Integrity and Barrier Function of the Epidermis Critically Depend on Glucosylceramide Synthesis*

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Ceramides are vital components of the water barrier in mammalian skin. Epidermis-specific, a major ceramide portion contains ω-hydroxy very long chain fatty acids (C30–C36). These ω-hydroxy ceramides (Cers) are found in the extracellular lamellae of the stratum corneum either as linoleyl acyl esters or protein bound. Glucosylceramide is the major glycosphingolipid of the epidermis. Synthesized from ceramide and UDP-glucose, it is thought to be itself an intracellular precursor and carrier for extracellular ω-hydroxy ceramides. To investigate whether GlcCer is an obligatory intermediate in ceramide metabolism to maintain epidermal barrier function, a mouse with an epidermis-specific glucosylceramide synthase (Ugcg) deficiency has been generated. Four days after birth animals devoid of GlcCer synthesis in keratinocytes showed a pronounced desquamation of the stratum corneum and extreme transepidermal water loss leading to death. The stratum corneum appeared as a thick unstructured mass. Lamellar bodies of the stratum granulosum did not display the usual ordered inner structure and were often irregularly arranged. Although the total amount of epidermal protein-bound ceramides remained unchanged, epidermal-free ω-hydroxy ceramides increased 4-fold and ω-hydroxy sphingomyelins, almost not detectable in wild type epidermis, emerged in quantities comparable with lost GlcCer. We conclude that the transient formation of GlcCer is vital for a regular arrangement of lipids and proteins in lamellar bodies and for the maintenance of the epidermal barrier.

Glucosylceramide synthase (Ugcg), catalyzing the initial step of glycosphingolipid synthesis (see Fig. 1A), is vital during embryogenesis as revealed by systemic deletion in mice (1). To investigate the different functions of glucosylceramide (GlcCer)4-based glycolipids in vivo, cell-specific deletions of this enzyme are indispensable. Successful deletion of glucosylceramide synthase in neural cells led to loss of brain gangliosides. Nevertheless, mice were born developing severe dysfunctions postnatally (2, 3). In this report we focus on the function of GlcCer in skin.

Land dwelling animals have a water and electrolyte barrier in the skin to prevent dehydration and electrolyte disturbances. Both membrane proteins of keratinocytes and lipids generated in the epidermis are important for maintenance of the skin barrier and, therefore, for the impermeability of the skin to water. Ceramides (Cers) constitute a major component of the lipid barrier. They are synthesized by amidation of sphingoid bases with long chain fatty acids at the endoplasmic reticulum. Conversion into GlcCer by Ugcg takes place at the cytoplasmic surface of the Golgi apparatus (4, 5).

GlcCers are the dominant glycosphingolipids of the epidermis and constitute ~4% of the total epidermal lipid mass (6). They are thought to act as an intracellular carrier for secreted ceramides and constitute the main components of lamellar bodies (LBs) (7–9). LBs are found in cells of the upper stratum granulosum and are extruded into the extracellular cleft of the stratum corneum interstices by exocytosis (10, 11) together with enzymes such as β-glucocerebrosidase. In the presence of its activator protein saposin C, β-glucocerebrosidase catalyzes the release of ceramides from GlcCer in the extracellular space (9, 12).

Besides Cers and GlcCers with a fatty acid chain length of 16–26 carbon atoms common to many cells, Cers and GlcCers with very long chain ω-hydroxy fatty acids containing up to 36 carbon atoms (OS-Cer and -GlcCer) form a major portion of Cer compounds in the epidermis. At the ω-position, many of the latter are additionally esterified with linoleic acid (EOS-Cer and -GlcCer) (6, 12); these OS-GlcCers can be covalently linked to proteins in the cornified layer (POS-Cer and in trace amounts, POS-GlcCer) obviously by transesterification of EOS-GlcCer (12, 13) to glutamine-glutamate rich regions of involucrin, envoplakin, and periplakin (14). EOS- and POS-Cers as well as their corresponding GlcCers seem to be important contributors to the extremely hydrophobic extracellular (O) and sphingosine (S); ESI-MS/AS, nanoelectrospray ionization tandem mass spectrometry; LB, lamellar body; NS, sphingolipids containing ω-hydroxy fatty acids (N) and sphingosine (S); OS, sphingolipids containing ω-hydroxy long chain fatty acids (O) and sphingosine (S); POS, sphingolipids containing protein-bound (P) ω-hydroxy long chain fatty acids (O) and sphingosine (S); SM, sphingomyelin.
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lipid lamellae of the stratum corneum (15). It has not been conclusively shown whether indeed all sphingolipid components (Cers, GlcCers, and sphingomyelins) or merely one or two of them are needed for maintenance of the epidermal water barrier. A deficiency of β-glucocerebrosidase or a deficiency of its sphingolipid activator protein saposin C in epidermis led to strong reduction of ceramides concomitant with an accumulation of GlcCers including the protein-bound fraction, suggesting GlcCer as a precursor of Cer, impairing the skin barrier function (7, 12). Whether glucosylceramide synthesis is obligatory for the lipid part of the skin barrier is not known. Therefore, a keratinocyte-specific deficiency of glucosylceramide synthase was generated.

