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The way we view cellular (glyco)sphingolipids

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Abstract

Mammalian cells synthesize ceramide in the endoplasmic reticulum (ER) and convert this to sphingomyelin and complex glycosphingolipids on the inner, non-cytosolic surface of Golgi cisternae. From there, these lipids travel towards the outer, non-cytosolic surface of the plasma membrane and all membranes of the endocytic system, where they are eventually degraded. At the basis of the selective, anterograde traffic out of the Golgi lies the propensity of the sphingolipids to self-aggregate with cholesterol into microdomains termed 'lipid rafts'. At the plasma membrane surface these rafts are thought to function as the scaffold for various types of (glyco) signaling domains of different protein and lipid composition that can co-exist on one and the same cell. In the past decade, various unexpected findings on the sites where sphingolipid-mediated events occur have thrown a new light on the localization and transport mechanisms of sphingolipids. These findings are largely based on biochemical experiments. Further progress in the field is hampered by a lack of morphological techniques to localize lipids with nanometer resolution. In the present paper, we critically evaluate the published data and discuss techniques and potential improvements.

Keywords: glycolipids, immuno electron microscopy, immunofluorescence microscopy, lipid topology, subcellular localization.

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Sphingolipids form a highly diverse class of lipids, characterized by a lipid backbone that consists of a long-chain sphingoid base that is mostly amide-linked to a fatty acid: ceramide. The polar headgroup of the most abundant sphingolipid in mammalian cells, sphingomyelin (SM), is phosphocholine. It is transferred from the cell's most abundant glycerophospholipid phosphatidylcholine (PC) onto ceramide by sphingomyelin synthase on the lumenal side of the Golgi membrane, giving rise to SM plus diacylglycerol. Both ceramide and diacylglycerol fulfill dual functions as metabolic precursors and as secondary messengers. The glycosphingolipids contain polar headgroups that contain one or more carbohydrates. The basic glycosphingolipid in every cell is glucosylceramide (GlcCer; Table 1), to which carbohydrates are added in a stepwise fashion on the lumenal surface of the Golgi. This yields glycosphingolipids containing one up to ten or more carbohydrates, which are often organized as branched chains. Glycosphingolipids containing sialic acids are termed gangliosides. Independently, a number of cell types synthesize galactosylceramide (GalCer), part of which is modified in the trans Golgi lumen to GalCer sulfate (sulfatide). These latter lipids are relevant for the proper function and stability of the myelin sheath, as evidenced by the tremor, late-onset paralysis and premature death of knock-out mice for the ceramide GalCer synthase. The GlcCer-based glycosphingolipids are crucial for mammalian development. Once again, this has become clear from studies on knock-out mice. Mice with null-alleles for the GlcCer synthase, causing a lack of all complex glycosphingolipids (Yamashita *et al.* 1999), die as embryos. Mice lacking more distal transferases display milder defects, which however, when translated to humans, would still be recognized as major inherited diseases (Proia 2003). For example, whereas mice lacking the GM3 synthase displayed enhanced insulin sensitivity (Yamashita *et al.* 2003), humans with this defect display infantile-onset symptomatic epilepsy syndrome (Simpson *et al.* 2004). While SM generally makes up some 15–20% of the lipids in plasma membranes, the levels of glycosphingolipids are generally much lower. However, particularly high levels of glycosphingolipids have been reported for apical membranes of epithelial cells (Simons and van Meer 1988), for myelin (Bosio *et al.* 1996; Coetzee *et al.* 1996) and neurons (Vanier 1999).

The molecular basis for the need for glycosphingolipids was originally supposed to reside in the tremendous variability in three-dimensional structure, that can be

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Abbreviations used: ER, endoplasmic reticulum; GlcCer, glucosylceramide; LacCer, lactosylceramide.

Table 1 Glycosphingolipid designation according to the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature[a], using the Svennerholm abbreviations for gangliosides[b]

Abbreviation	Name	Structure	Synonyms
GlcSph	Glucosylsphingosine	Glcβ1–Sph	Glucopsychosine
GalSph	Galactosylsphingosine	Galβ1–Sph	(galacto)Psychosine
GlcCer	Glucosylceramide	Glcβ1–Cer	Glucocerebroside
GalCer	Galactosylceramide	Galβ1–Cer	(galacto) Cerebroside
LacCer	Lactosylceramide	Galβ4Glcβ1–Cer	
SGalCer	Sulfogalactosylceramide	HSO_3-3Galβ1–Cer	Sulfatide, SM4
SLacCer	Sulfolactosylceramide	HSO_3-3Galβ4Glcβ1–Cer	SM3
Gb3	Globotriaosylceramide	Galα4Galβ4Glcβ1–Cer	Gb_3Cer
Gangliosides			
GM1a	II^3NeuAc–Gg_4Cer	Galβ3GalNAcβ4(Neu5Acα3)–Galβ4Glcβ1Cer	GM1
GM1b	IV^3NeuAc–Gg_4Cer	Neu5Acα3Galβ3GalNAcβ4–Galβ4Glcβ1Cer	G_{M1b}
GM3	II^3NeuAc–LacCer	Neu5Acα3Galβ4Glcβ1Cer	G_{M3}
GD3	$II^3$$NeuAc_2$–LacCer	Neu5Acα8Neu5Acα3–Galβ4Glcβ1Cer	G_{D3}
GM4	I^3NeuAc–GalCer	Neu5Acα3Galβ1Cer	G_{M4}

