Sorting of Newly Synthesized Galactosphingolipids to the Two Surface Domains of Epithelial Cells

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Abstract. The high concentration of glycosphingolipids on the apical surface of epithelial cells may be generated by selective transport from their site of synthesis to the cell surface. Previously, we showed that canine kidney MDCK and human intestinal Caco-2 cells converted a ceramide carrying the short fluorescent fatty acid *C_6-NBD* to glucosylceramide (GlcCer) and sphingomyelin (SM), and that GlcCer was preferentially transported to the apical surface as compared to SM. Here, we address the point that not all glycosphingolipid classes are apically enriched in epithelia. We show that a ceramide containing the 2-hydroxy fatty acid *C_6OH* was preferentially converted by MDCK and Caco-2 cells to galactosylceramide (GalCer) and its derivatives galabiosylceramide (Ga_2Cer) and sulfatide (SGalCer) as compared to SM and GlcCer—all endogenous lipid classes of these cells. Transport to the apical and basolateral cell surface was monitored by a BSA-depletion assay. In MDCK cells, GalCer reached the cell surface with a two- to sixfold lower apical/basolateral polarity than GlcCer. Remarkably, in Caco-2 cells GalCer and GlcCer displayed the same apical/basolateral polarity, but it was sixfold lower for lipids with a *C_6OH* chain than for *C_6-NBD* lipids. Therefore, the sorting of a sphingolipid appears to depend on lipid structure and cell type. We propose that the different ratios of gluco- and galactosphingolipid synthesis in the various epithelial tissues govern lipid sorting in the membrane of the trans Golgi network by dictating the composition of the domains from where vesicles bud to the apical and basolateral cell surface.

GLYCOSPHINGOLIPIDS form a highly polymorphic class of lipids. In mammalian cells they occur in a number of cellular membranes but are mainly expressed on the cell surface, where they are thought to play a role in cell signaling and recognition (17). The carbohydrate structures in most glycolipid series are based on the (Gal β1-4 Glc β1-) of lactosylceramide. Glucosylceramide (GlcCer)[1] therefore is the precursor for most glycolipid species. GlcCer itself has been implicated in growth and development (42). The other major monohexosylceramide, GalCer, serves as a precursor for only a few simple glycolipids, sulfatide (SGalCer), galabiosylceramide (Ga_2Cer), and the ganglioside sialo-galactosylceramide (I^3NeuAc-GalCer, or "G_{M4}").

While glycosphingolipids constitute only a few mole percent of the lipids in most membranes, they are major components of the apical plasma membrane of a number of epithelial cells in the gastrointestinal and urinary tracts and of myelin (reviewed in 33, 45, 47). In intestinal cells glycosphingolipids make up one third of the lipids in the apical membrane while the other two thirds consist of phospholipids and cholesterol. If restricted to the outer leaflet, the glycosphingolipids essentially cover the apical surface. The basolateral plasma membrane domain contains lower levels of glycosphingolipids and is enriched in the phospholipid PC and the (sphingo)phospholipid sphingomyelin (SM; 47).

Differences in the apical/basolateral polarity of the various lipids have also been documented for epithelial cells in culture. The distribution of the phospholipids PC and PE in MDCK cells was very similar to that found in intestinal tissue (see reference 47). To study the cellular processes responsible for these differences, we have developed an assay for transport of newly synthesized lipids to the two plasma membrane domains of epithelial cells in culture (53, 55, 56). Newly synthesized short-chain GlcCer was delivered preferentially to the apical surface in MDCK cells

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Glycosphingolipids are designated according to the IUPAC-IUB Commission on Biochemical Nomenclature. 1977. *Lipids.* 12:455–468, except for the use of SGalCer instead of I^3SO_3-GalCer.

1. *Abbreviations used in this paper*: C_6-, hexanoyl-; C_6OH, (D-2-hydroxy) hexanoyl-; Cer, ceramide; dh, dihydro; Ga_2Cer, galabiosylceramide, Gal α1-4 Gal β1-1 Cer; GalCer, galactosylceramide, Gal β1-1 Cer; GlcCer, glucosylceramide, Glc β1-1 Cer; HFA, 2-hydroxy fatty acid; NFA, normal (nonhydroxy) fatty acid; SGalCer, sulfatide, HSO_3-3 Gal β1-1 Cer; SM, sphingomyelin.

and in human intestinal Caco-2 cells while short-chain SM was not. The difference in polarity of delivery between GlcCer and SM in Caco-2 cells was sufficient to generate the apical enrichment of GlcCer over SM that has been observed in intestinal cells in vivo. Based on this observation, a model was formulated where glycosphingolipids aggregate into domains in the lumenal leaflet of the membrane of the TGN analogous to the sorting of membrane proteins (47). These lipid domains, which are presently also supposed to be enriched in proteins anchored to the membrane by a glycolipid tail (reviewed in reference 15), would then be transported by vesicles to the apical surface by an as yet unknown mechanism.

Although all present evidence supports the idea of lipid domains, lipid sorting in epithelia must involve more than the lateral segregation of apical GlcCer from basolateral SM and PC in the TGN because: (*a*) In human intestinal and kidney epithelial cells GalCer and not GlcCer is by far the major monohexosyl ceramide, while 40–60% of the total glycolipids are complex glycolipids notably SGalCer (7, 11, 46). In dog kidney (10) and dog kidney–derived MDCK cells (20, 37) the monohexosylceramide (40–60% of total glycolipid) contains some 20% GalCer. (*b*) Although the glycosphingolipids in MDCK cells overall contain a ceramide backbone composed of sphingosine (4-sphingenine) and nonhydroxy fatty acids (NFA; 20, 37), glycolipids in intestinal cells of rat and man mainly possess ceramides consisting of phytosphingosine (4-hydroxysphinganine) and 2-hydroxy fatty acids (HFA; 5, 11, 13, 19, 35). These additional hydroxyl groups in the ceramide moiety may well facilitate intermolecular hydrogen bonding between glycolipids (3). (*c*) In contrast to GlcCer, some complex glycosphingolipids were not found to be enriched on the apical surface in intestinal epithelial cells (13, 18, 24). This was also found for kidney cells (48), and the GalCer/GlcCer ratio was twofold lower on the apical than on the basolateral surface of MDCK (36). (*d*) Preferential transport of GlcCer over SM to the basolateral cell surface was observed in Fischer rat thyroid cells (60).

In the present study we extend our previous work on the intracellular transport and sorting of SM and GlcCer to galactose-containing glycosphingolipids. In colon cells, the polarity of these lipids is of relevance for medicine: GalCer and SGalCer have attracted attention as potential receptors for HIV (41, 58), while Ga_2Cer, like globotriosylceramide, plays a deleterious role as a receptor for Shiga toxin and verotoxin (9, 28). Our results show that the various glycosphingolipids are delivered to the epithelial cell surface with different apical/basolateral polarities. The observed differences, that depend on the structure of both the carbohydrate headgroup and the ceramide backbone, fit well with polarity data in the literature. The results refine the model of epithelial lipid sorting where apical and basolateral lipids are segregated by domain formation in the Golgi and provide insight into cellular trafficking of galactolipids.

Materials and Methods

Cells

MDCK cells, clones strain I (14) and strain II (30), were originally obtained from K. Simons (EMBL, Heidelberg, Germany). They were passaged and grown as 3- or 4-d-old monolayers on filters glued to the bottom of plastic rings (Transwell; Costar Corp., Cambridge, MA) as described before (54, 55). Caco-2 cells, clone PD7, were passaged as described (8) and used on day 7–15 in culture (56; but on Transwell filters in 10% FCS).

