Demonstration of Direct Glycosylation of Nondegradable Glucosylceramide Analogs in Cultured Cells*

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but not those of lactosylceramide, sialyl lactosylceramide. This observation is further confirmed by the fact that the cationic ionophore monensin, known to impede membrane flow from proximal to distal Golgi cisternae, inhibited the formation of complex ganglioside analogs.

The cationic ionophore monensin, known to impede membrane flow from proximal to distal Golgi cisternae, inhibited the formation of complex ganglioside analogs but not those of lactosylceramide, sialyl lactosylceramide (G₃₃) and disialyl lactosylceramide (G₆₃).

On the cell surface of vertebrate cells, glycosphingolipids (GSL)1 form cell-specific patterns which change specifically with cell differentiation, morphogenesis, and oncogenic transformation (for review see Refs. 1 and 2). Although being sur-
mised to be the place for the functional role of GSL, the plasma membrane is not the site of their metabolism. Rather, GSL are synthesized in the Golgi complex by sequential addition of monosaccharide units to ceramide and are degraded in lysosomes by the sequential removal of glycosyl residues starting from the nonreducing end. Thus, the maintenance of a balanced glycolipid profile requires a stringent control of metabolism and transport of GSL (for review see Refs. 3 and 4).

We have observed labeled and cell-specific glycosylation products when a radioactive or fluorescent analog of glucosylceramide had been administered to cultured cells. These products (analogs of globosides and gangliosides) could have been formed in the Golgi complex by direct glycosylation of the labeled glucosylceramide analogs or from the labeled ceramide analogs; the latter resulting from deglucosylation, most likely in lysosomes, of the former. Therefore, we have studied the metabolism of glucosylceramide analogs that contain sulfur in the glycosidic bond (Fig. 1) and are thus resistant to enzymatic deglucosylation. We will present data showing unambiguously that a direct glycosylation of these analogs of glucosylceramide takes place in various cultured cells leading to a cell-specific pattern of labeled glycolipid analogs.

EXPERIMENTAL PROCEDURES

Materials—SHSY5Y cells were kindly provided by Dr. H. Röser (Stuttgart, Germany). Thin layer chromatography plates (Silica Gel 60) were from Merck (Darmstadt, Germany). Defatted bovine serum albumin and monensin were from Sigma (Deisenhofen, Germany). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, horse serum, and Vibrio cholerae sialidase were from Boehringer (Mannheim, Germany). Galα₁β-galactosidase (5) and α-galactosidase (6) were prepared from human placenta (7), and Galα₂-activator protein was obtained as described (8). The glucosylthioceramide analogs contain either a radio carbon-labeled octyl (O⁻¹⁴C₅₆-Glc-S-Cer) or an 8-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminoceroyl residue (NBD-C₈-Glc-S-Cer) in place of a native acyl moiety and were prepared as described (9). The reference [O⁻¹⁴C₅₆-glycosphingolipids and NBD-C₈-glycosphingolipids as well as [O⁻¹⁴C₅₆-Gb₅₆S-Cer and NBD-C₈-Gb₅₆S-Cer were prepared according to Ref. 10 and were available in this laboratory. All other chemicals were of the highest purity available.

Preparation of the Glucosylthioceramides from BSA Complexes—An aliquot of a stock solution of the desired glucosylthioceramide (100 nmol) in methanol was dried, first under a stream of nitrogen and then in vacuo. The dried lipid was dissolved by first adding 20 μl of ethanol and then 1 ml of DMEM containing 7 mg of defatted BSA under vigorous stirring. The resulting clear solution was diluted with 9 ml of DMEM to yield a 10 μM lipid-BSA complex.

Cell Culture—Monolayer cultures of human skin fibroblasts obtained from biopsy of a male infant and rat neuroblastoma cells B 104 were cultured as described (11). Human neuroblastoma-derived SHSY5Y cells were grown as described (12). All cells were grown at 37°C in a water-saturated atmosphere of 5% CO₂ in air. Cultures were seeded with 7 × 10⁶ fibroblasts or B 104 in 25-cm² tissue culture flasks. The cultures were then grown for 1 to 2 days. For human neuroblastoma cells, 10⁶ SHSY5Y cells were seeded and grown for 4 days prior to use. Murine neuroblastoma cells were prepared essentially as described (13) and were plated onto poly(L-lysine)-coated 35-mm-diameter Petri
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Fig. 1. Structures of labeled glucosylthioceeramides. * denotes position of the radiocarbon.

