Homeostasis of Cell-surface Glycosphingolipid Content in B16 Melanoma Cells

EVIDENCE REVEALED BY AN ENDOGLYCOCERAMIDASE*

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This paper describes the homeostasis of glycosphingolipid (GSL) on the cell surface as revealed for the first time by an application of endoglycoceramidase (EGCase) capable of hydrolyzing the linkage between the oligosaccharide and the ceramide of various GSLs. When cell-surface GSLs of B16 melanoma cells were hydrolyzed by the action of EGCase, the synthesis of GSLs was found to increase transiently, possibly due to the activation of UDP-glucose:ceramide glucosyltransferase. As a result, the cell-surface GSL content was restored quickly to exactly the same level found without the EGCase treatment, if EGCase was removed from the cell culture. Treatment of erythrocytes with EGCase was found to increase the ceramide content of the plasma membrane. Surprisingly, however, in B16 cells the increase of membrane ceramide by EGCase caused the suppression of de novo ceramide production, resulting in maintenance of the ceramide content of B16 cells at the same level even after EGCase treatment. The signal for homeostatic regulation could be the ceramide released by the action of EGCase, since C2-ceramide was found to mimic in part the action of EGCase; it suppressed de novo production of ceramide and was directly converted to GSL, NeuAcα2,3Galβ1,4Glcβ1,1 N-acetylglucosamine (C2-ceramide GM3). Our finding demonstrates a novel form of homeostatic regulation coupled to the GSL-synthesizing system in mammalian cells for maintaining the contents of both cell-surface GSLs and free ceramide. Since many opportunistic pathogens were found to produce EGCase extracellularly, this restoration mechanism could also be present as a defense mechanism against microbial EGCase.

Glycosphingolipids (GSLs) are characteristic constituents of plasma membranes of mammalian cells and may modulate cell proliferation, differentiation, and cell-cell interaction (1). Although both the GSL content and the composition were found to change drastically during cellular differentiation and oncogenic transformation (2), under static conditions they could be kept constant in individual organelles, cell types, and organs, in spite of a continuous lipid flow between plasma membranes and intracellular organelles (3, 4). However, the molecular mechanism for maintaining the content and composition of cell-surface GSLs is presently not well understood.

Endoglycoceramidase (EGCase) is an enzyme that specifically hydrolyzes the linkage between oligosaccharide and ceramide of various GSLs (5, 6). By using EGCase with the assistance of its protein activator (7, 8), cell-surface GSLs of erythrocytes were found to be hydrolyzed specifically without damaging other cell membrane components (9, 10). We observed that the decrease of the GM3(NeuAc) content of B16 melanoma cells during EGCase treatment was much slower than GM3(NeuGc) of equine erythrocytes, although the initial velocity of EGCase toward GM3(NeuAc) is exactly the same as that toward GM3(NeuGc) (10). This observation motivated us to undertake this study. We report here a novel form of homeostatic regulation of cell-surface GSLs coupled to a GSL-synthesizing system that may include the activation of UDP-glucose ceramide glucosyltransferase.

Treatment of erythrocytes with EGCase was found to increase the ceramide content of the plasma membrane (9). Ceramide (11–14) and its metabolites, ceramide 1-phosphate (15), sphingosine (16), sphingosine 1-phosphate (17) and N,N-di-methylsphingosine (18), were found to evoke various physiological effects on various types of cells. Surprisingly, however, we also observed that in B16 cells the increase of membrane ceramide by EGCase causes the suppression of de novo ceramide production, with the result that the ceramide content in B16 cells is maintained at the same level even after EGCase treatment. This study clearly demonstrates the presence of a novel form of homeostasis at the cellular level, which maintains the content of both cell-surface GSLs and ceramide in mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibody M2590 and FITC-conjugated goat anti-mouse IgM were purchased from Cosmo Bio. Co. C2-ceramide was purchased from Matreya. [14C]Gal was obtained from DuPont NEN, and TLC and high performance TLC plates (silica gel 60) were from Merck, Germany. C2-ceramide GM3 was obtained from Wako Co., UDP-Glc from Nacalai Tesque, Inc., Japan, and C6 NBD-ceramide from Molecular Probes Co. c-thre-PDMP was kindly provided by Dr. J. Inokuchi of the Seikagaku Co., Japan.

Preparation of EGCase and Activator Protein—EGCase II was isolated from the culture supernatant of a Rhodococcus sp. M-750 as described in Ref. 6. Activator II, which specifically stimulates the activity of EGCase II, was isolated from a Rhodococcus sp. M-777 as described in Ref. 7. In this study, a 27.9-kDa polypeptide possessing
activity identical to that of the native activator II (69.2 kDa) was prepared from activator II by trypsin treatment followed by a trypsin inhibitor column as described in Ref. 8. This 27.9-kDa polypeptide and EGCase II were used for all experiments in this study and are referred to simply as activator and EGCase, respectively. The EGCase and the activator preparations used in this study each showed a single protein band after staining with Coomassie Brilliant Blue R. Both preparations contained no exoglycosidases, proteases, sphingomyelinase, and GlcTase.