In the epidermis the genes expressing keratins K5 and K14 are expressed in basal cells (16, 17). Because of its early expression in the basal layer, the keratin K14 promoter seems optimal to express Cre recombinase to achieve epidermis-specific gene deficiencies (18–20). K14-promoted Cre mice were bred with mice with a loxP flanked glucosylceramide synthase gene (2) to obtain a blockade of the initial step of the GlcCer biosynthesis in epidermal keratinocytes.

It is now shown that lack of GlcCer in the epidermis led to an irregular arrangement of the lamellar bodies in stratum granulosum cells and of extracellular lipids in the stratum corneum. Protein-bound ceramides were not reduced, and free and esterified α-hydroxy sphingomyelins were synthesized as functionally insufficient substitutes of GlcCer. Our data document for the first time that an intact stratum corneum with an ordered lipid arrangement depending on GlcCer-function is vital for skin barrier function.

EXPERIMENTAL PROCEDURES

Transgenic Animals—Mice with loxP-flanked exons 6–8 of the Ugcg gene locus were generated as described (2) (see Fig. 1B). Generation of mutant animals and experiments were performed according to federal laws for animal experiments and approved (Regierungspräsidium Karlsruhe, Germany).

For the generation of mice with the Ugcg gene deletion in the epidermis, homozygous floxed mice were crossed with K14-constitutive Cre animals (18). In a second mating step, heterozygous floxed mice with the respective K14-promoted Cre animals (18) were mated with mice with a loxP-flanked glucosylceramide synthase gene (2) to obtain a blockade of the initial step of the GlcCer biosynthesis in epidermal keratinocytes.

mRNA Isolation, Analysis, Profile—Skins were prepared from decapitated animals at different time points after birth. Adhering adipose tissue was removed. The epidermis was separated from the dermis after incubation with 0.5 M ammonium thiocyanate in 0.1 M potassium sodium hydrogen phosphate, pH 6.8, for 30 min on ice according to Diaz et al. (21).

Total mRNA was extracted using the RNA easy kit (Qiagen GmbH, Hilden, Germany) as described by the manufacturer. Quantitative real-time reverse transcription-PCR was done using the LC-fast DNA Master SYBR Green 1 kit PCR for the LightCycler (Roche Applied Science) as described, and glycer-aldehyde-3-phosphate dehydrogenase was used as reference gene (2).

Lipid Extraction—Skins were trypsinized in 0.25% trypsin for 16 h at 4°C. Epidermis was separated from dermis (22). Tissue was lyophilized, and glycosphingolipids were extracted from epidermis essentially according to Doering et al. (12).

In brief, ~5–10 mg of epidermis was extracted with 2 ml of chloroform/methanol/distilled water 30:60:8 by vol. The samples were treated under sonification at 50°C for 15 min and subsequently centrifuged at 3000 rpm for 10 min. Supernatant was taken, and pellets were extracted two additional times as described using the solvent mixtures chloroform/methanol/distilled water, 10:10:1, and chloroform/methanol, 2:1 by vol. Supernatants were combined with the previous fractions. For further purification, an aliquot of the extract pool was further purified by saponification under mild alkaline conditions followed by desalting using reversed phase RP-18 columns of 200 μl of volume as described (23, 24).

For the extraction of protein-bound sphingolipids, pellets, which were obtained after extraction above, were treated 3 times with 2 ml of 100% methanol at room temperature for 10 min followed by two additional extraction steps with 2 ml of 95% methanol in H2O at 60°C for 2 h to remove residual free lipids. The respective supernatants were taken after centrifugation as described, and the absence of free ceramides in the final supernatant was confirmed by nano-ESI-MS/MS analyses.

From the residual pellet, protein-bound sphingolipids were cleaved after treatment with 1 ml of 1 M KOH in 95% methanol at 60°C for 2 h. Supernatants were taken as described, neutralized with 1 M acetic acid, and dried and desalted using reversed phase RP-18 columns of 100 μl of volume as described (23).

TLC running solvents used were chloroform/methanol/water 65:25:4 (see Fig. 2, A and B) and chloroform/methanol/glacial acetic acid 190:9:1 (used twice; see Fig. 2, C and E). The amounts spotted corresponded to extracts from 1 mg of dry epidermis. Visualization was performed with 0.2% orcinol in 10% sulfuric acid, 120°C, 10 min (TLC, see Fig. 2A) and 10% CuSO4 in 8% H3PO4 at 180°C for 10 min (TLC, see Fig. 2, B, C, and E).

Quantification of sphingolipids was performed by nano-ESI-MS/MS using a precursor ion scan of m/z +184 for sphingomyelin (collision energy, 35 eV) (25) and of m/z +264 for cera-
buffered formaldehyde or zinc buffer. Tissue was embedded immersion-fixed at several time points with 4% phosphate-in paraffin wax. Sections of 3-
Linoleic acid-esterified sphingolipids were determined by subtraction of the amount of OS sphingolipids of the non-treated epidermal extracts from the respective amounts of OS sphingolipids evaluated in the saponified extracts (linoleic acid esterified-sphingolipid standards were not available). For determination of total GlcCer, mildly alkaline-treated epidermal extracts were quantified densitometrically using a Shimadzu CS-9301PC TLC-scanner (Shimadzu Europe, Duisburg, Germany).