[a]Chester (1998), [b]Svennerholm (1963).

generated by combining various carbohydrates in different combinations and orders and with different glycosidic bonds. Already early on, this variability suggested that the oligosaccharides on glycoproteins and glycosphingolipids might serve to mediate highly specific interactions between cells, cells and matrix and between cells and soluble signaling molecules (see Hakomori 2002). The high structural specificity is illustrated by the fact that many natural high-affinity antibodies have turned out to be directed against specific glycosphingolipids instead of proteins. An alternative role for sphingolipids was suggested by the lipid raft hypothesis in the late 1980s. The original hypothesis stated that glycosphingolipids in the lumenal leaflet of the membrane of the trans Golgi network self-aggregate and that epithelial cells selectively include these aggregates into budding transport vesicles destined for the apical plasma membrane domain of epithelial cells, sorting glycolipids with the apical proteins (van Meer *et al.* 1987). With the inclusion of SM and cholesterol, the hypothesis also nicely explains how cells enrich sphingolipids and cholesterol in the anterograde secretory pathway and thereby keep the endoplasmic reticulum (ER) concentrations of these lipids low (Fig. 1). The apical sorting of glycosphingolipids would then be superimposed on the general sorting of all sphingolipids and cholesterol towards the plasma membrane (van Meer 1989). Because the complex sphingolipids reside on the non-cytosolic surface they can reach other organelles by vesicular transfer only. The absence of a vesicular transport connection to mitochondria and peroxisomes then explains the general lack of SM and glycosphingolipids from these membranes as assessed biochemically (van Meer 1989). Because cholesterol readily flips across membranes and has a relatively high rate of monomeric transfer between membranes, its cellular distribution is determined essentially by its high affinity for sphingolipids (Wattenberg and Silbert 1983). (Glyco)sphingolipid-based rafts have received most attention for their roles in the signaling by immune receptors (Gomez-Mouton *et al.* 2001; Pierce 2002; Holowka *et al.* 2005; Barbat *et al.* 2007), in glycosignaling platforms on the cell surface (Hakomori 2002), and in other signal transduction events (see Mayor *et al.* 2006).

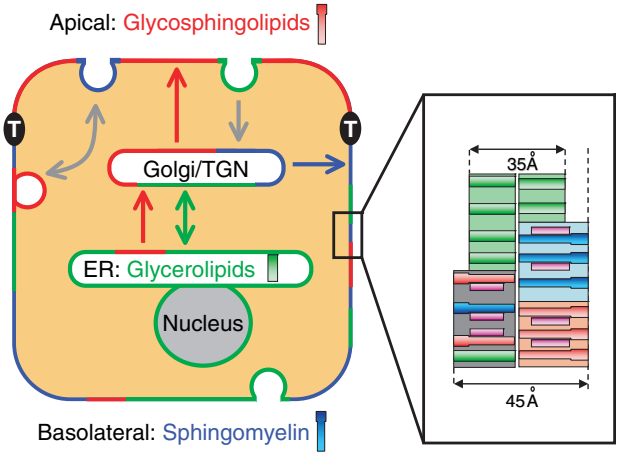

Fig. 1 Lipid sorting in intestinal epithelial cells (Simons and van Meer 1988; van Meer 1989). Glycosphingolipids (red) and cholesterol are enriched on the apical surface, whereas SM (blue) with cholesterol is preferentially transported to the basolateral surface. All three lipids are sorted in the anterograde direction, while unsaturated glycerophospholipids (green) are enriched in the retrograde pathway to the ER. The sorting occurs in the non-cytosolic leaflet of the various membranes, because the tight junctions (T) act as a barrier to lipid diffusion in the outer bilayer leaflet only. However, some sphingolipids are presumably present in the cytosolic leaflet. For the differences in membrane thickness (see Sprong *et al.* 2001).