Chemical Synthesis of Radiolabeled Ceramides

D-Erythro-[4,5-^{3}H]-Sphinganine. Synthesis was modified from the method of G. Brenner-Weiss (personal communication). An excess of 25 mg of D-erythro-sphingosine was dissolved in 2.5 ml methanol/water (4:1, vol/vol) in a screw-capped vial. After addition of 50 μl palladium acetate (50 mM in tetrahydrofuran) and 33 μl acetic acid, the mixture was flushed with N_2 and frozen in liquid N_2. 200 μl methanol/water (4:1, vol/vol) was added and frozen as a second layer on top. 2.3 μmol $NaB[^3H]_4$ (925 MBq) in 1 ml 0.4 M NaOH was added to the frozen mixture. The vial was flushed with N_2, closed, and thawed at room temperature under stirring. After 16 h the reaction mixture was centrifuged at 2,000 rpm for 5 min and the supernatant was concentrated in the rotary evaporator under addition of methanol/water and chloroform to remove excess tritium. Lipids were collected in the lower phase of a two-phase extraction (2), dried under N_2, and dissolved in chloroform/methanol (1:2, vol/vol).

D-erythro-[4,5-^{3}H]sphinganine was purified twice by thin layer chromatography (TLC) on silica 60 TLC-plates (20 × 20 cm^2) in chloroform/methanol/2 M NH_4OH (40:10:1, vol/vol). Products were detected with a β-camera (Birchover Instruments, Letchworth Herts, England) and identified by comigration with nonradioactive standards ($Rf_{sphingosine}$ = 0.40, $Rf_{sphinganine}$ = 0.28, visualized with 0.2% ninhydrin in ethanol). Sphinganine was extracted from the silica with chloroform/methanol (1:1, vol/vol) and chloroform/methanol/water (1:2.2:1, vol/vol). The lower phase of the Bligh and Dyer extraction (2) was dried under N_2 and dissolved in chloroform/methanol (1:2, vol/vol). Purity was checked by TLC (see above) and fluorography. The yield of the tritiation was 54 MBq D-erythro-[4,5-^{3}H]sphinganine with a calculated specific activity of 201 MBq/μmol.

Radiolabeled Ceramides. In a modification of an established method (39), ^{14}C-labeled hexanoic acid (0.58 mg; 0.15 MBq/ μmol), unlabeled hexanoic acid (0.58 mg), or (D,L-2-hydroxy)hexanoic acid (0.2 mg) were dissolved in dry ethylacetate (0.5 ml). Equimolar quantities of *N*-hydroxysuccinimide and dicyclohexylcarbodiimide were added and allowed to react overnight at room temperature in the dark. Thereafter, 1 ml of ethylacetate was added and the samples were centrifuged for 5 min at 2,000 rpm. The supernatant was dried under N_2, dissolved in freshly distilled tetrahydrofuran and added to D-erythro-sphingosine (1.5 mg), D-erythro-[3-^{3}H]sphingosine (1.3 MBq; 814 MBq/μmol) or D-erythro-[4,5-^{3}H]sphinganine (23 MBq; 201 MBq/μmol) in tetrahydrofuran to a final volume of 0.3 ml. After an overnight incubation at room temperature in the dark the samples were dried under N_2. The ceramide products were purified twice by preparative TLC in chloroform/methanol/acetic acid (90:2:8, vol/vol) for NFA-ceramides, or chloroform/methanol/25%NH_4OH (190:15:1, vol/vol) for HFA-ceramides. The products were detected with a β-camera. A minor part of the sample was run in a parallel lane and detected by spraying with 0.1% rhodamine 6G in methanol. Ceramides were scraped from the plate and extracted from the silica (2). The upper phase was washed once with chloroform. The lower phases were dried under N_2 and dissolved in chloroform/methanol (2:1). The D- and L-stereoisomers of the HFA-ceramides were separated by TLC in chloroform/methanol/acetic acid (90:2:8, vol/vol), followed by a run in diethylether. For products and specific activities, see Fig. 1 and Table I.

Lipid Analysis

Lipids were extracted from cells and media by a two-phase extraction (2). The upper phase contained 20 mM acetic acid and (for radiolabeled lipids) 120 mM KCl. After a chloroform wash, which was added to the lower phase, lipids remaining in the upper phase (SGalCer) were collected on SepPak C18 cartridges (Waters, Milford, MA), from which lipids were eluted with chloroform/methanol/water (1:2.2:0.1) and methanol. The organic (lower) phase was dried under N_2 and the lipids were applied to TLC plates, that had been dipped in 2.5% boric acid in methanol (25), dried, and activated by heating at 110°C for 30 min. They were developed in two dimensions: I. chloroform/methanol/ 25%NH_4OH/water (65:35:4:4, vol/vol), and II. chloroform/acetone/methanol/ acetic acid/water (50:20:10:10:5, vol/vol).

Fluorescent spots were detected under UV, scraped from the TLC

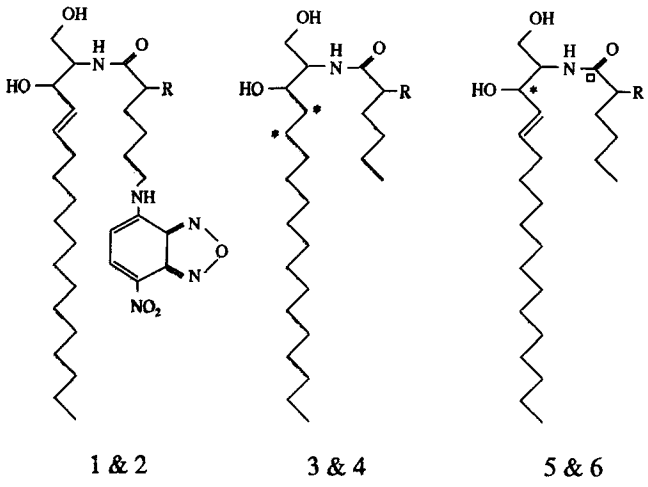

Figure 1. Chemical structure (38) of fluorescent and radiolabeled short-chain ceramides. (*1*) Cer(C_6-NBD), (*2*) Cer(C_6OH-NBD), (*3*) [^{3}H]dh-Cer(C_6), (*4*) [^{3}H]dh-Cer(C_6OH), (*5*) Cer([^{14}C]C_6), (*6*) [^{3}H]Cer(C_6OH). *1*, *2*, *5*, and *6* contain sphingosine (4-sphingenine), while *3* and *4* contain dihydrosphingosine (sphinganine) as the sphingoid base. (*R*) -H in *1*, *3*, and *5*; -OH in *2*, *4*, and *6*; the natural (D-2OH) and unnatural (L-2OH) stereoisomers were studied separately. (*) [^{3}H] in *3*, *4*, and *6*; (□) [^{14}C] in *5*.

plates, and the fluorescent lipid analogs were extracted from the silica in 2 ml chloroform/methanol/20 mM acetic acid (1:2.2:1, vol/vol) for 30 min. After pelleting the silica for 10 min at 1,500 rpm, fluorescence in the supernatant was quantified in a fluorimeter (Kontron, Zürich, Switzerland). Radiolabeled spots were detected by fluorography after dipping the TLC plates in 0.4% PPO in 2-methylnaphthalene with 10% xylene (4). Preflashed film (Kodak X-Omat S, France) was exposed to the TLC plates for 3 d at −80°C. The radioactive spots were scraped from the plates and the radioactivity was quantified by liquid scintillation counting in 0.3 ml Solulyte (J.T. Baker Chemicals, Deventer, The Netherlands) and 3 ml of Ultima Gold (Packard Instruments, Downers Grove, IL).

