Minireview

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Sphingolipid Transport: Rafts and Translocators*

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Until some 15 years ago, sphingolipids were generally believed to protect the cell surface against harmful factors in the environment by forming a mechanically stable and chemically resistant outer leaflet of the plasma membrane lipid bilayer. Furthermore, complex glycosphingolipids were found to be involved in specific functions like recognition and signaling (1). Whereas the first feature would depend on physical properties of the sphingolipids, the signaling functions involve specific interactions of the complex glycan structures on the glycosphingolipids with similar lipids on neighboring cells or with proteins. Since then, two findings have revolutionized the field. (i) Simple sphingolipid metabolites, like ceramide and sphingosine 1-phosphate, have been found to be important mediators in signaling cascades of apoptosis, proliferation, and stress responses (reviews by Hannun and Obeid (66) and Spiegel and Milstien (67)). (ii) It has been realized that ceramide-based lipids self-aggregate in cellular membranes to form a separate phase that is less fluid (liquid-ordered) than the bulk liquid-disordered phospholipids based on diacylglycerol. Sphingolipid-based microdomains or "rafts" were originally proposed to sort membrane proteins along the cellular pathways of membrane transport (2). Presently, most excitement focuses on their organizing functions in signal transduction (3).

Sphingolipids are synthesized in the ER[1] and the Golgi but are enriched in plasma membrane and endosomes where they perform many of their functions. Thus, sphingolipids travel between organelles. Transport occurs via transport vesicles and via monomeric transport through the cytosol. Furthermore, some sphingolipids efficiently translocate across cellular membranes. That transport is not random is clear from the heterogeneous distribution of sphingolipids over the cell; sphingolipids are virtually absent from mitochondria and the ER but constitute 20–35 mol % of the plasma membrane lipids (Table I). Furthermore, signaling pools of sphingolipids do not freely mix with pools of biosynthesis and degradation (reviews by Hannun and Obeid (66), Merrill (68), and Spiegel and Milstien (67)). The specificity in sphingolipid transport is the topic of the present review.

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[1] The abbreviations used are: ER, endoplasmic reticulum; GalCer, galactosylceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide; SM, sphingomyelin; TGN, trans-Golgi network; GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; GM3, Neu5Acα3Galβ4GlcCer; GM1, Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer; GD3, Neu5Acα8Neu5Acα3Galβ4GlcCer.

Biosynthetic Traffic and Lipid Translocators

Ceramide—The first steps in sphingolipid synthesis are the condensation of L-serine and palmitoyl-CoA to ketosphinganine and its reduction to sphinganine in the ER membrane. In yeast, these lipids do not feed into signaling pools (4), and exogenous sphingoid bases need to go through a cycle of phosphorylation and dephosphorylation before they can be utilized for ceramide synthesis (5). This suggests that sphingoid bases synthesized *de novo* are channeled through the pathway into ceramide without being able to escape. In yeast, ceramide is then converted to inositolphosphoceramide and the mannosyl derivatives mannosylinositolphosphoceramide and mannosyldiinositolphosphoceramide on the lumenal surface of the Golgi (6). In mammals, ceramide is utilized for the synthesis of glucosylceramide (GlcCer) on the cytosolic side of the Golgi, sphingomyelin (SM) on the lumenal surface of the Golgi, and in specialized cells, *e.g.* many epithelial cells, of galactosylceramide (GalCer) in the lumen of the ER (Fig. 1) (7). Because ceramide synthesis occurs on the cytosolic side of the ER, the rate of ceramide translocation toward the lumena of ER and Golgi affects the relative synthesis of the various products. If the $t_{1/2}$ of spontaneous ceramide translocation would be tens of minutes (8), this is slow compared with the vesicular transport between ER and Golgi (minutes). However, translocation may be faster in the unsaturated lipid environment of the ER. In addition, ER and Golgi may possess proteins that stimulate ceramide translocation. Ceramide transport to the site of SM synthesis can be inhibited under conditions where transport to the site of GlcCer synthesis and ER-Golgi vesicle transport are normal (9), and besides the vesicular pathway, a non-vesicular mechanism delivers ceramide to the Golgi in mammalian cells and yeast (10, 11). In yeast, this alternative pathway depends on ER-Golgi membrane contact and on a cytosolic factor and is energy-independent (11). Interestingly, close apposition of the ER to cisternae of the trans-Golgi has been observed in mammalian cells (12). In the model of Fig. 1, GlcCer synthase in the cis-Golgi receives ceramide via the vesicular pathway whereas GlcCer synthase and SM synthase in the trans-Golgi (13) receive ceramide from the ER via membrane contacts. Similar contacts have often been observed between ER and mitochondria (12). They may be responsible for the transfer of signaling ceramide to mitochondria. A mitochondrial ceramidase has been identified (Hannun and Obeid (66)).

