In a previous study we observed that long term (5 days) incubation with fumonisin B₁ (FB₁), an inhibitor of acylation of sphingoid long chain bases to (dihydro)-ceramide, resulted in morphological and biochemical changes in 3T3 fibroblasts (Meivar-Levy, I., Sabanay, H., Bershadsky, A. D., and Futerman, A. H. (1997) J. Biol. Chem. 272, 1558–1564). Among these were changes in the profile of synthesis of sphingolipids (SLs) and glycosphingolipids (GSLs). Whereas [³H]globotriaosylceramide ([³H]Gb₃) comprised 1.9% of the total [³H]SLs and [³H]GSLs synthesized in control cells, it increased to 16.5% in FB₁-treated cells. We now demonstrate by in vitro analysis that inhibition of ceramide synthesis by FB₁ for 5 days results in up-regulation of the activities of three enzymes in the pathway of Gb₃ synthesis, namely glucosylceramide, lactosylceramide, and Gb₃ synthases; up-regulation is due to an increase in Vₘₐₓ, with no change in Kᵣ values toward lipid substrates. Moreover, molecular analysis (reverse transcriptase-polymerase chain reaction) of glucosylceramide synthase indicated that this enzyme is up-regulated at the transcriptional level. No changes in either the Vₘₐₓ or Kᵣ values of sphingomyelin or of GM₁ synthase were detected after FB₁ treatment. Analysis of SL and GSL synthesis in cultured cells using [4,5-³H]sphinganine as a metabolic precursor demonstrated that at low substrate concentrations, Gb₃ synthesis is favored over GM₁ synthesis and glucosylceramide synthesis is favored over sphingomyelin synthesis, whereas the opposite is true at high substrate concentrations. These data demonstrate that GSL synthesis and in particular Gb₃ synthesis are tightly regulated in fibroblasts, presumably so as to maintain constant levels of Gb₃ on the cell surface.

Sphingolipids (SLs)³ and glycosphingolipids (GSLs) are ubiquitous and essential components of eukaryotic cell membranes (1). Significant variation exists in the types and levels of both acidic and neutral GSLs between different cells, and although most attention has been paid to the sialic-acidic containing GSLs, the gangliosides (2), neutral GSLs are also found at relatively high levels in a number of tissues (1, 3). Among these is globotriaosylceramide (Galα-1–4Galβ1–4Glcβ1-ceramide; Gb₃). Gb₃ is expressed in many types of human blood cells, including erythrocytes, lymphocytes, and platelets, and its expression is elevated in some lymphomas (4, 5). Sequential changes in the expression of Gb₃ and of other GSLs occurs during B cell differentiation due to sequential activation of the corresponding glycosyltransferases (6). No single function has been ascribed to Gb₃, although it has been implicated as a differentiation antigen for B lymphocytes, as the Pk blood group antigen (7), and as a marker for apoptosis of germinal center B-cells (8). In addition, although not a physiological function, Gb₃ acts as the cell surface receptor for Shiga toxin (9–11).

In a recent study, we demonstrated that upon inhibition of SL and GSL synthesis by fumonisin B₁ (FB₁), an inhibitor of (dihydro)ceramide synthesis (12), levels of Gb₃ synthesis were not reduced (13). Upon incubation of 3T3 fibroblasts for 5 days with 20 µM FB₁, the incorporation of [4,5-³H]sphinganine (a precursor of the synthesis of all SLs and GSLs (14)) into [³H]SLs was reduced by −70% (13). However, whereas [³H]sphingomyelin (SM) and total [³H]GSL synthesis decreased by 75 and 72%, respectively, [³H]Gb₃ synthesis unexpectedly increased after FB₁ treatment by −2.3-fold. This resulted in different profiles of [³H]SL synthesis, with [³H]Gb₃ comprising 1.9% of the total [³H]SLs synthesized in control cells but 16.5% in FB₁-treated cells (13).

