methylumbelliferone (4MU), 4MU-β-D-glucoside (4MUG), and sodium taurocholate were from Sigma Chemical Co. (St. Louis, MO). Bromoconduritol B epoxide (BrCBE) was kindly provided by Dr. Gunter Legler (Köln, Germany), and synthesized as described previously (26). Staphylococcal exfoliative toxin was from Toxin Technology (Sarasota, FL), and Bio-Rad Protein Assay Kit and bovine serum albumin (BSA) were from Bio-Rad (Richmond, CA). The multiprime DNA Labeling System was from Amersham International (Amersham, UK), while (α-^{32}P)dCTP (3000 Ci/mmol, 10 mCi/ml) was from NEN Research Products (Boston, MA). Minispin columns (G-50) were from Worthington Biochemical Corporation (Freehold, NJ). Molecular grade chemicals were from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific (Fairlawn, NJ). Oligo(dT)-cellulose, type 77F, was from Pharmacia LKB Biotechnology (Uppsala, Sweden), while nitrocellulose for transfers was from Schleicher and Schuell (Keene, NH). Murine β-GlcCer'ase cDNA was kindly provided by Dr. Raymond O'Neill at the NIH (27).

Preparation of epidermal samples

Hairless male mice (Hr/Hr) were purchased from Simonsen Laboratories (Gilroy, CA) and fed Purina mouse diet and water ad libitum; all animals were 8 to 12 weeks of age at the time of study. To prepare epidermal samples, animals were anesthetized and killed, skin samples were excised, and subcutaneous tissue was removed by scraping with a scalpel blade, as described previously (28). Epidermis was then separated from dermis by exposure of full thickness skin samples to dry heat (60°C for 60 sec) followed by mild scraping with a scalpel blade. No loss of β-glucosidase activity was evident due to heat exposure, as similar activities were observed after EDTA separation (10 mM, 60 min, 37°C). Moreover, human β-GlcCer'ase has been shown to be relatively heat-stabile (29). During all subsequent steps, tissue samples were maintained at 4°C, unless otherwise noted. Epidermal sheets were minced into small pieces (<1 mm^3), 10 volumes of phosphate-buffered saline (PBS) with 0.1 mM PMSF were added, and samples were homogenized using a Polytron PCU2 tissue homogenizer (15 sec × 3), followed by sonication (35%, 10 sec × 2) using a Fisher Sonic Probe Disembranator, Model 300 (Artec Corp, Farmingdale, NY).

For individual epidermal cell layer preparations, staphylococcal exfoliative toxin separation was used as described previously (3, 4). Groups of adult hairless mice, three to eleven each, were injected intradermally with 50 μg staphylococcal exfoliative toxin, dissolved in 100 μl PBS. After 2 h the mice were killed by cervical dislocation. The upper epidermis, comprising stratum corneum (SC) and stratum granulosum (SG) with some residual stratum spinosum (SS), was removed in one piece by gentle scraping with a surgical blade. The subjacent piece of skin, comprising lower epidermis, i.e., SB with some SS plus the dermis, was excised and immediately placed epidermal-side downward onto a covered Petri dish containing crushed ice. The undersurface of the skin pieces was scraped with a sharp scalpel blade to remove excess subcutaneous fat, and the pieces were immersed in 10 mM EDTA in Dulbecco's PBS, calcium (Ca^{2+})- and magnesium (Mg^{2+})-free (pH 7.4) at 37°C. After incubation for 30 min, the lower epidermis was peeled off the dermis by gentle scraping with a scalpel blade and dried on a paper towel. Upper and lower epidermal sheets then were weighed, minced into small pieces (<1 mm^3) with a blade, and stored in small plastic tubes overnight at −70°C. A small portion of each sheet from representative samples was processed for light microscopy to verify the site of the cleavage plane. Enzyme activity in whole epidermal samples was roughly equivalent to the sum of activities in the individual subfractions obtained with toxin treatment, indicating minimal-to-absent toxicity due to this separation technique.

β-Glucocerebrosidase has been shown to be firmly bound to lysosomal membranes, requiring nonionic detergents and bile salts for extraction of soluble enzyme (30). Therefore, in order to enhance the extraction of soluble enzyme, homogenates were incubated for 30 min (4°C) with or without detergent (Triton X-100 or Tween 20). Samples then were spun at 10,000 *g* (4°C) for 10 min, and, unless noted otherwise, the 10,000 *g* supernatant (epidermal extract) was used as the enzyme source in these studies. As the addition of PMSF (0.1 mM) and Tween 20 (0.05%) to the homogenization buffer improved the recovery of enzyme activity by 21% and 73%, respectively ($P < 0.001$), both were used in all subsequent enzyme preparations. In contrast, Triton X-100, DTT, 2-mercaptoethanol, and taurocholate did not enhance the recovery of enzyme activity (data not shown).

Extracutaneous tissue samples

Brain, liver, and spleen tissue samples were obtained from at least three separate animals immediately after skin excision. For enzymatic assays, samples were snap-frozen in liquid nitrogen and stored at −70°C overnight. Samples were resuspended in three volumes (by weight) of homogenization buffer and sonicated, and triplicate assays were performed, as described below.

