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Epithelial sphingolipid sorting allows for extensive variation of the fatty acyl chain and the sphingosine backbone

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In kidney MDCK and intestinal Caco-2 epithelial cells, glucosylceramide (GlcCer) and sphingomyelin (SPH) synthesized from the short-chain sphingolipid analogue *N*-6-[7-nitro-2,1,3-benzoxadiazol-4-yl]aminodecanoyl (C_6-NBD)-ceramide are delivered to the cell surface with apical/basolateral polarities of 2–4 and 0.6–0.9 respectively. We have tested how variations in the lipid backbone affect these polarities. First, the C_6-NBD moiety was replaced by a bare [^{14}C]octanoyl chain or by the even more bulky fluorophores 8-bimanoylthio-octanoyl (C_8-bimane) and 8-diethylaminocoumarin-octanoyl (C_8-DECA). In addition, the sphingosine in C_6-NBD-ceramide was changed in stereoconfiguration (L-*threo*) or saturation (dihydro). In all cases, GlcCer and SPH were produced and appeared on the cell surface at 37 °C, as assayed by back-exchange. The apical/basolateral polarity of the delivery of GlcCer was variable, but always exceeded 1. GlcCer was apically enriched over SPH (2–6 times for MDCK and 3–9 times for Caco-2). Even GlcCer synthesized from a highly water-soluble truncated ceramide (octanoyl-D-*erythro*-sphingosine analogue with C_8 backbone) was enriched apically by a factor of $\geqslant 2$ both in absolute polarity and compared with SPH. Sphingolipid sorting was quantitatively but not qualitatively affected by dramatic changes in the lipid backbone.

INTRODUCTION

The plasma membrane of epithelial cells is differentiated into an apical and a basolateral domain with unique protein and lipid compositions. Tight junctions separate the two and prevent intermixing of membrane components. Since for lipids the diffusion barrier is only present in the external bilayer leaflet, this is where the differences in lipid composition reside. To generate and maintain lipid polarity during the ongoing transport processes to and from each plasma membrane domain, apical and basolateral lipids must be segregated or sorted continuously (reviewed in [1,2]).

Previously, we have probed the generation of lipid polarity by studying the polarity of delivery of newly synthesized sphingolipids to the surface of dog kidney (MDCK) and human intestinal (Caco-2) epithelial cells [3,4]. The fluorescent *N*-6-[7-nitro-2,1,3-benzoxadiazol-4-yl]aminodecanoyl (C_6-NBD)-ceramide was used as a sphingolipid precursor. It readily partitions into cells, where it is metabolized to C_6-NBD-glucosylceramide (GlcCer) and C_6-NBD-sphingomyelin (SPH) [5,6], analogues of lipids that are enriched on the apical and basolateral surfaces respectively of epithelia *in vivo* [4]. Delivery to each cell surface could be monitored, as: (1) the short C_6-NBD acyl chain allowed depletion into the medium by BSA, and (2) in cell monolayers on filters, both surfaces are accessible. Indeed, GlcCer was preferentially targeted apically in terms of absolute amounts and also compared with SPH.

In the present study we tested how variations in the lipid chains affect preferential apical delivery of GlcCer and sorting of GlcCer from SPH. The NBD moiety was removed from the fatty acyl chain to exclude possible artefacts in our earlier sorting studies due to the bulky fluorescent NBD group. Secondly we tested whether 8-bimanoylthio-octanoyl (C_8-biamine)- and 8-diethylaminocoumarin-octanoyl (C_8-DECA)-lipids, which can be used in fluorescence co-localization and even resonance energy transfer studies with NBD-lipids [7,8], actually behave like NBD-lipids. Thirdly, the sphingosine backbone was modified to test the sorting mechanism for stereospecificity. Finally, both lipid chains were truncated to eight carbon atoms to test whether the hydrophobicity of the molecule was critical for sorting. All results were qualitatively similar to those obtained previously, which confirms the fundamental character of the observed sorting event.