In the current study, we examine the molecular mechanisms underlying the differences in profiles of SL and GSL synthesis after long term inhibition of ceramide synthesis by FB₁. By in vitro analyses, we demonstrate that three glycosyltransferases in the metabolic pathway leading to Gb₃ synthesis are up-regulated. In addition, in vivo analysis demonstrates that differences in Kᵣ values also result in a preference for Gb₃ synthesis when ceramide is synthesized at low levels. That fibroblasts specifically maintain cellular Gb₃ levels, in preference over other GSLs and SLs, adds weight to the idea that Gb₃ plays one or more essential, although as yet unidentified, physiological functions in fibroblasts and presumably in other cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

FB₁ was from the Division of Food Science and Technology (CSIR, Pretoria, South Africa). β-Sphinganine and lactosylceramide (LacCer) were from Matreya (Pleasant Gap, PA). N-Hexanoic acid (1.4Cl)n-hydroxysuccinimide ester (55 mCi/mmol) was from American Radioactive Chemicals (St. Louis, MO). CMP-N[(14C)sialic acid (294 mCi/mmol) and UDP-N[(14C)]Gal (261 mCi/mmol) were from Amersham International plc (Amersham, UK). Titan™ one tube RT-PCR system was from Boehringer Mannheim. Silica Gel 60 plates were from Merck. Ultima Gold was from Packard (Meriden, CT). Other chemicals were from Sigma, and solvents (analytical grade) were from Bio-Lab Laboratories Ltd. (Jerusalem, Israel).

**Cell Culture**

NIH 3T3 fibroblasts were cultured in Dulbecco's modified medium containing 10% calf serum and maintained in a water-saturated atmosphere of 5% CO₂. Cells were dissociated with trypsin/EDTA and plated in either 100-mm culture dishes for in vitro experiments or 60-mm
culture dishes for in vivo experiments, both at densities of ~5 × 10^4 cells/ml of medium. Medium was not changed before harvesting.

In Vitro Analysis of SL Synthesis

Preparation and Analysis of Lipids—N-[(1-[^14]C)Hexanoyl]-β-erythro-sphingosine ([^14]C)hexanoyl Cer) and [^14]C]hexanoyl GlcCer were synthesized by N-acetylation of sphingosine and glucosylphosphoinosine, respectively, using the N-hydroxysuccinimide ester of 1-[^14]C]hexanoic acid (15). [^14]C]Hexanoyl-lipids were prepared as a complex with defatted bovine serum albumin (molar ratio, 1:1) (16).

GlcCer Synthase (UDP-Glucose: N-Acylphosphoinosine β1,2-Galactosyltransferase)—Cells were washed three times with phosphate-buffered saline, harvested using a rubber policeman, and homogenized in a hand-held Potter-Elvehjem homogenizer in 1 ml of 250 mM sodium cacodylate buffer (pH 7.4) (TK buffer). Homogenates were used fresh. GlcCer synthase was assayed in homogenates (50 µg of protein) from control or FB1-treated fibroblasts. GlcCer synthase activity was assayed as described (22) with some modifications. Briefly, 200 µg of a cell homogenate was incubated with 10 µM [^14]C]hexanoyl GlcCer, 10 mM MgCl₂, 50 mM NaCl, and 5 mM UDP-Gal in total volume of 500 µl of 250 mM sucrose/10 mM Tris (pH 7.4) for 2 h at 37 °C.[^14]C]Hexanoyl lipids were extracted and analyzed as described above.

Gb₃ Synthase (UDP-Gal: Lactosylceramide 1,4-Galactosyltransferase)—Cells were homogenized in a hand-held Potter-Elvehjem homogenizer in 1 ml of 250 mM sodium cacodylate buffer (pH 5.9). Gb₃ synthase activity was assayed as described (22) with some modifications. The reaction mixture contained 500 µg of cell homogenate, 5–50 µM of LacCer, 0.3% Triton X-100, 10 mM MnCl₂, 1.4 µM UDP[^14]C]Gal (0.375 µCi), 50 mM sodium cacodylate buffer (pH 5.9) in a total volume of 1 ml. Incubations were performed for 4 h at 37 °C with vigorous shaking, and reactions were terminated by addition of 3 ml chloroform/methanol (2:1 v/v) and extracted (22). Lipids were separated by TLC using chloroform/methanol/CaCl₂ (60:35:8 v/v/v) as developing solvent. TLC plates were exposed to a[^14]C-sensitive imaging plate, lipids were recovered from the plates by scraping, and radioactivity was determined by liquid scintillation counting.

