During keratinocyte differentiation, the glycolipid, glucosylceramide (GlcCer), is thought to be synthesized, stored in intracellular lamellar granules and eventually extruded into the intercellular space where GlcCer is hydrolyzed to ceramide, a major component of the epidermal permeability barrier. Previous studies showed that GlcCer synthase (GCS) activity increases during keratinocyte differentiation; however, the mechanism by which GCS activity is regulated was not established. In the present study, we prepared anti-peptide antibodies and amplified cDNA probes based on the cDNA sequence for human GCS (Ichikawa, S., Sakiyama, H., Suzuki, G., Hidari, K. 1-P. J., and Hirabayashi, Y. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4638–4643) in order to study GCS expression during keratinocyte differentiation. Confluent human keratinocytes in culture were induced to terminally differentiate by elevation of Ca\(^{2+}\) in the medium without exogenous hormones or growth factors. GlcCer synthesis assayed in situ using a fluorescent ceramide analog increased ∼5-fold during keratinocyte differentiation, peaking at day 6. Fluorescence microscopy studies of living keratinocytes showed that fluorescent ceramide and/or its metabolites accumulated in the Golgi in undifferentiated cells but targeted to unique vesicular structures that may be derived from the trans-Golgi region. Expression of both GCS mRNA, a ∼3.8-kilobase transcript on Northern blots, and GCS protein, a ∼38-kDa polypeptide detected by Western blotting, increased dramatically (∼5-fold) during differentiation, reaching a maximum at about day 8. These results suggest that GCS is up-regulated at the transcriptional level during keratinocyte differentiation and provide the first direct evidence for GCS up-regulation in any cell type.

During keratinocyte growth and differentiation, glucosylceramide (GlcCer) is thought to be synthesized intracellularly and subsequently transported and stored in lamellar bodies. At later stages of the differentiation process, the contents of these granules are secreted into the stratum corneum by exocytosis, and the GlcCer is subsequently hydrolyzed by the enzyme β-glucocerebrosidase to ceramide (Cer), a major lipid constituent of the epidermal barrier (1, 2). Previous studies have demonstrated an increase of GlcCer synthase (GCS) activity during keratinocyte differentiation. GCS activity was measured in lysates of cultured human keratinocytes using radioactive or fluorescent substrates and was found to reach a maximum level 8 days after induction of the differentiation process (3). In fetal rats of gestational age 17–21 days, GCS activity in the epidermis peaks at day 19, prior to epidermal barrier competence (4). Parallel studies have also demonstrated that β-glucocerebrosidase activity and expression increase during keratinocyte differentiation and barrier formation (3, 4), although these events are not as closely correlated with keratinocyte differentiation as the induction of GCS activity. Although these studies indicate induction of GCS activity during keratinocyte differentiation, little is known about the regulation of this enzyme. In particular, it is not yet known whether the induction of GlcCer synthesis is due to regulation at the transcriptional or translational levels, to post-translational modifications of the enzyme, to alterations in GlcCer degradation, or to some other factor(s) that play a role in modulating GCS activity during differentiation. Recently the human cDNA sequence for GCS was identified (5), making possible the generation of new reagents to address some of these questions. In the present study, we have raised anti-peptide antibodies to human GCS suitable for Western blotting and have generated cDNA probes to GCS so that we could study GCS mRNA levels during differentiation. Using these tools we show that GCS expression is most likely up-regulated at the transcriptional level during keratinocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Normal human keratinocytes were isolated from neonatal foreskin specimens, and primary cultures were initiated and maintained in a replicative state using serum-free (“complete”) growth medium (MCDB153 medium (6) containing 0.1 mM Ca\(^{2+}\) and supplemented with 0.2% (v/v) bovine pituitary extract, epidermal growth factor (10 ng/ml), insulin (5 μg/ml), hydrocortisone (5 × 10\(^{-7}\) M), ephalnolamine (1 × 10\(^{-4}\) M), and phosphoethanolamine (1 × 10\(^{-4}\) M)) (7). Subconfluent keratinocytes from primary cultures were plated into secondary cultures at 1–10 × 10\(^{5}\) cells/cm\(^2\). At confluence (day 0), cells were washed several times with 1.5 mM Ca\(^{2+}\) MCDB153 medium without any growth factors, hormones, or bovine pituitary extract (“differentiation medium”) to initiate the differentiation process. The cultures were re-fed differentiation medium every other day (8). Cells were grown in 60-mm diameter tissue culture dishes for biochemical studies or on number 1 thickness glass coverslips for fluorescence microscopy. All cells were grown at 37 °C in a water-saturated atmo-
Glucosylceramide Synthase in Differentiating Keratinocytes