Cell Culture and Treatment with EGCase—B16 melanoma cells were grown in MEM supplemented with 10% FCS at 37°C in a humidified 95% air, 5% CO2 incubator. For the hydrolysis of cell-surface GSLs with EGCase, cells were seeded in 24-well microplates and preincubated for 3 h to attach the cells to the plate. After preincubation, the medium was replaced with 200 μl of fresh MEM supplemented with 5% FCS containing 200 nmoI of EGCase and incubated at 37°C in a CO2 incubator for the time indicated.

Determination of GM3 Hydrolysis of B16 Melanoma Cells and Erythrocytes—For the determination of GM3 hydrolysis of B16 cells, GM3 remaining in the cells after EGCase treatment was determined by high performance TLC as described in Ref. 9. Hydrolysis of cell-surface GM3 of erythrocytes was determined by the measurement of GM3 oligosaccharides released by the action of EGCase using high-performance anion-exchange chromatography with pulsed amperometric detection (Dionex) as shown in Ref. 9.

Metabolic Labeling—B16 melanoma cells before and after EGCase treatment were incubated with 200 μl of fresh MEM supplemented with 5% FCS containing 1 μCi of either [14C]Gal or [3H]Glc for the time indicated.

Extraction of GSLs and Ceramide and Analysis by TLC—B16 cells were harvested by centrifugation (800 rpm for 10 min) and washed twice with PBS. For extraction of GSLs, the cells were suspended in 750 μl of isopropyl alcohol/hexane/water (55:35:10, v/v) and subjected to sonication for 20 min and centrifuged at 13,000 rpm for 5 min. For extraction of ceramide, chloroform/methanol (2:1, v/v) was used. The supernatants obtained were dried under N2 gas and dissolved in 20 μl of chloroform/methanol (2:1, v/v) and applied to the TLC plate, which was then developed with chloroform/methanol/0.2% KCl (5:4:1, v/v). GSLs and chloroform/methanol/NH4OH (90:10:1, v/v) for ceramide. Each radioactive GSL and ceramide separated on a TLC plate was analyzed and quantified by an imaging analyzer (BAS1000 model, Fuji Film, Japan).

Staining of Cell-surface GM3 with a Specific Monoclonal Antibody—To determine the content of GM3, a specific monoclonal antibody M2590 (Pierce) with bovine serum albumin as the standard (20) was used. B16 melanoma cells were incubated at 37°C for 6 h in 400 μl of MEM containing 5% FCS with 40 milliunits of EGCase in the presence of 20 nmoI of activator.

RESULTS

Hydrolysis of Cell-surface GM3 of B16 Melanoma Cells and Its Restoration—The decrease in the content of GM3 (NeuAc) of B16 melanoma cells after EGCase treatment was found to occur much more slowly than that of GM3 (NeuGc) of equine erythrocytes (Fig. 1A), although the hydrolysis rate of GM3 (NeuAc) by EGCase was previously found to be identical to that of GM3 (NeuGc) (10). It was also noted that the GSLs of plasma membrane fractions of A431 cells were hydrolyzed much faster by EGCase than those of intact cells (22). These discrepancies of apparent hydrolysis rate, therefore, may arise because these cells or membranes either possess the synthetic pathway for GSLs or they do not. The GM3 content of B16 cells was thus examined immediately after EGCase treatment and also after re-culturing in MEM without the enzyme. It was of interest that the level of GM3 was restored to the same as that without EGCase treatment after 3–6 h when EGCase was removed from the cell culture, and the content of GM3 was strictly maintained at the same level, 0.8–0.9 nmol/106 cells, for 24 h (Fig. 1B). In order to examine whether restoration might occur on the cell surface of B16 cells, this factor was examined by flow cytometry after staining the cells with the monoclonal antibody M2590, which is specific to GM3 (19). The cell-surface GM3, which is an end product in B16 cells, was intensely stained with this monoclonal antibody (Fig. 2A). The content was reduced by EGCase treatment (Fig. 2D) but recovered quite rapidly after the removal of EGCase from the culture (Fig. 2E). After 6 h the cell-surface GM3 content of about 85% of the cells was completely restored to the original level before treatment with EGCase (Fig. 2F). It should be noted that either PDPD (23), an inhibitor of GSL synthesis, enhanced the disappearance of cell-surface GSLs by EGCase (Fig. 2C), whereas PDPD did not affect EGCase activity.