MATERIALS AND METHODS

Epithelial cell culture

Monolayers of MDCK strain II cells were grown on 0.4 μm-pore-size/4.7 cm^2 Transwell filters, and Caco-2 cells (passages 70–85) were grown on 0.45 μm-pore-size/4.7 cm^2 Transwell-COL filters (Costar, Cambridge, MA, U.S.A.), essentially as before [3,4]. Filters were suspended in polypropylene rings instead of the original 6-well cluster dish to optimize medium supply to the basal side of the filter. For experiments, filters contained 4.7×10^6 MDCK cells 3 days after plating, or 2.5×10^6 or 4.5×10^6 Caco-2 cells 7 or 12 days respectively after plating. Two filters were pooled for lipid analysis. One Caco-2 filter was used for radioactive lipid analyses.

Chemical synthesis and purification of fluorescent and radioactive ceramides

C_6-NBD-, C_8-bimane- and [^{14}C]octanoyl-ceramide were synthesized using D-*erythro*-sphingosine from bovine brain SPH plus 6-NBD-hexanoic acid, 8-bimane-octanoic acid or carboxy[^{14}C]octanoic acid (6 Ci/mol) respectively, by oxidation/reduction condensation with triphenylphosphine and 2,2′-dipyridyl disulphide [9]. C_6-NBD-dihydroceramide was prepared similarly using D-*erythro*-dihydrosphingosine. C_8-bimane-

Abbreviations used: C_6-NBD, *N*-6-[7-nitro-2,1,3-benzoxadiazol-4-yl]aminodecanoyl; C_8-bimane, 8-bimanoylthio-octanoyl; C_8-DECA, 8-diethylaminocoumarin-octanoyl; C_8/C_8-[^{3}H]ceramide, [2,3-$^3H_2$]-octanoyl-D-*erythro*-sphingosine analogue with a C_8 backbone; GlcCer, glucosylceramide; HBSS, Hanks' balanced salt solution without bicarbonate, containing 10 mM-Hepes, pH 7.4; PC, phosphatidylcholine; SPH, sphingomyelin.

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ceramide was also synthesized by acylation of D-*erythro*-sphingosine with the *N*-hydroxysuccinimide ester of 8-(methylmercapto)-mercapto-octanoic acid, followed by isolation of the protected ceramide, deblocking the thiol group with dithiothreitol and labelling with bromobimane [7,8]. C_8-DECA-ceramide was prepared by reacylation of D-*erythro*-sphingosine with the *N*-hydroxysuccinimide ester of 8-DECA-octanoic acid (according to ref. [10]). Ceramides were purified by preparative t.l.c. in chloroform/methanol/acetic acid (90:2:8, by vol.) [9]. Fluorescent ceramides were further purified by reversed-phase h.p.l.c. A 4 mm × 250 mm Spherisorb ODS-2 (5 μm) column (Pharmacia LKB, Uppsala, Sweden) was used with a chloroform/water/H_3PO_4 (850:150:1.5, by vol.) mobile phase. Samples were applied in 100 μl and were eluted at a flow rate of 0.75 ml/min, and the elution pattern was detected by measuring absorbance at 405 nm with a Uvicord SD detector. In all cases, three peaks were isolated. For C_6-NBD-ceramides, elution times were 18, 20 and 23 min. The first peak, the natural D-*erythro* stereoisomer [11], amounted to 80–95% of the fluorescence and was used for all experiments. The second peak co-eluted with L-*threo*-ceramide prepared enzymically from C_6-NBD-SPH as described by Koval & Pagano [12]. The third peak had the same retention time as D-*erythro*-C_6-NBD-dihydroceramide. [2,3-$^3H_2$]Octanoyl-C_8-D-*erythro*-sphingosine (C_8/C_8-[^{3}H]ceramide) was custom-synthesized from octanoic acid and $^3H_2$ (50 Ci/mmol; Amersham–Buchler, Braunschweig, Germany), as described [13].