Glucosylceramide—GlcCer synthesized on the cytosolic surface of the Golgi is partially converted to complex glycosphingolipids in the Golgi lumen (review by Kolter *et al.* (69)). Experiments with brefeldin A, which fuses the cis-medial Golgi with the ER, have suggested that the enzymes synthesizing lactosylceramide (LacCer; Fig. 1) and the first complex glycosphingolipids are in the early Golgi. However, the bulk of these events is thought to occur in the trans-Golgi or trans-Golgi network (TGN) *in vivo* (14, 15). GlcCer is probably translocated across the Golgi membrane by an energy-independent translocator (14, 16). Alternatively, GlcCer may be translocated toward the lumen by MDR1 P-glycoprotein, an ATP-binding cassette transporter that causes multidrug resistance (17). However, so far, translocation of GlcCer by MDR1 has only been proven for short chain analogs (18, 19). MDR1 is mostly found at the plasma membrane, where it may clear the cytosolic surface of GlcCer by translocation toward the exoplasmic leaflet. GlcCer has access to this cytosolic surface via the cytosolic side of transport vesicles or, alternatively, via monomeric transport throughout the cytosol (20), possibly mediated by the glycolipid transfer protein (21). An apical GlcCer translocator could thus enrich GlcCer on the apical as compared with the basolateral surface of epithelial cells, after which the difference in lipid composition between the two domains would be maintained by tight junctions acting as a barrier to lipid diffusion in the outer leaflet of the lipid bilayer (22). It is probably by a similar translocator that sphingosine 1-phosphate

TABLE I
Sphingolipid content of plasma membranes

Plasma membranes are manyfold enriched in sphingolipids and cholesterol as compared to the ER and the mitochondria (34, 61). Indeed, electron microscopy after immunolabeling demonstrated that the concentration of the complex glycosphingolipid Forssman antigen was 10-fold higher in the plasma membrane than in the ER, with no label over mitochondria and peroxisomes (53).

	Sphingolipids	Glycerophospholipids	Sterol
		mol / mol	
Intestinal epithelium			
Apical[a]	38	29	33
Basolateral[a]	19	56	25
Myelin[b]	28	28	44
Yeast plasma membrane[c]	16	36	48

[a] In this membrane, 50% of the sphingolipids were GlcCer, the rest consisted of the complex glycosphingolipid globoside and SM. The major glycerophospholipid, phosphatidylcholine, makes up 8 mol % of the lipids in the apical *versus* 29 mol % of the lipids in the basolateral membrane (62).

[b] The major sphingolipid in myelin is GalCer followed by sulfated GalCer and SM (63).

[c] Sphingolipids in *Saccharomyces cerevisiae* consist of nearly equal fractions of inositolphosphoceramide, mannosylinositolphosphoceramide, and mannosyldiinositolphosphoceramide (64). In addition, other yeasts generally contain GlcCer-based glycosphingolipids.