In Situ Metabolism and Fluorescence Microscopy Using BODIPY™-
Ceramide—N-[5-(5, 7-dimethyl BODIPY™-l-pentanoyl-d-erythro-
sphingosine (C₅–DMB-Cer or “BODIPY™-Ceramide,” Molecular Probes, Inc., Eugene OR) was prepared as a BSA complex in HMEM and stored at −20 °C as described previously (9). For in situ GCS and sphingomyelin (SM) synthase activity assays, keratinocytes at various stages of differentiation cultured on coverslips were washed with HMEM and then incubated with 2 μM C₅–DMB-Cer/BSA for 30 min at 37 °C. The cells were then washed with HMEM and either extracted immediately or further incubated for 90 min at 37 °C in the presence of 5% defatted BSA (DP-BSA, Intergen, Purchase, NY). In the latter case, the samples were subsequently incubated (six times for 10 min each) with 5% DP-BSA at 10 °C (“back-exchanged”) prior to lipid extraction (10). The cells were then washed with ice-cold HMEM and scraped from the culture dish, and the lipids were extracted, separated by thin layer chromatography, and quantified as described previously (11, 12). GCS and SM synthase activity values were back-exchanged to cellular protein (13).

For fluorescence microscopy, keratinocytes were incubated with 2 μM C₅–DMB-Cer/BSA and then back-exchanged as described above. Fluorescence microscopy was performed with an inverted microscope (IM-35, Carl Zeiss, Inc.) equipped with a Planapo 100× objective and epifluorescence optics. Samples were excited at 450–490 nm, and the fluorescence was observed at either 520 nm (green + red wavelengths) or at ≥590 nm (red wavelengths) (14).

Preparation of Antibodies against Human GCS and Western Blotting—A peptide with the sequence TISWTRGTRYLRCCGGTAAELDV corresponding to the 23 C-terminal amino acid residues of human GCS (5) was synthesized as an antigen for immunization. Specific antisera was raised in rabbits 8 weeks after inoculation with the synthesized peptide. For immunoprecipitation studies, antibodies were purified using the corresponding peptide antibodies linked to Aminolink Plus columns (Pierce). Purified antibodies were coupled to protein A Sepharose CL-4B beads (Sigma) using dimethylpimelimidate coupling as described (15). Keratinocyte cultures at the various stages of differentiation were washed with phosphate-buffered saline and then solubilized with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) (15) for 10 min at 4 °C. The cell extracts then were centrifuged at 12,000 × g for 5 min. The supernatants, corresponding to equal amounts of cellular protein, were incubated for 2 h with antibody-linked beads at room temperature with continuous rocking. The beads were spun down, and the pellet was washed with RIPA buffer (three times for 10 min each) at room temperature. The pellets were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer with 8 M urea, and the supernatants were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (16, 17). The membrane was incubated successively with rabbit polyclonal antisera (1:1,000) against human GCS and horseradish-peroxidase-labeled anti-rabbit IgG antibody (1:10,000; Boehringer Mannheim) and then visualized with the ECL detection system (Amersham Pharmacia Biotech).

Glucosylceramide Synthase cDNA—A 966-bp partial GCS cDNA corresponding to nucleotides 52–1017 of the published sequence of human GCS (5) was obtained from human skin fibroblasts, and GCS-specific primers were designed on the basis of human GCS cDNA sequences (5) and synthesized as follows: 5'-CTCTTCTTGGTGCTGTGGCTGATGC-3' (forward primer) and 5'-ACCTGGACACCCCTGAGTTGAATG-3' (reverse primer). The 966-bp PCR-amplified GCS cDNA fragment was cloned into the PCR II vector (Stratagene, La Jolla CA) using the GCG program (Genetic Computer Group, Inc., Madison WI) and the corresponding peptide antibodies linked to Aminolink Plus (Pierce). Purified antibodies were coupled to protein A Sepharose CL-4B beads using dimethylpimelimidate coupling as described (15). Keratinocyte cultures at the various stages of differentiation were washed with phosphate-buffered saline and then solubilized with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) (15) for 10 min at 4 °C. The cell extracts then were centrifuged at 12,000 × g for 5 min. The supernatants, corresponding to equal amounts of cellular protein, were incubated for 2 h with antibody-linked beads at room temperature with continuous rocking. The beads were spun down, and the pellet was washed with RIPA buffer (three times for 10 min each) at room temperature. The pellets were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer with 8 M urea, and the supernatants were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (16, 17). The membrane was incubated successively with rabbit polyclonal antisera (1:1,000) against human GCS and horseradish-peroxidase-labeled anti-rabbit IgG antibody (1:10,000; Boehringer Mannheim) and then visualized with the ECL detection system (Amersham Pharmacia Biotech).

