Use of photoactivatable sphingolipid analogues to monitor lipid transport in mammalian cells

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Photoactivatable derivatives of ceramide, glucosylceramide (GlcCer) and sphingomyelin {3-(*p*-azido-*m*-[¹²⁵I]iodophenyl)propionylceramide, 3-(*p*-azido-*m*-[¹²⁵I]iodophenyl)propionyl-GlcCer and 3-(*p*-azido-*m*-[¹²⁵I]iodophenyl)propionylsphingomyelin} were synthesized in an attempt to identify compartment-specific proteins involved in sphingolipid sorting or metabolism. In HT29 and BHK cells the ceramide analogue entered the cell by monomeric diffusion, as evidenced by the probe's efficient internalization at low temperature (4 °C). In contrast, the photoactivatable GlcCer was internalized only at elevated temperatures (37 °C), presumably reflecting an endocytic mechanism of uptake. The photoactivatable ceramide was mainly metabolized to the corresponding sphingomyelin analogue, but small amounts of GlcCer and galactosylceramide were also synthesized. The newly synthesized photoreactive sphingomyelin was subsequently

transported to the cell surface, a process that was effectively inhibited by the presence of brefeldin A. The incubation of cells with photoactivatable analogues at 4 °C, followed by illumination, led to the association of sphingolipid with a specific subset of proteins. The protein labelling pattern of ceramide differed from that of glucosylceramide. A further shift in labelling pattern was apparent when the cells were incubated with the lipid analogues at 37 °C. Moreover, most of the proteins labelled by photoreactive sphingomyelin seemed to be detergent-insoluble, which is indicative of a location in sphingolipid-rich microdomains at the plasma membrane. The potential of applying photoactivatable sphingolipids to further define and identify the role of distinct proteins in sphingolipid biosynthesis, transport and sorting, is discussed.

INTRODUCTION

Sphingolipids are predominantly located in the outer leaflet of the plasma membrane, where they are believed to serve regulatory functions in cell growth and differentiation, and to have a role in cell adhesion and recognition [1,2].

The ceramide backbone of sphingolipids is synthesized at the endoplasmic reticulum, from where it is transported to the Golgi apparatus. In the Golgi apparatus ceramide is metabolized to glycosphingolipids and sphingomyelin (SM), which are subsequently transported to the plasma membrane [3]. Ceramide is transported to the Golgi via a mechanism that has not yet been identified. Some evidence is, however, pointing to a non-vesicular transport pathway of the sphingolipid precursor from ER to Golgi ([4]; J. W. Kok, T. Babia, K. Klappe, G. Egea and D. Hoekstra, unpublished work). Transport of sphingolipids between Golgi stacks, and between the *trans*-Golgi network and the plasma membrane, is thought to be mediated primarily by a vesicular mechanism. In addition, available evidence indicates that, during endocytosis, sphingolipids are transported by a vesicular mechanism.

The use of fluorescent analogues has been an important tool in studying the intracellular transport and processing of sphingolipids. In particular fluorescence microscopy has contributed greatly to revealing that different sphingolipid analogues have characteristic pathways for intracellular transport. Sphingolipid sorting events have been demonstrated in both the endocytic [5] and the biosynthetic [6] pathways. Although the approach of using fluorescent lipid analogues is very useful for documenting the intracellular movement of lipids, it does not provide possi-

bilities for identifying potential proteins that might have a function in the metabolism or transport of sphingolipids.

The purpose of the present study was to develop an experimental approach that would allow the identification of such putative proteins. Therefore sphingolipid analogues were synthesized that bore a photoaffinity label. These sphingolipid analogues are stable in the dark but are converted by light into highly unstable compounds that can bind covalently to reactive groups located in the direct vicinity of the lipid probe. The photoactivatable sphingolipids that we synthesized were also labelled with ¹²⁵I, allowing the identification of the proteins bound by the lipid probe after irradiation. With the use of photoactivatable ceramide we show that the lipid analogues are recognized by sphingolipid-metabolizing enzymes, leading to the formation of the corresponding analogues of SM, glucosylceramide (GlcCer) and galactosylceramide (GalCer). Furthermore, by incubating the photoreactive sphingolipid analogues with various cell types, we were able to obtain a subset of labelled proteins that was lipid- and cell type-specific. The present approach indicates the potential for characterizing lipid-transporting proteins under defined conditions.

MATERIALS AND METHODS

Materials

Thallium trichloride, sphingomyelinase (from *Staphylococcus aureus*), D-sphingosylphosphocholine and 1β-D-glucosylsphingosine were from Sigma (St. Louis, MO, U.S.A.); [¹²⁵I]NaI was obtained from Amersham (Little Chalfont, Bucks., U.K.); 3-(*p*-

Abbreviations used: C₆-NBD-, 6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GalCer, galactosylceramide; GlcCer, glucosylceramide; HBSS, Hanks buffered salt solution; ¹²⁵I-N₃, 3-(*p*-azido-*m*-[¹²⁵I]iodophenyl)propionyl; [¹²⁵I]AIPP, 3-(*p*-azido-*m*-[¹²⁵I]iodophenyl)propionic acid; [¹²⁵I]AIPPS, 3-(*p*-azido-*m*-[¹²⁵I]iodophenyl)propionylsuccinimide; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; SM, sphingomyelin.

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Figure 1 Structural formula of photoactivatable ceramide ($^{125}\text{I-N}_3\text{-Cer}$)

aminophenyl)propionic acid was from ICN (Costa Mesa, CA, U.S.A.), kindly provided by Dr. Alan Schroit (University of Texas Houston, TX, U.S.A.); *N,N'*-dicyclohexylcarbodi-imide was from Merck (Schuchard, Germany); *N*-hydroxysuccinimide was from Fluka (Buchs, Switzerland). TLC was performed with silica gel 60 HPTLC plates from Merck.