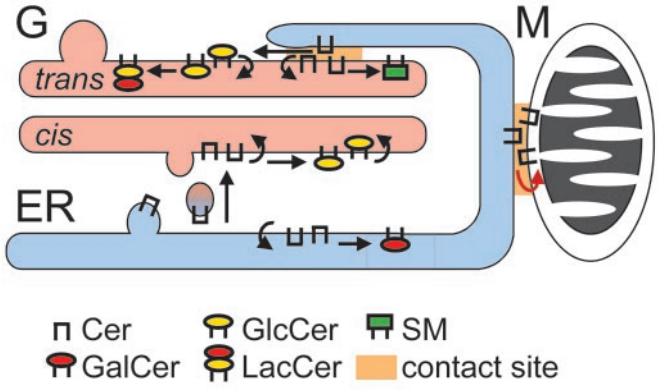

FIG. 1. **Sphingolipid synthesis and translocation in the Golgi.** Ceramide (*Cer*) from the cytosolic surface of the ER is converted to GalCer in the ER lumen or transported to the Golgi (*G*). The GlcCer synthase is found at two locations in the hepatocyte Golgi by sucrose gradient centrifugation (65). One peak colocalized with SM synthase, which was not relocated to the ER by brefeldin A in these cells (7), and thus probably situated in the trans-Golgi/TGN (13). Ceramide reaches the cis-Golgi by vesicular transport whereas ER-TGN contacts allow ceramide transport by exchange. These contacts have been suggested to be sites of general lipid exchange (12), which also holds for similar contacts between ER and mitochondria (*M*). GlcCer translocates toward the lumen of the Golgi, where it is galactosylated to LacCer. LacCer is the precursor for the various complex glycosphingolipid series. For simplicity, the seven cisternae of the Golgi have been reduced to just two.

after synthesis in the cytosol reaches the outside of the plasma membrane and is secreted.

Complex Sphingolipids and Sphingomyelin—GalCer synthesized in the ER lumen may flip toward the cytosolic surface (16), from where it has access to the same sites as GlcCer. In contrast, complex glycosphingolipids and SM synthesized in the lumen of the Golgi appear unable to translocate from the lumenal toward the cytosolic surface (14, 16). As a consequence, they can only leave the Golgi via the lumenal surface of transport vesicles (Fig. 1). This has been confirmed for the complex glycosphingolipid GM3 (sialyl-LacCer (23)), SM (7), and for the yeast inositol sphingolipids (24). The enrichment of complex glycosphingolipids and SM in the exoplasmic leaflet of the apical plasma membrane of epithelial cells (7) as compared with the basolateral surface (Table I) has led to the proposal that these sphingolipids self-aggregate at the site of budding of apical transport vesicles in the TGN (25). Basolateral vesicles would have lower sphingolipid levels but the same high concentration of cholesterol. Sphingolipid and cholesterol concentrations are low in the ER, implying that retrograde transport vesicles are devoid of

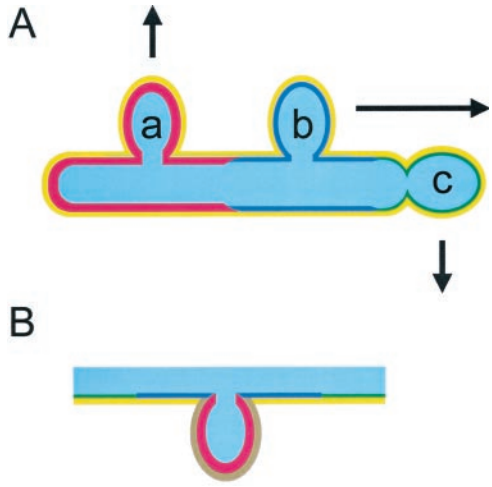

FIG. 2. **Lateral segregation of lipids into microdomains.** *A*, the Golgi complex of epithelial cells buds vesicles with at least three different lipid compositions: an apical composition, characterized by high levels of complex glycosphingolipids, SM, and cholesterol (*a*), a basolateral composition, having a high content of cholesterol (*b*), and an ER composition, with a low concentration of sphingolipids and cholesterol and a high concentration of unsaturated glycerophospholipids (34) (*c*). The three phases, displaying different thicknesses, must be recognized by the respective budding machineries in the cytosol, probably via membrane-spanning proteins. The segregation into three phases may occur in one single Golgi cisterna. *B*, caveolae. In an environment of glycerophospholipids (*green*), sphingolipid/cholesterol domains enriched in GPI proteins (*blue*) may contain subdomains enriched in GM1 (*red*; Ref. 39), with lipid domains enriched in caveolin and dually acylated kinases (*brown*) oriented toward the cytosol.

sphingolipids and cholesterol (22). This has been experimentally confirmed (26). These data led to the simple model for sphingolipid sorting of Fig. 2*A*. Sphingolipid rafts are thought to occur in the early Golgi (27), possibly even in the ER (28).