| Incorporation of radilabeled lipids | Incorporation of fluorescent lipids |
|------------------------------------|-----------------------------------|
| Fibroblasts                        | B 104 cells                       |
| SHSY5Y cells                       | Cerebellar cells                  |
| nmol lipid/mg protein              | nmol lipid/mg protein             |
| 23.7 ± 2.1                        | 13.2 ± 0.2                       |
| 12.0 ± 1.7                        | 33.8 ± 4.0                       |
| 32.8 ± 4.0                        | 57.3 ± 4.0                       |
| 37.2 ± 5.0                        |                                   |

RESULTS

Uptake of Labeled Glucosylthioceramides by Cultured Cells—We first examined the uptake of [14C]Glc-S-Cer and NBD-C8-Glc-S-Cer using equal concentrations of radioactive lipid-BSA and fluorescent lipid-BSA complexes. All cell types were incubated for 2 h at 37°C with a 10 μM concentration of either complex, washed, and further incubated for 20 h in DMEM devoid of the labeled lipid-BSA complexes, but containing 0.3% heat-inactivated fetal bovine serum or heat-inactivated horse serum in the case of murine cerebellar cells. During the 20-h incubation, some protein(s) of the sera extracted a considerable percentage of the glucosylthioceramide analogs and their metabolites from the plasma membrane of cells. Therefore, the labeled lipids were isolated from the incubation media, quantified, and combined with the lipid extract of the corresponding cells. The amount of cell-associated lipids is the sum of the lipids extracted by the medium and isolated from the cell pellet and is normalized to cellular protein. The data are means of three different experiments.

Identification of Labeled Metabolites—Glycosylation products were scraped from TLC plates and analyzed as follows. The radioactive analog of globotriaosylceramide from fibroblast extract was characterized by FAB-MS and identified by its degradation to the radioactive analogs of total lipid extracts were degraded by two subsequent actions of glycohydrolases (fibroblasts: β-hexosaminidases and α-galactosidase, SHSY5Y cells: β-hexosaminidases and sialidase, and murine cerebellar cells: sialidase and Gm(α2,3)-galactosidase) as outlined in Fig. 4. Briefly, extracted lipids were dissolved in a total volume of 330 μl of 85 mM sodium citrate buffer, pH 4.3, containing 2 mM taurodeoxycholate in addition to the respective enzymes. For β-hexosaminidases, sialidase, or α-β-galactosidase, 17,000, 1,300, 185, or 17 picokatal, respectively, were used. To assays containing β-hexosaminidases Gm(α2,3)-activator protein (1 unit according to Ref. 19) was also added. After 10 h at 37°C, each assay was split and one-half was treated further with the second enzyme as outlined in Fig. 4. The lipid extract of B 104 cells was treated with sialidase as described above for murine cerebellar cells.

TABLE I

Uptake of [14C]Glc-S-Cer and NBD-C8-Glc-S-Cer by four different cell types

Human skin fibroblasts, rat neuroblastoma B 104, human neuroblastoma SHSY5Y, and murine cerebellar cells were incubated with a 10 μM lipid-BSA complex of [14C]Glc-S-Cer and NBD-C8-Glc-S-Cer, respectively, in the presence of “Experimental Procedures.” The total amount of cell-associated lipids is the sum of the lipids extracted by the medium and isolated from the cell pellet and is normalized to cellular protein. The data are means of three different experiments.

| Human skin fibroblasts | B 104 cells |
|------------------------|-------------|
| 25.5 ± 2.1             | 13.2 ± 0.2  |
| 11.7 ± 1.7             | 23.1 ± 4.0  |

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Testing of the Incubation Media for Glycosyltransferase Activities—In a total assay volume of 100 μl of DMEM, pH 7.4, containing 50 nmol each of CMP-NeuAc, UDP-Gal, UDP-GalNAc, as well as 6 μl of heat-inactivated fetal bovine serum or horse serum, 5 nmol of [14C]Glc-S-Cer was incubated for 22 h at 37°C. After the addition of methanol (50 μl), salts and nucleotide sugars were removed according to Ref. 16. The lipids were analyzed by TLC (Fig. 3).

Miscellaneous Procedures—Fast atom bombardment mass spectrometry (FAB-MS) was carried as described (20) using a ZAB HF instrument (VG Analytical, Manchester, UK). Protein was quantified as described (15) using BSA as standard.
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Fig. 2. Postendocytotic glycosylation of $[^{14}C]_{C_8}$-Glc-S-Cer in various cell types. Human skin fibroblasts, rat neuroblastoma B 104, human neuroblastoma SHSY5Y, and murine cerebellar cells were incubated with $[^{14}C]_{C_8}$-Glc-S-Cer as described under "Experimental Procedures." In cases where monensin was used, this drug was present in a 1 μM concentration at all times of incubation. The incubation media were saved, and the cells were harvested with a rubber policeman. The lipids of cells and media were desalted and combined prior to separation by TLC using chloroform/methanol/15 mM calcium chloride (60:35:8, by volume) as developing system. The radioactive lipids were visualized by exposure to x-ray-sensitive film. In the right-hand margin, the mobilities of $[^{14}C]_{C_8}$-Glc-S-Cer and its glycosylation products, if produced in the respective cell type, is denoted by the standard abbreviation for GSL as outlined in Footnote 1. $x_1$, $x_2$, $x_3$, and $x_4$ denote the sulfates of $[^{14}C]_{C_8}$-Glc-S-Cer and the putative sulfates of the corresponding glycolipids of human fibroblasts, human neuroblastoma B 104 cells, and GM3, respectively.