RESULTS

Normal human keratinocytes were seeded into complete growth medium, grown to confluence, and induced to differentiate by switching to differentiation medium and elevating the Ca²⁺ concentration from 0.1 to 1.5 mM (see “Experimental Procedures” and Refs. 6 and 7). As previously shown (7, 8), this results in a population of differentiating keratinocytes containing lamellar granules and induces the expression of keratin 1, both of which are markers for keratinocyte differentiation.

Metabolism and Fluorescence Microscopy of C₅–DMB-Cer in Keratinocytes—We first examined the metabolism of C₅–DMB-Cer, a fluorescent Cer analog, in keratinocyte cultures using an in situ assay. At various time points following the switch to differentiating medium, cells were incubated for 30 min at 37 °C with C₅–DMB-Cer/BSA, and the fluorescent metabolites were quantified and normalized to total cellular protein. Formation of C₅–DMB-GlcCer increased markedly during differentiation, peaking at day 6 (~5-fold increase compared with day 0) following induction of differentiation, whereas the amount of C₅–DMB-SM that was formed remained relatively constant at each time point (Fig. 1).

For fluorescence microscopy, keratinocyte cultures were incubated with 2 μM C₅–DMB-Cer for 30 min at 37 °C, washed and further incubated with BSA for 90 min at 37 °C, and then back-exchanged (see “Experimental Procedures”) at low temperature to remove fluorescent lipids at the plasma membrane (10). These studies demonstrate a dramatic change in the distribution of intracellular fluorescent ceramide and sphingomyelin (Fig. 2). At confluence (day 0), fluorescence was concentrated in a perinuclear region that, based on previous studies in other cell types (9, 14), corresponds to the Golgi apparatus. The prominent red fluorescence further indicates that the lipid was concentrated in this organelle because the fluorescence emission of the BODIPY™-fluorophore shifts from green to red.
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wavelengths with increasing concentration (10, 14, 20, 21). In contrast, when cells from day 6 were used, the fluorescent lipid (and its metabolites) was widely distributed throughout the cytoplasm in punctate structures. Many of these “dot-like” bodies coincided with refractile structures that could be observed by phase microscopy (not shown). Metabolism studies using C$_5$-DMB-Cer under the conditions used for the microscopy studies revealed that day 6 differentiated cells accumulated ~3-fold more total C$_5$-DMB fluorescence than day 0 cells; however, the proportions of C$_5$-DMB-Cer, C$_5$-DMB-GlcCer, and C$_5$-DMB-SM present, respectively, were: day 0, 0.18 ± 0.06, 0.09 ± 0.01, and 0.54 ± 0.03 nmol/mg cell protein; and day 6, 0.50 ± 0.15, 0.33 ± 0.07, and 1.48 ± 0.36 nmol/mg cell protein.)

GCS Expression in Differentiating Keratinocytes—Polyclonal antibodies were raised against three different peptides that were synthesized using the predicted amino acid sequence of human GCS. For these antibodies, (i) each recognized a ~38-kDa polypeptide in rat liver Golgi membranes by immunoblotting, (ii) each immunoprecipitated this same ~38-kDa band, and (iii) two of these antibodies could be used to immunoprecipitate GCS activity from a detergent solubilized enzyme preparation from rat liver. Each of the three antibodies recognized GCS as a ~38-kDa polypeptide in human keratinocytes (data not shown). These results demonstrate that the ~38-kDa polypeptide corresponds to GCS. In the present study, one of the antibodies was used to examine the time course of GCS expression during differentiation of human keratinocytes (Fig. 3). In this experiment, cell lysates were prepared from keratinocytes at different stages of differentiation, and supernatants corresponding to equal amounts of cellular protein were immunoprecipitated with antibody-linked beads to enrich the samples for GCS. The immunoprecipitates were subjected to Western blotting with the same anti-GCS peptide antibody. As seen in Fig. 3, GCS protein expression was detectable in confluent cultures at day 0 and increased ~5-fold during keratinocyte differentiation, reaching a maximum at days 6–8 following the switch to differentiation medium.

