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Review

Sphingolipid management by an orchestra of lipid transfer proteins

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Abstract

The various membranes in eukaryotic cells have unique lipid compositions. Despite important discoveries in lipid research over recent decades, the basic principles by which cells define their membrane compositions are essentially unknown. Cells must sense the concentration of each lipid, integrate such signals and regulate the activity of their metabolic enzymes and transport routes to dynamically meet their needs in terms of membrane composition. Sphingolipids constitute a lipid category that is essential for eukaryotic life and appears to be key to differences in lipid composition. Here we discuss recent findings that assign an important role to lipid transfer proteins in the regulation of sphingolipid metabolism, organization and function.

Keywords: contact site; flippase; glycosphingolipids; lipid raft; sphingolipid sensor.

Introduction

The past decades have witnessed the identification of most eukaryotic enzymes and proteins involved in lipid synthesis, degradation and transport. However, the question as to how eukaryotic cells sense and control the lipid composition of their membranes still remains. To clarify this, it is necessary to identify the sensors and determine how they connect to – and regulate – metabolic enzymes. In addition, the enzymes need to be localized and, finally, we must understand how cells use and regulate lipid transport machineries to ensure specificity in lipid transport between cellular organelles (van Meer, 2005).

Sphingolipids comprise a small but vital fraction of membrane lipids in eukaryotes. They arose parallel to the appearance of eukaryotic life and are conserved from yeast to mammals, in which expression of phosphosphingolipids is indispensable for cell growth and survival (Wells and Lester, 1983; Hanada et al., 1992; Geta Tafesse et al., 2007). In contrast, glycosphingolipids are not required for the survival of individual cells (Ichikawa et al., 1994) but are essential for the development of multicellular organisms and the differentiation of distinct cell types. Mice deficient in glucosylceramide synthase, the first enzyme in complex glycolipid synthesis, die at an early embryonic stage (Yamashita et al., 1999) and mice deficient in galactosylceramide synthase show severe defects in brain function and in the male reproductive system (Bosio et al., 1996b; Coetzee et al., 1996a, 1998), emphasizing the role of galactolipids in myelination and spermatogenesis. Complex glycosphingolipids have been implicated in cell-cell contact and signaling events (Lisanti et al., 1994; Hakomori and Igarashi, 1995; Hakomori et al., 1998). Furthermore, glycolipids are highly enriched on the apical side of intestinal epithelial cells, where they protect the cell against the harsh chemical environment and the action of lipases (Simons and van Meer, 1988). Individual sphingolipids are known to be signaling molecules and are important mediators of survival, stress response and apoptosis (Hannun and Obeid, 2008). Both the expression of sphingolipids and their degradation are important. This is reflected in the incidence of sphingolipid storage disorders that are often accompanied by severe phenotypes (Futerman and van Meer, 2004; Jeyakumar et al., 2005; Sabourdy et al., 2008).

The functions of sphingolipids are largely related to the enormous structural variability of their headgroups, which allows a nearly unlimited number of specific interactions with glycoproteins and other glycosphingolipids. On the other hand, sphingolipids possess unique organization potential owing to their high affinity for each other. Because of their saturated nature and the possibility for hydrogen bonding, together with cholesterol they can form microdomains within a glycerophospholipid environment that are more ordered and rigid and thicken the membrane. Some proteins preferentially insert into these membrane domains, called lipid rafts, resulting in distinct specialized membrane environments. Therefore, lipid rafts can be utilized to sort proteins during vesicular transport or as platforms to recruit proteins, for example, in signaling (Grassme et al., 2007; Sengupta et al., 2007; van Meer et al., 2008).

Notwithstanding our increased insights into sphingolipid structure and function, the individual processes in which sphingolipids are involved and the mechanisms by which they affect cellular functions are poorly understood. The function of a lipid depends on its local concentration with time by which it can contribute to a defined membrane environment or directly interact with protein binding partners. To unravel the role of the many different sphingolipids, it is important to study their localization within the cell. As direct observation of sphingolipids *in vivo* is difficult owing to a lack of biosensors (Hoetzl et al., 2007), we must rely on information obtained from studies of their metabolism and transport.

Here we discuss recent advances in lipid research with respect to sphingolipid metabolism in terms of the enzymes involved in formation and transport. In particular, we highlight how cells orchestrate lipid transfer proteins in the creation of unique membrane lipid compositions and discuss mechanisms of lipid sensing.

Enzymes of sphingolipid synthesis

Sphingolipid synthesis is organized along the secretory pathway, indicating the functional significance of sphingolipids for membrane and protein transport. Reactions underlying the formation of sphingolipids have been known for many years and to date almost all enzymes of sphingolipid synthesis in mammals have been cloned, although only the yeast homologs are known for some (Figure 1; Dickson, 2008). Less is known about the overall regulation of sphingolipid metabolism, partly because of the lack of a grand scheme for the physiological functions of various sphingolipids; it remains unclear which lipid concentration has to be regulated, at what location and under which conditions.

