Fluorescent, short-chain C₆-NBD-sphingomyelin, but not C₆-NBD-glucosylceramide, is subject to extensive degradation in the plasma membrane: implications for signal transduction related to cell differentiation

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The involvement of the plasma membrane in the metabolism of the sphingolipids sphingomyelin (SM) and glucosylceramide (GlcCer) was studied, employing fluorescent short-chain analogues of these lipids, 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoylsphingosylphosphorylcholine (C₆-NBD-SM), C₆-NBD-GlcCer and their common biosynthetic precursor C₆-NBD-ceramide (C₆-NBD-Cer). Although these fluorescent short-chain analogues are metabolically active, some caution is to be taken in view of potential changes in biophysical/biochemical properties of the lipid compared with its natural counterpart. However, these short-chain analogues offer the advantage of studying the lipid metabolic enzymes in their natural environment, since detergent solubilization is not necessary for measuring their activity. These studies were carried out with several cell types, including two phenotypes (differing in state of differentiation) of HT29 cells. Degradation and biosynthesis of

C₆-NBD-SM and C₆-NBD-GlcCer were determined in intact cells, in their isolated plasma membranes, and in plasma membranes isolated from rat liver tissue. C₆-NBD-SM was found to be subject to extensive degradation in the plasma membrane, due to neutral sphingomyelinase (N-SMase) activity. The extent of C₆-NBD-SM hydrolysis showed a general cell-type dependence and turned out to be dependent on the state of cell differentiation, as revealed for HT29 cells. In undifferentiated HT29 cells N-SMase activity was at least threefold higher than in its differentiated counterpart. In contrast, in all cell types studied, very little if any biosynthesis of C₆-NBD-SM from the precursor C₆-NBD-Cer occurred. Moreover, in the case of C₆-NBD-GlcCer, neither hydrolytic nor synthetic activity was found to be associated with the plasma membrane. These results are discussed in the context of the involvement of the sphingolipids SM and GlcCer in signal transduction pathways in the plasma membrane.

INTRODUCTION

During internalization along the endocytic pathway, plasma membrane-derived sphingomyelin (SM) can be hydrolysed by the acidic sphingomyelinase (A-SMase), once it reaches the lysosomes. Apart from this lysosomal enzyme, which has an optimal activity at pH 4.5, a neutral sphingomyelinase (N-SMase) has been described with optimal activity at neutral pH [1–3]. This enzyme appears to be located on the outer (extracellular) leaflet of the plasma membrane [4–6]. Due to its activity and localization the N-SMase may be of great importance in signal transduction events, since it generates ceramide (Cer) in the plasma membrane. This sphingolipid intermediate has been shown to perform second messenger functions [7]. Moreover, a neutral ceramidase activity has been described to be present in the plasma membrane [5], which can further metabolize Cer to sphingosine (So), a potent inhibitor of protein kinase C (PKC) [8]. Cer, generated by N-SMase activity, may be re-utilized for synthesis of SM at the expense of phosphatidylcholine (PC), which donates the phosphorylcholine headgroup. PC is thus converted into diacylglycerol (DAG), a well-known activator of PKC. If SM hydrolysis and resynthesis are to be in balance, this generates a futile SM turnover cycle, the net result being the

formation of DAG from PC [4]. Given such a cycle, subtle changes in turnover rate could lead to the net formation of either So or DAG, which have opposite effects on PKC activity. Thus, such a mechanism would provide the cells with a system for fine tuning of the activity level of PKC.

The present study explores the likelihood of such an SM hydrolysis/resynthesis cycle. For this purpose we have used 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl (C₆-NBD)-sphingolipids, which can be readily inserted into the plasma membrane [9], a prerequisite for the availability of the substrate to the membrane-associated N-SMase. This property of the NBD-lipids allows us to study the N-SMase in its natural membrane environment since detergent solubilization is avoided. Furthermore, previous work in HT29 cells has shown that these C₆-NBD-sphingolipids are suitable probes for metabolic studies [10]. The results demonstrate that in contrast to the differentiated HT29 cell line, undifferentiated HT29 cells exhibit an extensive turnover of membrane-inserted C₆-NBD-SM. The extent of turnover was specifically related to the activity of a plasma membrane-associated N-SMase. Resynthesis of C₆-NBD-SM is very limited in all cell types studied. In marked contrast to the fate of C₆-NBD-SM, C₆-NBD-GlcCer is shown to be metabolically inert in the plasma membrane. These phenomena

Abbreviations used: AAP, adenine 9-β-D-arabinofuranoside 5'-monophosphate; A-SMase, acidic sphingomyelinase; BHK, baby hamster kidney; Cer, ceramide; C₆-NBD, 6-[N-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl or -hexanoic acid; DAG, diacylglycerol; DMEM, Dulbecco's modification of minimal essential medium; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTP, 2,2'-dithiopyridine; DTT, dithiothreitol; FCS, fetal-calf serum; GlcCer, glucosylceramide; HT29 G⁺ (rev), HT29 cells growing in the presence of glucose; NEM, N-ethylmaleimide; N-SMase, neutral sphingomyelinase; PC, phosphatidylcholine; PKC, protein kinase C; SM, sphingomyelin; So, sphingosine.

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have important consequences for the potential role of these (glyco-)sphingolipids in signal transduction [11,12].