β-Glucosidase assay

The assay method for β-glucosidase was modified from Mier and van den Hurk (31). Unless specified otherwise, all assays were carried out at 37°C in citrate–phosphate buffer (pH 5.6) with 5 mM (0.54%) sodium taurocholate, 0.5 mM 4MU-β-D-glucoside (4MUG) as substrate and a 60-min reaction time. Enzyme solutions in assay buffer (50 μl) were preheated in borosilicate glass culture tubes, and reactions were initiated by the addition of 50 μl substrate solution in assay buffer. The reaction was terminated by adding 1.25 ml of 200 mM carbonate–bicarbonate buffer (pH 10.5). The fluorescence (ex = 360 nm, em = 450 nm) was measured using a Perkin-Elmer spectrofluorimeter. A standard solution of 4-methylumbelliferone (4MU) (0–300 nM) in buffer was used for calibration of each assay. Protein concentration was measured using the Bradford procedure with bovine serum albumin as standard (32).

Enzymatic characterization was performed using epidermal tissue extracts (10,000 *g* supernatant) as follows. The protein concentration dependence was determined by diluting the extracts from 0 to 100 μg of protein per assay. The effect of pH on activity was tested with pH values from 2.8 to 6.4 using the McIlvaine citrate–phosphate buffer system (0.1 M citric acid, 0.2 M dibasic sodium phosphate) (33). Extracts were then diluted in water, and the substrate was dissolved in a twofold concentration of buffer, followed by initiation of reactions, as described above. The apparent K_m and V_{max} were determined by plotting the reciprocal of substrate concentration versus the reciprocal of enzyme activity (Lineweaver and Burk plots).

Comparisons of enzyme activity in epidermis, brain, liver, and spleen were performed using crude tissue homogenates. The total activities were also normalized with the DNA content for each tissue source, to estimate the relative activity per cell. DNA content was determined using the method of LaBarca and Paigen (34).

Tests for β-glucocerebrosidase versus nonspecific glucosidase activity

Effect of sodium taurocholate. In order to delineate between β-glucocerebrosidase (β-GlcCer'ase) and nonspecific β-glucosidase, the effect of sodium taurocholate on the hydrolysis of 4MU-β-D-glucoside was determined (12). First, the concentration-dependence of the taurocholate effect on activity was determined over the range of 0 to 500 mM included with the assay buffer. Second, the pH-dependence of the sodium taurocholate effect on enzyme activity was determined (12, 15–17) using citrate–phosphate buffer (pH 3.2 to 7.0), with added 5 mM (0.54%) sodium taurocholate.

In vitro inhibition. As a further test for the presence of β-glucocerebrosidase, assays were performed as described above with the inclusion of the specific inhibitor bromoconduritol B epoxide (BrCBE), ranging in concentration from 3×10^{-8} to 1×10^{-2} M. Assays were performed at pH 5.6 unless otherwise indicated. Inhibitors were added from stock solutions prepared fresh immediately prior to use. Estimates for K_i were obtained using Sigmaplot (Jandel Scientific, San Rafael, CA).

Isolation and analysis of epidermal mRNA

Total RNA was isolated by a variation of the guanidinium thiocyanate method (35). Briefly, epidermis (0.2–0.4 g from whole area of two mice) was homogenized in 4 ml solution A (4 M guanidinium thiocyanate, 24 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) using a ground-glass, hand-held homogenizer and then sonicated twice for 15 sec with a 30-sec intervening pause on ice. After the addition of acid phenol and chloroform–isoamyl alcohol 24:1, RNA was precipitated from the aqueous phase with isopropanol and subsequently washed and reprecipitated three times. The final pellet was dissolved in RNase-free water (RNA-H_2O) and heated at 65°C for 5 min; oligo(dT)-cellulose (10 mg) was then added and incubation was continued at room temperature for 30 min. The oligo(dT)-cellulose was washed four times in 5 ml high-salt buffer (0.5 M NaCl, 20 mM Tris, 1 mM EDTA, 0.2% SDS, pH 7.6) and transferred to microfuge tubes with 1 ml of low-salt buffer (0.1 M NaCl, 20 mM Tris, 1 mM EDTA, 0.1% SDS, pH 7.6). Poly A(+) RNA was eluted from the oligo(dT)-cellulose with three successive washes in RNA-H_2O warmed to 65°C. The

mRNA (in 0.6 ml RNA-H_2O) was precipitated from solution by adding 3 M sodium acetate (60 μl) and 1.2 ml 100% ethanol and incubating at −80°C for 1 h. mRNA was resuspended in 50 μl of RNA-H_2O and the absorbance was determined at 260 and 280 nm. Yields of 10–30 μg of epidermal poly A(+) RNA were obtained from the entire epidermis of two mice.

Brain, liver, and spleen tissue samples for polyA(+) mRNA preparations were obtained simultaneously with epidermal samples. Tissue samples were snap-frozen and stored at −70°C, and polyA(+) mRNA was isolated as described above. Similar to the epidermis, tissue samples from two separate animals were combined prior to homogenization, and mRNA was isolated in triplicate (n = 6 animals).