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2 Y. Hirabayashi, personal communication.
Activity of B16 Melanoma Cells after EGCase Treatment—
Since GSLs on the cell surface are quite stable and their turnover is very slow under static conditions, the restoration of cell-surface GSLs observed in this study might be due to a novel form of restoration mechanism coupled to GSL synthesis. Therefore, we examined the actual GSL synthesis of B16 cells with or without treatment by EGCase. When the GSL synthesis of B16 cells after EGCase treatment was evaluated by measuring the incorporation of [14C]Gal into GSL, the synthesis of GM3 and its precursors, ceramide monohexoside and lactosylceramide, was found to increase about 2-fold compared with that of control experiments without EGCase treatment (Fig. 3). At the same time it was observed that the UDP-Glc-ceramide glucosyltransferase (GlcTase) activity in the cell lysate of B16 melanoma cells was approximately doubled due to the treatment with EGCase (Fig. 4). Furthermore, it is significant that both GlcTase activity and GM3 synthesis decreased to the control level (Fig. 4, A and B) when the level of GM3 was restored to the level found before EGCase treatment (Fig. 2F). This result clearly indicates that the increase of GSL synthesis, possibly via GlcTase activation, is a transient phenomenon coupled to cell-surface GSL hydrolysis by EGCase. The activation of GlcTase seems to occur posttranslation, since even in the presence of cycloheximide an increase of GM3 synthesis was observed after EGCase treatment (data not shown).

This restoration mechanism could be present universally in mammalian cells, since an increase of GSL synthesis after EGCase treatment was observed not only in B16 cells but also in HL60 myelocytic leukemia cells and Swiss 3T3 fibroblasts.

Content and de Novo Synthesis of Ceramide in B16 Cells after EGCase Treatment—We examined the effect of EGCase on ceramide contents of B16 melanoma cells in which the ceramide portion of GSLs was first metabolically labeled with [14C]Ser. Fig. 5A shows the increase of ceramide in B16 cells after EGCase treatment in which de novo synthesis of ceramide was excluded from the estimation; B16 cells were treated with EGCase in the absence of [14C]Ser (found at 240% of the control, the value is the total of the upper and lower bands and the mean from triplicate determinations). It was noted that the ceramide of the upper band on TLC increased much more than that of lower band (Fig. 5A). According to a previous report (19), the upper band may be composed of C24:0 and C24:1 sphingosines, while both sphingosines may be d18:1. Interestingly, we observed that the ceramide content in B16 cells was not changed at all, even after EGCase treatment, when de novo synthesis of ceramide was included in the esti-
mation; B16 cells were incubated with EGCase in the presence of \([^{14}C]S\text{er}\) (found at 97% of the control, the value is the total of the upper and lower bands and the mean from triplicate determinations; Fig. 5B). De novo synthesis of ceramide was therefore examined independently with or without treatment with EGCase. It was confirmed that EGCase treatment markedly suppressed the de novo ceramide production, especially that of the upper band (found at 63% of the control, the value is the total of the upper and lower bands and the mean from triplicate determinations; Fig. 5C). This result showed that the increase of membrane ceramide in B16 cells by EGCase may cause the suppression of de novo ceramide production, resulting in maintenance of the ceramide content at the same level even after EGCase treatment. This result is in contrast to that in erythrocytes (8), in which the GSL-synthesizing system is lost after tenancy of the ceramide content at the same level even after GSl-synthesis and Ceramide—could not be directly transported to lysosomes for degradation. Inhibiting other components and releases the oligosaccharide EGCase specifically hydrolyzes the cell-surface GSLs without what is the signal molecule for this homeostatic regulation? The next question is, therefore, what is the signal molecule for this homeostatic regulation? EGCase treatment, the cells were washed with fresh medium, and then 1 \(\mu\text{C}\) of \([^{14}C]S\text{er}\) was added. De novo production of ceramide was determined after 3 h. C, control without EGCase; E, EGCase treatment; Cer, ceramide; DM, N,N-dimethylsphingosine; Sph, sphingosine.