MATERIALS AND METHODS

Materials

1- β -D-Glucosylsphingosine, sphingosylphosphorylcholine, D-sphingosine, *N*-ethylmaleimide (NEM), dextran, adenine 9- β -D-arabinofuranoside 5'-monophosphate (AAP) and merocyanine 540 were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. 6-[*N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)amino]-hexanoic acid (C_6 -NBD) was from Molecular Probes, OR, U.S.A. Dithiothreitol (DTT) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were from Boehringer-Mannheim, Germany. PMSF was from Merck, Darmstadt, Germany. Triphenylphosphine and 2,2'-dithiopyridine (DTP) were purchased from Janssen Chimica, Beerse, Belgium.

Synthesis of C_6 -NBD-labelled sphingolipids

C_6 -NBD-GlcCer, C_6 -NBD-SM and C_6 -NBD-Cer were synthesized from C_6 -NBD and 1- β -D-glucosylsphingosine, sphingosylphosphorylcholine and D-sphingosine respectively, as described previously [10].

Cell culture

Monolayer cultures of HT29 cells were grown in Dulbecco's minimal essential medium (DMEM; containing 25 mM glucose), supplemented with 10% (v/v) decompented (56 °C, 30 min) fetal-calf serum (FCS), in a water-saturated atmosphere of 5% CO₂/95% air. Cells were routinely seeded at a density of 6.6×10^4 cells/cm². During the exponential phase of growth, the culture medium was changed every 48 h. Under these conditions, cell confluency was reached after 7 days of culture. During the stationary phase, the medium was changed every 24 h, to avoid any nutrient exhaustion.

Isolation of plasma membranes from rat liver

Rat liver plasma membranes were isolated from rat liver according to the procedure of Wolters et al. [13]. The entire procedure was performed at 2 °C. Briefly, rat livers were minced, washed in 1.0 mM NaHCO₃ (pH 7.4) and homogenized in a dounce, with a loose-fitting pestle. The homogenate was diluted to 10% (w/v) with bicarbonate solution and filtered. After centrifugation (500 *g* for 5 min, followed by 1000 *g* for 10 min), the pellet was resuspended in 1.0 mM NaHCO₃ and recentrifuged at 1000 *g* for 10 min. The crude nuclear pellet thus obtained was resuspended in 4.5 vol. of 56% sucrose (w/w) and stirred for 15 min. The suspension was transferred to centrifuge tubes and overlaid with 0.66 vol. of 43% sucrose and 0.4 vol. of 36.5% sucrose. After centrifugation for 120 min at 65000 *g* in a swing-out rotor, the plasma membranes were recovered from the 36.5–43% interface, washed, and resuspended in 2 ml of 0.25 M sucrose.

Isolation of Golgi membranes from rat liver

Rat liver Golgi membranes were isolated according to the procedure of Croze and Morre [14]. The entire procedure was performed at 2 °C. After perfusion with an isotonic NaCl solution, rat livers were minced. Homogenization was done using 3 vol. of homogenizing buffer [0.5 M sucrose, 37.5 mM Tris,

pH 6.5, containing 5 mM MgCl₂, 2 mM EGTA, 1 mM PMSF and 1% (w/v) dextran] in a homogenizer with a loose-fitting Teflon pestle, until a homogenous suspension was obtained. Thereafter, the suspension was further homogenized in a homogenizer with a tight-fitting Teflon pestle (5 times), followed by filtration through a monodur PA 100 N gauze. The homogenate was diluted to 20% (w/v) in homogenizing buffer and centrifuged (20 min at 5000 *g*). The pellet was resuspended in homogenizing buffer and layered on 0.4 vol. of 1.2 M sucrose in centrifuge tubes. After centrifugation (45 min at 85000 *g*) the white band on the interface was collected, diluted threefold with PBS and centrifuged at 5500 *g* for 20 min. This PBS washing step was repeated once and finally the pellet was resuspended in 3 ml of 0.25 M sucrose, 25 mM KCl, 50 mM Tris, pH 7.4, buffer (protein concentration 1 mg/ml).

Isolation of plasma membranes from HT29 cells

Plasma membranes of undifferentiated HT29 G⁺ cells were isolated based on the procedure of Warren [15]. The entire procedure was performed at 2 °C. Approx. 5×10^8 cells were scraped with a rubber policeman in 0.16 M NaCl solution and centrifuged for 5 min at 900 *g*. The cell pellet was washed three times with 0.16 M NaCl by resuspension and centrifugation as above. The cell pellet was then resuspended in 50 mM Tris, pH 7.4, buffer to a concentration of 5×10^6 cells/ml and centrifuged as above. The Tris washing step was repeated once, after which the cell pellet was resuspended in Tris buffer at a concentration of 1×10^8 cells/ml. The cell suspension was mixed with an equal volume of Tris buffer and 0.1 vol. of 50 mM MgCl₂. After an incubation of 10 min (at 4 °C), the suspension was homogenized by 100 strokes in a tight dounce and centrifuged at 100000 *g* for 1 h. The pellet was resuspended in 8 ml of 20% sucrose and layered on top of a continuous sucrose gradient from 20% to 40% sucrose (w/w). All sucrose solutions contained 5 mM MgCl₂. The gradient was centrifuged for 20 h at 52000 *g*, followed by fractionation into six fractions. Each fraction was adjusted to 20% sucrose and pelleted at 100000 *g* for 1 h. Finally each pellet was resuspended in 1 ml of 20% sucrose. Fractions 4 (30.5% sucrose) and 5 (34.0% sucrose) were routinely used for metabolic assays. Plasma membranes of differentiated HT29 G⁺ rev cells were isolated according to a similar protocol, but with the following modifications. (a) The cells were removed from the plate by trypsinization instead of scraping, followed by centrifugation in DMEM (without FCS) to obtain a cell pellet. It should be noted that the trypsinization protocol did not affect the activity of the N-SMase, as tested with a metabolic assay employing C_6 -NBD-SM (see below). (b) The pellet obtained after the first ultracentrifugation (100000 *g*) was (after resuspension) layered on top of a discontinuous sucrose gradient (containing from top to bottom 1 vol. of 30%, 2 vol. of 35%, 1 vol. of 40%, 2 vol. of 45%, 2 vol. of 48% and 1 vol. of 50% sucrose). (c) After centrifugation the fractions of 30% and 35% sucrose were collected and centrifuged at 100000 *g* for 1 h. (d) The final pellet was resuspended in 2 ml of 20% sucrose.