Northern analysis. Aliquots of poly A(+) RNA (4–8 μg) were solubilized in 20 μl sample buffer (48% formamide, 2.1 M formaldehyde, 1.1 × MOPS buffer, 5.3% glycerol, and 0.027% bromophenol blue) and run on agarose/formaldehyde (1%/6.1%) gels for 3 h at 75 V. Ribosomal RNA bands were stained with acridine orange and photographed. RNA was transferred to nitrocellulose (0.45 μm pore size), subsequently fixed by baking at 80°C for 2 h and stored at −20°C until required for hybridization. The poly A(+) mRNA extracts from brain, liver, spleen, and epidermis were probed with murine cDNA probe for β-glucocerebrosidase. [^{32}P]dCTP-radiolabeled cDNA probes were prepared by random priming (Amersham Multiprime DNA Labelling System) with incubations at room temperature for 3–4 h. Blots were prehybridized at 42°C for 30 min in buffer F (50% formamide, 5 × SSPE, 1% SDS, 10% dextran sulfate, 100 μg/ml sheared salmon sperm DNA). Hybridization with radiolabeled probes was performed in buffer F overnight at 42°C, followed by a 30-min room temperature wash and a 60-min 65°C wash in wash buffer (0.2 × SSC, 0.1% SDS, pH 7.0). Air-dried blots were exposed to X-ray film, the film was developed, and bands were quantified by scanning densitometry.

RESULTS

Enzyme characteristics

Murine epidermal β-glucosidase activity was assessed using the supernatants of the 10,000 *g* pellets as the enzyme source ("epidermal extract"). Hydrolysis of 4MU-β-D-glucoside (4MUG) was proportional to incubation duration (0–3 h; r = 0.999) and protein concentration (0–500 μg/ml; r = 0.999) (data not shown). These results validate the 1-h assay time and protein concentrations used throughout the remainder of this study. Moreover, the constant rate of product formation was observed in the presence or absence of sodium taurocholate (not shown).

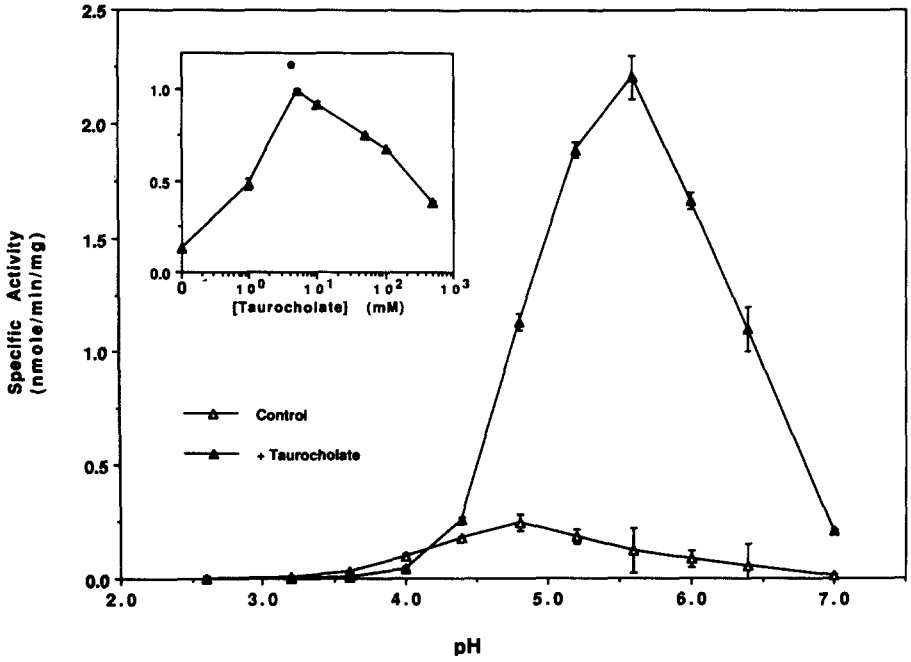

Fig. 1. The effect of taurocholate on epidermal β-glucosidase activity at various pH values. Plot of mean specific activity as pH of assay system was varied from 2.6 to 7.2 (±SD, n = 4). Epidermal extracts (10,000 *g* supernatant) were used as the protein source. In non-taurocholate controls (open triangles), the pH optimum was approximately 4.8, while addition of taurocholate (5 mM) shifted the pH optimum to pH 5.6. Stimulation of β-glucosidase activity at pH 5.6 was approximately 15-fold. Inset: Effect of sodium taurocholate on β-glucosidase activity. Plot of specific activity as taurocholate concentration was varied from 0 to 0.5 M (n = 3; ±SD). Peak activity was observed at 5 mM (0.54%) sodium taurocholate. Assay was performed at pH 5.6.

Furthermore, the activity of β-glucosidase was not influenced by added Ca^{2+}, Mn^{2+}, or Mg^{2+} (not shown). Finally, kinetic analysis was performed using epidermal extracts. Increasing concentrations of 4MUG substrate resulted in an apparent K_m of 0.93 ± 0.28 mM, with a V_{max} of 3.15 ± 0.51 nmol/mg per min (mean ± SD from four separate experiments).