Ceramide synthesis

Ceramide is the precursor for complex sphingolipids and its synthesis is localized to the cytosolic leaflet of the endoplasmic reticulum (ER), whereas most sphingolipid species are synthesized in the Golgi. Ceramide formation is divided into four steps and starts with the condensation of L-serine and palmitoyl-CoA to 3-ketosphinganine. This reaction is catalyzed by serine palmitoyl transferase (SPT). Three subunits of SPT have been identified in eukaryotes (Buede et al., 1991; Nagiec et al., 1994; Weiss and Stoffel, 1997; Hornemann et al., 2006). They are present in an oligomeric complex (Hornemann et al., 2007). It is not clear to what extent SPT3 contributes to *de novo* synthesis of 3-ketosphinganine, as it is differentially expressed in tissues (Hornemann et al., 2006). 3-Ketosphinganine reductase forms sphinganine (3KSR, Beeler et al., 1998), which is acylated by (dihydro)ceramide synthase (CerS, known as longevity assur-

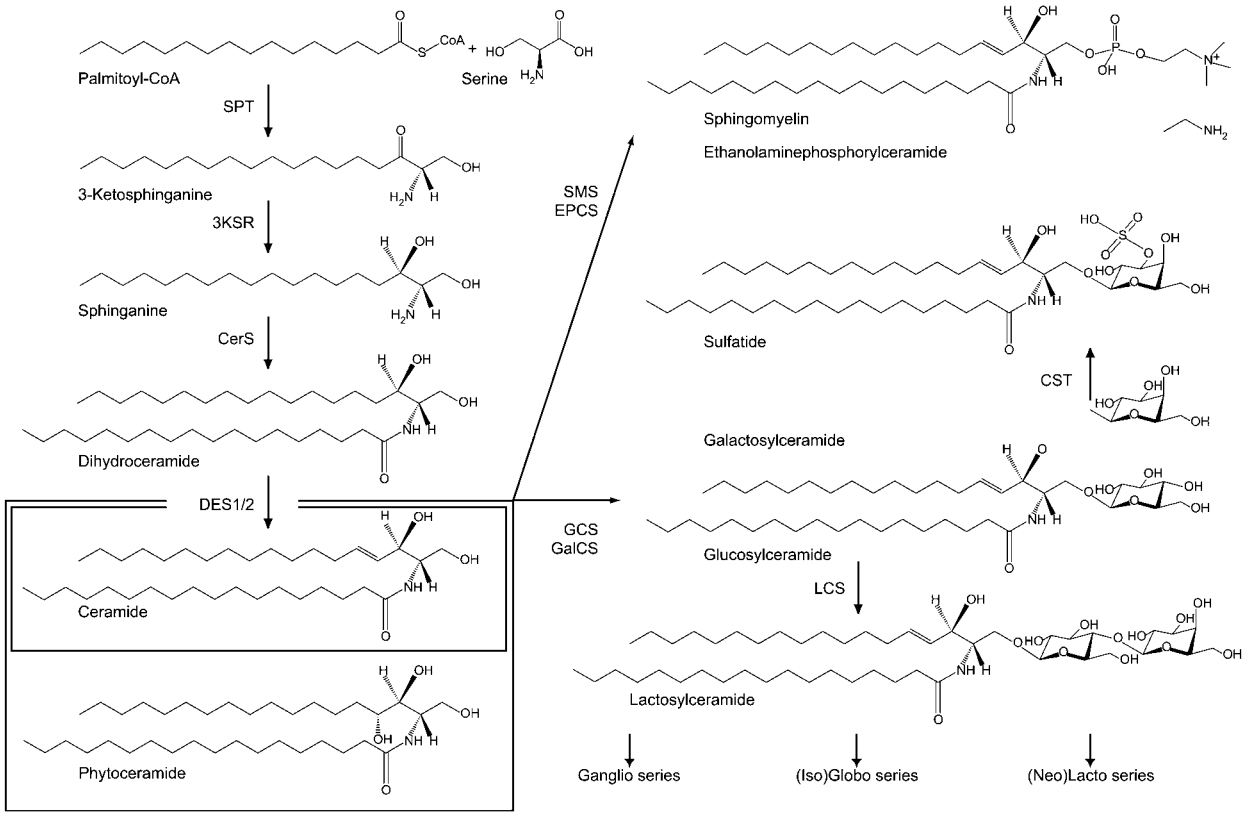

Figure 1 Mammalian sphingolipid synthesis.
The committed step in sphingolipid synthesis is the condensation of serine with palmitoyl-CoA to 3-ketosphinganine, which is reduced to sphinganine and acylated to dihydroceramide. Six (dihydro)ceramide synthase genes in man and mouse prefer acyl-CoAs of different chain lengths. Usually, dihydroceramide is converted to ceramide by introducing a double bond at the sphinganine C4. In some tissues dihydroceramide is hydroxylated instead to form phytoceramide, which is important in skin but is also found in brain, intestine and kidney. Ceramides can contain hydroxylated fatty acids, mainly in myelin galactosylceramide species. Ceramide is converted to glycolipids, sphingomyelin or the less abundant ethanolamine phosphorylceramide. Galactosylceramide, mainly found in myelin and epithelial cells of the kidney and intestine, can be sulfated to sulfatide. Glucosylceramide is (partially) converted to lactosylceramide, which is generally only found as an intermediate and further glycosylated. For further information on lipid structures or species, visit www.lipidmaps.org or www.sphingomap.org. SPT, serine palmitoyl transferase; 3KSR, 3-ketosphinganine reductase; CerS, (dihydro)ceramide synthase; GCS, glucosylceramide synthase; DES1/2, desaturase/hydroxylase; GalCS, galactosylceramide synthase; SMS, sphingomyelin synthase; EPCS, ethanolamine phosphorylceramide synthase; CST, cerebroside sulfotransferase; LCS, lactosylceramide synthase.