As an alternative procedure for plasma membrane isolation the method of Allan and Quinn [16] was employed, making use of merocyanine 540 to induce outward vesiculation of the plasma membrane. Cells were washed three times with PBS, followed by an incubation of 1 h at 37 °C with PBS containing 50 μ M merocyanine 540 (the solution was sonicated before addition to the cells). The medium was collected and centrifuged for 5 min at 500 *g* to remove cells, followed by centrifugation at 100000 *g* for 1 h to pellet the plasma membrane vesicles. The pellet was resuspended in Hanks' solution.

Metabolic assays

After washing, intact cells were incubated with 4 μ M C₆-NBD-lipid (added by ethanol injection in serum-free DMEM; see [17]) for 1 h at the indicated temperature. Occasionally, 20 mM NH₄Cl (diluted from a stock solution of 1 M in water) or 100 μ M AAP (diluted from a stock solution of 10 mM in water) was included in the medium. When required, a back-exchange to remove plasma membrane-associated lipid was subsequently performed, involving two incubations with 5% BSA at 2 °C (see also [17]). Isolated plasma membranes were incubated with 4 μ M C₆-NBD-lipid in PBS (pH 7) or PBS/acetate (pH 5) for 1 h at 37 °C, occasionally in the presence of 5 mM DTT or 1 mM NEM. Incubations with isolated Golgi membranes were performed in KCl/Hepes-buffer (140 mM KCl, 0.5 mM KH₂PO₄, 20 mM Hepes). In the case of glucosylceramide (GlcCer) formation from Cer, the incubations were performed in the presence of 10 mM Mn²⁺ and 10 mM UDP-glucose.

Lipid extraction, analysis and quantification

Lipids were extracted from cells or isolated membrane fractions by the procedure of Bligh and Dyer [18], with an average recovery of 93% for all NBD-lipids, irrespective of the nature of the headgroup. NBD-lipids were analysed by high-performance TLC (HPTLC) on silica gel 60 HPTLC plates (Merck), using CHCl₃/CH₃OH/20% (w/v) NH₄OH (14:6:1, by vol.) as the running solvent system. For quantification, individual spots were scraped from the plates and after addition of 3 ml of a 1% (v/v) Triton X-100 solution, shaken vigorously for 1 h at 37 °C, followed by centrifugation to remove silica particles. The C₆-NBD-lipids were quantified spectrophotometrically in a fluorometer (Perkin-Elmer-MPF-43 or SLM 8000) with an excitation wavelength of 465 nm and an emission wavelength of 530 nm, by reference to known amounts of C₆-NBD-PC. Corrections were made for differences in efficiency of removal of different types of lipids from the silica (the efficiency being 10% lower for C₆-NBD-SM compared with all other C₆-NBD-lipids).

Microscopy

Cells were grown on glass coverslips in 35-mm-diam. Petri dishes. Prior to experiments, the cells were cooled (30 min) and washed several times with ice-cold Hanks' solution. Control cells were subsequently labelled with 4 μ M C₆-NBD-SM for 30 min at 2 °C, washed four times with cold Hanks' buffer and thereafter incubated at 37 °C for 15 min, allowing internalization to occur. Finally, a back-exchange with 5% BSA at 2 °C was performed to remove the residual plasma membrane fluorescence, allowing visualization of the intracellular labelling pattern. In the case of dithionite quenching, cells were, prior to labelling and internalization of C₆-NBD-SM, incubated with Hanks' buffer containing 30 mM Na₂S₂O₄ (diluted from a stock solution of 1 M Na₂S₂O₄ in 1 M Tris, pH 10) for 30 min at 37 °C, allowing fluid-phase uptake of this NBD-quencher in endocytic compartments to occur. Hence, this procedure will result in loading of the endo/lysosomal system with the NBD-lipid quencher. Following this incubation the cells were extensively (15 times) washed with Hanks' buffer to remove all exogenous Na₂S₂O₄ from the incubation medium.

Fluorescence microscopy was performed with a Leitz Orthoplan microscope. Photomicrographs were taken at 10 s exposition times using Kodak T-max P3200 film that was processed at 6400 ASA.

Miscellaneous procedures

Protein was determined by the Lowry procedure, modified as described by Peterson [19]. BSA was used as a standard. The assays of organelle marker enzymes were done according to Scalera et al. ([20], acid phosphatase), Keeffe et al. ([21], alkaline phosphatase), Blanken and Van den Eynden ([22], galactosyl-transferase), Fleischer and Fleischer ([23], glucose-6-phosphatase), Scharschmidt et al. ([24], Na/K-ATPase) and Aronson and Touster ([25], phosphodiesterase).