Enzymatic Degradation of Galactolipids with Galactosidases

Lipids were mixed with 250 μg Na-taurocholate (in 50 μl chloroform/methanol, 2:1, vol/vol) and the solvent was evaporated. The samples were dissolved in 450 μl of 84 mM Na_2HPO_4, 58 mM citric acid, and 2 mM EDTA (pH 4.2), 0.15 U of α- or β-galactosidase was added (75 μl) and the mixture was incubated for 24 h at 37°C. Lipids were extracted and identified by TLC, by comparison with standards on borate-treated plates in solvent I (see above).

Transport Incubations

Transport of lipids to the epithelial cell surface was measured essentially as before (53, 56). In short, BSA complexes of the ceramides were prepared by injection of 10 μl of an ethanolic solution of the ceramides into Hanks' balanced salt solution without bicarbonate, 10 mM Hepes, pH 7.4 (HBSS'), containing 1% (wt/vol) BSA (HBSS' + BSA). Cell monolayers on filters were washed three times with HBSS' and placed in a six-well cluster dish. Each well contained 2 ml of a ceramide suspension in HBSS' + BSA and 1 ml was applied to the apical side. Cells were incubated at 37°C for 1 or 2 h, during which period short-chain lipids reaching the cell surface should be depleted into the medium by the BSA. After collection of the apical and basolateral medium, remaining short-chain lipids were washed from the cell surface in HBSS' + BSA for 30 min at 10°C, at which temperature sphingolipid transport to the cell surface is essentially blocked (56). The apical and basolateral washes were pooled with the corresponding 37°C media and the filter was cut from its ring, after which the lipids in media and cells were analyzed. For *C_6-NBD* analyses media and cells from two filters were combined.

To assess what part of the sphingolipids was synthesized on the cell surface, cells were incubated with 60 nM [^{3}H]dh-Cer(C_6OH) at 10°C with BSA present in the HBSS' on both sides to extract products from the cell surface (52). After a 3.5 h incubation the cells were washed with HBSS' + BSA for 30 min at 10°C, and the ^{3}H-lipids in the media and the cells were analyzed.

Calculation of Transport and Polarity

The transport of a short-chain lipid to the cell surface was expressed as the percentage of the total amount of that lipid in cells and media that was recovered in the BSA media. The apical/basolateral polarity of the delivery

*Table I. Biosynthesis of Sphingolipids from Various Precursor Ceramides**

Cells	Ceramide		SM	GlcCer	GalCer	Ga_2Cer	SGalCer	Total incorporation (percent of added precursor)
					(percent of total)			
MDCK I	NFA	Cer(C_6-NBD)‡¶	79	20				2
	HFA	[^{3}H]dh-Cer(C_6[D-2OH])	48	20	32			6
MDCK II	NFA	Cer(C_6-NBD)	86	14	0.5			1
		Cer([^{14}C]C_6)	84	16				2
		[^{3}H]dh-Cer(C_6)	100					4
	HFA	Cer(C_6[D-2OH]-NBD)§	28		72			2
		Cer(C_6[L-2OH]-NBD)§	100					1
		[^{3}H]Cer(C_6[D-2OH])	15	5	52	16	12	7
		[^{3}H]dh-Cer(C_6[D-2OH])	9	4	54	17	16	6
		[^{3}H]dh-Cer(C_6[L-2OH])§	100					4
Caco-2	NFA	Cer(C_6-NBD)¶	51	48	0.3			4
		Cer([^{14}C]C_6)	90	10				6
		[^{3}H]dh-Cer(C_6)	83	17				2
	HFA	[^{3}H]Cer(C_6[D-2OH])	13	20	48		16	7
		[^{3}H]dh-Cer(C_6[D-2OH])	17	9	56		18	7
		[^{3}H]dh-Cer(C_6[L-2OH])	100					10

*Cells on filters were incubated for 1 h at 37°C in the presence of Cer(C_6-NBD) (5 μM), Cer([^{14}C]C_6) (0.15 MBq/μmol; 15 μM), [^{3}H]ceramides (814 MBq/μmol; 15 nM) or [^{3}H]dh-ceramides (201 MBq/μmol; 60 nM) in both apical and basolateral medium. After the incubation lipids were extracted from the cells and the combined incubation media, and analyzed as described under Materials and Methods. In each case the data from a typical experiment are presented as percent of total labeled sphingolipid. 100%: no other spot identified.

‡25 μM.

§Ceramide was added to the apical surface only.

¶Traces (<1% of total) of two other lipids (L and G) were detected in Caco-2 (L) and MDCK I cells (L and G). They were identified by α- and β-galactosidase treatments as L: lactosylceramide (Gal β1-4 GlcCer) and G: globotriosylceramide (Gal α1-4 Galβ1-4 GlcCer).

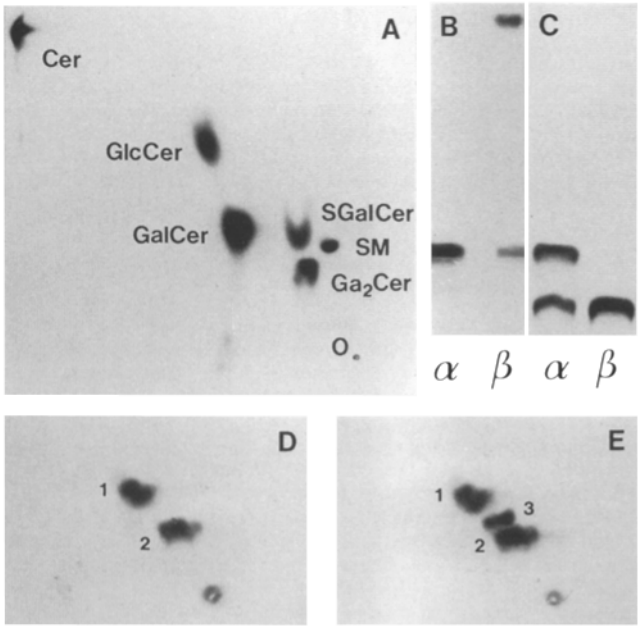

Figure 2. TLC autoradiograms of products from [^{3}H]dh-Cer(C_6OH) in MDCK II cells. (*A*) Cells were incubated for 1 h at 37°C (cf. Table I). Lipids were separated on 2D-TLC on borate-treated plates and visualized. *O*, origin. (*B*) GalCer was hydrolyzed by β-galactosidase (*β*) but not α-galactosidase (*α*) to ceramide. (*C*) Ga_2Cer was hydrolyzed by α-galactosidase (*α*) but not β-galactosidase (*β*) to GalCer. During a second hydrolysis again about half of the remaining Ga_2Cer was hydrolyzed (not shown). (*D*) [^{35}S]Sulfation of MDCK II cells (0.74 MBq, 20 pmol in 1 ml per dish, 6 h at 37°C) gave two spots, which by colocalization were identified as SGalCer (*1*) and sulfated lactosylceramide (*2*). (*E*) Addition of unlabeled dh-Cer(C_6OH) during [^{35}S]sulfation gave rise to a third ^{35}S-spot (*3*) which was also labeled with [^{3}H]dh-Cer(C_6OH) in *A*: SGalCer(C_6OH).

of a lipid was defined as the ratio of the amount recovered in the apical medium over that in basal medium. The relative polarity is the quotient of the polarities of two lipids, and is taken as a measure for lipid sorting (see reference 56).