ance gene, LASS; D'mello et al., 1994; Guillas et al., 2001; Schorling et al., 2001). In man and mouse, six different genes for CerS exhibit specificity for fatty acid chain length (Pewzner-Jung et al., 2006). The introduction of a double bond at sphinganine C4,5 by dihydroceramide desaturase 1 (DES1, Ternes et al., 2002) finally yields ceramide containing sphingosine as the sphingoid backbone. Most ceramide species in mammals contain sphingosine, but in skin, brain, intestine and kidney the sphinganine in dihydroceramides can be hydroxylated at C4 (phytoceramide), a reaction catalyzed by the bifunctional desaturase/hydroxylase DES2 in mouse or Sur2 in yeast (Haak et al., 1997; Ternes et al., 2002). In fact, most ceramide may be directly synthesized from sphingosine derived from sphingolipid breakdown in lysosomes via the salvage pathway (Kitatani et al., 2008).

Ceramide is readily converted to glycolipids or sphingomyelin

In kidney and intestinal epithelial cells, as well as in myelinating cells, (part of the) ceramide is consumed in the lumen of the ER for the formation of galactosylceramide by galactosylceramide synthase (GalCS, Bosio et al., 1996a; Coetzee et al., 1996b). The galactose headgroup is transferred from UDP-galactose to ceramide. UDP-galactose is imported into the lumen of the ER by UDP-galactose transporter (UGT). This transporter is normally localized to the *trans* side of the Golgi and supplies substrates for the glycosylation of proteins and lipids. However, in cells expressing GalCS, UGT is retained in the ER via a direct protein-protein interaction (Sprong et al., 2003). At the cytosolic side of the Golgi, glucosylceramide is formed by glucosylceramide synthase (GCS, Ichikawa et al., 1996) through addition of the glucose headgroup from UDP-glucose to ceramide. Because of its relatively small headgroup, ceramide can flip into the lumenal leaflet of the Golgi membrane, where it is consumed by sphingomyelin synthase, which transfers the headgroup from phosphatidylcholine to form sphingomyelin (SMS1, Huitema et al., 2004). At the exoplasmic leaflet of the plasma membrane, a second sphingomyelin synthase is involved in the re-synthesis of sphingomyelin from ceramide formed at the cell surface (SMS2, Huitema et al., 2004). Both enzymes are required for cell growth and homeostasis. The sphingomyelin synthase-related enzyme (SMSr, Huitema et al., 2004) may form ethanolamine phosphorylceramide, a minor lipid in mammals.

Complex glycosphingolipids

The next glycosylation step in the formation of glycolipids is the transfer of galactose from UDP-galactose to glucosylceramide. This reaction occurs in the lumen of the Golgi and is facilitated by lactosylceramide synthase (LCS, Nomura et al., 1998). Further addition of headgroups results in complex glycosphingolipids of, for example, the lacto-, globo- or ganglioseries (for an overview of glycosphingolipids we refer the interested reader to http://sphingolab.biology.gatech.edu/). For the addition of sugar headgroups, the expression and localization of sugar transferases are important, as well as the abundance of the activated sugar precursor, which requires the presence of its specific transporter. Cells expressing GalCS sometimes synthesize one or two complex glycosphingolipids based on galactosylceramide. In addition, part of the galactosylceramide is sulfated to sulfatide (3-sulfogalactosylceramide) by cerebroside sulfotransferase (CST; Honke et al., 1997; Hirahara et al., 2000).

Generation of signaling sphingolipids

The sphingolipid species identified as signaling lipids are either formed via *de novo* pathways or by degradation (Figure 2). Under normal conditions, ceramide formed *de novo* is converted to more complex sphingolipids, but under stress conditions it can accumulate, which leads to apoptotic signaling (Obeid et al., 1993; Grassme et al., 2007). Alternatively, sphingomyelin can be degraded to ceramide by the action of acid (lumenal) or neutral (cytosolic surfaces) sphingomyelinases (Schuchman et al., 1991; Hofmann et al., 2000; Tani and Hannun, 2007; Zeidan and Hannun, 2007). Acid ceramidase in lysosomes (Koch et al., 1996) and neutral ceramidase at the plasma membrane (Hwang et al., 2005) deacylate ceramide to sphingosine. Phosphorylation of sphingosine and ceramide to sphingosine-1-phosphate and ceramide-1-phosphate by their cognate kinases (Stoffel et al., 1970; Kohama et al., 1998; Sugiura et al., 2002) turns them into anti-apoptotic mediators. Together, these lipids constitute the sphingostat, a metabolic regulatory system that integrates all sorts of exogenous and endogenous input signals and balances apoptosis and proliferation (Hannun and Obeid, 2008). The hydrolysis of sphingosine-1-phosphate to ethanolamine phosphate and palmitaldehyde by the lyase abrogates signaling, and is the final step in sphingolipid degradation (Saba et al., 1997).