Discussion

The reason why mammalian cells possess a restoration system of cell-surface GSLs as described in this study remains to be clarified. EGCase has been isolated not only from microorganisms (5, 24), but also from leeches (25), earthworms (26), and clams (27). The functional significance of this system, however, may only be clarified after the presence of EGCase in mammals is clearly demonstrated, although the presence of the enzyme in rabbit mammary glands has been suggested (28, 29). We can indicate another possibility, given the fact that several strains of opportunistic pathogens were recently found to secrete EGCase into culture media. The microorganism isolated as an EGCase producer from land soil (5) was identified as Rhodococcus equi, which is an opportunistic pathogen in mammals (30). Recently it was found that an authentic strain of R. equi ATCC 6939 retained the ability to produce EGCase. R. equi is a Gram-positive actinomycete originally associated with a severe, often fatal pneumonia in foals. More recently, it was identified as an opportunistic pathogen in humans infected with the AIDS virus (31). Thus we examined the possibility of whether other opportunistic microorganisms can produce EGCase extracellularly. Surprisingly, many bacteria and actinomycetes, including opportunistic pathogens such as Rhodococcus eurythropolis, Rhodococcus rhodochrous, Corynebacterium hoagii, Corynebacterium mediolanum, Arthrobacter aureus, Brevibacterium sterolicum, and Nocardia globerula produce EGCase extracellularly. If such microbes succeeded in entering mammalian tissue, cell-surface GSLs and ceramide simultaneously. Oligosaccharide released from the cell could be easily diluted in the external medium, whereas the ceramide released could remain in the plasma membrane or might be incorporated into the cytosol. We thus examined the possibility of whether the ceramide released could be a signal molecule for homeostatic regulation using a cell-permeable short-chain ceramide, C2-ceramide (N-acetylsphingosine). As shown in Fig. 6A, C2-ceramide potently suppressed the de novo production of ceramide in B16 cells. Interestingly, C2-ceramide was found to rapidly convert the novel GSL as shown in Fig. 6B. The novel GSL conformed with standard C2-ceramide GM3 and was hydrolyzed by EGCase to produce sialyllactose (Fig. 6C). These results indicate that the novel GSL is a NeuAc-lactose N-acetylsphingosine (C2-ceramide GM3). Furthermore, we confirmed that natural ceramide from bovine brain (Sigma) increased GSL synthesis and suppressed de novo ceramide synthesis when it was added to B16 cell cultures in the concentration of 5 \(\mu\text{M}\), although the effect is slightly less than that by short-chain ceramide (data not shown).

These results strongly suggest that the ceramide released from GSLs by EGCase might be a signal for the homeostatic regulation of ceramide and cell-surface GSLs.
might be exposed to the action of EGCase. Cells therefore might possess the homeostatic regulation system for maintaining GSL content that was demonstrated in this study as a defense mechanism against microbial EGCase. Further investigations should reveal the relationship between pathogenicity and EGCase.

EGCase was found to efficiently hydrolyze the cell-surface GSLs of intact erythrocytes without damaging other cell membrane components (9, 10) and has recently been used for the analysis of functions of endogenous GSLs of A431 cells (21) and cultured cortical neurons (32). However, for cultured cells, EGCase appeared to hydrolyze cell-surface GSLs very slowly in comparison with erythrocytes (10) or the plasma membrane fraction of cultured cells (21). This paper may clarify the reason for this, i.e., the hydrolysis of cell-surface GSLs by EGCase evoked an increase of de novo GSL synthesis, possibly due to the activation of GlcTase, and thus cell-surface GSL supply could be reinforced markedly during EGCase treatment, preventing the loss of cell-surface GSLs.

Since ceramide is involved in cell regulation (12), its intracellular levels must be carefully regulated. We found in this study that the intracellular level of ceramide was maintained at the same level even after EGCase treatment due to the suppression of de novo synthesis of ceramide. The signal might be the ceramide released by the action of EGCase, since a cell-permeable analog of ceramide, C2-ceramide, also suppressed the de novo synthesis of ceramide. Furthermore, C2-ceramide was found to be converted to C2-ceramide GM3. This result strongly suggests that at least a part of the ceramide released from GSLs by EGCase might be transported directly to the Golgi apparatus where it could be converted to GM3 and thus could be finally recycled to plasma membrane. This hypothesis is consistent with the fact that the intracellular ceramide released by EGCase could not be converted to sphingosine or N,N-dimethylsphingosine (Fig. 5).

Slife et al. (33) have also reported that sphingosine was generated from sphingomyelin by sphingomyelinase treatment of rat liver plasma membranes, but not from GSLs by EGCase, although ceramide was produced from both enzyme treatments. Whether the intracellular metabolism of ceramide released from GSLs by EGCase in intact cells is different from that of sphingomyelin by sphingomyelinase should be carefully clarified, and this study is currently in progress in our laboratory.

Cell-permeable, synthetic short-chain ceramide was found to exert various physiological effects on different cell types; it induced the differentiation of HL-60 cells into monocyte-like cells (11), the cell-cycle arrest of Molt-4 cells (34), the programmed cell death of U937 cells (35), and it inhibited the endocytosis of Chinese hamster ovary cells (14). However, little is actually known of the intracellular metabolism of the short-chain ceramide. Interestingly, it was revealed in this study that C2-ceramide could be converted to C2-ceramide ganglioside GM3 in B16 cells. We have also confirmed that C2-ceramide could be converted to C2-ceramide GM3 in B16 cells. The synthesis of these GSLs with short-chain ceramide and the kinetics for their intracellular formation have been reported (36). These results lead us to the hypothesis that the physiological effects of short-chain ceramide reported so far may be attributed in part to the intracellular formation of short-chain ceramide gangliosides.

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