RESULTS AND DISCUSSION

SM degradation in liver plasma membranes

When plasma membranes isolated from rat liver were incubated with C₆-NBD-SM for 1 h at 37 °C, extensive degradation to C₆-NBD-Cer occurred, as shown in Table 1. This hydrolysis was due to the activity of the N-SMase located in the plasma membrane, since (i) highly purified plasma membranes were used, with little contamination of lysosomes, as shown in Table 2; (ii) the hydrolytic activity showed a pH optimum at basic pH, and, as shown in Table 3, was virtually abolished at pH 5 and not affected by NH₄Cl; (iii) as shown in Table 3, the hydrolytic activity was stimulated by Mg²⁺ (maximal stimulation occurred already at a concentration of 1 mM; cf. [1]) and 5 mM DTT, but was completely inhibited by 30 μ M Cu²⁺ (cf. [26]). On the other hand, when plasma membranes were incubated with C₆-NBD-Cer, biosynthesis of C₆-NBD-SM was very modest. However, in the latter case, it should be taken into account that, once synthesized, C₆-NBD-SM may be effectively hydrolysed to yield C₆-NBD-Cer again, as shown. To investigate this possibility, 1 mM NEM was included in the incubation mixture. As shown in Table 1, C₆-NBD-SM hydrolysis is virtually abolished by NEM. Thus, under these conditions, one can dissect synthesis and breakdown of C₆-NBD-SM and determine the maximal C₆-NBD-SM-synthetic capacity in the plasma membrane. Although the capacity almost doubled in the presence of NEM (Table 1), in absolute sense, and compared with its hydrolysis, C₆-NBD-SM biosynthesis in the plasma membrane remains marginal.

It should be noted that C₆-NBD-Cer can readily be used as a substrate for C₆-NBD-SM biosynthesis, both in intact cells [4,10] and isolated organelles. The latter was revealed when isolated liver Golgi's (see Table 2 for characterization) were incubated with C₆-NBD-Cer under conditions similar to those for plasma membranes. Compared with the rate in plasma membranes the rate of synthesis of C₆-NBD-SM in Golgi's is approx. 7-fold higher (Table 1), which is consistent with reported distributions of SM biosynthetic capacity over plasma membrane and Golgi in liver [27]. Furthermore, C₆-NBD-SM synthesis in Golgi membranes turned out to be only slightly affected by NEM, yielding a value of 84% (S.D. 10%) compared with control. The insensitivity of SM synthase activity *per se* towards NEM thus validates the use of this inhibitor of the N-SMase for the estimation of the C₆-NBD-SM-synthesis capacity of the plasma membrane. In conclusion, C₆-NBD-SM is subject to extensive degradation in liver plasma membranes, whereas within the same time interval its resynthesis from C₆-NBD-Cer is very limited. Therefore a balance between SM hydrolysis and synthesis, which is a prerequisite for the involvement of SM in a futile cycle leading to DAG production from PC, as has been suggested by several authors, seems rather unlikely ([4] and references therein). Hence, potential biologically active sphingolipid-derived molecules, like Cer and So, that are formed upon hydrolysis of SM, will probably be processed by other mechanisms, such as

Table 1 Degradation and synthesis of C₆-NBD-SM and C₆-NBD-GlcCer in liver organelles

Isolated liver plasma membranes (PM; 120 µg of protein per assay) or Golgi membranes (50 µg of protein per assay) were incubated with the C₆-NBD-derivatives of SM, GlcCer or Cer, as described in the Materials and methods section, in the absence or presence of 1 mM of NEM. The degradation C₆-NBD-SM or C₆-NBD-GlcCer to C₆-NBD-Cer or, alternatively, the biosynthesis of C₆-NBD-SM or C₆-NBD-GlcCer from C₆-NBD-Cer were measured and are expressed as the percentage of total extracted C₆-NBD-lipid. In the case of C₆-NBD-GlcCer formation from C₆-NBD-Cer the incubations were done in the presence of 10 mM Mn²⁺ and 10 mM UDP-glucose. Data are the means of triplicate measurements ± S.D. (n.d., not detectable; —, not measured).

	C ₆ -NBD-SM		C ₆ -NBD-GlcCer	
	Degradation	Biosynthesis	Degradation	Biosynthesis
PM (control)	53.3 ± 2.6	1.6 ± 0.1	0.5 ± 0.2	n.d.
PM (NEM)	4.1 ± 0.3	3.0 ± 0.1	—	—
Golgi (control)	—	11.6 ± 4.9	—	15.2 ± 9.2

Table 2 Characterization of liver Golgi and plasma membrane fractions

Liver Golgi and plasma membrane fractions were isolated as described in the Materials and methods section. Various organelle marker enzyme activities were determined in both fractions (phosphodiesterase for plasma membranes; galactosyltransferase for Golgi membranes; acid phosphatase for lysosomes and glucose-6-phosphatase for endoplasmic reticulum membranes). Data are expressed as relative enrichments (ratio of specific activity in isolated membrane fraction to specific activity in homogenate) ± S.D., and recovery (percentage of total homogenate) ± S.D. In parentheses is given the number of independent experiments. Note that plasma membrane fractions are not contaminated by Golgi membranes, whereas Golgi membranes are only slightly (10%) contaminated by plasma membranes.