β-Glucocerebrosidase (β-GlcCer'ase) versus nonspecific β-glucosidase

Three criteria have been utilized to distinguish nonspecific β-glucosidase from β-GlcCer'ase activity. The first involves the pH optimum for enzyme activity. Epidermal extracts displayed maximal activity at an acidic pH (4.8–5.8), with an optimum of pH 5.6 in the presence of taurocholate (**Fig. 1**), a feature characteristic of β-GlcCer'ase. Second, as reported previously in other tissues (12), we found a concentration-dependent activation of 4MUG hydrolysis in epidermal homogenates by added taurocholate at pH 5.6, with optimal in vitro activity at 5 mM (0.54%) (Fig. 1, inset). This activation by taurocholate was also pH-dependent (Fig. 1), as 4MUG hydrolysis increased approximately 15-fold over non-taurocholate controls at pH 5.6 (**Table 1**). In contrast, at the lower pHs characteristically associated with nonspecific β-glucosidase activity (12), taurocholate inhibited enzyme activity by 65% (Table 1).

The final criterion used to distinguish between the two enzymatic activities was the specific inhibition of β-GlcCer'ase with conduritol derivatives (13, 14). Epidermal β-glucosidase activity was uniquely sensitive to the bromo-derivative of conduritol B epoxide (BrCBE). In the absence of sodium taurocholate, the β-glucosidase activity in whole epidermal homogenate was 75% inhibited at BrCBE concentrations greater than 10^{-6} M (**Fig. 2**). In the presence of taurocholate, epidermal β-glucosidase activity was completely inhibited (>98%) by 10^{-5} M BrCBE (data not shown). Moreover, inhibition of activity in both supernatant and pellet fractions (following a 10,000 *g* centrifugation) resulted in IC_{50} values of approximately 1×10^{-7} M (data not shown). These data demonstrate that a majority (~75%) of epidermal enzymatic activity represents β-GlcCer'ase.

TABLE 1. pH dependence of sodium taurocholate effect on epidermal β-glucosidase

pH	Activity[a] Minus Taurocholate	Plus Taurocholate[b]	Change
	nmol/min/mg protein		%
5.6	0.166 ± 0.030	2.519 ± 0.225	+1515[c]
4.0	0.074 ± 0.007	0.025 ± 0.004	−65[d]

[a]Epidermal extracts (10,000 *g* supernatant) were used as enzyme source; values are mean ± SD (n ≥ 4).
[b]5 mM
[c,d]$P \leq 0.001$ versus control at same pH.

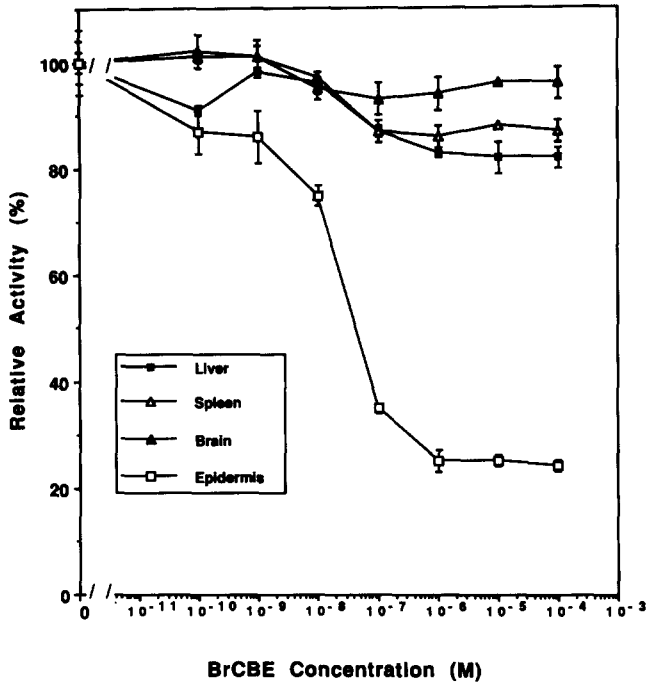

Fig. 2. BrCBE inhibition of β-glucosidase from various murine tissues. Plot of relative specific activity as BrCBE concentration increased from 0 to 1×10^{-4} M, with activity in absence of BrCBE (0.0 M) set to 100%. Each point represents triplicate determinations using tissue homogenates in absence of sodium taurocholate (±SD). At 1×10^{-6} to 1×10^{-4} M, epidermal β-glucosidase activity was 75% inhibited, while brain, liver, and spleen showed 6, 17, and 14% inhibition, respectively.

Activity in murine epidermis versus other tissues

We next compared the distribution of β-glucosidase activity in extracutaneous murine tissues to levels present in epidermis. Comparison of enzyme activity on a per mg DNA basis using cell homogenates showed nearly equivalent activity in epidermal, brain, and liver, with significantly lower activity in spleen (**Table 2**). As was observed with the epidermal extracts (Table 1), taurocholate again stimulated β-glucosidase activity in epidermal homogenates (Table 2). In contrast, enzyme activities in brain, liver, and spleen homogenates appeared to be variably diminished by taurocholate (Table 2). These results are consistent with epidermis containing a greater proportion of β-GlcCer'ase activity than the levels present in brain, liver, or spleen.