Fig. 3. Glycosylation products of $[^{14}C]_{C_8}$-Glc-S-Cer in fibroblast pellets and incubation media. Human skin fibroblasts were incubated with $[^{14}C]_{C_8}$-Glc-S-Cer as for Fig. 2. The lipids of cells and media were desalted and separated by TLC as for Fig. 2. To test for any glycosyltransferase activity in the incubation media, these media were incubated in the presence of $[^{14}C]_{C_8}$-Glc-S-Cer and nucleotide sugars as described under "Experimental Procedures." The radioactive lipids were visualized by exposure to x-ray-sensitive film. In the right-hand margin, the mobilities of $[^{14}C]_{C_8}$-Glc-S-Cer and of its glycosylation products is denoted by the standard abbreviation for GSL as outlined in Footnote 1.

Human skin fibroblasts, rat neuroblastoma B 104, human neuroblastoma SHSY5Y, and murine cerebellar cells were in-

FAB-MS. The structures of the other glycolipid analogs have been corroborated by sequential enzymatic degradation (Fig. 4). Thus, treatment of the glycosylation products of SHSY5Y cells with hexosaminidases resulted in a shift in both main bands to bands of analogs of G03 and G04 (Fig. 4, lane 6).

Subsequent sialidase treatment further shifted these bands to labeled lactosylceramide analog with the same property as an authentic synthesized lactosylceramide analog (reference lane), whereas the G01 analog remained unchanged (Fig. 4, lane 7). Similarly, sialidase treatment of glycosylation products of murine cerebellar cells led to an increase in the analogs of G01 and lactosylceramide (Fig. 4, lane 8) which on subsequent $\beta$-galactosidase treatment were converted mostly to the G02 analog and glucosylthioceramide, respectively (Fig. 4, lane 9). When human fibroblasts were treated with hexosaminidases, the bands of G01 and globotriaosylceramide analogs remained, whereas the bands of G02 and globotetraosylceramide analogs diminished (Fig. 4, lane 2). Subsequent hydrolysis with $\alpha$-galactosidase (Fig. 4, lane 3) converted the latter only slightly into the corresponding lactosylceramide. Therefore, the putative globotriaosylceramide analog was isolated from the TLC plate and treated with $\alpha$-galactosidase again. Fig. 5 demonstrates that about 50% of this lipid was hydrolyzed to lactosylceramide analog, confirming the data obtained by FAB-MS. Sialidase treatment of the lipid extract of B 104 cells diminished the analog of G03 while increasing the corresponding lactosylceramide. These results proved the structures of the glycosylation products of $[^{14}C]_{C_8}$-Glc-S-Cer.

The thick band just below glucosylthioceramide in all lipid extracts (denoted by $x_3$ in Figs. 2, 3, and 4) is formed predominantly in the process of cell incubation. After isolation from the TLC plate, its structure was shown by FAB-MS to be the oxidation product, i.e. the sulfoxide of $[^{14}C]_{C_8}$-Glc-S-Cer. When treated with trimethylsilyl iodide according to Ref. 17, the sulfoxide was reduced to $[^{14}C]_{C_8}$-Glc-S-Cer (Fig. 6, lane 3). This

Each cell type displayed a different glycosylation pattern. From comparison of the product bands to labeled glycolipid analogs with similar structures (reference lane) and from metabolic labeling of the endogenous glycolipid pattern of each cell type, it became immediately obvious that the undegradable radioactive glucosylceramide analog had been glycosylated to yield a cell-type specific pattern of glycolipid analogs. Thus, in fibroblasts, obviously those of globobiosides and GM3 were predominantly formed (Fig. 2, lane 2). In rat neuroblastoma B 104 cells, high amounts of the analogs of GM3 and smaller amounts of lactosylceramide were produced (Fig. 2, lane 4), whereas in murine cerebellar cells, labeled ganglioside analogs of GM3, G02, G01, G04, and G05, were present (Fig. 2, lane 8). These data agreed perfectly with the endogenous pattern obtained by metabolic labeling of the glycolipids of these cells (12). In human neuroblastoma SHSY5Y cells, the analogs of GM2 and G02, were the prevalent glycosylation products formed (Fig. 2, lane 6). This result again complies with the endogenous glycolipid pattern (12). We could also observe that the glycosylation products found in the incubation media were the same as those found in the respective cell pellets. This is demonstrated for fibroblasts in Fig. 3 (lanes 3 and 4). These patterns comply well with that of Fig. 2 (lane 2). In addition, we have shown that the incubation media containing 0.3% heat-inactivated fetal bovine serum were devoid of any detectable glycosyltransferase activity (Fig. 3, lane 2). The same holds for heat-inactivated horse serum (data not shown).