[^14]C]Sialic acid—[^14]C]Sialic acid (0.25 µCi) (diluted 1:5 in CMP-[^14]C]sialic acid) was used as a substrate for GM₃ synthase activity. The reaction mixture contained 500 µg of cell homogenate, 5–50 µM of LacCer, 0.2% Triton CF-54, 10 mM MnCl₂, 0.85 µM CMP-[^14]C]sialic acid (0.25 µCi) in a total volume of 1 ml. Incubations were performed for 4 h at 37 °C with vigorous shaking, and reactions were terminated by addition of 3 ml chloroform/methanol (2:1 v/v). Lipids were extracted and analyzed as described above for Gb₃ synthase.
In vivo analysis of SL metabolism

[4,5-³H]Sphingosine was synthesized by reduction of D-erythro-sphingosine with NaB[³H]₄ (10 Ci/mol) (14, 23, 24). After 24 h of incubation with [4,5-³H]sphingosine, cells were washed with phosphate-buffered saline, removed by scraping with a rubber policeman, and centrifuged (15,000 × g, 30 min, 4 °C). Protein was determined (18), and [³H]SLs/[³H]GSLs were extracted and analyzed exactly as described (13). Upon metabolism of [4,5-³H]dihydroceramide to [³H]sphingosine, we assume that 50% of the [³H] radioactivity is lost due to dehydrogenation of the 4,5-double bond; this was taken into account when quantifying [³H]GSL synthesis as described previously (14).

RT-PCR analysis of GlcCer synthase

Partial GlcCer synthase cDNA (~400 base pairs) based on the sequence of rat GlcCer synthase (28) was obtained from NIH 3T3 fibroblasts by RT-PCR using the following GlcCer synthase primers: 5′-TTGGTCGGCTCCGTGCTCTT-3′ (forward primer) and 5′-GACTCGTAT-TCCGCTATCAC-3′ (reverse primer). Total RNA was isolated by the TRI-Reagent protocol (Molecular Research Center Inc., Cincinnati, OH). RT-PCR products were resolved on 1.5% agarose-gels.

RESULTS

Five major SLs and GSLs are synthesized by 3T3 fibroblasts, namely SM, GlcCer, LacCer, Gb₃, and GM₃ (13). Using [4,5-³H]sphingosine as a precursor of SL and GSL synthesis, we previously observed that residual levels of each lipid differed after 5 days incubation with FB₁ (20 μM) (13), and unexpectedly, Gb₃ synthesis was not inhibited to any extent. No differences in residual levels of SL or GSL synthesis were observed after incubation with FB₁ for short times (i.e. 1 h).³

We have now systematically determined the activity of the five enzymes responsible for the synthesis of each lipid by in vitro analyses and analyzed whether differences in Km values between the different enzymes might account for the change in the profile of SL and GSL synthesis.

In vitro analysis of SL synthesis—Cultured fibroblasts were incubated with FB₁ (20 μM) for various times, removed from the culture dishes, and homogenized, and SM and GlcCer synthases were assayed in vitro using a short acyl chain radioactive analogue of ceramide, [¹⁴C]hexanoyl Cer (15, 17, 20). After 3 h of incubation of fibroblasts with FB₁, there was no difference in the activity of GlcCer synthase compared with control cells, but after 1 day, a 30% increase was detected (Fig. 1). As time of incubation with FB₁ increased, the activity of GlcCer synthase increased to a maximum of ~3-fold higher in cells incubated with FB₁ for 5 days compared with untreated cells (Fig. 1). Addition of FB₁ had no effect on GlcCer synthase activity or on the activity of any of the other enzymes assayed below, when added directly to the reaction mixture. The activity of SM synthase was much lower than GlcCer synthase in vitro, and no change in the level of SM synthase was detected even after 5 days of incubation with FB₁ (Fig. 1).