In order to estimate the relative amount of β-GlcCer'ase present in each tissue more precisely, we next compared the sensitivity of β-glucosidase activity in epidermal, brain, liver, and spleen homogenates to BrCBE. Previous studies have shown that approximately 18% of liver β-glucosidase activity is conduritol-sensitive, representing β-GlcCer'ase activity (36). As described

TABLE 2. β-Glucosidase activity in various murine tissue homogenates

Tissue	Activity/DNA[a]	Taurocholate[b]	Specific Activity[c]	% Change[d]
Epidermis	9.1 ± 0.4 (6)	−	0.26 ± 0.01	↑351[e]
		+	1.15 ± 0.04	
Brain	10.8 ± 1.7 (4)	−	0.47 ± 0.04	↓77[f]
		+	0.11 ± 0.01	
Liver	9.2 ± 3.2 (8)	−	0.21 ± 0.00	↓24[f]
		+	0.16 ± 0.01	
Spleen	1.8 ± 0.4 (4)	−	0.15 ± 0.00	↓41[f]
		+	0.09 ± 0.00	

[a]nmol/min/mg DNA; number of animals sampled in parentheses; each assayed in duplicate.
[b]5 mM sodium taurocholate.
[c]nmol/min/mg homogenate protein ± SD; n = 3; assayed at pH 4.5.
[d]Differences in activity (+ vs. −)/no taurocholate × 100.
[e]$P \leq 0.001$ (+ vs. − taurocholate).
[f]$P \leq 0.01$ (+ vs. − taurocholate for each tissue).

above, epidermal β-glucosidase activity was highly sensitive to BrCBE, with 75% of homogenate activity eliminated at 10^{-6} M (Fig. 2). In contrast, the other murine tissues displayed significantly less sensitivity to BrCBE. At a similar BrCBE concentration ($\geq 10^{-6}$ M, β-glucosidase activity of brain, liver, and spleen were inhibited 6%, 17%, and 14%, respectively (Fig. 2). These results strongly suggest that murine epidermis contains a uniquely high proportion of β-GlcCer'ase activity when compared to brain, liver, or spleen tissues.

Finally, Northern analyses of poly A(+) mRNA were performed to confirm β-GlcCer'ase expression in the epidermis and to again allow comparisons with other murine tissues (**Fig. 3**). Binding was observed in the appropriate range for murine β-GlcCer'ase mRNA (2.1–2.4 kb) (27). Comparison of poly A(+) mRNA extracts from murine epidermis, liver, and spleen, processed in parallel, revealed no apparent differences in oligonucleotide size. However, triplicate epidermal preparations consistently demonstrated a higher relative expression of poly(A)+ mRNA for β-GlcCer'ase than liver, brain, and spleen (Fig. 3). Together with the characterization studies above, these results confirm the presence of β-GlcCer'ase in murine epidermis. Moreover, these findings imply that, unique to the epidermis, the majority of β-glucosidase activity can be attributed specifically to β-GlcCer'ase.

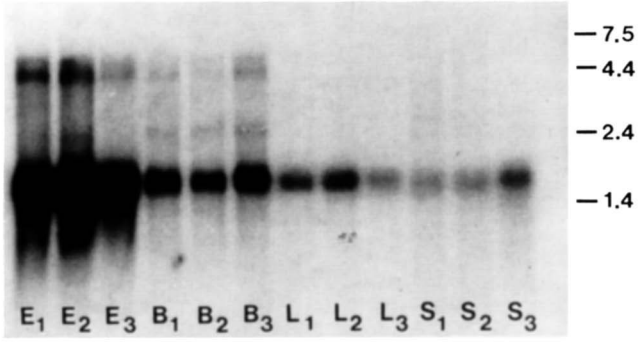

Fig. 3. Expression of β-glucocerebrosidase mRNA. Northern blots of poly(A)+ mRNA using a cDNA probe for murine β-glucocerebrosidase (see Methods). Triplicate poly(A)+ mRNA preparations (separate animals) for epidermis (E_1–E_3), brain (B_1–B_3), liver (L_1–L_3), and spleen (S_1–S_3) are shown with 6 μg poly (A)+ loaded on each lane.

Enzyme localization in relation to differentiation

We next determined the localization of enzyme activity in murine skin to assess whether β-GlcCer'ase expression is regulated in relation to cellular differentiation. As seen in **Table 3**, mean epidermal β-GlcCer'ase activity was at least twofold higher than the activity in dermis. Moreover, within the epidermis, mean β-GlcCer'ase activity increased progressively with cellular differentiation. Activity in the combined basal and spinosum layers was less than half of that present in the combined granulosum and corneum layers. The highest activity was found in stratum corneum, the most differentiated cell layer. These results demonstrate significant β-GlcCer'ase activity within the stratum corneum, a feature that correlates with the conversion of glucosylceramide to ceramide known to occur at this level of the epidermis.

DISCUSSION

Significant changes in lipid composition occur during the formation of the cutaneous permeability barrier. In the epidermis of terrestrial mammals, the content of glucosylceramides increases in the stratum granulosum, followed by the progressive elimination of glucosylceramides and appearance of ceramides from the stratum corneum (2–5). As the sphingoid base and N-acyl composition of both of these sphingolipids are virtually identical (6, 7), progressive deglycosylation may explain the emergence of ceramides. In contrast, in moist keratinizing epithelia, such as oral mucosa and marine cetacean epidermis, glucosylceramide content remains elevated in the

TABLE 3. Localization of β-glucocerebrosidase activity in murine skin

	Specific Activity[a]	n[b]	Percent versus Whole Epidermis
	nmol/min/mg protein		
Epidermis	2.60 ± 0.07	3;9	(100%)
Stratum basale/spinosum[c]	1.43 ± 0.05	7;21	55
Stratum granulosum/corneum[c]	3.17 ± 0.21	6;17	122
Stratum corneum[d]	4.09 ± 0.66	6;17	157
Dermis	1.20 ± 0.02	3;8	46