Identification of Glycosylation Products—The structures of some glycosylation products, i.e. globotriaosylceramide analog from fibroblasts and lactosylceramide, as well as GM3 analogs from rat neuroblastoma B 104 cells, have been confirmed by
sulfoxides could also be obtained by treating glucosylthioceramides with methanolic hydrogen peroxide. Under this condition, the corresponding sulfone is also produced. Both products could be separated by TLC (Fig. 6, lane 4 and 5) and their structure proven by FAB-MS. The faint bands seen between \([^{14}C]C_9\)Glc-S-Cer and its sulfoxide in Fig. 4 (denoted by \(y\) in lanes 6 and 7) may well represent this sulfone. The sulfoxide of \([^{14}C]C_9\)Glc-S-Cer also seems to be prone to glycosylation as most clearly demonstrated by the prominent band just below the \(G_{M3}\), analog for the B 104 cell extract (denoted by \(x_2\) in Fig. 2, lane 4).

Effect of Monensin on the Glycosylation of \([^{14}C]C_9\)Glc-S-Cer

In this paper we present, for the first time, an unambiguous proof for direct glycosylation of glucosylceramide analogs in various cultured cell types. In human fibroblasts, rat neuroblastoma B 104 cells, and murine cerebellar cells, the glycosylation pattern obtained was identical with the pattern that is obtained by metabolic labeling of these cells (14). Human neuroblastoma SHSY5Y cells also yielded a glycosylation pattern which completely agreed with the endogenous glycolipid pattern (12). The amount of the fluorescent glycosylation products was, however, much less than that of the radioactive anabolites (Table II) indicating that the fluorescent tag somehow inter-

**DISCUSSION**

In this paper we present, for the first time, an unambiguous proof for direct glycosylation of glucosylceramide analogs in various cultured cell types. In human fibroblasts, rat neuroblastoma B 104 cells, and murine cerebellar cells, the glycosylation pattern obtained was identical with the pattern that is obtained by metabolic labeling of these cells (14). Human neuroblastoma SHSY5Y cells also yielded a glycosylation pattern which completely agreed with the endogenous glycolipid pattern (12). The amount of the fluorescent glycosylation products was, however, much less than that of the radioactive anabolites (Table II) indicating that the fluorescent tag somehow inter-
For glycosphingolipids, the Golgi apparatus is the well accepted site for their biosynthesis. It is believed that lactosylceramide and gangliosides G_{M3} and G_{D3} are synthesized presumably at the proximal Golgi cisternae, and that the more complex glycosphingolipids are synthesized in distal Golgi membranes (23). The membrane flow from proximal to distal Golgi membranes is inhibited by monensin (for review, see Ref. 24). Thus, from our experiments with monensin, we infer that the glycosphingolipids were conveyed to Golgi cisternae that function in the early glycosylation steps in glycolipid biosynthesis, i.e. the formation of lactosylceramide and gangliosides G_{M3} and G_{D3}. The formation of analogs of lactosylceramide and more complex glycolipids. Also, the mode of uptake of the exogenous supplied glucosylceramide via liposomes may have directed this lipid to cellular compartments active in another glycosylation reaction, which is not deficient in Gaucher disease (27) or active in unspecific b-glucosidases that fall within the normal range of activity in patients with all forms of Gaucher disease (28).

An important question is whether native glucosylceramide and perhaps other glycosphingolipids molecules with long acyl chains would participate in the transport process we have observed for the labeled glucosylthioceramides. If so, would this have implications in terms of regulation of glycolipid biosynthesis? To address this question, we intend to perform studies employing undegradable analogs of glucosylceramide and other glycosphingolipids carrying long acyl chains in their ceramide portion. Even if the short chain glucosylceramide analogs should not mimic the endocytotic pathway of the endogenous glycolipids, they are valuable tools for revealing membrane transport that otherwise may be overlooked and that may be important for cellular events. Further experiments are needed to clarify this point.

Acknowledgments—We thank Dr. H. Rössner, University of Hohenheim, Germany, for the supply of SHSY5Y cells. We thank Martina Feldhoff for the preparation of murine cerebellar cells and for culturing fibroblasts, B 104, and SHSY5Y cells. We thank Dr. G. Pohlentz, University of Bonn, for recording the FAB-MS spectra.

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