Lipid transfer proteins in sphingolipid metabolism

The local concentration of a lipid depends on its synthesis and degradation, but lipids are not confined to the membranes where they are synthesized. Hence, lipid transport is an important parameter in defining a membrane environment. Because lipids are building blocks of membranes, there is a continuous bidirectional lipid flux between organelles via vesicular transport. However, eukaryotes contain various proteins in their cytosol that can solubilize lipids and transport them in a monomeric fashion across the aqueous phase. Although some of these lipid transfer proteins have been well characterized *in vitro,* their actual *in vivo* function remains largely unclear (Wirtz, 2006). The newest evidence suggests that facilitated monomeric exchange via lipid transfer proteins may occur only over short distances at so-called membrane contact sites, where it may be responsible for the rapid lipid exchange measured between the mitochondrial inner and outer membranes (Simbeni et al., 1990; Ardail et al., 1991) and between the mitochondrial outer membrane and the ER (Shiao et al., 1995; Achleitner et al., 1999), membranes that are not connected by vesicular transport.

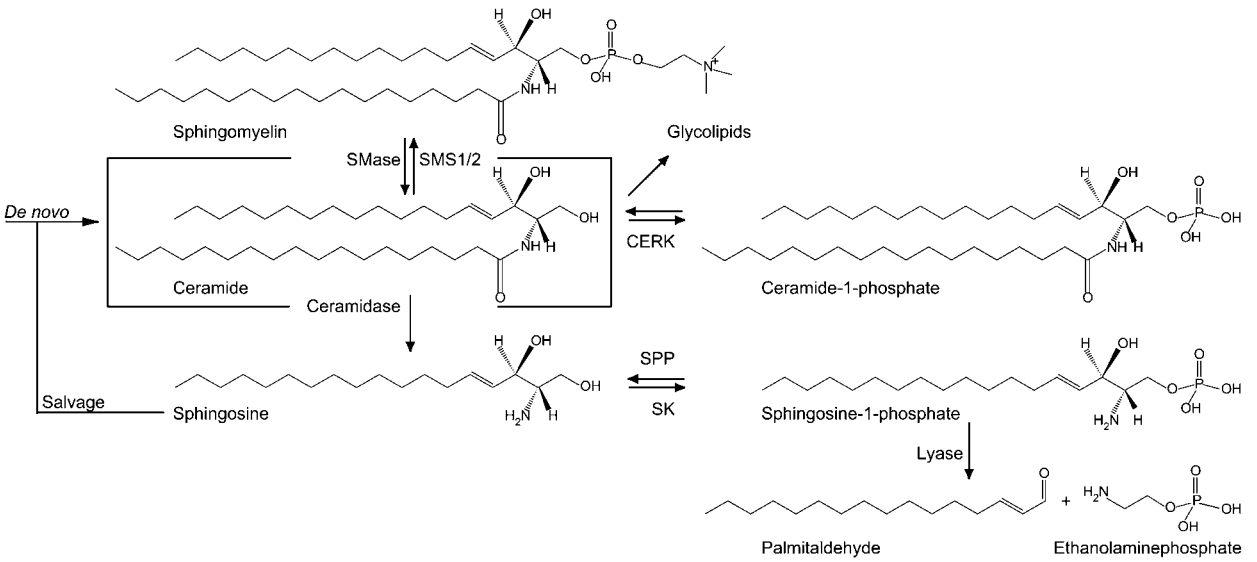

Figure 2 Generation of signaling sphingolipids.
Signaling pools of ceramide are either formed by *de novo* synthesis in the ER or through degradation of sphingomyelin at the cell surface, probably discriminating between different locations of ceramide signaling. Ceramide can be phosphorylated to ceramide-1-phosphate or degraded to sphingosine, which can be phosphorylated to sphingosine-1-phosphate or reused. SM deacylase, sphingomyelin deacylase; SMase, sphingomyelinase; CERK, ceramide kinase; SK, sphingosine kinase; SMS1/2, sphingomyelin synthase; SPP, sphingosine-1-phosphate phosphatase.

Finally, cell membranes are asymmetric, with one bilayer leaflet facing the cytosol and the opposite surface oriented towards the lumen of the organelle or the extracellular environment. Except for the ER membrane, which appears to have a symmetrical lipid distribution with rapid transbilayer equilibration of the common membrane lipids, the membranes of the secretory and endocytotic recycling routes have an asymmetric lipid distribution, with enrichment of the glycerophospholipids phosphatidylserine and phosphatidylethanolamine in the cytosolic and the sphingolipids and phosphatidylcholine in the non-cytosolic membrane leaflet (van Meer et al., 2008). The lipid asymmetry results from asymmetric synthesis and from a lack of free transbilayer movement for the common lipids in combination with active, unidirectional transport of specific lipids across the bilayer. In general, lipids containing small uncharged headgroups such as diacylglycerol, ceramide and cholesterol rapidly translocate spontaneously, whereas lipids with large hydrophilic headgroups need a protein machinery to move across. The best-characterized active translocation is that of phosphatidylserine and phosphatidylethanolamine towards the cytosolic surface by P4 P-type ATPases (Daleke, 2007). The effect on lipid asymmetry of translocation in the opposite direction by ABC transporters is unclear, because they seem to act as extruders rather than translocases (van Meer et al., 2006).