[a]Activities determined using homogenates. Significances: whole epidermis from dermis, $P < 0.01$; differences between stratum basale/spinosum vs. stratum corneum/granulosum, $P < 0.001$; stratum corneum/granulosum vs. stratum corneum, $P < 0.05$.
[b]n = Number of animals; number of determinations.
[c]Separated with the staphylococcal epidermolytic toxin (see Methods).
[d]Isolated by trypsinization (see Methods).

stratum corneum (37–39). Moreover, β-glucosidase activities reportedly are lower in oral epithelium (25) than epidermis. Whereas prior studies described β-glucosidase in various skin and mucosal preparations (18–25), it now appears certain from the present study that most of the epidermal activity is β-GlcCer'ase.

Three main criteria have been used in this study to distinguish between β-GlcCer'ase and nonspecific β-glucosidase activity (reviewed in references 10 and 15). First, we found epidermal β-glucosidase activity to be significantly stimulated by the addition of sodium taurocholate to standard assay mixtures (12). Second, our observation of a pH optimum for β-glucosidase of 5.6 agrees with reports of a pH optimum of 5.5–6.0 for purified β-GlcCer'ase in both human liver and spleen (12), and with previous reports of β-glucosidase activity in human skin (18–20) and epidermis (21, 31). However, our finding of only one pH optimum in murine epidermal extracts contrasts with human leukocyte β-glucosidase activity which exhibits dual pH optima at 4.0 and 5.6 (12, 40). Recent reports of more acidic pH optima (pH 3.8–4.7) in intact epidermal sheets (24, 25) may reflect differences in the starting tissues used (i.e., intact epidermis vs. epidermal extracts), buffer system, and/or the presence of sodium taurocholate.

Third, a significant percentage of epidermal β-glucosidase activity was BrCBE-inhibitable, both in the presence and absence of taurocholate (99 and 75%, respectively), suggesting that this activity primarily represents β-glucocerebrosidase. This exceptionally high level of BrCBE-inhibitable activity in epidermis is unique when contrasted with murine brain, liver, and spleen, which exhibited much lower levels of BrCBE-inhibitable activity. The high percentage of BrCBE-resistant activity in liver agrees with a previous study with CBE, but the values for brain and spleen tissues differ (36). This discrepancy is not likely due to the inhibitor used, as both BrCBE and CBE are active site-directed substrate analogues (41). Moreover, both compounds inhibited epidermal activity to a similar extent (data not shown), although the bromo-derivative was significantly more potent (IC_{50} values of 0.1 mM and 5 mM, respectively). The differences may reflect the use of cellular homogenates in these studies where other constituents, e.g., acidic lipids or saposins (42, 43), also could influence results.

We also have provided direct evidence that the epidermis has the capacity for abundant β-GlcCer'ase production. Northern analysis demonstrated not only expression of poly(A)+ mRNA for β-GlcCer'ase in murine epidermis, but also a higher recovery of β-GlcCer'ase mRNA (per μg poly (A)+ mRNA) from epidermis than from either murine brain, liver, or spleen. Together, these results show that β-GlcCer'ase accounts for the bulk of epidermal β-glucosidase activity, and that the epidermis may be uniquely enriched in this enzyme. Whether the epidermis contains a number of β-GlcCer'ase enzymes as observed in other tissues (44) is not yet known.

Prior biochemical and/or cytochemical studies have shown that several lipid catabolic enzymes including glycosidases (23, 45), sphingomyelinase (46), phospholipase A (31, 45, 47), triacylglycerol hydrolase (46), and steroid sulfatase (48) accumulate in the outer epidermis. We have now shown that GlcCer'ase activity also is concentrated in the outer epidermis. Moreover, several of these enzymes have been co-localized with their lipid substrates in epidermal lamellar bodies, and evidence has been presented that most appear to be delivered to membrane domains of the stratum corneum (45, 46). Whether β-GlcCer'ase likewise is localized to lamellar bodies and/or stratum corneum membrane domains is not yet known. Despite the localization data on these catabolic enzymes, a functional relationship to the epidermal permeability barrier has not been established. Our demonstration here of an apparent gradient for β-GlcCer'ase in the epidermis is consistent with the progressive replacement of glucosylceramides by ceramides during the transition from the nucleated to the cornified epidermal cell layers (2–5). Moreover, recent preliminary studies have shown that topical applications of BrCBE to

murine skin cause a progressive accumulation of glucosylceramides in the murine stratum corneum in association with immature intercellular membrane structures and an ineffective barrier (49). If substantiated, these studies lend further support to the concept that intercellular β-GlcCer'ase participates in the formation and maintenance of the epidermal permeability barrier.

This work was supported by National Institutes of Health grants AR-39448 and AR-19098, and the Medical Research Service, Veterans Administration. We gratefully acknowledge KAO Corporation (Japan) for support of Mr. Takagi as a Visiting Scientist. The authors thank Dr. Kenneth R. Feingold for helpful discussions and review of this manuscript and Dr. Mei-Jhy Su for assistance with Northern analysis. Finally, we thank Bil Chapman and Sally Michael for their adroit manuscript preparation.