In the past decade, various unexpected genetic and biochemical findings have opened new vistas on the organization of sphingolipids in the mammalian cell bringing up as many new sets of questions on the topology of the respective sphingolipids. (i) The simple glycosphingolipid glucosylceramide (GlcCer) is not synthesized in the lumenal leaflet of the Golgi membrane, but on the cytosolic surface (Ichikawa *et al.* 1996). Across which membrane does GlcCer translocate on its way into the Golgi lumen where it is required for the synthesis of higher glycosphingolipids? (ii) In contrast, the ceramide galactosyltransferase synthesizes galactosylceramide (GalCer) on the lumenal surface of the ER (Schulte and Stoffel 1993), where like all other lipids tested it has free access to the cytosolic surface by an energy-independent flip-flop process (Fig. 2). Do both GlcCer and GalCer redistribute to all other cytosolic surfaces via, for example, the cytosolic glycolipid transfer protein GLTP? (iii) Biochemically, complex glycosphingolipids and SM are found to some extent in the ER and even in the nuclear matrix (Albi and Viola Magni 2004; Hunt and Postle 2006). Are they really there and do they, as a consequence, have access to the cytosolic surface of the ER and that of other organelles as well? Recently, a non-lysosomal GlcCer degrading enzyme has been found in the ER (Boot *et al.* 2007). (iv) While mitochondria contain exceedingly low concentrations of (glyco)sphingolipids, in apoptotic cells SM and the ganglioside GD3 do seem to reach the mitochondria and to cause mitochondrial rupture and dysfunction (Rippo *et al.* 2000; Birbes *et al.* 2001; Garcia-Ruiz *et al.* 2002). Where exactly are these lipids located and how do these processes work? (v) The biosynthesis of SM and part of the GlcCer depends on transport of newly synthesized ceramide from the ER to the trans Golgi by the ceramide transfer protein CERT (Hanada *et al.* 2003). Does CERT act as a soluble carrier or as a contact-site protein, and where exactly does it deliver the ceramide? (vi) Finally, evidence has been presented that different types of microdomains can co-exist on the cell surface and that these domains have different (sphingo)lipid compositions (Gomez-Mouton *et al.* 2001; Brügger *et al.* 2004). How are these domains structured and do such domains exist along the vesicular transport pathways as well? These examples clearly illustrate the need for data on the location of (glyco)sphingolipids, if possible with nm resolution.

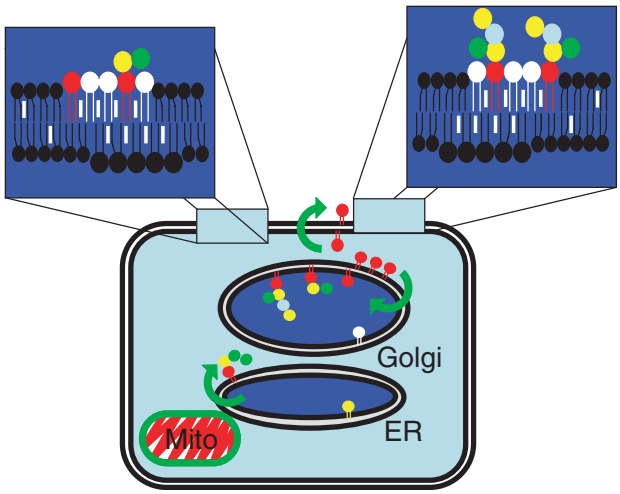

Fig. 2 Glucosylceramide (GlcCer; red) is synthesized on the cytosolic surface of the Golgi but is flipped to the lumenal surface of the Golgi, where it is converted to complex glycosphingolipids by the sequential addition of galactose (yellow) and sialic acid (green), to yield GM3. GM1 is synthesized by the further addition of N-acetylgalactosamine (light blue) and another galactose (Table 1). GM3 and GM1 occupy different domains on the surface of polarizing T cells, probably with SM (white). Some GD3 makes it back to the ER, where it can presumably flip freely across the membrane and now has access to, for example, mitochondria via contact sites.

Localization of (glyco)sphingolipids; the methods

So, what do we want to know about where the sphingolipid is located in the *in vivo* situation? In increasing order of resolution: (i) In which tissue or part of the tissue? (ii) In which cells? (iii) In which membranes in the cells? (iv) On which side of the membrane? (v) And, finally, at which lateral location within that membrane leaflet? At that point, we have reached the realm of chemistry and physics, where we can ask with which other membrane components our lipid interacts. Because natural lipids are not visible in the microscope (except for the very few that are autofluorescent), two general approaches and the combination of them have been followed to visualize them. (i) Lipids have been labeled by reagents that can be discriminated microscopically, mostly proteins that bind to a certain lipid, like antibodies, followed by fluorescent or gold-labeled secondary antibodies; (ii) analogs of lipids have been inserted into cells that are visible by themselves, because they are fluorescent, or can be visualized by the previous approach.

Labeling of endogenous lipids

Because of the remarkable antigenicity of glycosphingolipids, many anti-glycosphingolipid antibodies have been described. After binding to their target, they can be visualized by the traditional methods of immunofluorescence and immuno-electron microscopy. In comparison to the well-established immunolocalization of proteins, the localization of lipids is hampered by a number of special technical problems connected with their small size and hydrophobic nature. As a general principle, the burden is on the investigator to make sure that the interpretation adequately recognizes these problems. Only meaningful data can lay the solid foundation for the field that is needed before we can start to discuss the effects of lipids on the cellular organization, trafficking and signaling of proteins at the molecular level.