Quantification of fluorescent sphingolipid delivery to the cell surface

Fluorescent ceramide precursors were incorporated into cells for 0.5 h at 10 °C from liposomes [4] or BSA/ceramide complexes (1 ml of 5 μM-ceramide and defatted BSA [11]) in the apical medium, followed by three washes with Hanks' balanced salt solution without bicarbonate and containing 10 mM-Hepes, pH 7.4 (HBSS). The two methods gave identical levels of insertion, about 75 pmol of C_6-NBD-ceramide/10^6 MDCK cells. Delivery of fluorescent products to the plasma membrane was assayed during 1 h at 37 °C by continuous depletion from the surface by BSA in both apical and basal media (1%, w/v). This was followed by two back-exchanges against BSA-containing HBSS for 0.5 h at 10 °C. Fluorescent lipids were extracted from the apical medium at 37 °C plus apical medium at 10 °C, the pooled basal media, and the cell monolayer on the filter, after which they were quantitatively analysed by two-dimensional-h.p.t.l.c. and measurement of NBD fluorescence in the individual lipid spots as before [4]. At the end of the experiment about one-third of the remaining ceramide was recovered in the (apical) medium. The variation in levels of synthesis and transport was within 10% of the mean. Bimane and DECA fluorescences were quantified at 400 nm/460 nm by comparison with standards of bimane- or DECA-phosphatidylcholine (PC), which in turn were quantified by phosphate as before [3]. In a mock experiment the fluorescence background amounted to 0.4 pmol/10^6 MDCK cells, and to 0.8 pmol/10^6 Caco-2 cells.

Quantification of [^{14}C]octanoyl-sphingolipid delivery to the cell surface

[^{14}C]Octanoylceramide was added to cells as BSA complexes (43 μM for both [^{14}C]octanoylceramide and BSA) for 1 h at 10 °C. Cell-surface delivery was assayed as described above. Newly synthesized [^{14}C]octanoyl-lipids were located on h.p.t.l.c. plates by autoradiography, identified as SPH and GlcCer by comparison with authentic markers, and quantified in a mixture of 0.5 ml of Solulyte (J. T. Baker Chemicals B.V., Deventer, The Netherlands) and 5 ml of Ultima Gold (Packard Instrument Company, Downers Grove, IL, U.S.A.). The background was 50 c.p.m., equivalent to 4.7 pmol, or 0.5 pmol/10^6 MDCK cells and 1.9 pmol/10^6 Caco-2 cells in a mock experiment.

Quantification of C_8/C_8-[^{3}H]sphingolipid delivery to the cell surface

After three washes with HBSS, the cell monolayers were incubated for 1 h at 37 °C with 1 ml of HBSS containing 20 μCi of C_8/C_8-[^{3}H]ceramide/ml at the apical side and 2 ml of HBSS at the basolateral side. They were then washed with HBSS for 0.5 h at 10 °C. The pooled apical and pooled basal incubation media were run over a pre-wetted Sep-Pak cartridge (Waters, Millipore Corp., Milford, MA, U.S.A.). After a wash with 3 ml of water, C_8/C_8-[^{3}H]sphingolipids were eluted with 1.1 ml of 80% acetonitrile. Filters were extracted in 1 ml of methanol for 0.5 h at room temperature, and washed with 0.5 ml of methanol. The C_8/C_8-[^{3}H]sphingolipid extracts from media and cells were dried in a Speedvac concentrator, dissolved in methanol and applied to a t.l.c. plate. Plates were developed in butan-2-one/acetone/water/formic acid (30:3:5:0.1, by vol.) [14], dried, and dipped three times in 7% (w/v) 2,5-diphenyloxazole (PPO) in ether for fluorography. Two products were identified as C_8/C_8-[^{3}H]GlcCer and C_8/C_8-[^{3}H]SPH by their respective R_F values of 0.44 and 0.10 [13]. Bands were quantified by liquid scintillation counting in Ultima Gold. The background was 2000 c.p.m., equivalent to 0.03 pmol.