Manuscript received 20 February 1992 and in revised form 17 April 1992.

REFERENCES

1. Elias, P. M. 1983. Epidermal lipids, barrier function, and desquamation. *J. Invest. Dermatol.* **80:** 44–49.
2. Gray, G. M., and H. J. Yardley. 1975. Different populations of pig epidermal cells: isolation and lipid composition. *J. Lipid Res.* **16:** 441–447.
3. Elias, P. M., B. E. Brown, P. Fritsch, J. Goerke, S. Grayson, and J. White. 1979. Localization and composition of lipids in neonatal mouse stratum granulosum and stratum corneum. *J. Invest. Dermatol.* **73:** 339–348.
4. Lampe, M. A., M. L. Williams, and P. M. Elias. 1983. Human epidermal lipids: characterization and modulations during differentiation. *J. Lipid Res.* **24:** 131–140.
5. Cox, P., and C. A. Squier. 1986. Variation in lipids in different layers of porcine epidermis. *J. Invest. Dermatol.* **87:** 741–744.
6. Wertz, P. W., and D. T. Downing. 1983. Acylglucosylceramides of pig epidermis: structure determination. *J. Invest. Dermatol.* **24:** 753–758.
7. Wertz, P. W., D. C. Swartzendruber, K. C. Madison, and D. T. Downing. 1987. Composition and morphology of epidermal cyst lipids. *J. Invest. Dermatol.* **89:** 419–425.
8. Patrick, A. D. 1965. A deficiency of glucocerebrosidase in Gaucher's disease. *Biochem. J.* **97:** 17C–18C.
9. Brady, R. O., J. N. Kanfer, and D. Shapiro. 1965. Metabolism of glucocerebrosides II. Evidence of an enzymatic deficiency in Gaucher's disease. *Biochem. Biophys. Res. Commun.* **18:** 221–225.
10. Grabowsky, G. A., S. Gatt, and M. Horowitz. 1990. Acid β-glucosidase: enzymology and molecular biology of Gaucher disease. *Crit. Rev. Biochem. Mol. Biol.* **25:** 385–414.
11. Beutler, E. 1991. Gaucher's disease. *N. Engl. J. Med.* **325:** 1354–1360.
12. Peters, S. P., P. Coyle, and R. H. Glew. 1976. Differentiation of β-glucocerebrosidase from β-glucosidase in human tissues using sodium taurocholate. *Arch. Biochem. Biophys.* **175:** 569–582.
13. Daniels, L. B., R. H. Glew, N. S. Radin, and R. R. Vunnam. 1980. A revised fluorometric assay for Gaucher's disease using conduritol B epoxide with liver as the source of β-glucosidase. *Clin. Chim. Acta.* **106:** 155–163.
14. Daniels, L. B., P. J. Coyle, Y-B. Chiao, R. S. Labow, and R. H. Glew. 1981. Purification and characterization of a cytosolic broad specificity β-glucosidase from human liver. *J. Biol. Chem.* **256:** 13004–13013.
15. Glew, R. H., A. Basu, K. L. LaMarco, and E. M. Prence. 1988. Mammalian glucocerebrosidase: implications for Gaucher's disease. *Lab. Invest.* **58:** 5–25.
16. Chester, M. A., B. Hultberg, and P-A. Ockerman. 1976. The common identity of five glycosidases in human liver. *Biochim. Biophys. Acta.* **429:** 517–526.
17. Ben-Yoseph, Y., E. Shapira, D. Edelman, B. K. Burton, and H. L. Nadler. 1977. Purification and properties of neutral β-galactosidases from human liver. *Arch. Biochem. Biophys.* **184:** 373–379.
18. Ockerman, P. A. 1969. Acid hydrolases in human skin. *Acta Dermato.-Venereol.* **49:** 139–141.
19. Goldberg, M. F., E. Cotlier, L. G. Fichenscher, K. Kenyon, R. Enat, and S. A. Borowsky. 1971. Macular cherry-red spot, corneal clouding, and β-galactosidase deficiency. *Arch. Intern. Med.* **128:** 387–398.
20. Ohkawara, A., K. M. Halprin, J. R. Taylor, and V. Levine. 1972. Acid hydrolases in the human epidermis. *Br. J. Dermatol.* **83:** 450–459.
21. Mier, P. D., and J. J. M. A. Van Den Hurk. 1975. Lysosomal hydrolases of the epidermis. I. Glycosidases. *Br. J. Dermatol.* **93:** 1–10.
22. Wolff, K., and E. Schreiner. 1970. Epidermal lysosomes. Electron microscopic-cytochemical studies. *Arch. Dermatol.* **101:** 276–286.
23. Nemanic, M. K., J. S. Whitehead, and P. M. Elias. 1983. Alterations in membrane sugars during epidermal differentiation: visualization with lectins and role of glycosidases. *J. Histochem. Cytochem.* **31:** 887–897.
24. Wertz, P. W., and D. T. Downing. 1989. β-Glucosidase activity in porcine epidermis. *Biochim. Biophys. Acta.* **1001:** 115–119.
25. Chang, F., P. W. Wertz, and C. A. Squier. 1991. Comparison of glycosidase activities in epidermis, palatal epithelium, and buccal epithelium. *Comp. Biochem. Physiol.* **100B:** 137–139.
26. Legler, G. 1977. Glucosidases. *Methods Enzymol.* **46:** 368–381.
27. O'Neill, R. R., T. Tokoro, C. A. Kozak, and R. O. Brady. 1989. Comparison of the chromosomal localization of murine and human glucocerebrosidase genes and of the deduced amino acid sequences. *Proc. Natl. Acad. Sci. USA.* **86:** 5049–5053.
28. Holleran, W. M., K. R. Feingold, M. Mao-Qiang, W. N. Gao, J. M. Lee, and P. M. Elias. 1991. Regulation of epidermal sphingolipid synthesis by permeability barrier function. *J. Lipid Res.* **32:** 1151–1158.
29. Ohashi, T., C. M. Hong, S. Weiler, J. M. Tomich, J. M. F. G. Aerts, J. M. Tager, and J. A. Barranger. 1991. Characterization of human glucocerebrosidase from different mutant cells. *J. Biol. Chem.* **266:** 3661–3667.
30. Pentchev, P. G., R. O. Brady, S. R. Hibbert, A. E. Gal, and D. Shapiro. 1973. Isolation and characterization of glucocerebrosidase from human placental tissue. *J. Biol. Chem.* **248:** 5256–5261.
31. Mier, P. D., and J. J. M. A. Van Den Hurk. 1976. Lysosomal hydrolases of the epidermis. *Br. J. Dermatol.* **95:** 271–274.
32. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* **72:** 248–254.