Sphingomyelin synthesis is regulated by the ceramide transfer protein CERT

Sphingolipid synthesis is compartmentalized in the ER and Golgi. Whereas ceramide synthesis occurs at the ER, subsequent sphingolipids – apart from galactosylceramide – are synthesized in the Golgi. Consequently, for conversion to sphingomyelin and glucosylceramide, ceramide must be transported from the ER to the Golgi. An obvious transport mechanism would be the vesicular secretory pathway, and this route appears to be followed by a ceramide pool that is converted to glucosylceramide. However, numerous studies have indicated the involvement of a non-vesicular pathway in sphingomyelin synthesis (Hanada et al., 2007), culminating in the discovery of the cytosolic ceramide transfer protein CERT (Hanada et al., 2003).

The identification of CERT as a cytosolic protein required for sphingomyelin synthesis (Hanada et al., 2003) provided a mechanistic insight into the vesicle-independent transport of ceramide from the ER to the Golgi. CERT is expressed as two splice variants, $CERT_L$ and the more frequently expressed CERT, which lacks an internal 26-aa sequence. Both restore the wild-type phenotype in CERT mutant cells. The lipid transfer activity lies in the steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain of CERT. Its structure explains the lipid specificity (Kudo et al., 2008): ceramide is harbored in a hydrophobic cavity too narrow for lipids with bulky head groups. Furthermore, only ceramides containing a fatty acid $\leq$C18 fit adequately; longer fatty acyl chains are protected less efficiently, in line with the preferred ceramide species transferred by CERT *in vitro* (Kumagai et al., 2005).

The activity of CERT is mainly regulated by its localization, which depends on three factors: phosphatidylinositol-4-phosphate [PI(4)P] binding at the *trans*-Golgi (Hanada et al., 2003), VAMP-associated protein (VAP) binding at the ER (Kawano et al., 2006) and phosphorylation (Fugmann et al., 2007; Kumagai et al., 2007). CERT binds to the Golgi via its PH-domain that recognizes PI(4)P, like some other lipid transfer proteins. Transfer activity can be blocked by the point mutation G67E in the PH domain, which inhibits binding to PI(4)P (Hanada et al., 2003), or by inhibiting PI 4-kinase IIIβ, the kinase

mainly responsible for PI(4)P synthesis at the Golgi (Tóth et al., 2006). The association between CERT and the ER is mediated by VAP binding via CERT's FFAT motif (two phenylalanines in an acidic tract; Kawano et al., 2006). VAPs may act as a protein interaction platform in the ER, and, in general, protein-protein interaction with VAPs can also occur in a FFAT-independent manner. The dual binding specificity of CERT may locate the complex to ER-*trans* Golgi membrane contact sites (Mogelsvang et al., 2004), which would greatly stimulate its transfer efficiency (Figure 3).

Several serines and threonines in CERT can be phosphorylated. The region between the PH domain and the START domain contains a serine repeat with the typical sequence SXX′ (Raya et al., 1999; Kumagai et al., 2007). This motif is recognized by casein kinase I when a phosphorylated pSer/pThr is already present. Serine 132, the first serine in the serine repeat, could serve as such a residue and indeed was found to be phosphorylated independently by protein kinase D (PKD, Fugmann et al., 2007). Phosphorylation impedes membrane binding, transfer activity (Kumagai et al., 2007) and sphingomyelin synthesis. In the phosphorylated form, the PH and START domains of CERT bind to each other to yield an inactive conformation. It was shown that dephosphorylation of CERT is mediated by protein phosphatase 2Cε (PP2C, Saito et al., 2007) and restores membrane binding. Interestingly, PP2Cε is also a binding partner for VAP-A. Phosphorylation of CERT by PKD may serve as a type of feedback control for vesicular transport; PKD is recruited by diacylglycerol to the Golgi, where it is phosphorylated and activated by protein kinase Cη (PKC, Diaz Anel and Malhotra, 2005) and promotes vesicular transport (Baron and Malhotra, 2002). PKD phosphorylates and activates PI 4-kinase IIIβ to form PI(4)P (Hausser et al., 2005) and thereby recruits CERT to the Golgi (Tóth et al., 2006). Diacylglycerol formation via sphingomyelin synthesis then recruits more PKD in a positive feedback loop. At the same time, PKD phosphorylates CERT, which turns off lipid transfer, diacylglycerol production and PKD recruitment. CERT is one of the 15 members of the START domain family of proteins, including lipid transfer proteins for cholesterol (StAR and MLN64), phosphatidylcholine (PCTP), and phosphatidylcholine and phosphatidylethanolamine (StarD10), all of which are regulated by phosphorylation (Soccio and Breslow, 2003; see Fugmann et al., 2007).

The localization of CERT and sphingomyelin synthase to the *trans*-Golgi (Halter et al., 2007; Hanada et al., 2007) may explain the preferential use of ceramides transferred by CERT for sphingomyelin synthesis. It is less clear to what extent glycolipid synthesis depends on CERT (Hanada et al., 2003; Perry and Ridgway, 2006; Halter et al., 2007). It probably depends on whether the glucosylceramide synthase is located on the *cis*- (Futerman and Pagano, 1991) or *trans*-side of the Golgi (Halter et al., 2007), which may be cell-type-specific. The contribution of the CERT pathway may also be tissue-specific, because CERT is differentially expressed (Raya et al., 1999, 2000). CERT may be required for the formation of ceramide-1-phosphate at the Golgi (Lamour et al., 2007; Boath et al., 2008).