Access without loss or redistribution

The first and overriding condition that should be fulfilled by any method is that the method should not affect the feature that is to be studied, which in this case is the localization of the sphingolipid in the living cell. So the method should not change the level of the lipid or redistribute the lipid. The concentration of the lipid may change due to metabolism during the labeling procedure. This is especially realistic for signaling lipids which have a very high turnover. In addition, some enzymes are particularly resistant to fixatives. In these cases there are still various possibilities to record the *in vivo* situation: (i) to label *in vivo*, and study the label *in vivo* under a fluorescence microscope, (ii) to label *in vivo*, and fix the label instead of the lipid, and (iii) to freeze the situation by rapid freezing after which the lipids must be fixed in the frozen state.

A second problem is accessibility. Only glycosphingolipids on the cell surface can be labeled directly by exogenous antibodies. When one tries to approach intracellular lipids, problems arise that do generally not exist for protein targets. For protein localization, proteins are first fixed by aldehydes after which they are made accessible to the antibodies by opening the membranes. Because lipids are generally not fixed by aldehydes (and if they are, they mostly lose their antigenicity) special care must be taken to ensure that the opening method does not displace (or remove) the lipids. First of all, membranes have been opened by detergents or organic solvents like cold methanol or acetone. Such methods generally result in dissolution and release (Maneta-Peyret *et al.* 1999) and redistribution of lipids. This point has been convincingly made even at the level of tissues. The incubation with low concentrations of Triton X-100 resulted in the redistribution of glycosphingolipids between brain tissue samples that were co-processed in the same solution (Heffer-Lauc *et al.* 2005). Subcellular labeling patterns of lipids observed after such treatments must be verified by independent techniques. It should be realized that even a fixation procedure using aldehydes by itself can make membranes leaky to the labeling agent (Sugii *et al.* 2003). Such a change in the physical state of the membrane may well induce redistribution of lipids across, or in the plane of, the membrane or even between organelles.

A detergent-independent, general method to make proteins accessible for localization is the preparation of sections from frozen samples, frozen sections, which is followed by antibody labeling on the thawed sections. It is clear from lipid physics that cross-sectioned, and thereby opened, membranes have an inherent strong tendency to close. The lipids on the edge of the cross-sectioned lipid bilayer are inevitably unstable (Fig. 3), resulting in lipid redistribution across membranes and high background labeling over the section (van Genderen *et al.* 1991). A dramatic improvement in organelle-specific labeling on sections was observed after freeze-substitution: freezing the cells, followed by embedding in a polymer that is subsequently cross-linked by UV, all below 0°C. The complex glycosphingolipid Forssman antigen was found to reside in specific cellular membranes and to be absent from mitochondria and peroxisomes (van Genderen *et al.* 1991). First of all, this confirms that freeze-substitution does not redistribute the lipid over organelles, second, the results suggest that it is technically possible to address the question whether glycosphingolipids are present in mitochondria under specific conditions. The labeling density was high in the plasma membrane and in endosomes, threefold lower in the Golgi and 10-fold lower in the nuclear membrane. This fits the expectations from biochemical analyses, which suggested that this immunolocalization technique can be used to quantitate the surface density of the lipid in the various membranes. A later study using freeze-substitution (Parton 1994) even supplied sufficient resolution to conclude that the glycosphingolipid GM1 was not distributed uniformly over the plasma membrane but

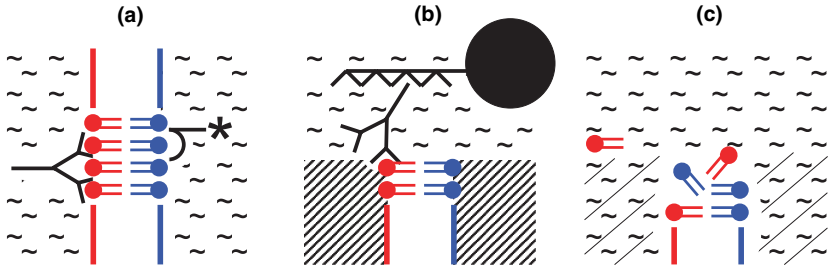

Fig. 3 The sidedness of lipid labeling. (a) Membrane impermeable probes can be added selectively from one side of the lipid bilayer. Examples are the exogenous addition of antibodies or toxins to the outer surface of intact cells (red; e.g., (Parton 1994), or the expression of cytosolic fluorescent PH domains to label phosphoinositides on cytosolic surfaces (blue; (Downes *et al.* 2005). Besides studying them by fluorescence, these labels can be fixed and processed for electron microscopy by a variety of techniques (thin frozen sections, plasma membrane sheets, fracture-label), whereby the label is visualized by pre- or post-embedding with, e.g., protein A-gold. (b) In freeze-substitution the samples are frozen, and the ice replaced by a polymer below 0°C. Thin sections are cut, and a primary reagent (antibody) followed by a secondary reagent (protein A-5 nm gold) labels lipids at the surface of the section. The gold does not reflect the sidedness of the lipid antigen. (c) In conventional thin frozen sections, the sample is thawed and labeled. As illustrated lipids at the interface will redistribute and some will spread. Labeling as in (b). While antibodies can penetrate the section, the gold label will not.

was concentrated approximately fourfold in non-coated invaginations, identified as caveolae. This was the first morphological evidence for the occurrence of glycosphingolipid-enriched domains in a mammalian plasma membrane. (Vielhaber *et al.* 2001b) successfully demonstrated a concentration of GlcCer in lamellar bodies of the skin. Excellent preservation of the galactosphingolipids GalCer and S-GalCer to myelin sheets in rat brain was reported as well (Kirschning *et al.* 1998). Still, there are many variables in the fixation and embedding steps of the method that must be optimized to minimize lipid loss (Maneta-Peyret *et al.* 1999), and redistribution.