To determine whether the increase in activity of GlcCer synthase was due to changes in the Vₘₐₓ or in the Km of the reaction, assays were performed using conditions in which the initial reaction rate (Vₜ₀) was linear with respect to time and to protein concentration and was not limited by substrate availability. After 5 days incubation with FB₁, the Vₘₐₓ of GlcCer synthase was 333 pmol/min/mg of protein, compared with 167 pmol/min/mg of protein in control cells (Fig. 2), but the Km values with respect to [¹⁴C]hexanoyl Cer were unchanged (6.3 μM in FB₁-treated cells and 6.5 μM in control cells (Fig. 2)). No change was detected in either the Vₘₐₓ or Km value of SM synthase after 5 days of incubation with FB₁ (not shown).

Because the cDNA encoding GlcCer synthase has recently been isolated (29), we analyzed the molecular mechanisms by which GlcCer synthase activity was regulated. RT-PCR analysis revealed that GlcCer synthase mRNA expression was increased in cells incubated with FB₁ for 5 days (Fig. 3) but not for 3 h (not shown). This demonstrates that GlcCer synthase is up-regulated at the transcriptional level upon long term incubation with FB₁.

We next examined the activity of LacCer synthase using [¹⁴C]hexanoyl GlcCer as substrate. The Vₘₐₓ of LacCer synthase also increased upon incubation with FB₁, for 5 days, from 143 pmol/min/mg of protein in control cells to 250 pmol/min/mg of protein in FB₁-treated cells (Fig. 4). The Km values with respect to [¹⁴C]hexanoyl GlcCer were unchanged (1.25 μM in FB₁-treated cells and 1.57 μM in control cells (Fig. 4)).

Fig. 3. RT-PCR analysis of GlcCer synthase. RNA was extracted from fibroblasts after 5 days of incubation with FB₁. RT-PCR products were resolved on 1.5% agarose gels using the indicated amounts of total RNA. Oligonucleotide markers (400–700 base pairs) are shown in the left-hand lane. Note that RT-PCR analysis was repeated three times, and in every case, more mRNA expression was observed in FB₁-treated than in control cells, although the extent of the increase differed between individual experiments.

Fig. 4. Kinetic analysis of LacCer synthase activity. A, Michaelis-Menten analysis of LacCer synthase in homogenates (200 μg of protein) from control (■) or FB₁-treated (□) fibroblasts. B, double reciprocal plot. Each point is the mean ± S.E. of 2–4 duplicate analyses of LacCer synthase activity.

³ I. Meivar-Levy and A. H. Futerman, unpublished observations.
larly, the activity of Gb3 synthase was 2.7-fold higher in FB1-treated versus control cells (Fig. 5), but in contrast, there was no increase in the V_{max} of GM3 synthase after FB1 treatment for 5 days (Fig. 6), although a small but statistically insignificant reduction in the activity of GM3 synthase was observed after FB1 treatment.

Incubation with FB1 causes depletion of ceramide from the synthetic pathway but also accumulation of sphinganine (12). Incubation of cultured cells directly with sphinganine (10 μM added each day for 5 days) had no effect on enzyme activity measured in vitro (GlcCer, SM, and LacCer synthesized), and co-incubation of FB1 together with sphinganine (10 μM added each day for 5 days) did not change the extent of enzyme up-regulation induced by FB1 (not shown). These results suggest that the effects observed are due to inhibition of ceramide synthesis rather than accumulation of sphinganine.

In Vivo Analysis of [3H]GSL and [3H]SM Synthesis—The data presented above demonstrate that long term inhibition (5 days) of ceramide synthesis results in up-regulation of the activity of three glycosyltransferases that use ceramide or downstream metabolites of ceramide as substrate, GlcCer, LacCer, and Gb3 synthases, but does not affect SM or GM3 synthases. We next examined whether flux to different branches of the SL and GSL synthesis pathway (see Fig. 8) is correlated with substrate levels by directly incubating cultured cells with varying amounts of [4,5-3H]sphinganine, a metabolic precursor of the five lipids analyzed above.