Materials

D-erythro-sphingosine, D,L-dihydrosphingosine, hexanoic acid, (D,L-2-hydroxy)hexanoic acid, [1-^{14}C]hexanoic acid, α- and β-galactosidases (from *Aspergillus niger* and jack beans, respectively), and BSA fraction V were purchased from Sigma Chemical Co. (St. Louis, MO). NaB[^{3}H]$_4$ and D-erythro-[3-^{3}H]-sphingosine were from Du Pont (Wilmington, DE). [^{35}S]Sulfate was obtained from Amersham Corp. (Amersham, UK). Cer(C_6-NBD) was from Molecular Probes (Eugene, OR). Cer(C_6[D-2OH]-NBD), and Cer(C_6[L-2OH]-NBD) were kindly donated by R. Pagano (Rochester, MN). SM(C_6-NBD), GlcCer(C_6-NBD), and GalCer(C_6-NBD) were synthesized as before (53). (C_6-NBD)lactosylceramide was a gift from J.W. Kok (University of Groningen, Groningen, The Netherlands), and SGalCer(C_{12}-NBD) was synthesized by S. Marchesini (University of Brescia, Brescia, Italy).

Results

MDCK and Caco-2 Cells Preferentially Use HFA-Ceramides for the Synthesis of Galactosphingolipids

The precursor ceramides used in the present study contained a C_6 fatty acid (Fig. 1). Because of this short-chain the ceramides can exchange between membranes across an aqueous phase, and the absence of a polar headgroup allows translocation across cellular membranes. When added to MDCK and Caco-2 cells, the ceramides entered the cells and reached the SM synthase and various glycosyltransferases as evidenced by the variety of products formed (Table I, Fig. 2 *A*). Clearly, the presence of 1% BSA, which was added to deplete short-chain lipid products from the cell surface (see below), did not prevent ceramide incorporation.

All ceramides were converted to SM (Table I). The various SM's were identified by comigration with a SM(C_6-NBD) standard in two-dimensional TLC, and by sensitivity to bacterial sphingomyelinase (not shown). Significant amounts of GlcCer were formed from Cer(C_6-NBD) (53, 56) and from the other ceramides containing an NFA, except from dh-Cer(C_6) in MDCK II as had been reported for dh-Cer(C_6-NBD) (55). GlcCer, as identified by comigration with a standard and by the fact that its synthesis was inhibited by the specific inhibitor of ceramide glucosyltransferase PDMP (10 μM for 1.5 h at 37°C; results not shown; 42), was also synthesized from the natural D-form of the HFA ceramides. As for the NBD-lipid (40), virtually no glycolipid synthesis from the L-forms was detected.

In striking contrast with the NFA-ceramides, which were preferentially glucosylated, the ceramides containing a D-2OH fatty acid gave rise to a main product that comigrated with a GalCer(C_6-NBD) standard. This product from [^{3}H]dh-Cer(C_6OH) in MDCK II cells was identified as GalCer as it could be hydrolyzed to ceramide by β-galactosidase (Fig. 2 *B*). A second new ^{3}H-spot on TLC comigrated with (C_6-NBD)lactosylceramide. Degradation by α-galactosidase, and not by β-galactosidase, to GalCer (Fig. 2 *C*) identified this product as Ga_2Cer (Gal α1-4 GalCer), an endogenous lipid in MDCK II (37). A third new [^{3}H]C_6OH product on the TLC plate (Fig. 2 *A*) migrated close to SGalCer(C_{12}-NBD). Additional support of it being SGalCer was: (*a*) its retention on a DEAE-Sephadex column (27); (*b*) inhibition of its synthesis (to ~ 15%) by 50 mM sodium chlorate after an 18 h chlorate pretreatment (1); (*c*) its labeling with [^{35}S]sulfate, when unlabeled Cer(C_6OH) was added to the incubation (Fig. 2, *D* and *E*); (*d*) lack of inhibition of its synthesis by the glucosyltransferase inhibitor PDMP (42), corroborating its identity as SGalCer (sulfatide, HSO_3-3GalCer) and not sulfated lactosylceramide.

As in MDCK II cells, in Caco-2 cells HFA-ceramides were preferentially converted to GalCer and SGalCer. Ga_2Cer was not observed. The synthesis of five different lipids from one precursor ceramide provided the opportunity to compare transport and sorting of these lipids independent of ceramide backbone, while the synthesis of the same lipid class from different ceramides created the possibility to study the dependence of transport and sorting on the lipid backbone.

Transport of Newly Synthesized Lipids to the Cell Surface

Synthesis of GalCer, GlcCer, and SM on the surface of Caco-2 cells, as assayed at 10°C (Materials and Methods), was negligible as was transport to the cell surface at 10°C.

*Table II. Polarity of Surface Delivery of Newly Synthesized Sphingolipids in MDCK I Cells**

Ceramide	Polarity of delivery (apical/basolateral)			Relative polarity	
	SM	GlcCer	GalCer	GlcCer/SM	GlcCer/GalCer
NFA Cer(C_6-NBD)	0.6 ± 0.1 (16)	1.2 ± 0.2 (16)		2.0 ± 0.4 (16)	
HFA [^{3}H]dh-Cer(C_6OH)	0.2 ± 0.0 (6)	0.7 ± 0.2 (6)	0.1 ± 0.0 (6)	2.7 ± 0.7 (6)	5.5 ± 1.4 (6)

The presence of ceramide at 37°C increased the signal in the sphingolipids. As synthesis occurred during the transport incubation, transport as percent in the medium after the 37°C incubation was somewhat lower than in previous experiments in which ceramide was added at 10°C and removed before warming to 37°C (53, 56). The fraction of SM, GlcCer, and GalCer extracted into the medium during the 1 h at 37°C (and the 10°C BSA-wash) was 50–60% vs. 60–70% in the earlier studies on MDCK and 40–50% vs. 50–60% for Caco-2. Delivery of the higher glycosphingolipids SGalCer(C_6OH) and Ga_2Cer(C_6OH) lagged behind delivery of GlcCer and GalCer, with 30–40% in the medium after 1 h at 37°C.

*Cell monolayers on filters were incubated with ceramide analogs in the concentrations mentioned in Table I at 37°C for 1 or 2 h (without differences), and the polarity of delivery to the cell surface was measured as described under Materials and Methods. Data are presented as mean±SD (number of samples). Relative polarity was calculated for each individual experiment.

Over 97% of each product of [^{3}H]dh-Cer(C_6OH) was found in the cells after 3.5 h at 10°C. However, 20 ± 1% (n = 4) of newly synthesized [^{3}H]dh-SM(C_6OH) was recovered in the basolateral HBSS' + BSA of MDCK II cells. This is in line with earlier findings using Cer(C_6-NBD) and has been interpreted as reflecting the presence of a SM synthase on the basolateral surface of MDCK II cells. The relative contribution of this enzyme to the total SM synthesis is thought to be of minor importance at 37°C as it was found to decrease at higher temperature (52).