Complex glycosphingolipid synthesis requires transmembrane translocation of glucosylceramide

The higher glycosphingolipids are synthesized in the Golgi lumen by the stepwise addition of sugars to glucosylceramide, which itself is synthesized at the cytosolic face of the Golgi (Ichikawa et al., 1996). Thus, both the activated sugars and glucosylceramide must be translocated towards the Golgi lumen. Because of the relatively large hydrophilic headgroup of glucosylceramide, translocation across the membrane is most likely protein-mediated. ABC transporters that translocate fluorescent glucosylceramide (van Helvoort et al., 1996) were proposed as candidate flippases (De Rosa et al., 2004), but natural glucosylceramide did not appear to be a substrate (Halter et al., 2007). Moreover, as stated above, ABC transporters with lipid substrates function as exporters rather than *bona fide* flippases (van Meer et al., 2006). Although glucosylceramide might translocate across the Golgi membrane directly, we have presented evidence that glucosylceramide flips across the ER membrane instead, and that the majority of the lipid flipped in the ER is directed to complex glycosphingolipid synthesis (Halter et al., 2007). Glucosylceramide flip across the ER is a tempting hypothesis, because conditions at the ER membrane facilitate lipid flip-flop. The ER membrane contains unsaturated lipids and has low amounts of sphingolipids and cholesterol, resulting in high membrane fluidity. Moreover, the ER is the location of membrane protein translocation, leading to a high concentration of transmembrane domains. In addition, signal peptides cleaved from secretory or membrane proteins reside in the membrane (Lyko et al., 1995) and may facilitate lipid flip (Boujaoude et al., 2001). Unexpectedly, glucosylceramide was also found to translocate across post-Golgi membranes by a mechanism that depended on the activity of the proton ATPase. However, this process did not contribute to higher glycosphingolipid synthesis (Halter et al., 2007) and must serve some other function.

Synthesis of higher glycosphingolipids is also regulated by lipid transfer proteins

Important information on the route of newly synthesized glucosylceramide from the cytosolic side of the Golgi to the site of complex glycosphingolipid synthesis in the Golgi lumen came from the finding that this process depends on the cytosolic protein FAPP2 (D'Angelo et al., 2007; Halter et al., 2007). FAPP2 (four phosphate adaptor protein 2, Godi et al., 2004) has a domain with homology (36% identity, 56% similarity) to the cytosolic glycolipid transfer protein GLTP (Abe et al., 1982; Metz and Radin, 1982). Crystal structure results (Malinina et al., 2004) showed that, compared to the START domain found in CERT and its family members, the GLTP domain harbors the lipid in a rather open 'sandwich' conformation that may allow potential protein-binding partners of glycosylceramide to bind to the GLTP-lipid complex. Both GLTP and FAPP2 transport a variety of glycolipids *in vitro* (Brown and Mattjus, 2007; D'Angelo et al., 2007). *In vivo*, glucosylceramide, synthesized on the cytosolic surface of the Golgi, and galactosylceramide, synthesized in the

ER lumen but having free access to the cytosolic surface of the ER (Burger et al., 1996), are likely substrates. The proteins should not have access to complex glycosphingolipids, which reside in the non-cytosolic leaflet of the Golgi and endocytotic membranes.

Via its PH domain with specificity for PI(4)P, FAPP2 binds to the *trans*-Golgi. Binding also involves ARF1 (Godi et al., 2004). The decrease in conversion of newly synthesized glucosylceramide to complex glycolipid upon FAPP2 knockdown implies FAPP2 involvement in glucosylceramide transport from its site of synthesis to its site of translocation (D'Angelo et al., 2007; Halter et al., 2007). Whereas D'Angelo et al. (2007) favored glucosylceramide synthesis at the *cis*-Golgi, FAPP2-mediated transport to the *trans*-Golgi and translocation across a *trans*-Golgi membrane, our evidence suggested glucosylceramide synthesis in the *trans*-Golgi followed by FAPP2-mediated retrograde transport to, and translocation across, the ER (Halter et al., 2007); further experiments are required to confirm this. FAPP2 activity may be regulated by phosphorylation. It contains several serine and threonine residues that could serve as phosphorylation sites.

Originally, FAPP2 (and FAPP1) was found to be important for vesicular transport from the Golgi to the plasma membrane (Godi et al., 2004; Vieira et al., 2005; D'Angelo et al., 2007). We observed no effect of FAPP2 knockdown on protein secretion or on transport of the complex glycosphingolipid GM3 to the surface of melanocytes (Halter et al., 2007). In addition, the lack of glycosphingolipids in melanocyte mutant cells did not affect protein transport to the cell surface (Ostermeyer et al., 1999). How FAPP2 is involved in protein transport to the (apical) plasma membrane of certain cells remains to be clarified.

Since reasonable amounts of PI(4)P are also found at the plasma membrane (Di Paolo and De Camilli, 2006), FAPP2 might be involved in the reported glucosylceramide transport to this location (Warnock et al., 1994). Indeed, FAPP2 knockdown decreased the transport of newly synthesized glucosylceramide to the cell surface. GLTP knockdown also had a partial effect on this transport (Halter et al., 2007). In contrast, GLTP knockdown did not affect complex glycosphingolipid synthesis (Tuuf and Mattjus, 2007). Genome analysis revealed a second GLTP gene in humans, but it is not transcriptionally active (Zou et al., 2008). In fungi and plants, GLTP homologs have been implicated in apoptosis (Saupe et al., 1994; Brodersen et al., 2002). However, the function of GLTP in mammalian cells still remains to be explored.