Synthesis of photoactivatable ceramide, GlcCer and SM

Synthesis of the heterobifunctional photoaffinity ligand *N*-{3-(*p*-azido-*m*-[^{125}I]iodophenyl)propionyl}succinimide ([^{125}I]AIPPS) was performed as described [7]. Briefly, [^{125}I]AIPPS was prepared by radioiodination of 3-(*p*-aminophenyl)propionic acid with carrier-free Na^{125}I (specific radioactivity approx. 2000 Ci/mmol) in the presence of thallium trichloride. All subsequent steps were performed under photographic red light to prevent the activation of the photoaffinity ligand. The azide, 3-(*p*-azido-*m*-[^{125}I]iodophenyl)propionic acid ([^{125}I]AIPP), was formed and was subsequently converted to the *N*-hydroxysuccinimide ester [^{125}I]AIPPS with *N,N'*-dicyclohexylcarbodi-imide.

Photoactivatable ceramide ($^{125}\text{I-N}_3\text{-Cer}$), photoactivatable GlcCer ($^{125}\text{I-N}_3\text{-GlcCer}$) and photoactivatable SM ($^{125}\text{I-N}_3\text{-SM}$) were prepared by mixing 300 μCi of [^{125}I]AIPPS (specific radioactivity approx. 2000 Ci/mmol) with 1 μmol of D-sphingosine, 1 β -D-glucosylsphingosine or sphingosylphosphorylcholine respectively in 100 μl of chloroform containing 2 μl of triethylamine. The reaction mixture was incubated for 20 h at 4 °C. After incubation, $^{125}\text{I-N}_3\text{-Cer}$ was purified by TLC with chloroform/methanol/acetic acid (90:2:8, by vol.) as a running solvent and [^{125}I]AIPPS and [^{125}I]AIPP as references. The product (R_F 0.59) was recovered from the silica by extraction with chloroform and chloroform/methanol (1:1, v/v). The structural formula of $^{125}\text{I-N}_3\text{-Cer}$ is depicted in Figure 1. $^{125}\text{I-N}_3\text{-GlcCer}$ (R_F 0.56) and $^{125}\text{I-N}_3\text{-SM}$ (R_F 0.09) were purified by TLC, with chloroform/methanol/25% (w/v) NH_4OH /water (70:30:4:1, by vol.) as the running solvent and [^{125}I]AIPPS and [^{125}I]AIPP as references. $^{125}\text{I-N}_3\text{-GlcCer}$ was extracted from the silica with chloroform/methanol (1:1, v/v) and $^{125}\text{I-N}_3\text{-SM}$ was extracted with methanol. The lipid products were identified by several criteria. The $^{125}\text{I-N}_3\text{-SM}$ spot was identified by treatment with sphingomyelinase (modification of the method of Gatt et al. [8]). To this end, a sample was scraped from the silica and was extracted three times with 1 ml of chloroform/methanol/0.1 M acetic acid (1:2:0.8, by vol.). The extract was dried under a stream of nitrogen and dissolved in 150 μl of 0.13% Triton X-100 in chloroform/methanol (2:1, v/v). The sample was dried again and dissolved in 200 μl of reaction buffer [250 mM Tris/HCl (pH 7.4)/12.5 mM MgCl_2] containing 2 μl of sphingomyelinase (13 units/ml), and

incubated for 90 min at 37 °C. After the incubation, 800 μl of water was added, a lipid extraction was performed and the chloroform phase was analysed by TLC. The sphingomyelinase treatment of the presumed $^{125}\text{I-N}_3\text{-SM}$ lipid spot resulted in a virtually complete degradation to $^{125}\text{I-N}_3\text{-Cer}$. Furthermore an analysis of the lipid products by TLC showed photoactivatable SM and GlcCer to co-migrate with natural and fluorescently tagged 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) SM and GlcCer respectively. Finally, the metabolism of acyl-chain ceramide analogues is usually restricted to their conversion to SM and GlcCer. These products were also recovered when photoactivatable ceramide was incubated with the cells. These two spots co-migrated with chemically synthesized $^{125}\text{I-N}_3\text{-GlcCer}$ and $^{125}\text{I-N}_3\text{-SM}$, thus supporting the identity of the precursor as being ceramide (see Figure 3).

Typical yields, as measured by the total recovery of radioactive iodine, were 40% for [^{125}I]AIPPS, 25% for $^{125}\text{I-N}_3\text{-Cer}$, 20% for $^{125}\text{I-N}_3\text{-GlcCer}$ and 15% for $^{125}\text{I-N}_3\text{-SM}$, after extraction from the silica.

Cell cultures

Baby hamster kidney (BHK) cells were maintained as monolayers at 37 °C in a humidified atmosphere of air/ CO_2 (19:1) in Glasgow minimal essential medium supplemented with 5% (v/v) fetal calf serum (FCS), 10% (v/v) tryptose phosphate broth, 2 mM L-glutamine, penicillin (100 i.u./ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). Monolayers of the human-derived adenocarcinoma cell line HT29 were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) FCS, penicillin and streptomycin. For the lipid uptake and protein labelling experiments, cells were plated in 35 mm Costar tissue culture dishes and grown to 90–100% confluence. For metabolic studies, cells were plated in 25 cm^2 culture flasks.

Ethanol injection of photoactivatable lipids

Photoreactive lipids were added to Hanks buffered salt solution (HBSS) or culture medium by means of ethanolic injection. Lipids from a stock solution in chloroform/methanol (2:1, v/v) were dried under nitrogen and solubilized in ethanol. The ethanolic solution was subsequently injected into culture medium or HBSS with vigorous vortex-mixing. The final concentration of ethanol did not exceed 0.8%.

Uptake and back-exchange of photoactivatable lipids

The cellular uptake of the lipid analogues was studied by incubating cells in the dark or under red light with approx. 100000 c.p.m. of photoactivatable sphingolipid/ml of HBSS at 4 or 37 °C. After the incubation, cells were washed extensively. When required, the fraction of photoreactive lipids present in the outer leaflet of the plasma membrane was then removed by a back-exchange procedure. For this the cells were incubated for 30 min at 4 °C with 5% (w/v) BSA in HBSS, followed by washing with HBSS. This procedure was repeated once.