Calculation of polarity and sorting

The polarity (apical/basolateral) of the delivery of each sphingolipid is defined as the ratio of the amounts of the lipid recovered in the apical and basal media: $GlcCer_{ap}/GlcCer_{bl}$ and SPH_{ap}/SPH_{bl}. The quotient of these ratios is termed the relative polarity (GlcCer/SPH), and is taken as a measure of sorting. Numbers > 1 represent an enrichment of GlcCer over SPH in the apical plasma membrane domain as compared with the basolateral domain [4]. The numbers are expressed as means ± S.D.; *n* represents the number of measurements.

Materials

BSA fraction V, defatted BSA, and [^{14}C]octanoic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 6-NBD-hexanoic acid, 8-bimane-octanoic acid and the *N*-hydroxysuccinimide ester of 8-DECA-octanoic acid were from Molecular Probes.

RESULTS

Variation of the fatty acyl chain

After incorporation of C_6-NBD-, C_8-bimane-, C_8-DECA- and [^{14}C]octanoyl-ceramides into confluent monolayers of MDCK or Caco-2 cells, two major products were synthesized that were characterized as GlcCer and SPH by t.l.c. The absolute and relative amounts of newly synthesized GlcCer and SPH were quite variable between the different ceramides and between MDCK and Caco-2 cells (Table 1). When incubations were performed at 10 °C in the presence of BSA in the apical and basal media, 88 ± 7% (n = 19) of both products was protected against BSA extraction, confirming that the bulk of each had been synthesized intracellularly. At 37 °C transport to the cell surface had occurred, as after 1 h, followed by two BSA washes at 10 °C, typically 80% of the products were recovered in the medium (Table 1). After a similar incubation, but in the absence of BSA, less than 10% was found in the medium (results not shown), indicating that the release of the sphingolipid analogues into the media required the presence of a scavenger.

For each precursor and cell type the apical/basolateral polarity

Table 1. Cell-surface delivery of sphingolipids with different fluorescent or radiolabelled fatty acyl chains during 1 h at 37 °C

Cell type	Ceramide	Synthesis (pmol/10^6 cells)		Transport (% in BSA medium)		Polarity (apical/basolateral)		Relative polarity (GlcCer/SPH)	*n*
		GlcCer	SPH	GlcCer	SPH	GlcCer	SPH		
MDCK	C_6-NBD-	4	19	86	80	1.7±0.3	0.9±0.3	2.0±0.5	16
	C_8-bimane-	2	5	83	68	3.2±0.6	1.3±0.3	2.6±1.1	8
	C_8-DECA-	2	11	90	90	5.9±3.7	1.0±0.4	5.8±3.6	9
	[^{14}C]Octanoyl-	2*	56*	70	76	1.3±0.2	0.6±0.1	2.4±0.5	4
Caco-2 (7 day old)	C_6-NBD-	15	26	71	56	3.1±0.4	0.9±0.2	3.8±1.1	10
	C_8-bimane-	2	17	82	70	6.6±3.9	0.9±0.3	9.4±8.0	4
	C_8-DECA-	1	11	86	74	3.8±2.0	0.6±0.1	6.1±1.7	3
	[^{14}C]Octanoyl-	31*	175*	42	38	3.0±1.7	0.5±0.2	6.2±3.7	6

*Eight times more [^{14}]C]octanoyl-ceramide was added to allow reliable quantification of the GlcCer product.