	Relative enrichment	Recovery
Golgi membrane fraction		
Phosphodiesterase	7.15 ± 4.42 (4)	1.30 ± 0.67 (4)
Galactosyltransferase	74.17 ± 20.40 (3)	15.13 ± 7.08 (3)
Acid phosphatase	1.90 ± 0.36 (3)	0.37 ± 0.06 (3)
Glucose-6-phosphatase	0.80 (1)	0.10 (1)
Protein	—	0.20 ± 0.04 (3)
Plasma membrane fraction		
Phosphodiesterase	19.80 ± 9.14 (3)	8.27 ± 6.36 (3)
Galactosyltransferase	0.05 (2)	0.02 (2)
Acid phosphatase	1.00 (2)	0.45 (2)
Glucose-6-phosphatase	0.35 (2)	0.15 (2)
Protein	—	0.40 (2)

Table 3 Effect of various treatments on N-SMase activity in liver plasma membranes

Isolated liver plasma membranes (25–50 µg of protein per assay) were incubated with C₆-NBD-SM, as described in the Materials and methods section. The formation of C₆-NBD-Cer was measured in plasma membranes in PBS at pH 7 (control), supplemented with either 20 mM NH₄Cl, 5 mM DTT, 1 mM Mg²⁺ (at which concentration maximal stimulation already occurred) or 30 µM Cu²⁺, or in membranes suspended in PBS/acetate buffer at pH 5. Data are expressed as the relative N-SMase activity, which represents the percentage of C₆-NBD-Cer formation in treated samples divided by the percentage of C₆-NBD-Cer formation in control samples (control = 100%). Data are the average values of two or more measurements.

Treatment	Relative N-SMase activity (%)
pH 5	7
NH ₄ Cl	101
DTT	143
Mg ²⁺	142
Cu ²⁺	4

Table 4 Intracellular C₆-NBD-lipid composition in HT29 G⁺ cells after incubation with C₆-NBD-SM or C₆-NBD-GlcCer

HT29 G⁺ cells were incubated with C₆-NBD-SM or C₆-NBD-GlcCer for 1 h at 37 °C, followed by a back-exchange at 2 °C to remove the plasma membrane NBD-lipid pool (see the Materials and methods section). The intracellular NBD-lipid pool was extracted, separated on TLC and quantified. Data are expressed as percentages of total NBD-lipid. Values are the means of triplicate measurements ± S.D. Note that intracellular C₆-NBD-GlcCer is still largely intact, whereas C₆-NBD-SM is extensively converted into C₆-NBD-Cer before or during internalization.

	C ₆ -NBD-SM	C ₆ -NBD-GlcCer
C ₆ -NBD-Cer	73.80 ± 0.75	14.10 ± 1.10
C ₆ -NBD-SM	17.30 ± 0.87	2.90 ± 0.45
C ₆ -NBD-GlcCer	8.90 ± 0.53	83.00 ± 0.75

metabolism along other pathways (e.g. phosphorylation) and/or (endocytic) transport.

The metabolism of GlcCer and SM is closely related in the sense that Cer is the immediate precursor or product in the biosynthesis and upon hydrolysis, respectively, of either sphingolipid. In the context of the present study it was therefore of interest to investigate whether the glycolipid is also subject to any metabolism at the cell surface, in particular since there is some evidence indicating that this sphingolipid is involved in proliferation of cells via modulation of signal transduction pathways [11,12]. It was not established, however, whether GlcCer itself or a breakdown product, like Cer and/or So, was responsible for these effects. When isolated liver plasma membranes were incubated with C₆-NBD-GlcCer, virtually no hydrolysis occurred (Table 1). Conversely, when incubated with C₆-NBD-Cer, in the presence of UDP-glucose and Mn²⁺, no synthesis of C₆-NBD-GlcCer could be detected. Again, under similar conditions synthesis did occur in isolated Golgi membranes (Table 1). In conclusion, if GlcCer is implicated in signal transduction, it seems likely that its action is not propagated by way of breakdown products. It should be noted that with the approach of C₆-NBD-sphingolipids the effects of deacylation, yielding lysosphingolipids, cannot be monitored, since C₆-NBD-Cer does not appear to be a good substrate for ceramidase activity [9].

SM degradation related to cell differentiation

When intact undifferentiated HT29 G⁺ cells were incubated with either C₆-NBD-SM or C₆-NBD-GlcCer at 37 °C for 1 h, only in

Figure 1 Temperature-dependent intracellular accumulation of C₆-NBD-lipids in different cell types upon incubation with C₆-NBD-SM or C₆-NBD-GlcCer

HT29 G⁺ cells (a, c), HT29 G⁺ rev cells (b), or BHK cells (d) were incubated with C₆-NBD-SM (a, b, d) or C₆-NBD-GlcCer (c) for 1 h at the indicated temperatures, followed by a back-exchange at 2 °C to remove the plasma membrane NBD-lipid pool (see the Materials and methods section). The remaining, intracellular NBD-lipid pool was extracted, separated on TLC and quantified. Data are expressed as pmol of NBD-lipid (C₆-NBD-Cer, C₆-NBD-SM or C₆-NBD-GlcCer) per mg of protein. Values are the means of duplicate measurements.

the case of C₆-NBD-SM did (extensive) hydrolysis occur, yielding C₆-NBD-Cer. This was revealed when cells were incubated with the respective C₆-NBD-sphingolipids, followed by a BSA back-exchange at 2 °C to remove the plasma membrane pool of NBD-lipid. When the cells were incubated with C₆-NBD-SM, the intracellular pool was largely made up of C₆-NBD-Cer, resulting from the breakdown of C₆-NBD-SM (Table 4). In the case of the NBD-glycolipid, the intracellular pool consisted mostly of intact C₆-NBD-GlcCer. To discriminate between N-SMase activity (plasma membrane) and A-SMase activity (lysosomes) as the cause of C₆-NBD-SM hydrolysis, the following experiments were performed. Based on the notion that endocytic uptake of NBD-sphingolipids is severely reduced when the incubation temperature is lowered, the temperature-dependent formation and intracellular accumulation of C₆-NBD-Cer were determined, as shown in Figure 1(a). It should be noted that C₆-NBD-SM cannot translocate across the plasma membrane and can therefore only gain intracellular access by virtue of endocytosis. C₆-NBD-Cer, on the other hand, can translocate and readily gains intracellular access by diffusion, also at low temperature (2 °C). The results demonstrated that (i) at all temperatures the intracellular pool of C₆-NBD-Cer far exceeds that of C₆-NBD-SM and (ii) even at temperatures of 10 °C or less, when endocytic uptake of C₆-NBD-SM is negligible, appreciable amounts of C₆-NBD-Cer were found intracellularly. Thus, at these temperatures the C₆-NBD-Cer formed results from hydrolysis of C₆-NBD-SM in the plasma membrane by the N-SMase. It can be assumed that also at 37 °C, when N-SMase activity is expected to be even higher, a large part of the hydrolysis of C₆-NBD-SM is accounted for by the N-SMase.