Upon incubation with 30 nM [4,5-3H]sphinganine, [3H]Gb3 comprised 1.9% of the total [3H]SLs and [3H]GSLs synthesized (see also Ref. 13), but at 0.6 nM [4,5-3H]sphinganine, [3H]Gb3 comprised 9.0% (Table I). Because [3H]GM3 comprised 25% of total [3H]SLs and [3H]GSLs synthesized at both high and low [4,5-3H]sphinganine concentrations (Table I), the ratio of [3H]Gb3 to [3H]GM3 synthesis increased ~4-fold as [4,5-3H]sphinganine decreased (Fig. 7A). In addition, whereas [3H]SM comprised 59% of the total [3H]SLs synthesized at 30 nM [4,5-3H]sphinganine, it comprised only 43% at 0.6 nM [4,5-3H]sphinganine (Table I), and as a result the ratio of [3H]GSLs to [3H]SM synthesis increased by ~2-fold as [4,5-3H]sphinganine decreased (Fig. 7B). These results demonstrate that the amount of substrate shunted to one or the other branch of the synthesis pathway varies depending on substrate concentration, with a preference for Gb3 versus GM3 synthesis at low substrate levels.

Analysis of GSL Turnover—To determine whether GSL turnover is also affected upon long term incubation with FB1, we analyzed acid glucosylceramidase activity in an in vitro assay.

**Table I**

| Sphinganine | [3H]GlcCer | [3H]SM | [3H]LacCer | [3H]Gb3 | [3H]GM3 |
|-------------|------------|--------|------------|--------|--------|
| 30          | 8.4 (141 ± 7.2) | 58.8 (985 ± 51.8) | 5.8 (97.1 ± 3.7) | 1.9 (31.6 ± 2.5) | 25.1 (420.8 ± 23.9) |
| 15          | 12.3 (86.4 ± 9.2) | 54.3 (381.5 ± 42.34) | 5.4 (37.7 ± 3.5) | 3.2 (22.5 ± 3.1) | 24.8 (174.5 ± 18.4) |
| 6           | 10.4 (30.7 ± 5.6) | 55.3 (162.8 ± 19.1) | 6.5 (19.3 ± 2.2) | 5.2 (15.2 ± 3.1) | 22.5 (66.3 ± 13) |
| 3           | 9.9 (15.5 ± 2.0) | 55.0 (86.4 ± 11.2) | 5.9 (9.2 ± 1.7) | 4.4 (6.8 ± 1.7) | 24.9 (39.0 ± 4.1) |
| 1.2         | 13.3 (8.0 ± 1.6) | 49.1 (29.4 ± 2.4) | 6.6 (3.9 ± 0.8) | 7.1 (4.3 ± 0.4) | 23.9 (14.3 ± 1.4) |
| 0.6         | 13.6 (4.3 ± 0.4) | 42.9 (13.5 ± 1.1) | 9.7 (3.1 ± 0.4) | 9.0 (2.8 ± 0.2) | 24.8 (7.8 ± 1.1) |

Sphinganine levels refer to total [4,5-3H]-sphinganine added to the medium. The total amount of cell-associated 3H radioactivity (derived from [4,5-3H]-sphinganine) is 4.5 pmol/10⁶ cells for 0.6 nM [4,5-3H]-sphinganine, and 240 pmol/10⁶ cells for 30 nM [4,5-3H]-sphinganine. Of this, TLC analysis indicated that only ~1% is unmetabolized, free [4,5-3H]-sphinganine. Therefore, for all six concentrations of [4,5-3H]-sphinganine in the medium, levels of cell-associated [4,5-3H]-sphinganine are lower than the normal concentration of sphinganine in cells (~5–10 pmol/10⁶ cells).
using [14C]hexanoyl GlcCer (30) as substrate. No significant change in activity was observed for up to 9 days of incubation with FB1 (e.g., glucosylceramidase activity in control cells after 4 days of incubation was 516 ± 1 pmol/min/mg of protein and in FB1-treated cells was 529 ± 45 pmol/min/mg of protein, and after 7 days it was 618 ± 139 and 635 ± 90 in control and FB1-treated cells, respectively).