**Skin Permeability Assay**—Skin permeability was tested by Lucifer yellow diffusion essentially as described (27, 28). In brief, animals at P4 were sacrificed with CO2, briefly washed with 70% ethanol, and incubated in 1 nm Lucifer yellow (Sigma) dissolved in isotonic NaCl at 37 °C for 1 h. The solution was removed, and bodies were incubated for one additional hour at 37 °C in the dark. Subsequently, skin sections were mounted, and slices with a thickness of 5 μm were prepared. Nuclear counterstaining was performed using DRAQ5 (Alexis, San Diego, CA), and slices were investigated by confocal laser microscopy (Leica, Wetzlar, Germany).

**Determination of Transepidermal Water Loss**—Transepidermal water loss was measured using a Tewameter® TM 300 (Courage-Khazaka Electronics, Cologne, Germany) as described (27).

**Light Microscopy and Immunohistochemistry**—Skin of snout, ear, and midline abdomen (two pieces of each tissue type) of Ugcg^floxed^/K14Cre mouse and their respective controls were immersion-fixed at several time points with 4% phosphate-buffered formaldehyde or zinc buffer. Tissue was embedded in paraffin wax. Sections of 3-μm thickness were stained by hematoxylin (HE), periodic acid-Schiff (PAS), and Goldner trichrome. Tissues from same locations were immediately frozen in isopentane precooled with liquid nitrogen. Cryo-conserved skin sections (5 μm) were used for indirect immunofluorescence microscopy for membrane-associated proteins and cytoskeleton components (27, 29).

Primary antibodies were as follows: 1) guinea pig antibodies against keratins K5 (1:2000), K14 (1:3000), K17 (1:2000), K2e (1:2000), Ha5 (1:5000), Hb5 (1:1000), and K6hf (1:2000) (mutant), were born according to Mendelian inheritance constituting ~25% of the litter, indicating no prenatal death.

Southern blot analysis showed an approximately 70% deletion of the Ugcg gene locus in the epidermis of newborn mice at P0 (Fig. 1D). An almost complete deletion of the gene was observed at P4 (Fig. 1D), and minimal mRNA expression could be demonstrated using real time reverse transcription-PCR (Fig. 1E). Residual Ugcg DNA and mRNA might be explained by expression in non-keratinocyte cells in which the K14 promoter is not active, e.g., Langerhans cells.

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**RESULTS**

**Skin-specific Glycosphingolipid Deletion in Mice**—Offspring with the deleted Ugcg gene in the epidermis, Ugcg^floxed^/K14Cre (mutant), were born according to Mendelian inheritance constituting ~25% of the litter, indicating no prenatal death.

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**Glucosylceramide Synthase Was Deleted in the Epidermis of Ugcg-deficient Mice**—DNA and mRNA levels of the glucosylceramide synthase were negligibly low in epidermis of mutant mice at P4 (Fig. 1, D and E). The enzyme product, GlcCer, was determined at different days after birth. The total GlcCer content in epidermis of control animals decreased approximately by 40% within the first 4 days after birth (Fig. 2, A and D, controls).

Compared with controls, a significant decrease (42%) of GlcCer could be seen in the epidermis of animals with Ugcg gene deletion at P0, \( p < 0.01 \) (Fig. 2, A and D, P0). Two days
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A.

B.

C.

D.

E.

FIGURE 1. GlcCer synthesis pathway, cloning strategy and genotyping of Ugcg<sup>fl/fl</sup>/K14Cre mice. A, glucosylceramide biosynthesis from ceramide and UDP-activated glucose is catalyzed by Ugcg. B, The Ugcg gene deletion was initialized by generating mice containing homozygous Ugcg-flxed alleles (f/f) in combination with a K14Cre transgene. The K14-promoter driven Cre-recombinase deleted both loxP-flanked alleles in Ugcg-mice. kb, kilobases. C, mouse tail biopsies from mutant animals were characterized by PCR analysis using a triple primer pair for the detection of the Ugcg-wild type and floxed and null-alleles as well as for the K14Cre transgene. * due to the induction of the null allele in epidermis only, complete tail biopsies of mutant mice show a faint band for the null mutation. D, Ugcg gene deletion in epidermis was verified by Southern blot analysis after separation of epidermis from dermis at the age of P0 and P4. A 4.3-kilobase BglII fragment indicated the gene deletion. E, quantitative real time reverse transcription-PCR of the epidermis from mutant animals at P4. Ugcg-mRNA decreased 96%; n = 3. Residual Ugcg-DNA and mRNA might be explained by expression in non-keratinocyte cells in which the K14 promoter is not active. f, flox; +, wild type.

After birth, epidermal GlcCer was reduced by 80% in Ugcg-deficient mice compared with their respective controls, p < 0.0009 (Fig. 2, A and D, P2). The highest degree of GlcCer reduction of ~95%, which correlated well with very low DNA and mRNA levels of Ugcg, could be measured shortly before most animals died at P4 and P5, p < 0.0002 (Fig. 2, A and D, P4).