One other method to gain access to the cytosolic surface of the plasma membrane is the preparation of plasma membrane sheets. Labeling such sheets with gold-labeled antibodies, followed by imaging and pattern analysis allowed an assessment of the clustered appearance of lipid-anchored kinases on the cytosolic surface (Plowman *et al.* 2005), and an assessment of the lateral distribution of phosphoinositides and phosphatidylserine, lipids restricted to the cytosolic surface, should not be problematic: An analysis of the distribution of prelabeled GM1 (in the outer leaflet) showed a difference between the random distribution in the control and the clustering occurring after cross-linking (Wilson *et al.* 2004).

A final problem of accessibility is shielding of the lipid by either proteins or by other lipids. This particularly applies to lipids that are deeply embedded in the lipid bilayer and expose only a small headgroup. A clear example is the observation that the cholesterol–binding agent filipin does not bind to cholesterol in coated vesicles until after removal of the clathrin coat (McGookey *et al.* 1983; Steer *et al.* 1984). This predicts that agents proposed to label lipids like ceramide (Vielhaber *et al.* 2001a) may not label their target with similar efficiencies in different membranes or different membrane domains. The labeling of SM by the SM-binding toxin lysenin, or probes derived thereof, depended on the lipid environment. The presence of glycolipids inhibited binding (Ishitsuka *et al.* 2005). Such probes can therefore not be used for the quantitative assessment of the distribution of a lipid over various locations, because the absence of labeling does necessarily mean that the lipid target is not present.

Labeling without redistribution

A subsequent set of problems with the microscopic localization of glycosphingolipids is connected to the nature of the labeling reagents. First of all, the agent should be specific. A standard test for the specificity of the label for a certain glycosphingolipid is a binding assay on TLC plates, but it is impossible to test all lipids for cross reactivity. Moreover, a lipid is presented differently on a TLC plate and in a biomembrane: the possibility exists that a protein specific in a TLC assay cross-recognizes other epitopes in the cellular context. The ideal test to exclude such cross-reactivity is to study the same cells but lacking this specific glycosphingolipid (van Genderen *et al.* 1991). This is now facilitated by the availability of a cell line without glycosphingolipids (Ichikawa *et al.* 1996). Second, the agent should have a high affinity for the lipid in order to increase the labeling density. The affinity increases when the labeling reagent is multivalent, which takes us to the next problem: because lipids are not fixed by routine fixation protocols, they can be redistributed by multivalent ligands abolishing their original distribution pattern. This is especially true when these primary ligands are subsequently labeled by secondary ligands that are multivalent by themselves. Labeling of the complex glycosphingolipids globoside and Forssman antigen by an antibody, or labeling of GM1 by cholera toxin, followed by tetra- or pentavalent protein A or by a second antibody resulted in clustering (Tillack *et al.* 1983; Spiegel *et al.* 1984; Butor *et al.* 1991; Fujimoto 1996). The artificial clustering by the second protein label could be prevented by fixation of the first label before addition of the second (Butor *et al.* 1991; Fujimoto 1996). A disturbance of the lipid distribution may also occur as a consequence of the fact that the reagent changes an essential physical property of the lipid. The label is generally big as compared to the target lipid and may change its diffusion co-efficient. Especially, labels that insert into the membrane like (agents based on) membrane penetrating toxins, e.g., lysenin for SM and perfringolysin for cholesterol, are likely to interfere with those lipid–lipid interactions that determine their phase behavior, and thereby affect their lateral distribution. Whereas the cellular dynamics of phosphoinositides have been studied extensively using the (green fluorescent protein-coupled) truncated binding domains (PH-, PX-, ENTH-, and FYVE-domains) of proteins that bind specifically to distinct phosphoinositides (Downes *et al.* 2005), remarkably few probes (antibodies/toxins) are available that bind one (glyco)sphingolipid molecule with sufficient affinity to study singular (glyco)sphingolipids.

Finally, the resolution of the labeling is limited by the dimensions of the labeling reagent: whereas the cross-sectional area of a typical lipid is 0.6 nm^2, the diameter of the gold particles is 5–10 nm or more, and the linker between the antigenic lipid and the gold particle again has a length of >10 nm (Fig. 3). It is therefore of paramount importance to devise monovalent high-affinity reagents of a limited size. Still, it is clear that the size of the electron microscopic reagents by themselves makes it impossible to define the sidedness of an epitope on membranes by the post-embedding of thin sections (Fig. 3). Protocols must be used where the accessibility to the labeling reagent is limited to one side of the membrane only. This can be achieved by labeling intact membranes (or cells) prior to freezing, embedding or cutting. Alternative approaches that have been applied to lipids are the immunolabeling of membrane sheets (Wilson *et al.* 2004), and the fracture-label technique, which is immunolabeling of membrane halves produced by freeze-fracture (Barbosa and Pinto da Silva 1983).