GCS mRNA Expression during Keratinocyte Differentiation—We next used a 966-bp cDNA probe to GCS to examine the time course of mRNA expression during keratinocyte differentiation. GCS mRNA expression was detected as a 3.8-kilobase transcript on Northern blots at culture confluence (day 0) and increased steadily following the switch to differentiation medium, reaching a peak at about 8 days (Fig. 4). A similar but earlier increase in expression was observed for keratin 1 mRNA, an early marker for terminal differentiation in keratinocytes (8, 19), whereas the expression of keratin 14 mRNA, a basal keratinocyte proliferative marker (19), was relatively unchanged in stratifying cultures undergoing differentiation. GAPDH mRNA was used for normalization of these data.

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Fig. 2. Intracellular distribution of C$_5$-DMB-Cer and its metabolites in human keratinocytes. Undifferentiated (day 0) and differentiated (day 6) human keratinocytes were incubated with C$_5$-DMB-Cer/BSA for 30 min at 37 °C, washed with HMEM, and further incubated for 90 min at 37 °C in the presence of 5% DF-BSA. The samples were then incubated with 5% DF-BSA at 10 °C and viewed under the fluorescence microscope ($\lambda_{\text{em}} = 470–490$ nm) using filter combinations appropriate for Green + Red ($\lambda_{\text{em}} \geq 520$ nm) or Red fluorescence ($\lambda_{\text{em}} \geq 590$ nm). The latter results from membranes containing relatively high concentrations of the BODIPY$_{581/591}$ lipid. Note the prominent perinuclear labeling at day 0, corresponding to the Golgi apparatus (9, 14, 20), and the widely dispersed punctate labeling at day 6. (Biochemical analysis of cells incubated under the same conditions as used for microscopy showed that the amounts of C$_5$-DMB-Cer, C$_5$-DMB-GlcCer, and C$_5$-DMB-SM present, respectively, were: day 0, 0.18 ± 0.06, 0.09 ± 0.01, and 0.54 ± 0.03 nmol/mg cell protein; and day 6, 0.50 ± 0.15, 0.33 ± 0.07, and 1.48 ± 0.36 nmol/mg cell protein.)

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2 D. L. Marks, P. Paul, Y. Kamisaka, and R. E. Pagano, manuscript in preparation.
Keratinocyte cultures were induced to differentiate as described under "Experimental Procedures." At each time point, cell lysates were prepared, and the GCS protein was detected by Western blotting (see "Experimental Procedures"). Equal amounts of cell protein were loaded per lane. A, a representative Western blot for GCS at days 0–10. B, quantitation of Western blots for GCS by densitometry. Data are the means ± S.D. of three different experiments.

**Fig. 3. Expression of GCS in differentiating human keratinocytes.** Keratinocyte cultures were induced to differentiate as described under "Experimental Procedures." Poly(A)⁺ RNA was prepared from the cultures at the indicated time points. A, Northern blotting was performed using a cDNA probe for human GCS, keratin 1, keratin 14, and GAPDH as indicated. B, quantitative data for GCS (●) and keratin 14 (○) mRNA expression normalized by GAPDH expression. Data are the means of two representative Northern blots and are relative values in which the maximum level of expression for each transcript was defined as 100%.

**Fig. 4. Expression of GCS mRNA during keratinocyte differentiation.** Keratinocyte cultures were induced to differentiate as described under "Experimental Procedures." Poly(A)⁺ RNA was prepared from the cultures at the indicated time points. A, Northern blotting was performed using a cDNA probe for human GCS, keratin 1, keratin 14, and GAPDH as indicated. B, quantitative data for GCS (●) and keratin 14 (○) mRNA expression normalized by GAPDH expression. Data are the means of two representative Northern blots and are relative values in which the maximum level of expression for each transcript was defined as 100%.

**Discussion**

Keratinocyte differentiation in terrestrial mammals is characterized by a programmed sequence of biochemical and morphological changes that eventually produce the epidermal permeability barrier (1, 22). During terminal differentiation of keratinocytes into a stratified epithelium, complex changes in lipid synthesis and organization occur. Keratinocytes become enriched in specific lipids and form lamellar granules that are thought to be enriched in the glycolipid, GlcCer. Upon extrusion of the lamellar granules into intercellular spaces at the junction of viable granular layer and nonviable cell layers of the stratum corneum, GlcCer is converted to Cer, which becomes a major component of the epidermal permeability barrier. Thus, the production of GlcCer by GCS plays a critical role in the formation of the functional cornified epithelium. In the present study we demonstrate that GCS activity, mRNA, and protein expression increase in coordination with differentiation in a defined human keratinocyte culture model.