Metabolism of photoactivatable ceramide

For metabolic studies, with $^{125}\text{I-N}_3\text{-Cer}$ as a sphingolipid precursor, the lipid was added to culture medium without FCS to a final activity of 0.1–0.5 $\mu\text{Ci}/\text{ml}$. Activation of the probe was avoided during the whole procedure of incubation and lipid analysis by keeping the samples in the dark or under red light. After an incubation at 37 °C, the medium was removed, the cells were washed and a back-exchange of plasma membrane-located

Figure 2 Uptake of photoactivatable lipids

HT29 cells were incubated in the dark with approx. 100 000 c.p.m. of $^{125}\text{I-N}_3\text{-Cer}$ or $^{125}\text{I-N}_3\text{-GlcCer}$ /ml HBSS at 4 or 37 °C for various periods. After the incubation the cells were washed and the lipid analogues present at the outer leaflet of the plasma membrane were removed by back-exchange with 5% (w/v) BSA in HBSS at 4 °C. The lipid analogues from the plasma membrane (●) and the intracellular compartment (▲) were quantified by counting for radioactivity. To avoid extensive metabolism during the incubations at 37 °C, the uptake was monitored for only 60 min. Note the different time scales on the 4 °C and the 37 °C incubations. Each data point represents the average of two independent experiments.

lipid was performed. The radioactive lipids from the medium, the plasma membrane and the intracellular pool were extracted by the procedure of Bligh and Dyer [9] and analysed by TLC, with chloroform/methanol/25% (w/v) NH_4OH /water (70:30:4:1, by vol.) as the running solvent and the synthesized $^{125}\text{I-N}_3\text{-Cer}$, $^{125}\text{I-N}_3\text{-GlcCer}$ and $^{125}\text{I-N}_3\text{-SM}$ as references. The radioactive lipid spots were detected by autoradiography and counted for radioactivity with an LKB γ -counter. The sphingomyelin spot was identified by treatment with sphingomyelinase as described above. To distinguish between $^{125}\text{I-N}_3\text{-GalCer}$ and $^{125}\text{I-N}_3\text{-GlcCer}$ as two of the formed monoglycosylceramides, the TLC system as described by van Meer et al. [6], with the use of borate-impregnated TLC plates, was employed.

Photoactivation experiments

Cells were washed with cold HBSS and incubated with 1–2 $\mu\text{Ci/ml}$ photoactivatable lipid for 30 min at 4 °C in HBSS. Until activation the experiments were performed in the dark or under red light. After the incubation the non-incorporated label was removed and the cells were washed with cold HBSS. In the 37 °C incubations the cold HBSS was subsequently replaced by

HBSS at 37 °C. After the incubations the warm HBSS was replaced again by ice-cold HBSS. When indicated, a back-exchange was performed. For photoactivation the cells were scraped gently from the culture dish, with a Costar cell scraper and 1 ml of PBS, and transferred to an ice-water-cooled 1 cm quartz cuvette. The photoaffinity label was activated by irradiating the cells at a distance of 10 cm with an Osram 100 W/2 super-pressure mercury lamp, for 5 min under constant mixing. In initial experiments, cells were not brought into suspension for irradiation but rather were grown on glass coverslips that were irradiated by placing the slips in a PBS-containing cuvette. The protein labelling profile obtained in this case did not differ from that obtained for the scraped cells. For practical reasons, therefore, scraped cells were used in further studies. After irradiation, the cells were spun down and the pellet was resuspended in 10 μl of PBS, sonicated for 1 min in a bath sonicator and treated with 2 μl of DNase I (1 mg/ml) for 30 min at 37 °C. The protein content of the samples was determined by the method of Peterson [10]. Protein samples were solubilized in SDS sample buffer and equal amounts of protein (100–200 μg) were separated on a 10% (w/v) polyacrylamide gel with the system of Laemmli [11]. The gels were stained with Coomassie Brilliant

Blue R-250 and dried. Radiolabelled proteins were identified by autoradiography at -80°C with Kodak X-OMAT film.

Isolation of the low-density detergent-insoluble membrane fraction

The low-density detergent-insoluble fraction was isolated from BHK cells by the method of Brown and Rose [12]. Briefly, BHK cells, grown to confluence on a 145 mm culture dish, were labelled with ^{125}I - N_3 -SM as described above. After photoactivation, the cell pellet was extracted with 1 ml of TNE buffer [25 mM Tris (pH 7.5)/150 mM NaCl/5 mM EDTA], containing 1% (v/v) Triton X-100 and 1 mM PMSF, for 30 min on ice. The lysate was homogenized with 10 strokes of a Dounce homogenizer and adjusted to 40% (w/v) sucrose by using 80% (w/v) sucrose in TNE buffer. The lysate was transferred to an ultracentrifuge tube and a sucrose step gradient consisting of 7 ml of 38% (w/v) sucrose in TNE buffer and 2.5 ml of 5% (w/v) sucrose in TNE buffer was layered on top of the lysate. The gradients were centrifuged for 18 h at 200000 g in a Beckman SW41 rotor at 4°C . The insoluble material at the 5%/38% interface was harvested, diluted with TNE buffer and pelleted. The Triton X-100-insoluble material was solubilized in SDS sample buffer and labelled proteins were analysed as described above.

RESULTS

Uptake of photoactivatable lipids

To characterize and compare the uptake of ^{125}I - N_3 -Cer and ^{125}I - N_3 -GlcCer, cells were incubated with the lipids at 4°C and at 37°C (Figure 2). The cellular distribution of the lipids after incubation was monitored by separating the plasma membrane pool from the intracellular pool by a back-exchange procedure, which removed the lipid analogue from the plasma membrane. Activation of the photoaffinity probe was avoided during the incubations by keeping the cells in the dark.