After the cells had been incubated with short-chain ceramides at 37°C in the presence of BSA, up to 60% of the various sphingolipid products were found in the medium, reflecting transport from their site of synthesis to the cell surface and extraction from the apical and basolateral surface by the BSA. The depletion rates by BSA were the same for SM, GlcCer, GalCer, and Ga_2Cer. This was established in an experiment, where MDCK II cells synthesized these lipids from [^{3}H]dh-Cer(C_6OH) at 37°C in the absence of BSA, after which [^{3}H]C_6OH lipids were depleted from the apical cell surface by BSA in two 30 min incubations at 10°C. For each ^{3}H-lipid 88% of the exchangeable amount of that lipid on the apical surface was depleted in the first incubation, and 12% in the second.

Newly Synthesized GalCer Is Enriched over GlcCer on the Basolateral Surface of MDCK Cells

The apical/basolateral polarities with which GlcCer and SM reached the surface of the two plasma membrane domains of MDCK cells are similar to those reported previously for the C_6-*NBD* lipids (53–55), although the experimental protocol was slightly different (Tables II and III). In both MDCK I and II the relative polarity GlcCer/SM was 2.0 for the C_6-*NBD* lipids. Similarly, it was 2.2 for the $[^{14}C]C_6$ chain in MDCK II. Although the absolute polarity of delivery of SM and GlcCer tended to be lower for the HFA-lipids than for the NFA-lipids, the relative polarity GlcCer/SM was similar for C_6-*OH* and C_6.

Interestingly, GalCer did not reach the MDCK cell surface with the same apical/basolateral polarity as GlcCer: It was delivered more basolaterally than GlcCer especially in MDCK I (sixfold) but also in MDCK II. In initial experiments on MDCK II using Cer(C_6[D-2OH]-NBD), fluorescent GalCer was more basolateral than SM. In subsequent experiments, the relative polarity GlcCer/GalCer was 2.8 for the C_6-*NBD* analogs, 2.0 for the C_6OH analogs, and 1.4 for the dihydro-C_6OH species. The GalCer derivatives SGalCer and Ga_2Cer were delivered to the MDCK II cell surface with a polarity very similar to that of GalCer itself (Table III).

Newly Synthesized GalCer Behaves Like GlcCer in Caco-2 Cells

In the Caco-2 clone PD7 the relative polarity GlcCer/SM for both the C_6-*NBD* and the $[^{14}C]C_6$ chain of 12.7 (Table IV) was much higher than in MDCK (Table II and III) and about two times higher than the values reported for the parent Caco-2 line (55, 56). A difference in the polarity of GlcCer and SM of about 2 was observed for the C_6OH species. In contrast to the situation in MDCK cells, no sorting between GlcCer and GalCer was observed in Caco-2 as reflected by the value of 1 for the relative polarity GlcCer/GalCer for the C_6-*NBD*, C_6OH, and the dihydroC_6OH analogs (Table IV). However, in Caco-2 cells the presence of the C_6OH chain caused a drastic shift of both glycolipids but not of SM to the basolateral sur-

*Table III. Polarity of Surface Delivery of Newly Synthesized Sphingolipids in MDCK II Cells**

Ceramide	Polarity of delivery (apical/basolateral)					Relative polarity	
	SM	GlcCer	GalCer	SGalCer	Ga_2Cer	GlcCer/SM	GlcCer/GalCer
NFA Cer(C_6-NBD)[‡§]	0.8 ± 0.1 (12)	1.5 ± 0.3 (12)	0.6 ± 0.3 (8)			2.0 ± 0.5 (12)	2.8 ± 1.3 (8)
Cer($[^{14}C]C_6$)	0.6 ± 0.1 (4)	1.2 ± 0.1 (4)				2.2 ± 0.4 (4)	
HFA [^{3}H]Cer(C_6-OH)	0.4 ± 0.1 (4)	1.4 ± 0.2 (4)	0.7 ± 0.1 (4)	0.5 ± 0.2 (4)	0.6 ± 0.1 (4)	3.7 ± 1.1 (4)	2.0 ± 0.2 (4)
[^{3}H]dh-Cer(C_6-OH)	0.4 ± 0.1 (14)	1.0 ± 0.3 (13)	0.7 ± 0.1 (14)	0.6 ± 0.2 (5)	0.5 ± 0.1 (14)	2.6 ± 0.4 (13)	1.4 ± 0.2 (13)

*See the legend to Table II, but all incubations 1 h 37°C.

‡5, 10, and 15 μM.

§The side of ceramide addition has been reported to influence the resulting apical/basolateral polarity of the products (54). Indeed the absolute polarity of delivery of SM(C_6-NBD) and GlcCer(C_6-NBD) was intermediate between the absolute polarities found after apical addition, 0.8 and 1.7, and after basolateral addition alone, 0.6 and 1.2 (54). Still, the difference in polarity between SM(C_6-NBD) and GlcCer(C_6-NBD) as expressed by the relative polarity GlcCer/SM was 2 in all cases.

*Table IV. Polarity of Surface Delivery of Newly Synthesized Sphingolipids in Caco-2 Cells**

Ceramide	Polarity of delivery (apical/basolateral)				Relative polarity	
	SM	GlcCer	GalCer	SGalCer	GlcCer/SM	GlcCer/GalCer
NFA Cer(C_6-NBD)	0.3 ± 0.1 (13)	2.7 ± 1.1 (13)	2.4 ± 1.0 (7)		12.7 ± 7.7 (13)	1.1 ± 0.4 (7)
Cer([^{14}C]C_6)	0.2 ± 0.1 (11)	1.6 ± 0.6 (11)			12.7 ± 7.1 (11)	
HFA [^{3}H]Cer(C_6OH)	0.2 ± 0.0 (8)	0.4 ± 0.1 (8)	0.4 ± 0.1 (8)	0.3 ± 0.2 (4)	2.0 ± 0.6 (8)	1.0 ± 0.2 (8)
[^{3}H]dh-Cer(C_6OH)	0.2 ± 0.1 (17)	0.4 ± 0.1 (12)	0.4 ± 0.1 (15)	0.1 ± 0.1 (7)	2.4 ± 1.1 (12)	1.1 ± 0.3 (12)

*See the legend to Table II.

face. Finally, SGalCer produced after incubation of the cells with [^{3}H]dh-Cer(C_6OH) was fourfold more basolateral than the corresponding GalCer.

Two types of experiments were performed to assess whether the short-chain analogs behaved as probes or influenced the system under study. First, [^{3}H]Cer(C_6OH) was added to MDCK II and Caco-2 cells in the presence of a 100-fold higher concentration (5 μM) of unlabeled Cer(C_6OH). No effects were observed on the relative amount of synthesis of the various ^{3}H-lipids and on the polarity of delivery of these lipids to the cell surface (data not shown). When the concentration of (C_6-NBD)Cer was increased from 5–25 μM, a slight reduction of 10–20% was observed in transport of SM and GlcCer to the surface of MDCK and Caco-2 cells, without effect on polarities of delivery. Second, we investigated whether the differences in polarity between the various *C_6-NBD-* and *C_6OH* sphingolipids (Tables III and IV) could be measured simultaneously in the same cells. For this, MDCK II or Caco-2 cells were incubated with a mixture of 5 μM Cer(C_6-NBD) and 60 nM [^{3}H]dh-Cer(C_6OH) and synthesis and transport to the cell surface was analyzed for the NBD-products in one set of cells and for the ^{3}H-lipids in a parallel set. The results of this direct comparison were identical to those in Tables III and IV (not shown), implying that the differences between the *C_6-NBD-* and *C_6OH* lipids were not caused by an effect of one of the short-chain ceramides (or their products) on the cellular machinery responsible for the transport and sorting of sphingolipids to the two epithelial cell surfaces.