Our study is consistent with previous work that demonstrated an increase in GCS activity during keratinocyte differentiation by assaying enzymatic activity in cell homogenates using various Cer substrates (3); however, the present study is the first to demonstrate the up-regulation of GCS mRNA and protein expression during keratinocyte differentiation. Our results suggest that GCS is up-regulated at the transcriptional level during keratinocyte differentiation. We found that although GCS mRNA (Fig. 4) and protein expression (Fig. 3) peaked at day 8 of differentiation, GCS activity (Fig. 1) in situ was highest at day 6. Based on these data we speculate that after day 6, increased levels of β-glucocerebrosidase may degrade newly formed GlcCer during the in situ assay, causing a decrease in apparent GCS activity at day 8. This would be consistent with the reported increase in β-glucocerebrosidase activity observed during late stages of keratinocyte differentiation in culture and during epidermal development (3, 4).

In the previous study by Sando et al. (3), GCS activity increased with keratinocyte differentiation induced by a complex regimen of medium changes, including serum addition. Previous findings by us (7, 8) and others (23, 24) have shown that serum markedly alters both keratinocyte proliferation and differentiation, including expression of various differentiation-related proteins and organelles such as lamellar bodies. In the present study, cells were grown in serum-free culture medium, and differentiation was induced by growth to confluence and a switch to defined medium containing elevated Ca²⁺ concentration and the absence of exogenous growth factors and hormones. Thus, our study demonstrates that the increase in GCS expression observed during keratinocyte differentiation does not require the presence of any added hormones or growth factors, although the removal of specific factors (present in complete medium) and culture confluence may act as triggers to stimulate both GCS expression and differentiation. Future studies using this defined and easy to manipulate culture system will allow us to examine the effects of various factors (e.g. vitamins, lipids, antioxidants, and growth factors) that may play a role in regulating keratinocyte differentiation and GCS function without interference by undefined serum factors.

In the present study, we used a BODIPY™-labeled analog of ceramide, C₇-DMB-Cer, for both in situ assays of GCS activity and morphologic studies of sphingolipid distribution in differentiating keratinocytes. This fluorescent ceramide analog exhibits a shift in fluorescence emission from green to red wavelengths as its concentration in membranes is increased. Thus, we were able to clearly observe the regions of highest fluorescence accumulation in keratinocytes by fluorescence microscopy. When undifferentiated keratinocytes were incubated with C₇-DMB-Cer, fluorescence was concentrated in perinuclear sacs and vesicle-like structures, consistent with the targeting of fluorescent ceramide and/or its metabolites to the Golgi complex, as observed in other cell types (9, 14, 20). However, with increasing differentiation the distribution of C₇-DMB fluorescence was altered markedly, becoming predomi-
nanty localized to large vesicle-like structures distributed throughout the cells. We and others have previously shown that when fluorescent ceramides are added to cells, the fluorescent probe accumulates in the membranes of the trans-Golgi or trans-Golgi network (TGN), probably due to a specific affinity of fluorescent ceramides for these Golgi membranes (14, 25, 26). Based on these observations, we suggest that the fluorescent vesicles observed in differentiated keratinocytes are derived from the trans-Golgi or TGN and that this compartment is extensively modified in differentiated keratinocytes to function in the production of lamellar bodies. This conclusion is consistent with recent observations that differentiated keratinocytes of the stratum granulosum possess a widely dispersed trans-Golgi-like network closely associated with interconnected lamellar bodies (27). Work is in progress in our laboratory to characterize the structures labeled by C6-DMB in differentiated keratinocytes by immunolocalization of TGN38 (28) and other Golgi markers and determine the relationship of these structures to lamellar bodies.

In the meantime, several findings in our study should stimulate new directions in the fields of both glycosphingolipid function and keratinocyte differentiation. First, our differentiation model in which no exogenous hormones or growth factors are needed to stimulate both differentiation and GCS expression represents an ideal system to study what mechanisms (e.g. Ca2+; hormones, and autocrine factors) are involved in the regulation of GCS expression. Indeed, although the cDNA for human GCS has been cloned, presently there is nothing known about the mechanisms of regulation (e.g. promoter regions and relevant transcription factors) of GCS expression. Second, we found that GCS expression increased in coordination with the level of differentiation, but it is unknown whether GCS is required as part of the normal differentiation program. Disruption of GCS expression (e.g. using antisense oligonucleotides or transfection with mutant GCS constructs) or use of specific inhibitors to block GCS activity (29) during keratinocyte differentiation would allow us to determine whether GCS expression or activity is necessary for keratinocyte differentiation. It will also be of interest to use the tools described in this paper to study the localization of GCS (e.g. by immunolocalization) and the mechanisms by which GlcCer is targeted to lamellar granules in keratinocytes.

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Up-regulation of Glucosylceramide Synthase Expression and Activity during Human Keratinocyte Differentiation

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