In contrast, a similar experiment performed with C₆-NBD-

Table 5 C₆-NBD-SM hydrolysis by N-SMase under conditions of A-SMase inhibition in HT29 G⁺ cells

HT29 G⁺ cells were incubated with C₆-NBD-SM for 1 h at 37 °C, in DMEM (without FCS) with either 20 mM NH₄Cl or 100 μM AAP, to inhibit the A-SMase, or without inhibitors (control). The total NBD-lipid pool was extracted, separated on TLC and quantified. Individual NBD-lipids are expressed as percentages of total NBD-lipid. Values are the means of triplicate measurements ± S.D.

	Lipid content (% of total NBD-lipid)		
	Control	NH ₄ Cl	AAP
C ₆ -NBD-Cer	76.10 ± 1.62	59.20 ± 2.91	53.20 ± 2.11
C ₆ -NBD-SM	15.80 ± 1.10	30.80 ± 2.77	38.00 ± 2.50
C ₆ -NBD-GlcCer	8.10 ± 1.00	10.00 ± 0.96	8.80 ± 2.17

GlcCer revealed temperature-dependent internalization of the glycolipid, but no formation of C₆-NBD-Cer (Figure 1c). This result is thus consistent with the observations of degradation of C₆-NBD-SM, but not of C₆-NBD-GlcCer, in rat liver plasma membrane (see above). To obtain further evidence for the involvement of the N-SMase, another type of experiment was performed in which internalization of SM was allowed to proceed at 37 °C. In parallel, a similar incubation was carried out in the presence of either 20 mM NH₄Cl, which inhibits lysosomal degradation by increasing the intralysosomal pH [28], or 100 μM AAP (a potent inhibitor of the A-SMase [29]). As shown in Table 5, in the presence of either inhibitor only a slight decrease in the percentage of C₆-NBD-Cer formed was seen. This observation

Figure 2 Organelle-marker distribution and pH-dependent C₆-NBD-SM hydrolysis along the sucrose gradient

(a) Analysis of the activities of Na/K-ATPase (●), acid phosphatase (Δ) and galactosyl-transferase (○) as markers for plasma membranes, lysosomes and Golgi respectively in six fractions of a continuous sucrose gradient. The enzyme activities are normalized to the fraction with maximal activity. This gradient separation is part of the plasma membrane isolation protocol (see the Materials and methods section) for HT29 G⁺ cells and shows that fractions 4 (30.5% sucrose) and 5 (34.0% sucrose) are relatively enriched in plasma membranes. Therefore, these fractions are routinely used for metabolic assays. (b) C₆-NBD-Cer formation (as a percentage of total NBD-lipid) was determined along the same gradient as in (a), either at pH 7 (N-SMase; ●) or at pH 5 (A-SMase; ○). A sample (10 μg of protein) of each of the six fractions is incubated with C₆-NBD-SM for 1 h at 37 °C (see the Materials and methods section). Note that N-SMase activity is optimal in fractions 4 and 5, coinciding with high Na/K-ATPase activity. Lysosomal contribution to SM hydrolysis in these fractions is low, since hydrolysis can be largely reduced by incubation in buffer with pH 5, in which the N-SMase is inactive. Values are the averages of duplicate measurements.

strongly indicates that the majority of Cer found intracellularly after a 37 °C incubation must have resulted from N-SMase activity in the plasma membrane.

Since it is difficult to rigorously establish the rate of N-SMase activity in intact cells, due to the contribution of the A-SMase in the lysosomes, plasma membranes of HT29 G⁺ cells were isolated on sucrose gradients. As shown in Figure 2, the peak of C₆-NBD-SM hydrolysis (Figure 2b) on such a gradient coincides with that of the plasma membrane marker Na/K-ATPase (Figure 2a), which is well separated from both lysosomal and Golgi markers. Furthermore, when the hydrolysis of C₆-NBD-SM along the gradient is examined at pH 5 the peak activity is highly reduced (Figure 2b; cf. Table 3), again indicating the involvement of the N-SMase. Absence of appreciable A-SMase activity in sucrose fraction 2 (which shows the highest acid phosphatase activity, indicating the presence of lysosomes) could be a result of low intrinsic activity of the A-SMase. Alternatively, it could result from the inability of the C₆-NBD-SM substrate to reach the internal leaflet of isolated lysosomes, where conversion into C₆-NBD-Cer most likely occurs.

As shown in Figure 3, in isolated HT29 G⁺ plasma membranes a very high extent of C₆-NBD-SM hydrolysis was observed after a 1 h incubation at 37 °C. Similar results were obtained using a

Figure 3 N-SMase activity in HT29 G⁺ versus HT29 G⁺ rev cells, as a function of protein concentration

Plasma membranes were isolated from HT29 G⁺ or HT29 G⁺ rev cells (see the Materials and methods section). Samples containing different amounts of protein were tested in metabolic assays for N-SMase activity by incubating them with C₆-NBD-SM for 1 h at 37 °C (see the Materials and methods section). C₆-NBD-Cer formation as a percentage of total NBD-lipid is depicted as a function of the protein concentration. Note that N-SMase activity in HT29 G⁺ cells (●) is at least threefold higher than the activity in HT29 G⁺ rev cells (○). Values are the means of two sets of measurements from two independent isolations.