Discussion

The present paper reports the basic finding that in MDCK and Caco-2 cells ceramides with a hydroxy fatty acid are preferentially converted to galactosphingolipids. The galactosphingolipids reached the epithelial cell surface with a lower apical/basolateral polarity than GlcCer in MDCK but not in Caco-2 cells. The data provide new insights in the mechanism by which the differences in lipid composition between the apical and basolateral cell surface are generated.

Glycosphingolipid Biosynthesis

GalCer is an abundant lipid in MDCK II cells (20, 37). However, only little GalCer was synthesized from NFA-ceramides (Table I). GalCer synthesis could be boosted dramatically by feeding the cells short-chain HFA-ceramides. This is in agreement with the reported preference of the myelin galactosyl-transferase for HFA-ceramides (34). After synthesis, HFA-GalCer was efficiently converted to Ga_2Cer and SGalCer. These lipids are endogenous to MDCK II, but as NFA- rather than HFA-derivatives (37) suggesting a low rate of fatty acid hydroxylation in MDCK II as opposed to kidney in vivo (23). MDCK I cells possess lower GalCer levels than MDCK II (20), and indeed were less efficient in converting HFA-ceramide to GalCer (Table I). The relatively high synthesis of SM and GlcCer from HFA-ceramide is probably an in vitro artifact. In bovine kidney, where GalCer contains only HFA, exclusively NFA-SM was found (23). Endogenous long-chain HFA-ceramide is made in the ER and, in contrast to the short-chain HFA-ceramides used here, cannot spontaneously diffuse through the aqueous phase. It will first encounter the HFA-ceramide galactosyltransferase which has been localized to the ER (43, 44, 49) and apparently little reaches the Golgi where the SM synthase and glucosyl-transferase are located (see 16, 21, 22). Also Caco-2 cells produced high levels of GalCer and SGalCer from HFA-ceramide but not from NFA-ceramide (Table I). Indeed, a high ratio of GalCer and SGalCer to GlcCer and a high content of HFA has been reported for human intestinal cells (11, 35, 46).

Topology of Sphingolipid Synthesis and Mechanism of Transport

SM (16, 21, 22), lactosylceramide (see reference 26), and SGalCer (50) are synthesized in the lumen of the Golgi and are unable to translocate across the Golgi membrane. Thus, after synthesis they are expected to reach the cell surface by vesicular transport, which has been experimentally confirmed for gangliosides (32, 59). It is less clear how GlcCer and GalCer are transported to the cell surface. In liver, the glucosyltransferase is oriented towards the cytosol (see 22) and the myelin galactosyl-transferase is localized in the ER lumen (43, 44, 49). We have confirmed these topologies for MDCK cells and we have measured in vitro that newly synthesized GlcCer and GalCer have access to the cytosolic side of the membrane (6, 22). From there they can theoretically exchange to the cytosolic leaflet of the plasma membrane but translocation towards the outer leaflet has not been demonstrated (cf. 57). More likely, GlcCer and GalCer pass through the lumen of the Golgi before reaching the cell surface. Indeed, translocation of GlcCer and GalCer across the Golgi membrane has been shown to occur (6, 26). In the present study, the synthesis of Ga_2Cer and SGalCer (Tables III and IV) also points to translocation of GalCer across the Golgi membrane. Recycling of GalCer from the cell sur-

face is very unlikely, because any GalCer reaching the cell surface is immediately extracted by the BSA in the medium. Still, it remains to be shown that passage of GlcCer and GalCer through the Golgi lumen is an obligatory step in their transport to the cell surface.

Selectivity in Sphingolipid Transport

A useful parameter to assess sorting of one lipid from another is the relative polarity, a comparison of the apical/basolateral polarity with which they reach the cell surface (56). It is independent from the apical and basolateral surface areas (and from the nature of the intracellular transport; 51). The one common observation on the sorting of newly synthesized sphingolipids between MDCK (Tables II and III) and Caco-2 (Table IV) was that GlcCer analogs are found enriched over the corresponding SM in the apical domain. This is in line with previous results (53, 56).

In both MDCK lines, HFA-GlcCer and HFA-SM were more basolateral than their NFA counterparts but sorting between them was maintained. Strikingly, HFA-GalCer was transported more basolaterally than GlcCer with the same backbone, sixfold in MDCK I and twofold in MDCK II. The present data are in good agreement with independent evidence that the GlcCer/GalCer ratio in the apical plasma membrane in MDCK II cells is twofold higher than in the basolateral membrane (36). In MDCK II, SGalCer and Ga_2Cer displayed polarities similar to GalCer suggesting that the galactosyl group may be an important sorting determinant in these cells. A different pattern of sphingolipid sorting was observed in Caco-2 cells. (*a*) Sorting of NFA-GlcCer from SM was more efficient with relative polarities >12. (*b*) GlcCer was not sorted from GalCer, independent of fatty acyl chain. (*c*) HFA-containing GlcCer and GalCer had a polarity of delivery four- to sixfold lower than the NFA-analogs. In Caco-2 cells, the fatty acyl chain rather than the headgroup seems the major determinant in the partitioning of GlcCer and GalCer into the apical domain. (*d*) SGalCer had a lower polarity than GalCer, which suggests a role for sulfate as an additional basolateral determinant.

Epithelial Sorting by Domain Formation in the TGN

If the lipids destined for the apical and basolateral cell surface pass through the lumen of the TGN, they must be sorted by lateral segregation into apical and basolateral precursor domains (15, 47, 53). In support of such a sorting mechanism, sphingolipids have the capability to form intermolecular hydrogen bonds in contrast to the glycerophospholipids like PC (3). Glyco-sphingolipids have a higher capacity for hydrogen bonding than SM, in line with the sorting of GlcCer from SM that was observed under all conditions. In addition, the orientation of the hydroxyls in the carbohydrate contributes to the intra- and intermolecular hydrogen bonding by influencing the orientation of the sugar-headgroup (38). The observed sorting of GlcCer from GalCer can be explained by a difference in the aggregation properties due to the orientation of the hydroxyl in the 4 position of the carbohydrate, equatorial in GlcCer and axial in GalCer. Indeed, differences in miscibility with phosphatidylcholine have been reported between GlcCer and GalCer (31). Also additional hydroxyls in the ceramide backbone will change intra- and intermolecular hydrogen bonding (3). Molecules with a 2-hydroxyl group on the fatty acid were clearly sorted from NFA species, especially in Caco-2 cells (Table IV). An effect of additional hydroxyl moieties in the sphingoid base, like in the abundant phytosphingosine, can also be expected. Finally, differences in the structure of the endogenous lipids between MDCK and Caco-2 cells (see Introduction) may greatly influence the sorting of individual glycosphingolipids. At present, insufficient data are available concerning the lipid composition of the lumenal leaflet of the TGN membrane in the two cell types and concerning the phase behavior of complex lipid mixtures to explain the observed sphingolipid sorting.