When HT29 cells were incubated with ^{125}I - N_3 -GlcCer at 4°C , the plasma membrane pool of the lipid increased with time until it reached a steady-state value after 60 min (Figure 2C). The intracellular pool of GlcCer, defined as the non-back-exchangeable fraction, remained very low. Even after a 3 h incubation, the fraction never exceeded 5% of total cell-associated radioactivity, implying that the lipid could not enter the cell at 4°C . During incubation at 37°C with ^{125}I - N_3 -GlcCer (Figure 2D), the plasma membrane reached a steady state within 30 min, whereas over the same period the intracellular pool increased almost linearly, showing a tendency to level off after longer incubation times. Lipid extraction and TLC analysis of medium and cells after a 60 min incubation period revealed that little if any metabolism of photoactivatable GlcCer had occurred. Less than 10% of the total radioactivity was recovered as ^{125}I - N_3 -Cer; the remainder was recovered as ^{125}I - N_3 -GlcCer.

The cellular uptake of ^{125}I - N_3 -Cer seemed to differ entirely from that of ^{125}I - N_3 -GlcCer. At 4°C the plasma membrane pool reaches a maximum after 60 min. In contrast with ^{125}I - N_3 -GlcCer, the intracellular pool increased with time during the 3 h incubation. At 37°C , the plasma membrane and intracellular pool of ^{125}I - N_3 -Cer increased with the same kinetics.

Metabolism of photoactivatable ceramide in HT29 cells

To determine whether the photoreactive ceramide was processed by the cells as a sphingolipid precursor, HT29 cells were incubated with ^{125}I - N_3 -Cer for 24 h at 37°C and the presence of sphingolipids in the incubation medium and cells was determined by lipid extraction and analysis on TLC. From the extract of the

Table 1 Metabolism of ^{125}I - N_3 -Cer in HT29 cells

HT29 cells were incubated with 0.5 μCi of ^{125}I - N_3 -Cer in DMEM without FCS at 37°C . After 24 h the cells were washed and back-exchanged. The radioactive lipid metabolites from the incubation medium (Medium), the back-exchange medium (Plasma membrane) and the cells (Intracellular) were extracted and analysed by TLC as described in the Materials and methods section. The levels of GlcCer in the intracellular fraction and the breakdown product AIPP in the plasma membrane and intracellular fraction were below the detection level. The photoactivatable lipids were not illuminated during the entire experiment. Results are means $\pm$ S.E.M. for three independent experiments.

Metabolite	Radioactivity (% of total)		
	Intracellular	Plasma membrane	Medium
Ceramide	17.3 ± 1	18.8 ± 2	24.0 ± 2.2
GlcCer	—	0.1 ± 0.01	0.5 ± 0.1
GalCer	1.4 ± 0.1	0.5 ± 0.05	0.2 ± 0.05
SM	16.8 ± 1.7	19.1 ± 0.6	2.7 ± 0.3
AIPP	—	—	1.0 ± 0.2

Figure 3 Metabolism of photoactivatable ceramide

HT29 cells were incubated with 0.5 μCi of ^{125}I - N_3 -Cer in DMEM without FCS at 37°C . After 24 h, the radioactive lipid metabolites from the incubation medium (MED), the plasma membrane (PM) and the intracellular pool (IC) were extracted and analysed by TLC. The photoactivatable lipids were not illuminated during the entire experiment.

incubation medium, four (occasionally five) radioactive products were recovered, whereas in the cellular extract three radiolabelled compounds appeared. As shown in Table 1 and Figure 3, the products detected in the incubation medium were identified as ^{125}I - N_3 -Cer, ^{125}I - N_3 -GlcCer and photoreactive sphingomyelin (^{125}I - N_3 -SM). The fifth spot, which was observed only occasionally, had the same R_F as [^{125}I]AIPP (R_F 0.41). We therefore conclude that this spot represents a breakdown product of ^{125}I - N_3 -Cer. The cell-associated products were identified as ^{125}I - N_3 -Cer, ^{125}I - N_3 -GalCer and ^{125}I - N_3 -SM. The biosynthesis of these products was not cell type-specific; similar results were obtained in a differentiated subtype of the HT29 cell line and in BHK cells. Also in these cells, ^{125}I - N_3 -SM was the major product; minor amounts of ^{125}I - N_3 -GalCer and ^{125}I - N_3 -GlcCer were synthesized, the latter of which was recovered almost completely from the incubation medium. ^{125}I - N_3 -GlcCer was not expelled into the medium immediately after synthesis. When the synthesis and distribution of the metabolites of ^{125}I -

Figure 4 Time-dependent distribution of photoactivatable ceramide metabolites

HT29 cells were washed with HBSS and labelled at 37 °C for 60 min with 0.3 μ Ci of 125 I- N_3 -Cer/ml of DMEM without FCS. Then the 125 I- N_3 -Cer was removed and replaced by unlabelled DMEM without FCS. The time scale indicates the time after the addition of 125 I- N_3 -Cer. The results were plotted as a percentage of the total synthesized radioactive lipid in the intracellular compartment (▲), the plasma membrane (●) and the medium (■) at the indicated time points. Results are averages of two independent experiments.

N_3 -Cer were studied as a function of time, we found that at early times (2.5–7 h after the addition of 125 I- N_3 -Cer), 125 I- N_3 -GlcCer could be detected in the three different pools. Only after 24 h was GlcCer found exclusively in the medium (Figure 4). The distribution of the other metabolites seemed to be different: the intracellular 125 I- N_3 -SM pool remained more or less constant, whereas the plasma membrane pool increased in time. Intriguingly, for 125 I- N_3 -GalCer, only the intracellular pool increased and neither significant transport to the plasma membrane nor expulsion of the lipid into the medium occurred (Figure 4). To characterize further the behaviour of the photoactivatable sphingolipids, in particular the nature of their intracellular flow, we next examined the effect of brefeldin A on this process.