Vesicular transport of sphingolipids

After synthesis in the Golgi lumen, sphingomyelin and complex glycosphingolipids do not have access to the cytosolic leaflet and therefore can be transported exclusively via vesicular transport. As sphingolipids and cholesterol are enriched at the plasma membrane, they must be preferably transported by anterograde routes (van Meer, 1989). Indeed, they were found to be largely excluded from retrograde carriers (Brügger et al., 2000). In epithelial cells, transport of sphingolipids is specialized because of the presence of two distinct plasma membrane compartments. In these cells, glycosphingolipids are enriched at the apical side, whereas the basolateral side has a normal plasma membrane composition (van Meer and Simons, 1988). How glycosphingolipids are sorted in apical transport routes is poorly understood, but it must involve their lateral enrichment at the budding site of the apical precursor vesicles. Their large capacity for hydrogen bonding may induce their segregation from other membrane lipids in the lumenal leaflet of the Golgi membrane (van Meer and Simons, 1988). Such enrichment has been observed for lactosylceramide during recycling through the lumen of endosomes (Sharma et al., 2003), and may also occur during the budding of vesicles (or viruses) away from the cytosol into the lumen of late endosomes (Wubbolts et al., 2003; Trajkovic et al., 2008) or into the extracellular medium. There is accumulated evidence that lateral segregation on short time and length scales is a property of the plasma membrane surface, and that small domains or rafts can coalesce when they are stabilized by physical parameters such as curvature or membrane stress or by a change in lipid composition via sphingomyelin hydrolysis and ceramide production (van Meer et al., 2008).

Sphingolipid transfer proteins in the endo-lysosomal lumen

Proteins with lipid transfer activity *in vitro* have also been purified from the lumen of endo/lysosomes. In general, these proteins are involved in presenting glycosphingolipids to degradative soluble enzymes (Kolter and Sandhoff, 2005). These saposins are apparently most efficient in mobilizing glycosphingolipids from the intralumenal vesicles in multivesicular endosomes (Locatelli-Hoops et al., 2006). A different function of saposins in the endo/lysosomal lumen appears to be the insertion of glycolipids into the antigen-binding groove of antigen-presenting CD1b and -1d proteins (Winau et al., 2004; Yuan et al., 2007). A soluble CD1 molecule, CD1e, is a dedicated glycolipid transfer protein involved in loading antigenic lipids onto CD1b (Tourne et al., 2008). It has been reported that the cholesterol transporter Niemann-Pick type C2 protein (Liou et al., 2006; Babalola et al., 2007) transports a glycolipid (Schrantz et al., 2007), although this could not be confirmed (Konrad Sandhoff, Bonn, personal communication).

Regulation of local lipid composition

To create a specific lipid environment, lipid metabolism and transport must be coordinated, but the basic layout of how the cell orchestrates the activity of metabolic enzymes is not known. Many reports in the literature connect specific tissues, conditions, signaling pathways and molecules to the occurrence and (local) concentration of a specific sphingolipid, and results reflect a common complex regulation at the transcriptional and post-transcriptional levels of the enzymes of sphingolipid metabolism.

ER-Golgi crosstalk

A special feature of the sphingolipid system is the control of substrate supply via lipid transfer proteins, one of