Proteins anchored to the membrane by a glycosylphosphatidylinositol tail (GPI-proteins) are transported to the apical surface of MDCK and Caco-2 cells. They may collect into the putative apical precursor domain in the TGN by a direct interaction with the glycosphingolipids (reviewed in reference 15). As MDCK and Caco-2 cells display differences in lipid sorting, the two cell types may utilize different lipid mixtures to create a domain of similar physical characteristics. Apparently, these are recognized by the GPI-proteins much more efficiently than by any individual glycosphingolipid. As yet we have no idea how a domain on the lumenal side of the TGN membrane is recognized as having an apical destination. An interesting observation is that one protein thought to be involved in epithelial sorting has been found to show homology to lectins (12). Interestingly, a lactose-binding lectin has been reported to be secreted apically by MDCK cells (29), but whether it passes through the lumen of the TGN where it could interact with lactosylceramide is unknown. In this context, it cannot be excluded that individual short-chain lipid molecules partition into the Golgi lumen (although arguments have been provided to suggest that the bulk of each short-chain lipid resides in membranes; 55) where they could bind to, and be transported by, carbohydrate receptors. The fact that a 100-fold increase in the concentration of the short-chain lipids did not influence the polarities of delivery seems to argue against an important role for such a one-to-one lipid–receptor interaction.

The preferential delivery of GlcCer over SM to the apical surface has been widely interpreted as implying self-aggregation of (all) glycosphingolipids into an apical precursor domain (see reference 15). The present data show that epithelial sphingolipid sorting is more complex. No single glycosphingolipid is exclusively delivered to the apical cell surface, some even have a basolateral preference. In addition, different cell types sort lipids differently. Much attention is focused on elucidating the signals in proteins responsible for their targeting and for their interaction with components of the transport machinery. Sorting of lipids presents a different set of questions. The answers may provide original angles into problems of protein sorting as well.

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References

1. Bansal, R., and S.E. Pfeiffer. 1994. Inhibition of protein and lipid sulfation in oligodendrocytes blocks biological responses to FGF-2 and retards cytoarchitectural maturation, but not developmental lineage progression. *Dev. Biol.* 162:511–524.
2. Bligh, E.G., and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.
3. Boggs, J.M. 1987. Lipid intermolecular hydrogen bonding: influence on structural organization and membrane function. *Biochim. Biophys. Acta.* 906:353–404.
4. Bonner, W.M., and J.D. Stedman. 1978. Efficient fluorography of ^{3}H and ^{14}C on thin layers. *Anal. Biochem.* 89:247–256.
5. Breimer, M.E., G.C. Hansson, K.-A. Karlsson, and H. Leffler. 1982. Glycosphingolipids of rat tissues. Different composition of epithelial and nonepithelial cells of small intestine. *J. Biol. Chem.* 257:557–568.
6. Burger, K.N.J., P. van der Bijl, and G. van Meer. 1996. The topology of sphingolipid galactosyltransferases in ER and Golgi implies that monohexosyl sphingolipids undergo transbilayer movement. *J. Cell Biol.* In press.
7. Carter, H.E., P. Johnson, and E.J. Weber. 1965. Glycolipids. *Annu. Rev. Biochem.* 34:109–142.
8. Chantret, I., A. Rodolosse, A. Barbat, E. Dussaulx, E. Brot-Laroche, A. Zweibaum, and M. Rousset. 1994. Differential expression of sucrase-isomaltase in clones isolated from early and late passages of the cell line Caco–2: evidence for a glucose-dependent negative regulation. *J. Cell Sci.* 107:213–225.
9. Cohen, A., G.E. Hannigan, B.R.G. Williams, and C.A. Lingwood. 1987. Roles of globotriosyl- and galabiosylceramide in verotoxin binding and high affinity interferon receptor. *J. Biol. Chem.* 262:17088–17091.
10. Esselman, W.J., J.R. Ackermann, and C.C. Sweeley. 1973. Glycosphingolipids of membrane fractions from normal and transplanted canine kidney. *J. Biol. Chem.* 248:7310–7317.
11. Falk, K.-E., K.-A. Karlsson, H. Leffler, and B.E. Samuelsson. 1979. Specific pattern of glycosphingolipids enriched in a mucosa scraping of human small intestine. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 101:273–276.
12. Fiedler, K., R.G. Parton, R. Kellner, T. Etzold, and K. Simons. 1994. Vip36, a novel component of glycolipid rafts and exocytic carrier vesicles in epithelial cells. *EMBO J.* 13:1729–1740.
13. Forstner, G.G., and J.R. Wherrett. 1973. Plasma membrane and mucosal glycosphingolipids in the rat intestine. *Biochim. Biophys. Acta.* 306:446–459.
14. Fuller, S., C.-H. von Bonsdorff, and K. Simons. 1984. Vesicular stomatitis virus infects and matures only through the basolateral surface of the polarized epithelial cell line, MDCK. *Cell.* 38:65–77.
15. Futerman, A.H. 1995. Inhibition of sphingolipid synthesis: effects on glycosphingolipid–GPI-anchored protein microdomains. *Trends Cell Biol.* 5: 377–380.
16. Futerman, A.H., B. Stieger, A.L. Hubbard, and R.E. Pagano. 1990. Sphingomyelin synthesis in rat liver occurs predominantly at the cis and medial cisternae of the Golgi apparatus. *J. Biol. Chem.* 265:8650–8657.
17. Hakomori, S.-i. 1990. Bifunctional role of glycosphingolipids. *J. Biol. Chem.* 265:18713–18716.
18. Hansson, G.C. 1983. The subcellular localization of the glycosphingolipids in the epithelial cells of rat small intestine. *Biochim. Biophys. Acta.* 733: 295–299.
19. Hansson, G.C., K.-A. Karlsson, and J. Thurin. 1984. Glycosphingolipid patterns of the epithelial and non-epithelial compartments of rat large intestine. *Biochim. Biophys. Acta.* 792:281–292.
20. Hansson, G.C., K. Simons, and G. van Meer. 1986. Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. *EMBO J.* 5:483–489.
21. Jeckel, D., A. Karrenbauer, R. Birk, R.R. Schmidt, and F. Wieland. 1990. Sphingomyelin is synthesized in the cis Golgi. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 261:155–157.
22. Jeckel, D., A. Karrenbauer, K.N.J. Burger, G. van Meer, and F. Wieland. 1992. Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. *J. Cell Biol.* 117:259–267.
23. Karlsson, K.-A., B.E. Samuelsson, and G.O. Steen. 1973. The sphingolipid composition of bovine kidney cortex, medulla and papilla. *Biochim. Biophys. Acta.* 316:317–335.
24. Kawai, K., M. Fujita, and M. Nakao. 1974. Lipid components of two different regions of an intestinal epithelial cell membrane of mouse. *Biochim. Biophys. Acta.* 369:222–233.
25. Kean, E.L. 1966. Separation of gluco- and galactocerebrosides by means of borate thin-layer chromatography. *J. Lipid Res.* 7:449–452.
26. Lannert, H., C. Bünning, D. Jeckel, and F.T. Wieland. 1994. Lactosylceramide is synthesized in the lumen of the Golgi apparatus. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 342:91–96.
27. Ledeen, R.W., R.K. Yu, and L.F. Eng. 1973. Gangliosides of human myelin: sialosylgalactosylceramide (G7) as a major component. *J. Neurochem.* 21:829–839.
28. Lindberg, A.A., J.E. Brown, N. Strömberg, M. Westling-Ryd, J.E. Schultz, and K.-A. Karlsson. 1987. Identification of the carbohydrate receptor for Shiga toxin produced by Shigella dysenteriae type 1. *J. Biol. Chem.* 262: 1779–1785.
29. Lindstedt, R., G. Apodaca, S.H. Barondes, K.E. Mostov, and H. Leffler. 1993. Apical secretion of a cytosolic protein by Madin-Darby canine kidney cells. Evidence for polarized release of an endogenous lectin by a nonclassical secretory pathway. *J. Biol. Chem.* 268:11750–11757.
30. Louvard, D. 1980. Apical membrane aminopeptidase appears at sites of cell-cell contact in cultured epithelial cells. *Proc. Natl. Acad. Sci. USA.* 77:4132–4136.
31. Maggio, B., T. Ariga, J.M. Sturtevant, and R.K. Yu. 1985. Thermotropic behavior of binary mixtures of dipalmitoylphosphatidylcholine and glycosphingolipids in aqueous dispersions. *Biochim. Biophys. Acta.* 818:1–12.
32. Miller-Podraza, H., and P.H. Fishman. 1982. Translocation of newly synthesized gangliosides to the cell surface. *Biochemistry.* 21:3265–3270.
33. Morell, P., R.H. Quarles, and W.T. Norton. 1994. Myelin formation, structure, and biochemistry. Basic Neurochemistry. Molecular, cellular, and medical aspects. G.J. Siegel, B.W. Agranoff, R.W. Albers, and P.B. Molinoff, editors. Raven Press, New York. 117–143.
34. Morell, P., and N.S. Radin. 1969. Synthesis of cerebroside by brain from uridine diphosphate galactose and ceramide containing hydroxy fatty acid. *Biochemistry.* 8:506–512.
35. Natomi, H., T. Saitoh, K. Sugano, M. Iwamori, M. Fukayama, and Y. Nagai. 1993. Systematic analysis of glycosphingolipids in the human gastrointestinal tract: enrichment of sulfatides with hydroxylated longer-chain fatty acids in the gastric and duodenal mucosa. *Lipids.* 28:737–742.
36. Nichols, G.E., T. Shiraishi, and W.W. Young, Jr. 1988. Polarity of neutral glycolipids, gangliosides, and sulfated lipids in MDCK epithelial cells. *J. Lipid Res.* 29:1205–1213.
37. Niimura, Y., and I. Ishizuka. 1986. Glycosphingolipid composition of a renal cell line (MDCK) and its ouabain resistant mutant. *J. Biochem.* 100: 825–835.
38. Nyholm, P.-G., and I. Pascher. 1993. Steric presentation and recognition of the saccharide chains of glycolipids at the cell surface: favoured conformations of the saccharide–lipid linkage calculated using molecular mechanics (MM3). *Int. J. Biol. Macromol.* 15:43–51.
39. Ong, D.E., and R.N. Brady. 1972. Synthesis of ceramides using N-hydroxysuccinimide esters. *J. Lipid Res.* 13:819–822.
40. Pagano, R.E., and O.C. Martin. 1988. A series of fluorescent N-acylsphingosines: synthesis, physical properties, and studies in cultured cells. *Biochemistry.* 27:4439–4445.
41. Parmantier, E., M. Monge, M. Yagello, F. Cabon, C. Demerens, J.-C. Gluckman, and B. Zalc. 1995. HIV-1 envelope glycoprotein gp120 does not bind to galactosylceramide-expressing rat oligodendrocytes. *Virology.* 206:1084–1091.
42. Radin, N.S. 1994. Glucosylceramide in the nervous system — a mini-review. *Neurochem. Res.* 19:533–540.
43. Schaeren-Wiemers, N., P. van der Bijl, and M.E. Schwab. 1995. The UDP-galactose: ceramide galactosyltransferase: expression pattern in oligodendrocytes and Schwann cells during myelination and substrate preference for hydroxyceramide. *J. Neurochem.* 65:2267–2278.
44. Schulte, S., and W. Stoffel. 1993. Ceramide UDPgalactosyltransferase from myelinating rat brain: purification, cloning, and expression. *Proc. Natl. Acad. Sci. USA.* 90:10265–10269.
45. Shayman, J.A., and N.S. Radin. 1991. Structure and function of renal glycosphingolipids. *Am. J. Physiol.* 260:F291–F302.
46. Siddiqui, B., J.S. Whitehead, and Y.S. Kim. 1978. Glycosphingolipids in human colonic adenocarcinoma. *J. Biol. Chem.* 253:2168–2175.
47. Simons, K., and G. van Meer. 1988. Lipid sorting in epithelial cells. *Biochemistry.* 27:6197–6202.
48. Spiegel, S., R. Blumenthal, P.H. Fishman, and J.S. Handler. 1985. Gangliosides do not move from apical to basolateral plasma membrane in cultured epithelial cells. *Biochim. Biophys. Acta.* 821:310–318.
49. Stahl, N., H. Jurevics, P. Morell, K. Suzuki, and B. Popko. 1994. Isolation, characterization, and expression of cDNA clones that encode rat UDP-galactose: ceramide galactosyltransferase. *J. Neurosci. Res.* 38:234–242.
50. Tennekoon, G., M. Zaruba, and J. Wolinsky. 1983. Topography of cerebroside sulfotransferase in Golgi-enriched vesicles from rat brain. *J. Cell Biol.* 97:1107–1112.
51. van Genderen, I.L., and G. van Meer. 1993. Lipid sorting — measurement and interpretation. *Biochem. Soc. Trans.* 21:235–239.
52. van Helvoort, A.L.B., W. van't Hof, T. Ritsema, A. Sandra, and G. van Meer. 1994. Conversion of diacylglycerol to phosphatidylcholine on the basolateral surface of epithelial (MDCK) cells. Evidence for the reverse action of the sphingomyelin synthase. *J. Biol. Chem.* 269:1763–1769.