Free (Fig. 2, B and C) and protein-bound epidermal sphingolipids (Fig. 2E) were analyzed by TLC. In the SM pattern a slight decrease of the slow migrating SM band could be seen in Ugcg-deficient epidermis at P2/P4 (Fig. 2B) that correlates with a 50% decrease of α-hydroxy palmitoyl containing sphingomyelin measured by mass spectrometry. In the ceramide pattern slight quantitative differences occurred (Fig. 2C). A TLC of the protein-bound epidermal lipids reflected no striking changes in lipid compositions between wild type and mutant mice (Fig. 2E).

To analyze changes of sphingolipid subpopulations grouped according to their fatty acid residues, the C18-sphingosine (d18:1)-based sphingolipids of free extractable and protein-bound lipid extracts of wild type and Ugcg-deleted epidermis were quantitatively analyzed by nano-ESI-MS/MS at the age of P4. The sum of GlcCer<sub>d18:1</sub> with all its different fatty acid moieties, as already seen by TLC (Fig. 2, A and D) was similarly reduced. No OS- and EOS-GlcCers<sub>d18:1</sub> were detectable in these mutant mice (p < 0.0009 for NS/AS and p < 0.03 for EOS; Fig. 2F). Ceramides<sub>d18:1</sub>, nonhydroxy as well as α-hydroxy long chain ceramides<sub>d18:1</sub> (NS/AS) were not altered in mutant mice (Fig. 2G) with the exception of the single species of Cer (d18:1,18:0) which increased 6–10-fold from hardly detectable amounts (<30 ng/mg dry weight) in wild type epidermis (data not shown). However, OS-ceramides<sub>d18:1</sub> significantly accumulated about 4-fold in epidermal extracts, p < 0.002. Surprisingly, no differences could be seen within EOS as well as the POS-ceramides<sub>d18:1</sub> (Fig. 2G). Because of the latter findings and the fact that GlcCer-synthase deficiency in neuronal cells leads to enhanced SM production (2), possible alterations in SM content were investigated in epidermal extracts. OS- and EOS-SMs<sub>d18:1</sub> which in control animals were only detectable in trace amounts, increased in mutant mice significantly (Fig. 2H), resulting in roughly 2-fold the amount of the sum of OS- and EOS-GlcCers<sub>d18:1</sub> of wild type mice. However, protein-bound SM could not be detected by ESI-MS/MS.

K14 Promoted Ugcg-deficient Mice Displayed Strong Skin Alterations—Mutant mice could not be distinguished from their respective control littermates immediately after birth, showing no skin abnormalities or differences in size at P0 (Fig. 3, C versus A, control). By days P3/P4, skin desquamation was observed in joints, periorally, and in parts of skin of the tail. Peeling of the cornified layer increased quickly, progressing to desquamation covering large areas of the body at P4/P5 (Fig. 3, D, Ugcg-deficient, and B, control). HE staining of different skin regions demonstrated that skin desquamation exclusively involved the stratum corneum (Fig. 3D2, compare D1 intact...
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Concomitantly with the detachment of the stratum corneum, a loss of barrier function with a drastic increase of transepidermal water loss in mutant mice could be demonstrated at P4 \( (p < 0.0001; \text{Fig. 3E}) \). Furthermore, a significant increase of \( 15.5 \pm 2.2\% \) of the blood hemocrit levels could be measured in Ugcg\(^{\text{floxflox}}/\text{floxflox}/\text{K14Cre} \) mice at developmental stage P4/P5 \( (p = 0.0017, n = 3 \text{each}) \).

The drastic increase in transepidermal water loss during postnatal development went along with a decrease in body weight. Neonatally, the body weight increased for both, mutant and control animals until P2 (Fig. 3F). Starting from P3, the weight in mutant mice stagnated and during further development decreased. Compared with their respective control littermates, the bodyweight at P4 was only half that of controls \( (p < 0.0001; \text{Fig. 3F}) \). As a result of water loss all mutant animals died postnatally within 5 days (Fig. 3G).

Structural Analysis Revealed Severe Abnormalities in the Skin of Ugcg\(^{\text{floxflox}}/\text{floxflox}/\text{K14Cre} \) Mice—No significant differences in epidermal morphology between newborn controls (Fig. 4A) and mutant animals could be seen at birth (Fig. 4B). All epidermal layers, the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum, appeared normal. In addition, Ki67 staining showed no alteration in proliferation of basal cells (Fig. 4, F, mutant, versus E, control). In the following days, epidermal defects appeared such as an absence of granules in the stratum granulosum at P4 in mutant mice (Fig. 4D). The epidermis, including stratum corneum, broadened compared with control mice (Fig. 4C). Ki67-positive proliferating cells increased significantly in the stratum basale and could also be seen in the lower stratum spinosum (Fig. 4, H, mutant, versus G, control (green brackets)).