Fluorescent lipid analogs

Thirty years ago Simoni and colleagues demonstrated how parinaric acid, a family of unnatural fluorescent C18 fatty acids with 4 conjugated *cis*- and *trans*-double bonds can be used to study physical properties of fatty acids and phospholipids *in vitro* and *in vivo* (Sklar *et al.* 1975; Rintoul and Simoni 1977). Since then, a variety of fluorescent lipophilic dyes have been applied that unlike parinaric acid are visible by fluorescence microscopy. They have been used in numerous cases to study physical properties of cellular membranes and to unravel transport pathways of cellular lipids, for example the barrier properties of epithelial tight junctions (Dragsten *et al.* 1981; van Meer and Simons 1986). However, an important breakthrough in this field was the broad application by (Pagano *et al.* 1981) of visible fluorescent lipids that sufficiently resembled the natural mammalian lipids in structure to be recognized by the cellular metabolic pathways. Because these analogs carried a short fluorescent and relatively polar fatty acid (C6-NBD), they were rather well soluble in water, which property enabled the investigators to efficiently deliver them to, or deplete them from, the cell surface. Over the years, these probes provided a wealth of topological data (Pagano and Sleight 1985) and laid the basis for the lipid raft hypothesis (van Meer *et al.* 1987).

By the time that it became clear that the C6-NBD lipids did not faithfully reflect the phase properties of especially the raft forming sphingolipids (Wang and Silvius 2000), a new fluorescent fatty acid analog was introduced, C5-Bodipy, that is more hydrophobic and has the attractive possibility that its fluorescence is concentration-dependent (Pagano *et al.* 1991). Its use has provided information on glycolipid concentration into microdomains in the endocytic pathway (Sharma *et al.* 2003), to which picture also other fluorescent probes had contributed [reviewed in Mukherjee and Maxfield (2000); Barbat *et al.* (2007)]. Still, the phase properties of even the Bodipy-sphingolipids are different from those of their natural analogs (Wang and Silvius 2000), and the question remains how their localization in cells relates to that of the natural sphingolipids.

A more recent breakthrough in the application of fluorescent analogs of the cellular lipids takes us back full circle to the use of parinaric acids. In the intervening 30 years new delivery technologies and improved microscopy have potentiated the use for cell biology of pyrene fatty acids, as being more stable analogs than the parinaric acids while maintaining close-to-natural properties (Somerharju 2002). However, the parinaric acids have now been rendered suitable for light microscopy by the chemical addition of yet another conjugated double bond, which brings their excitation and emission wavelengths in the visible part of the spectrum. These fluorescent fatty acids are the first ones to be efficiently incorporated into cellular sphingolipids, illustrating their closest possible resemblance to the natural fatty acids and offering optimal possibilities to study the location and behavior of natural sphingolipids (Kuerschner *et al.* 2005). Still, there are numerous practical limitations to their broad application. The cunning combinations of biochemical, biophysical, and microscopic experiments devised over the years by numerous investigators for the application of other fluorescent lipid analogs should help to surmount these limitations and develop their utmost potential for the characterization of the topology of glycosphingolipids.

Glycosphingolipid topology in mammalian cells

Glycosphingolipids and SM have been found to be exposed on the surface of the cell by a number of biochemical approaches including enzymatic degradation, enzymatic and non-enzymatic oxidation, and the binding of antibodies and toxins. These data have led to the general impression that 60–70% of the cellular SM and a large fraction of all glycosphingolipids are exposed on the cell surface, i.e., in the outer, non-cytosolic leaflet of the plasma membrane (see van Meer and Holthuis 2000; Sillence *et al.* 2000). This fits the overall idea that glycosphingolipids and SM are synthesized in the lumenal leaflet of the Golgi membrane and are transported via the inner leaflet of membrane vesicles (Fig. 2). As predicted from this idea these lipids follow the endocytotic recycling pathways and are also present on the lumenal side of membranes of endosomes and lysosomes (van Genderen *et al.* 1991), where they are finally degraded (Kolter and Sandhoff 2005).

Diffusion barrier-based lipid macrodomains

It has been realized for a long time that lipids on the surface of some cells display a polarized distribution. This is particularly true for epithelial cells from the intestine and the kidney where glycolipids or SM were found to be at least fourfold enriched in the apical domain of the continuous plasma membrane that surrounds those cells (see Simons and van Meer 1988; Holthuis *et al.* 2001). This situation is maintained by the proteinaceous tight junctions, which form a barrier to lipid diffusion in the outer leaflet of the plasma membrane. Thus, the differences in lipid composition reside in the outer leaflet and the glycosphingolipids and SM are enriched in the outer leaflet of the apical membrane [discussed in Simons and van Meer (1988)]. The other striking example of stable lipid macrodomains is found on the surface of sperm cells. In this case, the evidence points at a proteinaceous diffusion barrier between the acrosomal and post-acrosomal domain (James *et al.* 2004; Selvaraj *et al.* 2006).