Table 2 Effect of brefeldin A on the synthesis and transport of the metabolites of photoactivatable ceramide

HT29 cells were incubated with 0.5 μ Ci of 125 I- N_3 -Cer in DMEM without FCS in the absence or the presence of 2 μ g/ml brefeldin A for 24 h at 37 °C. The results are expressed as a percentage of the total synthesized radioactive lipid in the intracellular compartment, the plasma membrane and the medium. The levels of GlcCer were below detection level in the intracellular fraction of control cells. Results are averages from two independent experiments.

Fraction	Metabolite	Radioactivity (% of total)	
		Control	Brefeldin A
Incubation medium	SM	2.4	2.1
	GlcCer	0.3	0.4
	GalCer	0.2	0.2
Plasma membrane	SM	15.3	1.2
	GlcCer	0.1	0.1
	GalCer	0.1	0.1
Intracellular compartment	SM	18.4	23.2
	GlcCer	—	1.0
	GalCer	1.3	1.0

Brefeldin A inhibits transport of sphingomyelin

The fungal metabolite brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the ER [13], and can be used as an inhibitor of vesicular transport.

Labelling of the Golgi with the fluorescent vital stain 6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoylsphingosine (C_6 -NBD-Cer) showed that in HT29 cells the Golgi was disrupted within 30 min after treatment with brefeldin A (2 μ g/ml; results not shown). This result is in agreement with studies performed by others [14]. In the present study, HT29 cells were incubated for 24 h at 37 °C with 125 I- N_3 -Cer in the presence of 2 μ g/ml brefeldin A, after which the radioactive sphingolipids from the incubation medium, the plasma membrane and the intracellular pool were extracted and analysed. Brefeldin A inhibited the synthesis of 125 I- N_3 -GalCer (approx. 10 %) and 125 I- N_3 -SM (approx. 25 %), whereas the 125 I- N_3 -GlcCer synthesis was stimulated by 3.7-fold. However, a more pronounced effect of brefeldin A was seen on the transport of the sphingolipids. The plasma membrane pool of 125 I- N_3 -SM decreased to less than 10 % of the control value and a substantial intracellular accumulation of 125 I- N_3 -GlcCer was observed (Table 2). In BHK cells, brefeldin A had a stimulating effect (approx. 20 %) on the synthesis of both sphingomyelin and GlcCer (results not shown). The effect on lipid transport was not studied in these cells.

Photoactivatable ceramide and GlcCer label a restricted subset of proteins in HT29 and BHK cells

The following experiments were performed to determine whether specific proteins might associate with the various photoreactive analogues during their internalization and subsequent trafficking, as described above.

Radiolabelling of proteins with the photoactivatable 125 I- N_3 -Cer and 125 I- N_3 -GlcCer was performed at 4 and 37 °C. As shown above, at 4 °C 125 I- N_3 -Cer can enter the cell, whereas 125 I- N_3 -GlcCer remains at the outer leaflet of the plasma membrane. At 37 °C both lipids enter the cell, with similar initial rates, although the mechanism of uptake probably differs in that most of the ceramide will enter by monomeric translocation [15], whereas GlcCer is primarily internalized along the endocytic pathway [16]. By incubating the cells with 125 I- N_3 -Cer and 125 I- N_3 -GlcCer

Figure 5 Photoaffinity labelling of HT29 proteins with $^{125}\text{I-N}_3\text{-GlcCer}$ and $^{125}\text{I-N}_3\text{-Cer}$

HT29 cells were labelled with $^{125}\text{I-N}_3\text{-GlcCer}$ or $^{125}\text{I-N}_3\text{-Cer}$ as described in the Materials and methods section. After photoactivation, equal amounts of total cellular protein (150 μg) were loaded on an SDS/polyacrylamide gel. (A) Total cellular protein and proteins labelled with $^{125}\text{I-N}_3\text{-GlcCer}$: lane 1, Coomassie Brilliant Blue staining of total cellular protein; lane 2, labelling with $^{125}\text{I-N}_3\text{-GlcCer}$ at 4 °C; lane 3, labelling with $^{125}\text{I-N}_3\text{-GlcCer}$ after a 30 min incubation at 37 °C and a subsequent back-exchange. (B) Labelling of HT29 proteins with $^{125}\text{I-N}_3\text{-Cer}$: lane 1, labelling with $^{125}\text{I-N}_3\text{-Cer}$ at 4 °C; lane 2, after a 30 min incubation at 37 °C; lane 3, after a 30 min incubation at 37 °C and a subsequent back-exchange. (C) Densitometric scans of the autoradiograms in (A). The solid line represents protein labelling with $^{125}\text{I-N}_3\text{-GlcCer}$ at 4 °C [lane 2 in (A)]; the dotted line represents labelling at 37 °C [lane 3 in (A)]. The large peak, left of the 14 kDa marker, represents either the $^{125}\text{I-N}_3\text{-GlcCer}$ that has been cross-linked to other lipids or non-cross-linked lipid analogue. (D) Densitometric scans of the autoradiograms in (B). The solid line represents protein labelling with $^{125}\text{I-N}_3\text{-Cer}$ at 4 °C [lane 1 in (B)]; the dotted line represents labelling at 37 °C [lane 2 in (B)]; the broken line represents labelling obtained after a 30 min incubation at 37 °C followed by a back-exchange [lane 3 in (B)]. Abbreviation: kD, kDa.

at different temperatures before illuminating the lipid probe, we examined whether the differences in localization were reflected in different protein labelling profiles. Control experiments in which the cells were not irradiated by UV were performed to ensure that the photoreactive lipids displayed specificity and became activated only when desired, rather than that labelling occurred non-specifically during the incubation or analysis procedures. In these control experiments, cells were incubated for 30 min at 37 °C with $^{125}\text{I-N}_3\text{-Cer}$ or $^{125}\text{I-N}_3\text{-GlcCer}$ and the potential presence of labelled proteins was determined without photoactivation as described for conditions under which irradiation had taken place. In these experiments, no labelled proteins were detected. However, at the gel front a radioactive spot was seen that most probably represents the non-cross-linked photoactivatable lipid (see Figure 5).