At P4, a drastic increase of apoptosis (terminal dUTP nick-end labeling assay) could be observed at the interphase of stratum granulosum and stratum corneum (red dotted line, Fig. 4I), in contrast to normal skin from age-matched littermates with only few labeled apoptotic cells (red dotted line, Fig. 4I). Furthermore, in P4/P5 mutant mice the number of monocytes, macrophages, and dendritic cells was elevated in the dermis.
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FIGURE 3. Phenotypical analysis of control and Ugcg<sup>flu</sup>/K14Cre mice. A–D, photographs of control littermates (A and B) and mutant mice (C and D) at P0 (newborn, A and C) and at P4 (B and D). Shortly after birth Ugcg<sup>flu</sup>/K14Cre mice could not be distinguished from their respective controls. Postnatally at P4 mutant animals showed severe skin desquamation (D) and did not gain weight. B1–D3, HE staining of skin sections from control (B1) and mutant mice (D1–D3) as indicated. Bars, 100 μm. D1, reflects a region in which the epidermal stratum granulosum (sg)-stratum corneum (sc) junction is not yet dissociated fully. Peeled skin consists solely of cells of the stratum corneum layer (sc, D2). Cell layers under the peeled skin showed a complete loss of the stratum corneum cell layer. Solely the stratum granulosum and the stratum spinosum (sg and ss) layers could be seen (D3). B1′–D3′, Lucifer yellow diffusion was restricted to the outer stratum corneum layer in wild type (B1′) and in regions of mutant animals without detachment of the stratum corneum (D1′). A destruction of the skin permeability barrier was observed in regions in which the stratum corneum layer was lost, as indicated by Lucifer yellow diffusion throughout the whole skin (D3′). E, mutant mice showed a highly significant increase of transepidermal water loss at P4 (p < 0.0001); n = 4 animals each, controls and Ugcg-deficient. F, mutant animals displayed significant less weight as their respective control littermates at P4. G, All mutant animals died postnatally within 5 days.

Moreover, in the normally parakeratotic ear epidermis, keratin K2 (formerly K2e) was positive in some keratinocytes of the stratum spinosum in the stratum granulosum of control mice (Fig. 5G, white bracket, sg). In mutant mice K2 staining was greatly enhanced in numerous cells of the middle and upper stratum spinosum (Fig. 5G′; yellow bracket, ss<sup>+</sup>-sg).

We also investigated the expression of hair follicle specific keratin, characterizing the differentiation of hair follicle keratinocytes and trichocytes, as hair follicles appeared atrophic. The keratins K75 (formerly K6hf), specific for the companion layer (Fig. 5, cl) and hair medulla (med), K71 (formerly K6irs1),...
specific for the three layers, Henle, Huxley, and IRS cuticle of the hair inner root sheath (IRS), and the hair keratins K35 (formerly Ha5) and K85 (formerly Hb5), which are expressed in the hair cuticle and hair matrix/cortex (for review, see Ref. 30) were studied. Although in the mutant animals the hair follicles were considerably smaller and reduced in number compared with wild type, the expression of the epithelial keratins K75 (Fig. 5, H–I) and K71 (Fig. 5, J) as well as the hair keratins K35 (Fig. 5, K) and K85 (data not shown) indicated that all of these structures of the hair follicles were normally developed (compare with Figs. 5, H–J) at least in terms of the expression of these structural proteins. K75 is precisely seen in the hair companion layer (Fig. 5, H and H′, cl) and medulla (Fig. 5H, med), and K71 is seen in all the three inner root sheath compartments (Fig. 5, I and I′, irs) while leaving the interfollicular skin negative; similar observations were made for the hair keratins K35 and K85, restricted to the hair fiber (hf) cuticle and hair matrix/cortex (Fig. 5, J and J′).

The stage of cornification of the epidermal keratinocytes was investigated by the immunostaining of filaggrin (fil, Fig. 5, K and K′), loricrin (lor, Fig. 5, L and L′), and transglutaminase I (tg1, Fig. 5, M and M′); all of these proteins were seen in their typical localizations in both the control (Fig. 5, K–M) and the mutant (Fig. 5, K′–M′) animals, with filaggrin and transglu-
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Ugcg-contr.  

Ugcg^f/f/K14Cre
taminase I in the stratum spinosum and stratum granulosum and loricrin in the stratum granulosum alone. The only consistent difference was that the width of immune positivity was higher in the snout (Fig. 5, K’–M’) and ventral skin (data not shown) of Ugcg-deficient epidermis, indicative of their hyperkeratinization.

Immunohistochemical staining of the desmosomal protein, desmosplakin (dp), was not altered in the various Ugcg-deficient tissues when comparing the staining pattern with that of the control mice (representatively dp-staining of snout is shown (Fig. 5, N and N’). Immunolabeling of the tight junction proteins claudin-1, cingulin, occludin and protein ZO-1 appeared in their typical, wild-type localization (31) i.e. claudin-1 (cl-1, Fig. 5, O and O’), and protein ZO-1 (data not shown) could be detected at the margins of cells of the lower strata, including those of the stratum basale of control (Fig. 5O, white bracket) and mutant mice (Fig. 5O’, yellow bracket); cingulin (cing, Fig. 5P) and occludin (data not shown) were restricted to the cell margins of keratinocytes of the stratum granulosum in the control animals (sg, Fig. 5P) but often were positive in the upper stratum spinosum (uss+sg, Fig. 5P’) of Ugcg-deficient mice.