Table 2. Cell-surface delivery of C_6-NBD-sphingolipids with different sphingosine backbones in Caco-2 cells (12 days old) during 1 h at 37 °C

C_6-NBD-ceramide	Synthesis (pmol/10^6 cells)		Transport (% in BSA medium)		Polarity (apical/basolateral)		Relative polarity (GlcCer/SPH)	*n*
	GlcCer	SPH	GlcCer	SPH	GlcCer	SPH		
D-*erythro*-	10	19	39	50	4.2±1.7	0.6±0.1	7.5±2.5	8
L-*threo*-	3	19	45	52	4.0±1.6	0.9±0.3	4.6±0.9	6
D-*erythro*-dihydro-	6	4	22	43	1.7±0.4	0.6±0.1	3.4±0.1	4

Table 3. Release of C_8/C_8-[^{3}H]sphingolipids into the medium during 1 h at 37 °C

Cell type	Synthesis (pmol/10^6 cells)		Transport (% in medium)		Polarity (apical/basolateral)		Relative polarity (GlcCer/SPH)	*n*
	GlcCer	SPH	GlcCer	SPH	GlcCer	SPH		
MDCK	0.3	1.8	79	71	2.0±0.3	0.7±0.1	2.9±0.2	6
Caco-2 (7 days old)	2.2	5.5	95	65	2.7±1.1	1.0±0.2	2.6±0.7	4

of delivery at 37 °C was very different between GlcCer and SPH (Table 1). The polarity of GlcCer was larger than unity in all cases, implying apical enrichment, whereas SPH was generally non-polarized in MDCK and more basolateral in Caco-2. This difference is reflected in the value of the relative polarity (GlcCer/SPH), which exceeded 2 in all cases; all analogues were sorted. [^{14}C]Octanoyl-GlcCer, the most natural analogue studied, was convincingly sorted to the apical domain as compared with [^{14}C]octanoyl-SPH (Table 1). This shows that the sorting observed in previous studies was not an artefact of the NBD moiety, and validates the use of the fluorescent NBD lipids to probe sorting.

Variation of the sphingolipid backbone

In a new series of experiments, we investigated the influence of the long-chain base on epithelial sphingolipid sorting. Caco-2 cells synthesized both GlcCer and SPH not only from the D-*erythro*- but also from the L-*threo*- stereoisomer of C_6-NBD-ceramide and also from C6-NBD-dihydroceramide (Table 2). This was in contrast to MDCK cells (and fibroblasts [11]), where no GlcCer synthesis from the latter two precursors was observed. Less than 1% of the SPH formed from L-*threo*-ceramide was D-*erythro*-C_6-NBD-SPH, as analysed by the t.l.c. procedure of Koval & Pagano [12]. From this it was calculated that at most about 5% of the newly synthesized GlcCer could have been due to contamination of the L-*threo*-ceramide with D-*erythro*-ceramide, the other 95% being true L-*threo*-C_6-NBD-GlcCer. Evidence that the biosynthetic products derived from C_6-NBD-dihydroceramide still contained the saturated long-chain base was found by reversed-phase h.p.l.c. analysis. The retention volumes of C_6-NBD-dihydroceramide and its GlcCer and SPH analogues were 1.4 times greater than those of the C_6-NBD-D-*erythro*-sphingolipids.

In Caco-2 cells, for each backbone, the apical/basolateral polarity of delivery of GlcCer was greater than 1, whereas that of SPH was below 1. The value for the relative polarity displayed an apical enrichment of GlcCer over SPH by a factor 3–8. The fact that the relative polarity of the natural D-*erythro*-C_6-NBD-ceramide in these 12-day-old cells (Table 2) was higher than in 7-day-old cells (Table 1) confirms earlier observations [4].

Decreases in the length of both sphingosine backbone and acyl chain

The water-soluble C_8/C_8-[^{3}H]ceramide was directly incorporated from HBSS into MDCK and Caco-2 cells. The two major biosynthetic products were identified as C_8/C_8-[^{3}H]GlcCer and

C_8/C_8-[^{3}H]SPH by t.l.c., as described in the Materials and methods section. Experiments at 0 °C proved that both products were predominantly synthesized intracellularly. About 15% of the total truncated sphingolipids was recovered from the media after 4 h at 0 °C, whereas after 1 h at 37 °C (and a 0.5 h wash at 10 °C), this was about 80% (Table 3). The same fraction of the products was recovered from the media as compared with the cells whether or not the media contained BSA (results not shown).