When HT29 cells were incubated at 4 °C with $^{125}\text{I-N}_3\text{-GlcCer}$ and subsequently irradiated with UV, protein analysis revealed the presence of two very prominently labelled protein bands (Figure 5A, lane 2), located at approx. 20 and 100 kDa. Some minor labelling of several other proteins was also observed (Figures 5A and 5C). When before irradiation a back-exchange was performed, which removes at least 95 % of the cell-associated $^{125}\text{I-N}_3\text{-GlcCer}$ (Figure 2), a similar labelling pattern was observed, although much longer exposure times of the autoradiogram were needed to detect the labelled proteins (results not shown). This indicates that the small percentage of $^{125}\text{I-N}_3\text{-GlcCer}$ that remained cell-associated after the back-exchange was not interacting with a specific protein, which would prevent its removal from the plasma membrane by BSA. When cells were allowed to internalize $^{125}\text{I-N}_3\text{-GlcCer}$ for 30 min at 37 °C and

Figure 6 Photoaffinity labelling of proteins of BHK cells with ^{125}I - N_3 -GlcCer and ^{125}I - N_3 -Cer

BHK cells were labelled with ^{125}I - N_3 -GlcCer or ^{125}I - N_3 -Cer as described in the Materials and methods section. After photoactivation, equal amounts of total cellular protein (150 μg) were loaded on an SDS/polyacrylamide gel. (A) Labelling of cells with ^{125}I - N_3 -GlcCer: lane 1, labelling with ^{125}I - N_3 -GlcCer at 4 °C; lane 2, labelling with ^{125}I - N_3 -GlcCer after a 5 min incubation at 37 °C and a subsequent back-exchange. (B) Labelling of cells with ^{125}I - N_3 -Cer: lane 1, labelling with ^{125}I - N_3 -Cer at 4 °C; lane 2, after a 5 min incubation at 37 °C and a subsequent back-exchange. For reasons of clarity, the autoradiograms showing the 5 min incubations at 37 °C were exposed to film for longer than the autoradiograms showing the incubations at 4 °C. (C) Densitometric scans of the autoradiograms in (A). The solid line represents protein labelling with ^{125}I - N_3 -GlcCer at 4 °C [lane 1 in (A)]; the dotted line represents labelling at 37 °C [lane 2 in (A)]. (D) Densitometric scans of the autoradiograms in (B). The solid line represents protein labelling with ^{125}I - N_3 -Cer at 4 °C [lane 1 in (B)]; the dotted line represents labelling at 37 °C [lane 2 in (B)]. Abbreviation: kD, kDa.

when the plasma membrane was subsequently depleted from the lipid by a back-exchange before the probe was irradiated, a distinct shift in protein labelling was observed (Figure 5A, lane 3). Proteins that were labelled by ^{125}I - N_3 -GlcCer after the 4 °C incubation also appeared after the 37 °C incubation, as judged by their apparent molecular masses of 20 and 100 kDa on an SDS gel, suggesting that the close association of protein and lipid remained after internalization. However, at the elevated temperature an additional set of proteins with apparent molecular masses of 60–65 kDa and 24 kDa became intensely radiolabelled. Minor labelling of a larger set of proteins compared with the labelling profile at 4 °C was also observed. Interestingly, none of the labelled proteins represented major proteins, as revealed by Coomassie Brilliant Blue staining of a total cell protein fraction (Figure 5A, lane 1).

The labelling profile of HT29 cells that had been incubated at 4 °C with ^{125}I - N_3 -GlcCer was specific for this lipid; when cells were incubated with ^{125}I - N_3 -Cer at 4 °C (Figure 5B, lane 1;

Figure 5D, continuous line), substantial labelling was apparent only in the 100 kDa region, whereas labelling in the 20 kDa region, as observed for ^{125}I - N_3 -GlcCer, was minor (Figure 5A, lane 2). However, incubation of HT29 cells with ^{125}I - N_3 -Cer at 37 °C resulted in a labelling profile that had similarities with the profile of the 37 °C incubation with ^{125}I - N_3 -GlcCer, showing prominent labelling of the 100 kDa, the 65–50 kDa region and labelling of proteins of 24 and 20 kDa. When the plasma membrane of these cells was subsequently depleted of photoreactive lipid before photoactivation, the prominent labelling in the 100 kDa region observed in cells labelled with ^{125}I - N_3 -GlcCer was no longer apparent (Figure 5B, lane 3). In addition, labelling in the 20–30 kDa regions disappeared.

To determine whether the labelling profiles that we obtained in the HT29 cell line were cell type-specific, BHK cells were analysed in a similar fashion (Figure 6). After an incubation at 4 °C, ^{125}I - N_3 -GlcCer labelled proteins in the 20 and 100 kDa regions in BHK cells, as it did in HT29 cells (Figure 6A, lane 1; Figure 6C,

Figure 7 Triton X-100-insoluble low-density fraction, isolated from BHK cells, contains most of the proteins labelled by $^{125}\text{I-N}_3\text{-SM}$

BHK cells were labelled with $^{125}\text{I-N}_3\text{-SM}$ as described in the Materials and methods section. The Triton X-100-insoluble low-density fraction was then isolated and the total cellular proteins fraction, the Triton X-100-insoluble fraction, and proteins that were soluble in Triton X-100 were separated by SDS/PAGE. Because the relative labelling intensity of the Triton X-100 insoluble fraction was much higher than that of the other fractions, only 40 μg of protein in this fraction was applied to the gel, whereas 200 μg protein was loaded for the other fractions. (A) Coomassie Brilliant Blue-stained proteins and the autoradiographic representation of labelled proteins in these fractions: lane 1, total cell lysate, Coomassie-stained; lane 2, total cell lysate, autoradiogram; lane 3, Triton X-100-insoluble fraction, Coomassie-stained; lane 4, Triton X-100-insoluble fraction, autoradiogram; lane 5, Triton X-100-soluble proteins, Coomassie-stained; lane 6, Triton X-100-soluble proteins, autoradiogram. (B) Densitometric scans of the autoradiogram in (A). The solid line represents the total cell lysate (lane 2); the dotted line represents the Triton X-100-insoluble low-density fraction (lane 4); the broken line represents the Triton X-100-soluble proteins (lane 6). Abbreviation: kD, kDa.

continuous line). Additionally, a group of proteins in the 40–45 and 30 kDa regions were labelled. Except for prominent labelling of the 100 kDa band, the labelling profile of $^{125}\text{I-N}_3\text{-GlcCer}$ after internalization (at 37 °C) and subsequent lipid depletion of the plasma membrane (Figure 6A, lane 2), differed markedly from the profile obtained at 4 °C. The specificity of this labelling is further emphasized when the pattern is compared with that obtained for HT29 cells (e.g. Figure 5A, lane 2, compared with Figure 6A, lane 1).