Sphingolipid/Cholesterol Rafts

A large body of evidence supports the notion that the lipids of eukaryotic plasma membranes display a heterogeneous lateral distribution. Biophysical studies on model membranes have firmly established the principles by which mixtures of sphingolipids, unsaturated glycerophospholipids, and cholesterol can segregate into two fluid phases, where the sphingolipids and part of the cholesterol segregate into a "liquid-ordered" domain from the unsaturated lipids in a "liquid-disordered" phase. At the same time, these studies have delimited the applicability of detergents in the cold to isolate the domains as detergent-insoluble remnants that float in sucrose gradients (3). A number of questions concerning the structural characteristics of the liquid-ordered domains remain to be solved.

(*a*) What percentage of the cell surface is occupied by rafts? The diameter of sphingolipid/cholesterol rafts on the outer surface of the plasma membrane has been estimated by a number of approaches to be small (tens to hundreds of nm) compared with that of cells (tens of μm) and to occupy some 10% of the cell surface (29, 30). In contrast, sphingolipids constitute 20–50% of the polar lipids of the plasma membrane (Table I) where they are concentrated in the outer bilayer leaflet. Thus, they completely cover the apical surface of epithelial cells, whereas the relative occupancy will be close to 40% in non-epithelial cells. In support of the latter, roughly one-half of the plasma membrane resisted extraction by cold detergent (31, 32). In a monolayer consisting of apical membrane lipids from kidney, only 50% was covered by liquid-ordered rafts whereas the outer leaflet of the apical membrane would consist exclusively of sphingolipids (33).

(*b*) However, in the experimental monolayer the lipids of the outer and inner leaflets of the plasma membrane mixed, and the domain properties of the lipids of the cytosolic leaflet are unknown. From the fact that dually acylated proteins colocalize with the sphingolipid/cholesterol domains as measured by various techniques, it is assumed that liquid-ordered rafts exist in the cytosolic leaflet of the plasma membrane as well. Of the phosphatidylserine, confined to the cytosolic leaflet by the aminophospholipid translocase, 70% may be disaturated (34), whereas in yeast PI contained

some disaturated species (35). These lipids could thus form the basis for a liquid-ordered phase. In pure lipid membranes, rafts on one side of the membrane perfectly match rafts in the opposite leaflet (36). However, from the low concentration of raft-lipids in the cytosolic leaflet it is unlikely that cytosolic rafts fully complement rafts in the outer leaflet of the plasma membrane.

(*c*) If the rafts measured by biophysical techniques are different from the rafts as defined by detergent insolubility (see question *a*), does this imply that different types of raft exist within a single membrane? Indeed, studies locating the gangliosides GM1, GM3, and GD3, various proteins with a glycosylphosphatidylinositol (GPI) anchor, and caveolin have clearly established that different liquid-ordered domains co-exist on the cell surface (see Refs. 37 and 38). Small ganglioside-rich microdomains can exist within larger ordered domains in both natural and model membranes (39, 40). Caveolae are examples of such "super" rafts being coupled to cytosolic rafts as defined by the acylated kinases (Fig. 2*B*). Cytosolic rafts may colocalize with each type of domain in the outer leaflet or with only one of the various types of domains. Coupling may involve caveolin or membrane-spanning proteins or may depend on phase-coupling between the opposed lipid domains.

(*d*) By what mechanisms do membrane proteins locate to domains? One determinant may be a long transmembrane domain that would fit the thicker raft (41, 42). Membranes in cells occur in at least three thicknesses (Fig. 2*A*). The ER has the thickness of a pure phospholipid bilayer (hydrophobic thickness of some 3.5 nm), the liquid-disordered phase of the plasma membrane displays the thickness of phospholipid plus cholesterol (4 nm), and the sphingolipid rafts may be 4.5–5.5 nm thick (42). Because the thickness of a raft depends on whether it is matched by a cytosolic raft and on the length of the amide-linked fatty acids, the various types of raft may display a distinct thickness and recruit unique sets of proteins. The mechanism of raft association of proteins with multiple transmembrane domains and of protein-protein complexes is more difficult to understand. A GPI anchor targets proteins to specific rafts. The mechanism is not clear. They can be displaced from rafts by gangliosides (see Ref. 33). A reduction of mobility by for example binding of a multivalent ligand also stimulates raft association. Acylation is a signal for raft localization on the cytosolic side (43).