Transmission Electron Microscopy—Ultrastructural differences in skin of mutant animals were investigated postnatally at day P4. Hyperplasia of the epidermal cell layers could be confirmed. The stratum corneum was extremely broadened without clear cut layers (Fig. 6A, control, versus D, mutant). In addition, as already seen by light microscopy (Fig. 4D), a drastic reduction of almost all granules in the stratum granulosum cell layer was observed in Ugcg-deficient epidermis. Furthermore, numerous apoptotic cells could be detected in stratum granulosum and stratum corneum of mutant mice (Fig. 6, D and E, asterisks; compare terminal dUTP nick-end labeling assay, Fig. 4, I and J). Moreover, autophagy could be seen in some cells of the stratum granulosum of mutant mice (Fig. 6E, inset).

In Ugcg-deficient epidermis, lamellar bodies displayed an irregular arrangement of lipid lamellae (Fig. 6, C, control versus F, Ugcg<sup>flex/flex</sup>/K14Cre<sup>+</sup>), and extrusion of lamellar sheets into the stratum corneum interphase was disordered. In addition, in some instances accumulation of lipid depots could be observed in the interstices of the stratum corneum as well as between single corneocytes of the stratum corneum (data not shown).

However, alterations in desmosomes and tight junctions could not be detected in mutant mice (data not shown). These findings corroborated the immunofluorescence data (Fig. 5, N’–P’ versus N–P, control).

**DISCUSSION**

**Epidermal GlcCer Deletion Is Complete Shortly after Birth—** At birth the epidermis of mice, with floxed alleles for glucosylceramide synthase and with K14Cre transgene, still contained the glucosylceramide synthase gene, although K14 promoter activity has been observed to start at E9.5 during ectoderm development (17). It has been shown that K14 promoter-driven Cre recombinase activity appeared in a mosaic pattern at E15 and reached strong ubiquitous expression in the basal cell layer of the epidermis and in the outer root sheaths of the hair follicles after birth (18). Accordingly GlcCer content in the epidermis of Ugcg-deficient mice decreased to 20% of wild type control values 2 days after birth and to ~5% at P4. This remnant of GlcCer might be due to expression of glucosylceramide synthase in cells of epidermis other than keratinocytes (Langerhans cells, melanocytes) or appendage structures negative for K14 (e.g. hair fiber trichocytes). Therefore, a focal incomplete deficiency of glucosylceramide synthase down to 4% could still be detected at P4 and would be plausible.

The fall in GlcCer in wild type mice after birth is due to an increased degradation by β-glucocerebrosidase, which cleaves GlcCer into glucose and ceramide. Its activity rises between E17 and birth by 5-fold (32), and the distribution of the enzyme changes from patchy epidermal expression to a ubiquitous pattern at birth (32). Similarly, this degradation probably takes place in mutant mice. However, they show a drastic decrease of