C_8/C_8-[^{3}H]GlcCer was preferentially delivered into the apical medium. The apical/basolateral polarity of delivery was 2.0 and 2.7 for MDCK and Caco-2 cells respectively, versus 0.7 and 1.0 for C_8/C_8-[^{3}H]SPH. This resulted in relative polarities (GlcCer/SPH) of 2.9 and 2.6 (Table 3). Thus the C_8/C_8-[^{3}H]sphingolipids were essentially sorted like all other analogues (Tables 1 and 2).

DISCUSSION

The structure of the precursor ceramide and the cell type strongly influenced the ratio of synthesis of GlcCer and SPH. After incubations with L-*threo*-C_6-NBD-ceramide or dihydro-C_6-NBD-ceramide, no GlcCer at all was observed in MDCK cells, as in BHK cells and V79 fibroblasts [11], but considerable amounts were produced in Caco-2 cells. SPH synthesis also depended on ceramide and cell type, with, for example, a 4-fold reduction for C_8-bimane-ceramide as compared with C_6-NBD-ceramide in MDCK cells (Table 1). Such differences could be due to changed membrane/water/BSA partition coefficients and exchange rates for the various ceramides. However, the fact that the effects were not the same for MDCK and Caco-2 cells suggests that, in addition, structural differences between ceramides differentially affected recognition by the glucosyltransferases and the sphingomyelin synthases of the two cell lines.

Our assay for the polarity of epithelial lipid transport compares the delivery of GlcCer and SPH to the two cell surfaces. GlcCer polarity was always greater than 1. That of SPH tended to be below 1. With an apical/basolateral surface ratio of about 1 for MDCK cells under the present conditions [15], this results in an apical enrichment for GlcCer and a slight basolateral enrichment for SPH. Polarity can now be compared between the various GlcCer analogues, and between the various SPHs. By this criterion, C_8-bimane- and C8-DECA-GlcCer were sorted most efficiently. A sorting parameter independent of the areas of the two cell surface domains is the relative polarity (GlcCer/SPH), which represents the apical enrichment of GlcCer over SPH. Without knowing the surface areas in Caco-2 cells, comparison of these values allows us to conclude that sorting in Caco-2 cells was significantly more efficient than in MDCK cells. The relative polarity was $\geqslant 2$ in all cases. Also by this parameter, C_8-bimane- and C_6-DECA-sphingolipids were sorted better than the [^{14}C]octanoyl and C_6-NBD analogues.

Evidence in favour of a vesicular mode of transport of SPH and GlcCer from the Golgi to the cell surface has been presented [3–6,13,14,16–18]. Based on this, a simple mechanism for epithelial lipid sorting has been proposed [2]. Its central feature is the lateral segregation of newly synthesized apical from basolateral lipids in the lumenal leaflet of an intracellular sorting compartment. Driven by intermolecular hydrogen bonding, glycosphingolipids aggregate into microdomains that subsequently bud into apical transport vesicles. As a consequence of the withdrawal of the glycosphingolipids the sites of basolateral vesicle budding become enriched in phospholipids, notably PC. Sorting of membrane proteins by a similar segregation mechanism has been assigned to the last Golgi compartment on the way out, and this trans Golgi network has also been proposed as the site of epithelial lipid sorting.

In the context of the lipid microdomain model, an apical enrichment of GlcCer *per se* does not imply selective channelling of GlcCer into the apical pathway, since the amount of membrane flux to the two surfaces is not known. For example, with an apical pathway carrying twice as much membrane compared with the basolateral route, a GlcCer polarity of 2 would indicate the absence of intracellular GlcCer sorting. Still, the polarity can be used as a sorting parameter in the comparison of GlcCer analogues with different lipid tails. A second measure of intracellular sorting is the relative polarity (GlcCer/SPH), which is independent of the membrane flux along the two pathways, as this is the same for the two lipids. In terms of the model, the GlcCer analogue with the highest polarity would experience the most efficient aggregation into the lipid microdomain, while the sphingolipid analogues with the highest relative polarity would most efficiently combine aggregation of GlcCer with exclusion of SPH. So, in fact, the question would be how changes in the lipid tails modulate microdomain formation of GlcCer both in absolute terms and compared with SPH.