53. van Meer, G., E.H.K. Stelzer, R.W. Wijnaendts-van-Resandt, and K. Simons. 1987. Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. *J. Cell Biol.* 105:1623–1635.
54. van Meer, G., and W. van't Hof. 1993. Epithelial sphingolipid sorting is insensitive to reorganization of the Golgi by nocodazole, but is abolished by monensin in MDCK cells and by brefeldin A in Caco-2 cells. *J. Cell Sci.* 104:833–842.
55. van't Hof, W., J. Silvius, F. Wieland, and G. van Meer. 1992. Epithelial sphingolipid sorting allows for extensive variation of the fatty acyl chain and the sphingosine backbone. *Biochem. J.* 283:913–917.
56. van't Hof, W., and G. van Meer. 1990. Generation of lipid polarity in intestinal epithelial (Caco-2) cells: sphingolipid synthesis in the Golgi complex and sorting before vesicular traffic to the plasma membrane. *J. Cell Biol.* 111:977–986.
57. Warnock, D.E., M.S. Lutz, W.A. Blackburn, W.W. Young, Jr., and J.U. Baenziger. 1994. Transport of newly synthesized glucosylceramide to the plasma membrane by a non-Golgi pathway. *Proc. Natl. Acad. Sci. USA.* 91:2708–2712.
58. Yahi, N., J.-M. Sabatier, S. Baghdiguian, F. Gonzalez-Scarano, and J. Fantini. 1995. Synthetic multimeric peptides derived from the principal neutralization domain (V3 loop) of human immunodeficiency virus type 1 (HIV-1) gp120 bind to galactosylceramide and block HIV-1 infection in a human CD4-negative mucosal epithelial cell line. *J. Virol.* 69:320–325.
59. Young, W.W. Jr., M.S. Lutz, and W.A. Blackburn. 1992. Endogenous glycosphingolipids move to the cell surface at a rate consistent with bulk flow estimates. *J. Biol. Chem.* 267:12011–12015.
60. Zurzolo, C., W. van't Hof, G. van Meer, and E. Rodriguez-Boulan. 1994. VIP21/caveolin, glycosphingolipid clusters and the sorting of glycosylphosphatidylinositol-anchored proteins in epithelial cells. *EMBO J.* 13:42–53.