Microdomains based on lipid–lipid interactions

Based on the enrichment of glycosphingolipids on the outside of the apical membrane and the preferential apical transport of various fluorescent glycosphingolipids as compared to sphingomyelin analogs in kidney and intestinal

epithelial cells, it was proposed that these epithelial cells sort their lipids by the segregation of glycosphingolipids (and cholesterol) away from SM and phosphatidylcholine (plus cholesterol) in the lumenal leaflet of the trans Golgi network (van Meer *et al.* 1987; van 't Hof *et al.* 1992). It was then suggested that such lipid-based domains might exist at the cell surface where they could be involved in signal transduction (Lisanti *et al.* 1994). This original suggestion concerned stable structures, the caveolae, for which it was shown at that time that they were enriched in the glycosphingolipid GM1 (Parton 1994) by the application of cholera toxin on freeze-substituted samples, which was confirmed by photo-crosslinking (Fra *et al.* 1995). Stable glycosphingolipid-enriched domains may also exist in various types of glycosynapses (Hakomori 2002), e.g., non-caveolar glycosphingolipid signaling domains enriched in GM3 (Iwabuchi *et al.* 1998), and point contacts between neuronal growth cones and extracellular matrix (Negreiros *et al.* 2003). However, studies using a variety of biophysical techniques have suggested that microdomains of specific lipid compositions exist but that they are generally small and transient (Pike 2006), unless they are stabilized by an increase in order. Large scale lipid segregation may be induced by a change in curvature (Roux *et al.* 2005), or by changes occurring in membranes during signal transduction, like the generation of ceramide by a sphingomyelinase (Bollinger *et al.* 2005). Other factors causing lipid raft coalescence into stable platforms are the relatively subtle changes in lipid raft partitioning of multichain immune recognition receptors following signal initiation by ligand-mediated receptor crosslinking (Pierce 2002; Holowka *et al.* 2005). In contrast, a different role for lipid rafts, namely as carriers for signaling molecules, has been suggested during the formation of the immunological synapse between antigen-presenting cells and T cells (Saito and Yokosuka 2006).

Sorice *et al.* (1997) properly post-fixed anti-GM3 antibodies before addition of the secondary antibodies to prevent redistribution of the primary antibodies upon cross-linking by the secondary IgM, and observed, by fluorescence and electron microscopy, large, 300 nm clusters of GM3 molecules that covered close to 50% of the surface of T cells. When (Spiegel *et al.* 1984) clustered GM1 into a cap on the lymphocyte surface via cholera toxin, anti-toxin antibodies and protein A, exogenously added (fluorescent) GM3 co-capped with GM1 suggesting that the two gangliosides resided in the same plasma membrane domains. However, Gomez-Mouton *et al.* (2001) reported that during T cell polarization GM1 and GM3 segregated into specific lipid rafts at the uropod and at the leading edge, respectively (Fig. 2). In polarizing MCF-7 adenocarcinoma cells GM1 moved to the leading edge (Manes *et al.* 1999), which probably also represents the uropod in these fibroblast-like cells (Gomez-Mouton *et al.* 2001). Finally, partial co-localization of GM1- and GM3- enriched domains was reported by Barbat *et al.* (2007) after the induction of CD4 signaling in the absence of T cell receptor engagement. However, electron microscopy and proper sample preparation protocols will be required to resolve whether there was real co-localization under these conditions. The same applies to the observation that GM1 and GD3 did not co-localize on cerebellar neurons (Vyas *et al.* 2001). Kiyokawa *et al.* (2005) concluded that GM1-rich membrane domains are spatially distinct from SM-rich domains on Jurkat T cells. However, because the SM-specific probe used, lysenin, does not bind to SM in the presence of glycosphingolipids (Ishitsuka *et al.* 2005), the possibility exists that (a high concentration of) SM was present in the GM1 rafts.

Evidence for the co-existence of different types of lipid rafts has also been reported for surface of neuronal cells. Two typical raft marker proteins, the glycosylphosphatidylinositol-anchored prion and Thy-1 proteins, occupied different domains. While prion protein occurred at high density in domains located primarily at the cell body, Thy-1 was clustered in separate domains mainly on neurites covering half of their surface (Madore *et al.* 1999). Affinity-purification of the two types of rafts from detergent-resistant membranes showed that prion protein rafts contained fivefold higher levels of glucosylceramide than Thy-1 rafts (Brügger *et al.* 2004).