The lipid microdomain model has proposed hydrogen bonding as the driving force in glycosphingolipid aggregation. It has been proposed that the carbohydrate moiety of these lipids drastically enhances their hydrogen bonding capability [19]. Because hydrogen bonding between molecules decreases greatly when the distance between them increases, the effect of the various acyl chains on sorting (Table 1) was compared with their influence on molecular surface area. The surface area in a lipid monolayer at the air–water interface was compared between a natural PC, di-$C_{18:1}$, and fluorescent PCs containing $C_{16:0}$ in the *sn*-1 position. PC was used instead of GlcCer to allow for accurate quantification by phosphate. The surface area per molecule at the physiological surface pressure (30 mN/m) was 63.8 Å^2 for di-$C_{18:1}$-PC, 74.6 Å^2 for C_6-NBD-PC, 77.3 Å^2 for C_8-bimane-PC and 85.4 Å^2 for C_8-DECA-PC (1 Å = 0.1 nm) (R. Demel, personal communication). The GlcCer polarity was highest for the probes possessing the largest surface area. In terms of the sorting model this implies that the probe groups not only allowed substantial preservation of normal lipid–lipid hydrogen bonding. They must have contributed to the hydrogen bonding themselves or potentiated the hydrogen-bonding ability of the probe headgroup (e.g. by altering or restricting the headgroup conformation) to a degree sufficient to overcome the negative effects of the measured increase in distance between the molecules. This would predict some increase in hydrogen bonding of the SPH as well. A modest increase in the polarity of DECA- and bimane-SPH was indeed observed. Also, the relative polarity was highest for the bimane and DECA probes. Apparently, the fluorescent groups enhanced hydrogen bonding of GlcCer more than of SPH. This supposes an interaction of the fluorescent moiety with the glucose headgroup, which would be in line with the notion that the NBD group at the end of an acyl chain loops back to the membrane surface [20].

In contrast to the preference of the GlcCer synthase for the natural D-*erythro*- versus the L-*threo*- stereoisomer, no difference in the sorting of C_6-NBD- stereoisomers was found. The configuration at the hydroxy group at position 3 of the sphingosine base appears irrelevant for sorting. In contrast, the polarity of dihydro-GlcCer, and thereby the relative polarity, was decreased. This suggests some positive influence of the sphingosine double bond on GlcCer microdomain formation, possibly by a reduction in chain mobility and thereby surface area.

GlcCer and SPH produced from the truncated C_8/C_8-[^{3}H]ceramide were also sorted intracellularly, as the polarity of release for C_8/C_8-[^{3}H]GlcCer was 2.0–2.7, versus 0.7–1.0 for C_8/C_8-[^{3}H]SPH (Table 3). In the context of the lipid microdomain model this was unexpected, because the high water-solubility of

these probes would seem to predict that in the Golgi the C_8/C_8 molecules were present in the luminal fluid. The observation could, therefore, support alternative models for lipid sorting, for instance the collection of GlcCer by a lectin into the apical pathway. However, the distribution of C_8/C_8-lipids between the membrane and fluid phase in the Golgi is unknown. As the volume/surface ratio is roughly 10^6 times lower for the lumen of the Golgi than for the cell surface in our experimental set-up (cf. ref. [21]), the truncated C_8/C_8-[^{3}H]-GlcCer and -SPH may well be predominantly membrane-associated in the sorting compartment, and therefore be able to participate in lipid microdomain formation. It will be necessary to synthesize analogues with greater water-solubility to resolve this issue.

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