**FIGURE 5. Differentiation of keratinocytes of Ugcg<sup>flex/flex</sup>/K14Cre<sup>+</sup> and respective controls (GCS<sup>flex/flex</sup>/K14Cre<sup>+</sup>), characterized by expression of keratins, cornified envelope proteins, and proteins of cell-cell junctions as desmosomes and tight junctions using immunofluorescence microscopy.** The new nomenclature for designations of keratins is used (48) with the former names given in brackets. Immunostaining of keratins in control (letter) and mutant (letter plus apostrophe mark hair follicles) mouse tissues, A–B’, staining of keratin K14 (14) in snout skin (A/A’ and B/B’ are the respective phase contrast micrographs). Whereas the K14 staining is restricted to the stratum basale (sb) of control mice, it is also detected in the stratum spinosum of mutant mice (yellow brackets). Furthermore, the outer root sheaths of the hair follicles (open red arrows in A) are positive for K14. Asterisks mark hair follicles. C–E’, staining of keratin K6 (C/C’; 6) in ventral skin, K16 (D/D’; 16) and K17 (E/E’; 17). Keratins K6, K16, and K17 are not found in the interfollicular epidermis of wild type control mice (white brackets in C–E), but staining of these keratins in mutant mice includes all epidermal layers (yellow brackets in C–E). The outer root sheaths (red open arrows in C–E) and sebaceous glands (white open arrows in C–E) of both types of mice are generally positive for these keratins. F–G, keratin K10 (F/F’; 10) is found in all suprabasal cells (stratum spinosum (ss) and stratum granulosum (sg)) of snout skin of control (F, white bracket) and with a considerably increased height of both strata in mutant mice (F’, yellow bracket). In addition, the stratum corneum is labeled. Keratin K2 (formerly K2e; G/G’; 2) is expressed in sg cells of the parakeratotic ear skin control (G, white bracket) and strongly in all suprabasal layers (ss+sg) of those tissues of mutant mice (G’, yellow bracket). Note again that in mutant mice stratum spinosum and stratum granulosum layers are considerably higher. Note that hair follicles (asterisks) and sebaceous glands (white open arrows) are negative for both keratins. H–J, specific staining of K75 (formerly K66; H/H’; 75) in the hair follicle companion layer (c) and medulla (md) in ventral skin of both wild type and mutant mice. Immunostaining of K71 (formerly K61; I/I’; 71) in the hair follicle inner root sheath (irs) and of hair keratin K35 (formerly Ha5; J/J’; 35) in the hair fiber (hf) of both control and mutant mice. K–M’, staining of filaggrin (K’/f); fil, loricin (L’/l); lor, and transglutaminase 1 (M’/t1), typical proteins of the cornified envelope formation process, in snout epidermis. Filaggrin is normally expressed in strata spinosum (ss) and strata granulosum (sg) of normal (K, ss+sg, white brackets) and the higher stratified skin of mutant mice (K’, ss+sg, yellow brackets). The hair follicle (asterisks) outer and inner root sheaths are positive (open arrows). Loricin and transglutaminase-1 can be detected in the stratum granulosum (sg) of control mice (sg; l and M), in mutant mice these proteins are commonly seen in uppermost stratum spinosum (uss) and stratum granulosum (sg) (L’, M’). Both proteins are negative in the hair follicle compartments (asterisks). Background staining in the dermal tissue (d, M/M’) is due to the use of mouse primary antibodies against transglutaminase 1 (t1) on mouse tissues. N/N’, desmosplakin (dp) is stained in same patterns at the desmosomes of snout skin in all the interfollicular (white brackets in wild type mice; yellow brackets in mutant mice) and follicular (asterisks) parts of such epidermis. O–P’, claudin-1 (O/O’; cl-1) is found in the cell-cell borders of all layers of the skin, whereas the staining of cingulin (P/P’; cing) in snout is restricted to the stratum granulosum of wild type mice (P, sg, white brackets); this protein is also detectable in upper stratum spinosum and granulosum of mutant animals (P’; uss+sg, yellow bracket). The presence of both proteins is indicative for an intact tight junction barrier in both mice (white brackets in wild type mice; yellow brackets in mutant mice). Blue staining, 4’,6-diamidino-2-phenylindole-stained nuclei. Bars, 50 μm.
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FIGURE 6. Transmission electron micrographs of ventral skin sections. A–C, control skin sections; D–F, sections from skin of mutant mice. Granules from the stratum granulosum cell layer (sg) can only be seen as tiny dots in the epidermis of mutant mice 4 days after birth (D and E compared with A and B, controls). ss, strata spinosum; sb, stratum basale; sc, stratum corneum; LD, lamina densa. The number of apoptotic bodies in Ugcg-deficient epidermis (D and E, asterisks) is significantly increased and can even be observed in the stratum corneum. C, inset, autophagocytosis was observed in epidermal sheaths of Ugcg-deficient epidermis. C and F, lamellar bodies (arrows) could be found in both control epidermis (C) and epidermis of mutant mice at the border to the stratum corneum (F). However, in Ugcg-deficient epidermis the shape of lamellar bodies was irregular, and the arrangement of their lamellae within the bodies was not strictly parallel to each other.

Glucosylceramide (GlcCer) levels compared with control animals in the same time period. The significantly additional decrease consequently has to be attributed to the lack of GlcCer synthesis postnatally.

Absence of GlcCer Leads to Severe Skin Defects—Concomitant with the reduction of GlcCer content in the epidermis, mice showed severe signs of failure of the skin water barrier along with detachment of the stratum corneum, leading to death of all animals 5 days post-partum. An altered ceramide metabolism apparently led to this failure of skin function. Ceramides are synthesized in the endoplasmic reticulum and enzymatically released from SM or GlcCer in the extracellular space.

During late embryonic development GlcCer content in mouse embryo skin decreases in favor of the formation of ceramide by the action of β-glucocerebrosidases (33). This change in the lipid component profile takes place predominantly during differentiation of cells of the stratum granulosum to the stratum corneum layer. In the corneal layer a proportional decrease of GlcCer and a corresponding increase of ceramide could be described (34, 35). GlcCer is enriched in lamellar bodies (8, 36), from which lipid lamellae are sequentially extruded to the corneocyte interstices (10, 15). In the presence of its activator protein saposin C, β-glucocerebrosidase catalyzes the release of ceramides from GlcCer after extrusion of LB into the extracellular space.

In mice in which β-glucocerebrosidase was deleted, free- and protein-bound ceramide content of the skin decreased significantly due to the storage of corresponding GlcCer (Gaucher mice) (7, 37). The absence of β-glucocerebrosidase led to altered intercellular lamellar membranes and drastically increased epidermal water loss (37). In a related study animals were generated that lacked the activator protein necessary for proper action of β-glucocerebrosidase (12). In these mice especially, the protein-bound ceramides decreased, whereas protein-bound GlcCer increased. Again, this animal model displayed a disrupted skin water barrier.

Surprisingly, in our mutant mice lacking epidermal GlcCer, the amount of protein-bound ceramides was not significantly altered in epidermal extracts. In the absence of GlcCer synthesis, the corresponding non-esterified ω-hydroxy long-chain fatty acid-ceramides (OS) and linoleic acid-esterified ω-hydroxy ceramides (EOS) appeared to be shuttled into the SM pathway, whereas in wild type animals keratinocytes expressed these SMs (OS/EOS) if at all, only in tiny amounts (<0.5%) (see also Ref. 38). Despite this backup-pathway, OS-ceramides significantly increased in epidermal extracts of mutant mice by 4-fold. This result suggested that OS-ceramides obviously have to be processed via glucosylation to maintain normal OS-sphingolipid levels.