**DISCUSSION**

The data presented above demonstrate that although only a minor GSL compared with GM3, fibroblasts strive to maintain constant levels of Gb3. When levels of metabolic precursors are low, Gb3 levels are preserved by two mechanisms (Fig. 8), namely (i) up-regulation of the activity of the three glycosyltransferases in the pathway of Gb3 synthesis and (ii) shunting LacCer to Gb3 synthesis rather than to GM3 synthesis. Together, this results in maintenance of Gb3 levels even when levels of metabolic precursors are low. In addition, GSL synthesis is preferred to SM synthesis when ceramide synthesis is low (Fig. 8) (see also Ref. 31). A similar dual mechanism of regulation has been suggested for GM2 and GD2 synthases in human cancer cell lines (32), where expression of GD2 synthase is regulated by levels of its immediate precursor, GD3. Regulation of glycolipid expression by substrate availability has also been observed during development and oncogenesis (33, 34).

Molecular analysis demonstrated that GlcCer synthase is up-regulated at the transcriptional level resulting in increased production of the glycosyltransferase rather than by post-translational modification of existing enzyme. This is supported by the observation that relatively long times of incubation with FB1 (>1 day) were required to detect changes in $V_{\text{max}}$ values. Similarly, GlcCer synthase is induced during keratinocyte differentiation (35) due to transcriptional up-regulation (36). Interestingly, upon long term incubation with FB1, the activity of three glycosyltransferases (GlcCer, LacCer, and Gb3 synthases) is increased, presumably by coordinate regulation at the transcriptional level. At this time, it is not possible to determine whether Gb3 synthase is also regulated at the transcriptional level because this enzyme has not been cloned, although a partial purification of enzyme activity was reported from rat liver (37).

We do not know whether up-regulation of the glycosyltransferase activities is a result of inhibition of ceramide synthesis.
per se or of reduction of GM3 and Gb3 levels. It is well documented that cells respond in various ways to changes in ceramide levels, but most cellular responses are due to increased production of ceramide at the cell surface due to degradation of SM in ceramide-mediated signaling pathways (38) rather than ceramide depletion in the endoplasmic reticulum where it is synthesized (23, 39). Alternatively, fibroblasts may respond to changes in levels of the lipid products (GM3 and Gb3). Depletion of GM3 in fibroblasts results in changes in the actin cytoskeleton and in a block in cell proliferation and DNA synthesis (13), and one of these effects could be an initial signaling event in glycosyltransferase up-regulation. Although we cannot distinguish between biochemical effects due to depletion of GM3 compared with Gb3, it is nevertheless apparent that fibroblasts ascribe particular importance to maintaining Gb3 levels.

That fibroblasts go to such length to maintain cellular Gb3 levels implies that Gb3 plays a key role in fibroblast function. Gb3 is expressed in a restricted set of hematopoietic cells and is considered both a differentiation antigen (40) and more recently as a regulator of apoptosis during differentiation of the hematopoietic system (41). No single function has been ascribed to Gb3 in fibroblasts. We assume that the function of Gb3 is related to its localization at the cell surface and are testing the possibility that Gb3 is found in GSL-enriched domains on the cell surface, as may be the case for both GM3 (42) and GM1 (43). Because a number of transducer molecules also appear to be found in these GSL-enriched domains, such as c-Src, Ras, Rho, and focal adhesion kinase, the presence of Gb3 in these domains might suggest a regulatory role in signaling events related to cytoskeletal organization; indeed, we have recently shown that GM3 mediates events associated with assembly of the actin cytoskeleton (13). This possible role has not been tested for Gb3, but if Gb3 is also enriched in these domains, it will be of importance to determine the mode of interaction of these two structurally similar GSLs with both signaling molecules and with the underlying cytoskeleton.

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