33. McIlvaine, T. C. 1921. A buffer solution for colorimetric comparisons. *J. Biol. Chem.* **49:** 183–186.
34. LaBarca, C., and K. Paigen. 1980. A simple, rapid and sensitive DNA assay procedure. *Anal. Biochem.* **102:** 544–352.
35. Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
36. Hara, A., and N. S. Radin. 1979. Destruction and resynthesis of mouse β-glucosidases. *Biochim. Biophys. Acta.* **582:** 412–422.
37. Squier, C. A., P. S. Cox, P. W. Wertz, and D. T. Downing. 1986. The lipid composition of porcine epidermis and oral epithelium. *Arch. Oral Biol.* **31:** 741–747.
38. Squier, C. A., P. W. Wertz, and P. Cox. 1991. Thin-layer chromatographic analysis of lipids in different layers of porcine epidermis and oral epithelia. *Arch. Oral Biol.* **36:** 647–653.
39. Elias, P. M., G. K. Menon, S. Grayson, B. E. Brown, and S. J. Rehfeld. 1987. Avian sebokeratocytes and marine mammal lipokeratinocytes: structural, lipid biochemical and functional considerations. *Am. J. Anat.* **180:** 161–177.
40. Beutler, E., and W. Kuhl. 1970. The diagnosis of the adult type of Gaucher's disease and its carrier state by demonstration of deficiency of β-glucosidase activity in peripheral blood leukocytes. *J. Lab. Clin. Med.* **76:** 747–755.
41. Legler, G., and E. Bieberich. 1988. Active site-directed inhibition of a cytosolic β-glucosidase from calf liver by bromoconduritol B epoxide and bromoconduritol F. *Arch. Biochem. Biophys.* **260:** 437–442.
42. Glew, R. H. 1988. Activation of human spleen glucocerebrosidases by monoacylglycol sulfates and diacylglycerol sulfates. *Arch. Biochem. Biophys.* **262:** 345–353.
43. Morimoto, S., Y. Kishimoto, J. Tomich, S. Weiler, T. Ohashi, J. A. Barranger, K. A. Kretz, and J. S. O'Brien. 1990. Interaction of saposins, acidic lipids, and glucosylceramidase. *J. Biol. Chem.* **265:** 1933–1937.
44. Aerts, M. F. G., W. E. Donker-Koopman, C. Laar, S. Brull, G. J. Murray, D. A. Wenger, J. A. Barranger, J. M. Tager, and A. W. Schram. 1987. Relationship between the two immunologically distinguishable forms of glucocerebrosidase in tissue extracts. *Eur. J. Biochem.* **163:** 583–589.
45. Freinkel, R. K., and T. N. Traczyk. 1985. Lipid composition and acid hydrolase content of lamellar granules of fetal rat epidermis. *J. Invest. Dermatol.* **85:** 295–298.
46. Menon, G. K., S. Grayson, and P. M. Elias. 1986. Cytochemical and biochemical localization of lipase and sphingomyelinase activity in mammalian epidermis. *J. Invest. Dermatol.* **86:** 591–597.
47. Freinkel, R. K., and T. N. Traczyk. 1983. Acid hydrolases of the epidermis: subcellular localization and relation to cornification. *J. Invest. Dermatol.* **80:** 441–446.
48. Elias, P. M., M. L. Williams, M. E. Maloney, J. A. Bonifas, B. E. Brown, S. Grayson, and E. H. Epstein, Jr. 1984. Stratum corneum lipids in disorders of cornification: steroid sulfatase and cholesterol sulfate in normal desquamation and the pathogenesis of recessive X-linked ichthyosis. *J. Clin. Invest.* **74:** 1414–1421.
49. Holleran, P. M., Y. Takagi, G. K. Menon, K. R. Feingold, and P. M. Elias. 1992. Processing of epidermal glucosylceramides is required for mammalian permeability barrier function. *Clin. Res.* **40:** 73A.