With regard to the protein bound ceramides, two possibilities cannot be excluded. There might be differences of POS-Cer in stratum corneum between wild type and mutant mice that are not detectable by the values obtained from total epidermal extracts. The turnover of POS-Cer is a slow process because the continued renewal of epidermis needs about 7 days in wild type mice (39). Therefore, it could very well be that measured POS-Cer levels at P4 have, at least to a certain extent, their origin from an earlier time point at which synthesis of EOS/POS-GlcCer still took place.

Disordered Arrangement of Lipid Lamellae by Lack of GlcCer—In addition, OS- and EOS-SMs apparently cannot qualitatively substitute for corresponding GlcCers. Using electron microscopy, an abnormal arrangement of lamellae in lamellar bodies could be seen in cells of the stratum granulosum of Ugcg-deficient epidermis. The loss of geometry in LB might be explained in several ways. Sphingomyelin is synthesized at the luminal side, whereas GlcCer is produced at the cytosolic side of the Golgi. Because of the different topology of the synthesis of these two compounds, lamellar arrangement of lipids within the LB might be altered during GlcCer deficiency. A change in the polarity of the lamellae might also occur. Compared with glucose, the zwitterionic phosphocholine head group of SM may have caused a stronger repulsion of the lamellae, resulting in disorganization of lamellae within the LB. Furthermore, a 4-fold increase of free OS-ceramide also could affect the stability of the sandwich-like structure of the extracellular lamellae. In this structure the linoleyl portion of EOS ceramides sticks out of the crystalline into the fluid sublattice (10). Increased free ceramides, therefore, should dilute the sublattices connecting EOS-ceramides per area. Similar alterations in LB appearance were observed in mice in which GlcCer syn-
thesis was partially inhibited by \textit{d,L\text{-}threo\text{-}1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl} (40). However, analysis of sphingolipid alterations had not been performed in this study. GlcCer may have exerted additional effects on LB with regard to sorting of proteins and electrolyte composition (41, 42). Until now, this effect has not been characterized in detail.

As a consequence, lipid-lamellae were not extruded in an orderly fashion to the extracellular cleft as indicated by droplet-like structures in the stratum corneum. The severe disturbance of lamellar membranes apparently changed the interaction between corneal proteins and lipids to such an extent that the stratum corneum appeared as a broadened, compact, homogeneous mass. Its mechanical properties were such that it easily detached from the stratum granulosum after mechanical strain. In mutant mice the skin covering joints was primarily afflicted, constituting the nidus for large area denuding of the corneal layer. These areas had an extreme increase in water permeability. Therefore, the stratum corneum is an essential component of the skin barrier independent of occluding junctions in the cellular part of the epidermis. The structural composition of the lamellar bodies seemed to determine whether the lipid lamellae of the stratum corneum are regularly arranged. GlcCer is needed to achieve this result.

These findings demonstrate an interacting evolutionary aspect of the skin barrier in land-bound vertebrates. Skin barrier function is of such vital importance that different systems were built up to assure water impermeability; that is, a multitude of proteins for elaborate construction of hydrophobic occluding junctions and different lipids including ceramides for an intricate layering of lipids. The interdependence of these systems makes skin barrier vulnerable to default in case one component fails (43).

Compensatory Mechanisms to Loss of Water Barrier—Several phenomena were observed that might be interpreted as reactive and compensatory mechanisms. As signs of hyperkeratosis, the distribution of keratins K14, K5, and K10 was considerably broader and was also indicative of an enhanced cell proliferation in epidermis of mutant mice. Keratins might play a role in the sequestration of 14-3-3 and thereby indirectly modulate cell cycle progression (44, 45). Keratins K6 and K16 and K17, which are only expressed in single keratinocytes of control skin, showed a strong staining in tissue from mutant mice, indicating physical stress.

Characteristic proteins of the hair follicle in mutant mice showed a regular distribution; follicles seemed to develop normally. However, hairs were obviously not able to enter the stratum corneum; this might be due to the tightly clustered stratum corneum; this might be due to the tightly clustered stratum corneum; this might be due to the tightly clustered stratum corneum; this might be due to the tightly clustered stratum corneum. Besides lipids, transglutaminases are important for the corneous envelope as they may catalyze the formation of stable isopeptide bonds during cornified envelope assembly (47). In mutant mice, transglutaminase-1 was similarly expressed as in controls.

At present it is not known whether a deficiency or a decrease of glucosylceramide synthase in the epidermis of man can occur and whether this would lead to disease. A total deletion of the enzyme is lethal during mouse embryogenesis (1). This study has shown that disruption of glucosylceramide synthase in epidermal GlcCer synthase-deficient mice induced multiple alterations in skin leading to a breakdown of barrier function and death due to dehydration. GlcCer was necessary for correct lamellar body formation and for regular metabolism and arrangement of lipids in the stratum corneum. The stratum corneum was essential to maintain the water barrier of the skin.

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