Finally, as shown in Figures 6(B) and 6(D), labelling obtained in BHK cells with $^{125}\text{I-N}_3\text{-Cer}$ displayed a pattern that, apart from labelling of the 100 kDa region, was quite distinct from that obtained with $^{125}\text{I-N}_3\text{-GlcCer}$. In addition, the profile changed

substantially when the incubation temperature was elevated (Figure 6B, lane 2). Interestingly, the pattern thus obtained was only marginally related to that of GlcCer. This emphasizes the specificity of the molecular environment of both lipids.

Photoactivatable SM accumulates in a detergent-insoluble fraction of BHK cells

As shown above, the photoactivatable ceramide was mainly metabolized to sphingomyelin, which was subsequently transported to the plasma membrane. It has been shown that at low temperature, a large fraction of total SM from MDCK cell lysates can be recovered in a low-density detergent-insoluble vesicle fraction that is enriched in (glyco)sphingolipids, cholesterol and glycosylphosphatidylinositol-anchored proteins [12]. It has been suggested that these detergent-insoluble complexes represent membrane microdomains that are involved in apical membrane sorting and are related to or similar to caveolae, i.e. specialized microdomains at the plasma membrane [17]. Indeed, the plasma membrane origin of the detergent-insoluble fraction has been demonstrated in numerous cell lines, including fibroblasts [17,18]. However, a potential artifact in identifying microdomain-specific proteins arises when, during detergent extraction, proteins co-purify and associate with the detergent-insoluble vesicle fraction. To explore the possibility of circumventing this problem, we used photoreactive sphingomyelin ($^{125}\text{I-N}_3\text{-SM}$) to label proteins associated in sphingolipid-rich domains *in situ*, before detergent extraction. From control, i.e. non-labelled BHK cells, which have cognate apical and basolateral transport routes [19], the low-density detergent-insoluble fraction was isolated as described in the Materials and methods section. In this fraction 28 ± 1.4 (S.E.M.) % of the total SM pool was recovered, whereas only 7 ± 2 % of the total phosphatidylcholine pool and 0.3 ± 0.04 % of total protein pool were recovered in the same fraction. When BHK cells were labelled at 4 °C with $^{125}\text{I-N}_3\text{-SM}$, this lipid, as was found for $^{125}\text{I-N}_3\text{-GlcCer}$, remained at the outer leaflet of the plasma membrane (see Figure 2) and therefore labels only plasma membrane proteins on activation of the probe. When the detergent-insoluble fraction was isolated from such treated cells, we recovered 15 % of the total $^{125}\text{I-N}_3\text{-SM}$ -associated radioactivity in this fraction. Furthermore most of the plasma membrane proteins that were found to be labelled in the total cell lysate (Figure 7A, lane 2; Figure 7B, continuous line), were insoluble in detergent, although the protein labelling pattern of the detergent-insoluble fraction (Figure 7A, lane 4; Figure 7B, dotted line) was somewhat different from that of labelled proteins in a total cell lysate. In this respect it is interesting to note that a protein of 30 kDa was clearly labelled in the detergent-insoluble fraction, while it was barely distinguishable in the total cell lysate.

DISCUSSION

In the present study we have synthesized novel, photoactivatable sphingolipid analogues. Evidence has been provided that demonstrates that the analogues are metabolically active and that they are transported and handled by the cells in a manner similar to their widely used fluorescent counterparts (e.g. NBD analogues), emphasizing a versatile potential for cell biological studies. The results indicate that application of these probes might pave the way for developing novel strategies to identify proteins that regulate specifically with sphingolipid trafficking.

In contrast with a fairly detailed knowledge of molecular mechanisms involved in the specific sorting of membrane proteins, such knowledge about specific routing of lipids is entirely obscure. Sorting of distinct lipids evidently occurs by a mech-

anism that differs from bulk flow. For example, GlcCer has been reported to be sorted in the endocytic pathway in undifferentiated HT29 cells, as well as in the biosynthetic pathway in polarized epithelial cells. It has been proposed that the formation of patches and/or domains in which particular lipids accumulate might constitute a mechanism that triggers specific sorting and ensuing targeting [12,20]. It is unclear whether physical interactions between lipids or specific interaction with proteins could have a role in such a mechanism. This study was initiated to elucidate whether distinct proteins can act as lipid chaperones. The present approach allows a direct determination of proteins adjacent to the lipid, whereas others [21] have made use of an indirect procedure, with fluorescent lipid analogues and a photoactivatable protein-directed probe. The versatile use of the direct approach has been demonstrated in studies aimed at identifying the aminophospholipid translocase [22].

As shown in Figure 2, the properties of the synthesized analogues are reminiscent of the short-chain fluorescent NBD analogues. The ceramide analogue enters cells by rapid translocation across the membrane at temperatures as low as 4 °C, which is indicative of a transfer as a free monomer [15,23]. In contrast, the photoactivatable GlcCer derivative, very similarly to what is observed for the NBD-labelled analogue, enters the cell only at elevated temperatures, i.e. presumably along the pathway of receptor-mediated endocytosis [16]. In addition, analogously to observations with the C₆-NBD analogues, there is no reutilization of degradation products. Thus [¹²⁵I]AIPP is rapidly removed from the cells and expelled into the medium, its fate being similar to that of C₆-NBD [3].