Table 6 Effect of various sulphhydryl reagents on HT29 (G⁺ versus G⁺ rev) plasma membrane N-SMase activity

Plasma membranes (10 μg of protein per assay) isolated from either HT29 G⁺ or HT29 G⁺ rev cells were incubated with C₆-NBD-SM (see the Materials and methods section). The formation of C₆-NBD-Cer was measured in control plasma membranes, or when the incubations were performed in the presence of 1 mM of NEM, DTNB, DTP, or DTT. Data are expressed as the relative N-SMase activity, which represents the percentage of C₆-NBD-Cer formation in treated samples divided by the percentage of C₆-NBD-Cer formation in control samples (control = 100%). Data are the average values of three or more measurements. Values in parentheses are S.D.

Treatment	Relative N-SMase activity (%)	
	HT29 G ⁺	HT29 G ⁺ rev
NEM	18 (9.8)	62 (4.1)
DTNB	24 (9.5)	88 (12)
DTP	3 (2.1)	53 (3.5)
DTT	150 (45)	116 (2.0)

different protocol for the isolation of plasma membranes [16] with the aid of merocyanine 540 (results not shown). This method has the advantage that plasma membrane fractions are isolated by vesiculation of the cellular membrane, thus excluding any contamination with, for instance, lysosomes. However, the yield is rather low and the preparation does not necessarily represent a random plasma membrane fraction since vesiculation may occur preferentially at specific membrane domains. Nevertheless, in spite of these drawbacks, the extent of hydrolysis of C₆-NBD-SM in this membrane fraction was found to be 81.4% in 1 h at 37 °C. Also in this case, breakdown of C₆-NBD-GlcCer was not detectable. N-SMase inhibition experiments in HT29 G⁺ plasma membranes employing NEM revealed that the activity is not reduced as much as in liver plasma membranes (Table 6). However, as in rat liver plasma membranes (Table 1), rather low C₆-NBD-SM biosynthetic capacity was detected which, similarly, showed a tendency to increase in the presence of NEM (1.85 ± 0.53% C₆-NBD-SM formation in NEM-treated plasma

membranes compared with $0.87 \pm 0.42\%$ in control plasma membranes).

Interestingly, when the N-SMase was examined in differentiated HT29 G⁺ reversed cells (cf. [10]), its activity was found to be much lower than in the undifferentiated cells (Figures 1b and 3). In intact cells, temperature-dependence experiments showed a lower production and a diminished intracellular accumulation of C₆-NBD-Cer (Figure 1b). Furthermore, in isolated plasma membranes, the extent of hydrolysis after 1 h at 37 °C was at least threefold lower than that in plasma membranes of the undifferentiated counterpart (Figure 3). Interestingly, compared with N-SMase in differentiated HT29 G⁺ rev cells, the N-SMase in undifferentiated HT29 G⁺ cells showed a different susceptibility to the sulphhydryl reagents NEM, DTNB and DTP and the S-S reducing reagent DTT (Table 6). This could indicate that different cell-type-specific subtypes of N-SMase exist. Alternatively, it cannot be excluded that differences may arise from cell-type-dependent differences in the molecular environment of the N-SMase, which in turn could affect the susceptibility to the action of sulphhydryl reagents. It should be noted that in the case of differentiated HT29 G⁺ reversed cells, due to the reduced effectiveness of NEM to inhibit C₆-NBD-SM hydrolysis, a contribution by SM synthase activity to the NBD-lipid turnover could not be rigorously excluded, as was done for undifferentiated HT29 G⁺ cells and liver plasma membranes.

In order to set these results in a broader cell-biological perspective, C₆-NBD-SM hydrolysis was also measured in baby hamster kidney (BHK) fibroblasts. As shown in Figure 1(d), the N-SMase activity in BHK cells more closely resembles that of (differentiated) HT29 G⁺ reversed cells, yielding relatively low amounts of C₆-NBD-Cer. In conclusion, when comparing several types of cultured cell lines, the extent of C₆-NBD-SM hydrolysis appears to be exceptionally prominent in undifferentiated HT29 G⁺ cells.

Cell biological consequences

The result that undifferentiated HT29 G⁺ cells showed an exceptionally high extent of SM hydrolysis in the plasma membrane is quite compatible with previous studies on C₆-NBD-sphingolipid metabolism in HT29 cells [10]. These studies indicated that in undifferentiated HT29 G⁺ cells an extensive conversion takes place of C₆-NBD-SM into C₆-NBD-GlcCer (via C₆-NBD-Cer), which occurs to a much lesser extent in the differentiated counterpart. As a result, these metabolic conversions yield a high C₆-NBD-GlcCer/SM ratio in HT29 G⁺ cells compared with HT29 G⁺ reversed cells. The present work indicates that the conversion of C₆-NBD-SM into C₆-NBD-Cer, serving as a precursor for C₆-NBD-GlcCer biosynthesis, in undifferentiated HT29 G⁺ cells primarily involves the N-SMase in the plasma membrane. Indeed, other studies have revealed that during every endocytic cycle from the plasma membrane to endosomes and back to the plasma membrane most of the C₆-NBD-sphingolipid recycles intact, while only a minor fraction is diverted to the lysosomes [4,30,31]. Therefore, regardless of its activity, the A-SMase probably contributes only marginally to C₆-NBD-SM turnover. Thus, when comparing the endocytic internalization and recycling of C₆-NBD-GlcCer and C₆-NBD-SM, one has to take into account that C₆-NBD-GlcCer will remain largely intact, whereas C₆-NBD-SM is degraded. Considering endocytic trafficking, previous studies have shown that the two lipids are sorted during internalization, C₆-NBD-GlcCer being transported to the Golgi and C₆-NBD-SM ending up in the endo/lysosomal system [32]. Apart from a localization of C₆-NBD-SM in endocytic compartments, one might also envision in