Intracellular sphingolipids

In line with its synthesis in the lumen of the Golgi, the complex glycosphingolipid Forssman antigen was found in the Golgi, the plasma membrane and endosomal membranes, but not in mitochondria and peroxisomes (van Genderen *et al.* 1991). Obviously, high concentrations of glycosphingolipids can accumulate in endocytotic organelles in cells from patients suffering from a variety of storage diseases (Futerman and van Meer 2004). In addition, a low density of Forssman was observed over the ER and nuclear membrane, demonstrating that complex glycosphingolipids can be retrogradely transported to the ER (van Genderen *et al.* 1991). A number of glycolipid-binding toxins like cholera toxin (GM1) and Shiga toxin (Gb3) need to be transported back to the ER where their active subunit passes the membrane into the cytosol. It is not clear whether such transport reflects the natural retrograde transport of glycolipids or that it is induced by the pentameric toxins (Falguieres *et al.* 2006; Chinnapen *et al.* 2007), but GM1 has been found in the nuclear membrane by itself (Ledeen and Wu 2006).

Although mitochondria generally contain very low levels of SM (van Meer 1989), a remarkable increase has been reported for cancer cells (see Holthuis *et al.* 2001) and mitochondrial SM appears to be the precursor for the apoptotic mitochondrial ceramide (Birbes *et al.* 2001). Likewise, by immuno-electron microscopy mitochondria were negative for Forssman labeling (van Genderen *et al.*

1991) and for the disialo-ganglioside GD3 (Rippo *et al.* 2000; Garcia-Ruiz *et al.* 2002). However, mitochondrial GD3 labeling was found in Fas- and ceramide-induced apoptosis in lymphocytes (Rippo *et al.* 2000) and in TNF-α-treated hepatocytes (Garcia-Ruiz *et al.* 2002). Unfortunately, these papers did not provide data on the GD3 labeling of the other cellular organelles leaving many unanswered questions concerning methodological controls and possible mechanisms of how GD3 got into the mitochondria. In any case, if the GD3 was originally synthesized in the lumen of the proximal Golgi (Uliana *et al.* 2006), it must have crossed a cellular membrane. This transmembrane translocation may have occurred as a consequence of the signaling at the plasma membrane (by the elusive scramblase?) or after retrograde traffic to the ER. Like all lipids tested so far GD3 may spontaneously translocate across the ER membrane. Subsequently, from the cytosolic surface of the ER it may reach the mitochondria, either via the ER-mitochondria contact sites or via monomeric transport through the cytosol, possibly stimulated by, e.g., the glycolipid transfer protein.

The fact that complex glycosphingolipids like Forssman glycolipid have been located to the ER and nuclear membrane and the indiscriminate flip-flop of lipids across the ER membrane suggests that SM and glycosphingolipids in general may occur to some extent in the cytosolic bilayer leaflet of the secretory and endocytotic organelles. Glucosylceramide is a special case in that it is synthesized on the cytosolic surface of the Golgi. Although many interactions between glycosphingolipids and cytosolic proteins have been reported over the years, a number of these studies were carried out *in vitro* and have not been validated in intact cells. In addition, when microscopic co-localization between glycosphingolipids and cytoskeletal elements was observed (i) often redistribution of the glycosphingolipid during sample preparation and labeling has not been thoroughly excluded, and (ii) the glycosphingolipids may have resided in the lumen of transport vesicles attached to the cytoskeleton (Gillard *et al.* 1993). Still, glycosphingolipids could in principle be present in complexes with proteins in the cytosol and nuclear matrix. A growing body of evidence supports the presence of intact phosphoinositides in the nuclear matrix, where they are thought to take part in signaling (Irvine 2006), and the same may be the case for SM and glycosphingolipids (Ledeen and Wu 2006).

A special case are the sphingolipids ceramide, ceramide-1-phosphate, sphingosine and sphingosine-1-phosphate which occur both as metabolic intermediates and as signaling lipids. It would be very important to be able to follow their local concentrations in time. However, there are several technical problems: (i) They are very sensitive to metabolic turnover (even during sample preparation), (ii) The single chain sphingosine and sphingosine-1-phosphate rapidly exchange as monomers through the aqueous phase, (iii) ceramide spontaneously flips across membranes, and finally (Rippo *et al.* 2000) there are only limited tools for their localization, like anti-ceramide antibodies (Vielhaber *et al.* 2001a) and it has not been established how well they label ceramide when it is present in different lipid and protein environments.

Perspectives

Glycosphingolipids and SM fulfill important functions within specialized domains of cellular membranes and in their interactions with proteins in, on and outside membranes. A combination of biological, physical, and chemical approaches is required to uncover these functions and the underlying molecular interactions. Morphology may be a great help. However, the traditional methodology is full of pitfalls. Notably, the specificity of the tools (Yanagisawa *et al.* 2006) and the fixation protocol (Schwarz and Futerman 1996; Heffer-Lauc *et al.* 2005) have been problematic. It is a tremendous challenge to try and overcome these methodological problems, and to be aware of them is a good start. All eukaryotes have sphingolipids and they display a bewildering range of structures. Evolution must have used their chemical and physical potential and endowed them with vital functions. Let's uncover them.

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