When the cells were incubated with photoactivatable ceramide at 37 °C, the sphingolipid precursor was mainly metabolized to sphingomyelin and only very small amounts (less than 4 % of the total cell-associated radioactive lipid products) of the glycosphingolipids GlcCer and GalCer were synthesized. Note that the ability of the photoactivatable precursor to enter the biosynthetic pathway can be exploited for further characterization of sphingolipid processing in the Golgi apparatus. As was shown by Pütz and Schwarzmann [24], Golgi labelling by ceramide derivatives occurred only when these analogues could be metabolized. The preferential synthesis of sphingomyelin is not specific for the ceramide analogue that we used, but has also been reported when fluorescent C₅-DMB-ceramide [23] and other precursor ceramides [25] that were modified at their fatty acid chain were employed. This feature can thus be exploited, as an alternative to the use of specific inhibitors of lipid synthesis, to identify the nature of the lipid (SM or GlcCer) that associates with protein in the biosynthetic pathway, when photoactivatable ceramide is used as a precursor. The discrepancy in net biosynthesis of SM compared with GlcCer is not usually observed when C₆-NBD-Cer is used as the sphingolipid precursor. However, when the sphingolipid synthesis *de novo* was determined in Chinese hamster ovary (CHO) cells by using tritiated palmitate, incorporation into SM was more than 10-fold higher than into GlcCer [26].

In this study we also demonstrate that the cellular distribution of the metabolites of photoactivatable ceramide differs for each metabolite. This implies that each of the photoreactive products formed, i.e. SM, GalCer and GlcCer, is recognized by the cells as such and apparently processed in a specific lipid-species-dependent manner. Because, in molecular terms, GlcCer and GalCer differ only in the orientation of one hydroxy group, these observations would suggest a substantial degree of specificity in the (intra)cellular processing of these lipids. This specificity would entail that GlcCer is governed to enter a transport pathway to the cell surface, whereas GalCer is not. Remarkably, once at the cell surface, the pool of labelled GlcCer disappears, in

contrast with the cell-associated pools of SM (also present at the cell surface) and GalCer. These results correlate with those described by Warnock et al. [26], who showed, in a pulse-chase experiment with [³H]glucose-labelled GlcCer, that the pool of newly synthesized GlcCer appearing in the plasma membrane of CHO cells via a Golgi-independent pathway can decrease rapidly. The extent of inhibition of GlcCer transport (approx. 50 %) that we observe in the presence of brefeldin A is of interest in this context, as it could suggest that part of the photoreactive GlcCer also acquires access to the cell surface via a non-vesicular, Golgi-independent pathway, whereas another part is transported by a vesicular transport mechanism. Moreover, under similar conditions, essentially the entire fraction of newly synthesized SM does not reach the cell surface. These results indicate that SM transport is primarily vesicular. This notion is of major relevance in the context of suggestions, derived from studies with NBD-labelled SM and brefeldin A treatment, that a fraction (10–20 %) of the lipid analogue might reach the cell surface in a non-vesicular manner [27]. It is more likely that NBD-labelled SM is artificially translocated across the Golgi membrane, as induced by brefeldin A treatment, and reaches the cell surface by monomeric transfer. The present results support the concept that SM transport to the cell surface is by means of vesicular transport, consistent with some studies reported previously [26,28]. The effect of brefeldin A on SM metabolism and transport has been studied in some detail by others. Brefeldin A was reported to stimulate SM synthesis in BHK [28], CHO cells [26,29] and hepatocytes [30]. In contrast with these results, there have been reports of hydrolysis of SM in HL60 cells [31] and inhibition of incorporation of radioactive precursors into SM in cultured neurons [32] by brefeldin A. These conflicting results might be explained partly by cell type-dependent effects of brefeldin A, as our results show that brefeldin A can inhibit synthesis of SM in HT29 cells, whereas in BHK cells a small stimulation was found.

The ultimate goal of applying photoactivatable probes is to identify proteins that might be crucial in lipid sorting and transport. Given the similarity in their behaviour as previously observed for NBD-labelled analogues, and given the detailed knowledge of the intracellular flow of the former, the present work should provide a basis for revealing protein involvement, if any. First of all, cell type-dependent differences in protein labelling pattern were apparent when BHK cells were compared with HT29. Secondly, labelling profiles were also remarkably dependent on the lipid species examined, although similarities were seen as well. Thirdly, only a restricted number of proteins became labelled, whereas the labelled proteins commonly found were not proteins that were abundantly present in the cell. This would suggest a distinct degree of labelling specificity. Interestingly, the labelling pattern of photoactivatable ceramide, which potentially distributes over intracellular membranes at 4 °C, seems to be relatively simple compared with the labelling pattern of photoactivatable GlcCer, which is located only at the plasma membrane at this temperature. Particularly prominent is the labelling of a 100 kDa protein in all cases. Intriguingly, this protein seems to accompany GlcCer during early endocytic events, whereas no association with ceramide is seen after raising the temperature and when performing a back-exchange before photoactivation. Furthermore in BHK cells, GlcCer, but not ceramide, was specifically associated with a protein fraction centred on 40 kDa.

The present approach, employing the newly synthesized photoreactive sphingolipid analogues, including their metabolically susceptible precursor ceramide, should enable the determination of whether specific proteins recruit sphingolipids (or vice versa) in Triton X-100-resistant microdomains [12]. Potential sphingo-

lipid-transporting proteins might also be identified in this manner [33]. As we have shown here, photoaffinity-labelled sphingomyelin is accumulating in a Triton X-100-insoluble fraction, and many plasma membrane proteins labelled by this analogue were also found to be Triton X-100-insoluble. Experiments are currently in progress to identify further, under carefully controlled conditions, the nature of the labelled proteins and their significance in sphingolipid processing.

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