(*e*) What are the physical properties that determine the affinity of a certain lipid for rafts? Although this question has been answered for model membranes of simple lipid compositions (3), biomembranes contain mixtures of 50–100 lipid species and various types of rafts. This means that many aspects of lipid-lipid immiscibility in these membranes remain to be resolved. Fluorescent reporter molecules have been helpful in spotting lipid sorting events. However, mostly it is not clear to what extent such a molecule mimics a natural lipid.

Uptake into the Cell

Plasma membrane sphingolipids are continuously taken up into the cell via the membrane flux of endocytosis. In addition, lipids on the cytosolic surface may transfer to other membranes as monomers.

Non-vesicular Uptake from the Plasma Membrane—Most sphingolipids in the exoplasmic leaflet of the plasma membrane bilayer have no access to the cytosolic side under resting conditions. One exception is sphingosine. When added exogenously to cells or when produced in lysosomes, it spontaneously translocates to the cytosolic surface and equilibrates with intracellular membranes. It has been suggested that during cell stimulation, SM may translocate via a scramblase protein to the cytosolic surface where it is then hydrolyzed to ceramide by a neutral sphingomyelinase (see Refs. 44 and 45). It is not fully clear how this ceramide reaches sites where it is reutilized for synthesis of SM and GlcCer. Surprisingly, a Golgi protein with lipid transfer specificity for SM strongly stimulated SM resynthesis (46). Ceramide appears unable to leave the lumen of the lysosome (47), possibly due to its inability to leave the internal membranes where it is produced. Exogenous sphingosine 1-phosphate, which binds to specific cell surface receptors, apparently translocates to the cytosolic surface via the ABC transporter CFTR, the cystic fibrosis transmembrane conductance regulator (48). In addition, galactosylsphingosine and glucosylsphingosine, when added to cells, are acylated probably after translocation toward the cytosolic surface. After translocation, lysosphingolipids

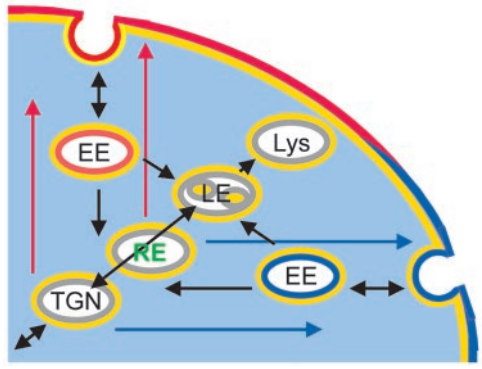

FIG. 3. **Endocytotic recycling of sphingolipids.** Sphingolipids can be endocytosed via clathrin-dependent and -independent pathways. From early endosomes (*EE*) they are recycled to the plasma membrane or shuttled to the recycling endosome (*RE*), the late endosome (*LE*), or the TGN. Endosomes and Golgi are connected via a bidirectional vesicular route. In epithelial cells, one leg of the system is connected to the apical and one to the basolateral surface.

can freely move through the cell due to their high off-rate from membranes, whereas the resulting GalCer and GlcCer may fulfill functions on the cytosolic surface (49).

Only in one study of many, an exogenous GlcCer (analog) was reported to flip toward the cytosolic surface of the plasma membrane (see Refs. 7 and 50). It is not clear whether complex glycosphingolipids ever reach the cytosolic surface of the plasma membrane, nor is it clear what would be their fate. Interestingly, specific interactions of glycosphingolipids have been reported with cytosolic proteins like calmodulin (51). In addition, if gangliosides reach mitochondria during signaling events (52), they must first have reached a cytosolic surface (Fig. 1).