Figure 4 Internalization of C₆-NBD-SM in HT29 G⁺ cells preloaded with Na₂S₂O₄

(a) C₆-NBD-SM was allowed to be internalized in HT29 G⁺ cells for 15 min at 37 °C, followed by a back-exchange at 2 °C, to remove plasma membrane-associated fluorescence (see the Materials and methods section). The resulting intracellular fluorescence labelling pattern is one of endo/lysosomal nature (cf. [32]). (b) HT29 G⁺ cells were, prior to the labelling protocol described in (a), incubated with 30 mM Na₂S₂O₄ and allowed to internalize this NBD-quencher by fluid-phase endocytosis for 30 min at 37 °C. This procedure will result in loading of the endo/lysosomal system with the NBD-quencher. When C₆-NBD-SM was next internalized into the endo/lysosomal system, the fluorescence was quenched. As a result, no punctate fluorescence can be seen in (b). However, a weak Golgi labelling becomes apparent now (arrowheads; cf. [32]), which represents C₆-NBD-Cer and the anabolic products C₆-NBD-SM and C₆-NBD-GlcCer synthesized in the Golgi. The C₆-NBD-Cer has been formed in the plasma membrane from C₆-NBD-SM by the N-SMase and has gained intracellular access by translocation and diffusion, followed by accumulation in the Golgi and metabolic conversion to products. In this way the C₆-NBD-Cer does not come in contact with the NBD-quencher. Bar, 10 μm.

view of the present results that at least part of the C₆-NBD-Cer resulting from C₆-NBD-SM hydrolysis finds its way to the Golgi apparatus, as is the case when cells are incubated directly with C₆-NBD-Cer [33]. Indeed, this appears to be the case, as shown in Figure 4. The normal fluorescence labelling pattern observed after C₆-NBD-SM endocytosis is one of punctate endosomal appearance (Figure 4a; cf. [32]). However, when cells are preloaded with Na₂S₂O₄, which is taken up into endosomes by fluid-phase endocytosis, the NBD-fluorescence of the NBD-sphingolipid molecules in the endosomes is quenched. As a result, Golgi labelling can now be seen (Figure 4b). Evidently, under control conditions (cf. Figure 4a) this Golgi labelling is too weak to be discerned among the more bright endosomal fluorescence.

Apart from an apparent need to convert C₆-NBD-SM into C₆-NBD-GlcCer, the plasma membrane-localized hydrolysis of C₆-NBD-SM may serve another purpose in HT29 G⁺ cells. The products of N-SMase (Cer) and ceramidase (So) may function in modulating signal transduction at the plasma membrane, resulting in modification of cellular responses such as proliferation and/or differentiation [34,35]. However, the formation of large amounts of C₆-NBD-Cer in the plasma membrane of undifferentiated, highly proliferative HT29 G⁺ cells is not in accordance with the generally accepted hypothesis that Cer is inhibitory to proliferation [34]. When added exogenously to HT29 G⁺ cells, C₆-NBD-Cer is able to inhibit cell proliferation (J. W. Kok and D. Hoekstra, unpublished work). This apparent contradiction may well be related to the (in)ability of the C₆-NBD-Cer to reach its intracellular target. Therefore, intracellular transport and topology of C₆-NBD-Cer formation could be important factors determining its cellular actions. For instance, the recently postulated cytosolic N-SMase [36] may be responsible for the generation of a signalling-active ceramide pool in HT29 cells. In this case turnover of C₆-NBD-SM to C₆-NBD-Cer by the exoplasmic N-SMase could merely be a metabolic event, not related to modulation of cellular processes such as proliferation. As far as the extensive production of C₆-NBD-GlcCer in un-

differentiated HT29 G⁺ cells is concerned, this may be related to the postulated stimulation of cell proliferation by GlcCer [11,12].

It is important to note that all metabolic experiments in this study were carried out with (fluorescent) short-chain analogues of sphingolipids. Extrapolation of these results to the fate of their natural counterparts should be performed with caution, given (potential) differences in biophysical/biochemical properties of these short-chain analogues, compared with endogenous cellular sphingolipids. However, many studies in the field of sphingolipid signalling have employed short-chain (non-fluorescent) sphingolipid analogues to monitor effects on cellular responses [37,38]. Similarly, also these studies should be interpreted with caution, not only because of changed properties of the sphingolipid molecule. In addition, these short-chain analogues may well be subject to metabolism, in a similar fashion to the NBD-derivatized lipids. Furthermore, changes in endogenous sphingolipid metabolism as a result of the addition of short-chain sphingolipid analogues to cells should be considered as a potential mechanism underlying cell-biological responses. Finally, as discussed, the topology of sphingolipid turnover and intracellular trafficking may well be of critical importance in eliciting cell-biological responses. The potential role of SM hydrolysis products and GlcCer as bioactive lipids modulating signal transduction related to cell growth and differentiation and the underlying mechanisms are currently being investigated in our laboratory.

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