Endocytosis—Like other lipids, sphingolipids follow the bulk membrane flow through the exocytotic and endocytotic vesicular transport pathways. From studies on the transport of (mainly) membrane proteins a complex pattern of pathways and compartments has been identified (Fig. 3). Sphingolipids have been shown to pass through each of these compartments. High concentrations of complex glycosphingolipids have been observed in the internal membranes of late endosomes (53, 54), most likely the site of their degradation. On the other hand, studies on the transport and Golgi glycosylation of exogenous glycosphingolipid (analogs) have established that most sphingolipids recycle from the early (sorting) endosomes, the late endosomes, and the recycling endosomes to the plasma membrane. At the same time, a fraction of the complex sphingolipids, but particularly GlcCer, reaches the Golgi complex (7). The latter is also true for the glycolipid-binding toxins like cholera and Shiga toxin and *Escherichia coli* verotoxin. From the Golgi, the toxin-glycolipid complexes follow the retrograde pathway all the way to the ER, where the active subunit is translocated across the membrane into the cytosol (*e.g.* Ref. 55). Also in the absence of toxin, a small fraction of the complex glycosphingolipids reaches the ER (53).

Although ample evidence supports lipid sorting by domains in the various endocytotic organelles, most of this evidence is derived from using lipid analogs, toxins, antibodies and virus bound to glycosphingolipids, and GPI-proteins as raft markers. The quantitative behavior of the natural lipids and the size of the various pathways remain to be established. Analogs of LacCer and globoside were endocytosed by a clathrin-independent subclass of the vesicles that took up SM, indicating sorting at the plasma membrane (56). The two pathways led to different classes of early endosomes, both of which had a connection to the Golgi. Both clathrin-dependent and -independent pathways are followed by glycolipid-bound toxins (*e.g.* Refs. 55 and 56). The clathrin-independent pathway of toxin transport has been suggested to provide very efficient access to the Golgi for GPI-proteins (55) and might be a raft pathway. Interestingly, a rise in the cellular cholesterol concentration misrouted LacCer to the lysosomes. The latter situation was also encountered in a number of sphingolipid storage diseases (see Ref. 56). Either high cholesterol levels abolished the clathrin-independent pathway to the Golgi, or alternatively, high

cholesterol affected the partitioning of the LacCer analog into the proper membrane domain. The biophysical basis for domain-mediated sorting in the endosomes, notably the interaction between the various domains and coat proteins, remains to be established.

Endocytotic lipid sorting is particularly interesting in epithelial cells, as these display a transcellular vesicular pathway but at the same time need to maintain the difference in apical and basolateral lipid composition. In initial studies in Madin-Darby canine kidney cells no specificity was observed in the transcytosis of SM and GlcCer analogs, which allowed for their use as bulk membrane markers (7). However, sorting between these analogs was observed in hepatocyte-derived HepG2 cells. It was concluded that the lipids are sorted by lateral segregation in an apical endosome, termed the "subapical compartment" (see Ref. 57) or "apical recycling compartment" (58), which functionally resembles the recycling endosome in non-epithelial cells. Under normal conditions in fully polarized hepatocytes, a GlcCer analog was recycled to the bile canalicular surface and SM to the basolateral surface (see Ref. 57), and evidence was provided for transient activation during polarity development of a pathway for SM to the bile canalicular surface by protein kinase A (59). Transcytosis of GalCer from the apical to the basolateral membrane of enterocytes has been held responsible for allowing the passage of human immunodeficiency virus across the intestinal epithelium (see Ref. 60).

Perspectives

The exciting developments in the fields of sphingolipid-mediated signal transduction and sphingolipid-mediated protein sorting have led to a tremendous activity in the studies of sphingolipid organization, especially the structural role of sphingolipids in membrane rafts. It is now being realized that such rafts exist in most cellular membranes. To fully grasp raft function, it will be necessary to identify and characterize the different types of raft, to follow their fate in time, and to understand the role of the various sphingolipids in their structure. Although one important challenge will be to unravel the biophysical complexity of lipid mixtures, it will be most important to define the interactions between sphingolipids and proteins. These are proteins involved in signaling but also proteins involved in vesicular transport. Except for structural functions, sphingolipids serve regulatory functions in their own right. Because sphingolipid functions are governed by the enzymes that make, break, and transport the sphingolipids, another major challenge will be to identify these enzymes and establish how their activity is regulated in the living cell.

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