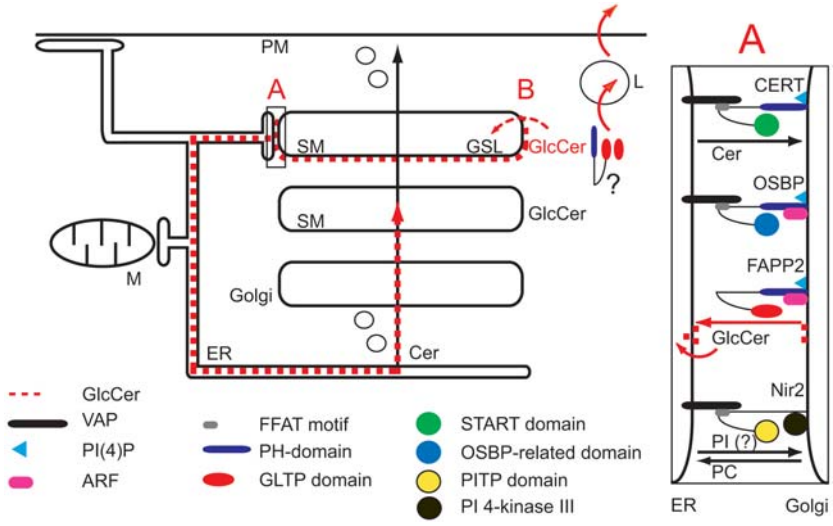

Figure 3 Integration of sphingolipid transport at ER-Golgi contact sites.
Newly synthesized ceramide (Cer) is transported from the ER to the Golgi by CERT or vesicles for sphingomyelin (SM) and glucosylceramide (GlcCer) formation. GlcCer must translocate into the lumen, where it is converted to complex glycosphingolipids (GSL). (A) One major pathway is GlcCer transport to the ER by the lipid transfer protein FAPP2. There it translocates and is transported by vesicles to the Golgi, where it is converted to lactosylceramide and GM3 (Halter et al., 2007). One prediction of this model is that GlcCer is concentrated in the anterograde vesicle pathway from the ER, which may exclude phosphatidylinositol (PI) and, in the Golgi, ceramide. (B) Translocation directly at the Golgi has also been proposed (D'Angelo et al., 2007), but occurred less efficiently than at ER membranes *in vitro* (Halter et al., 2007). GlcCer can also be transported through the cytosol, perhaps by FAPP2 or GLTP, to the plasma membrane (PM) or lysosomes (L), where it translocates to the lumenal side. Cytosolic transfer proteins have no access to SM and GSL in the lumen. Many lipid transfer proteins share the START domain for lipid transfer activity, whereas others have the OSBP-related domain. Glycolipid transfer proteins have a GLTP domain, which displays a novel folding motif. Transport by lipid transfer proteins may occur efficiently at membrane contact sites. The lipid transfer proteins CERT and OSBP bind to two organelles, the ER in a VAP-dependent manner through a FFAT motif and the Golgi via the PH domain for PI(4)P, which is enriched in the *trans*-Golgi. PI(4)P levels at the Golgi are regulated by the PI/PC exchange protein Nir2, which binds to the ER via its FFAT motif. FAPP2 binds to the Golgi via its PH domain and transports GlcCer to the ER. OSBP and FAPP2 also interact with the small GTPase ARF on the Golgi. Together, these characteristics interconnect the metabolism of phosphoglycerolipids, sphingolipids and cholesterol and link these to vesicular transport.

which (CERT) works between the ER and Golgi and may be present at contact sites between these organelles (Figure 3). It attaches via its PH domain to the *trans*-Golgi via a molecule that is itself highly regulated, PI(4)P. On the ER side, CERT binds to VAPs in the ER via an FFAT motif. In addition, CERT is regulated via phosphorylation by different types of kinases. Surprisingly, OSBP, a lipid transfer protein of a different family, the 12-member ORP family (Perry and Ridgway, 2006), shares with CERT its binding to PI(4)P on the *trans*-Golgi, its regulatory phosphorylation and its FFAT-domain-mediated binding to the ER. In addition, phosphatidylinositol/phosphatidylcholine-binding/transfer protein Nir2 of the three-member Nir family is phosphorylated (Litvak et al., 2002) and binds to ER VAPs via its FFAT domain (Amarilio et al., 2005). OSBP was required for sterol-dependent activation of CERT (Levine and Munro, 2002; Perry and Ridgway, 2006), whereas Nir2 recruits the PI 4-kinase IIIβ to the Golgi (Aikawa et al., 1999), producing the PI(4)P required for Golgi binding of both CERT and OSBP. This emphasizes that a complex lipid transport system at the ER-Golgi interface integrates the metabolic regulation of multiple lipid classes, sphingolipids, glycerophospholipids and sterols. Via a variety of kinases, the various transfer proteins are embedded in the cellular signalome.

In addition to PI(4)P, the binding of OSBP to the *trans*-Golgi involves ARF (Perry and Ridgway, 2006). Thus, FAPP2 has the same binding site as OSBP on the *trans*-Golgi, suggesting that FAPP2 is involved in transport at the same *trans*-Golgi-ER contact sites. PH domains can dimerize (Klein et al., 1998) and thereby enhance binding to the cognate phosphoinositide at the membrane. However, it is not clear whether this also occurs among lipid transfer proteins. The FAPP2 homolog FAPP1 is a protein of unknown function that contains a PI(4)P PH domain but lacks the GLTP domain. *In vivo*, over-expression of PH domains competes with binding of other PH-domain-containing proteins (Godi et al., 2004). On the other hand, FAPP1 could dimerize with a lipid transfer protein such as FAPP2 and thus enhance its binding properties. Remarkably, *trans*-Golgi ARF1 is an essential part of the binding; in addition, membrane attachment of ARF is regulated, which links FAPP binding to vesicular traffic.

Lipid transfer proteins: composition sensors?

An element missing from the lipid regulation network is sensors for the bulk lipid composition. It has been suggested that some lipid transfer proteins with their corresponding specificity could actually be sensors. For example, PITPβ is specifically bound to the Golgi (van Tiel et al., 2002) and has an *in vitro* transfer activity towards phosphatidylinositol and phosphatidylcholine, as well as sphingomyelin (de Vries et al., 1995). Instead of acting as a transfer protein, it might sense the concentration of sphingomyelin in the cytosolic surface

(which should normally be very low). Because of the high affinity of GLTP for glycosphingolipids (Neumann et al., 2008) and since the amount of GLTP binding to membranes reflects the glycosphingolipid concentration *in vitro* (Rao et al., 2005), GLTP might act as a sensor for glycolipids instead of being a genuine transfer protein. However, it remains unclear how such a sensor would pass on a signal to an effector that would translate the signal into a change in membrane composition.

Perspectives

Under all conditions, cells appear to maintain the unique lipid composition and transbilayer organization of their various membranes, which therefore seem essential for eukaryotic cells. For this, the concentration of individual lipid species has to be sensed and translated into synthesis, degradation or transport. How this is achieved is only understood in a few specific cases. The best-characterized system is the regulation of cholesterol homeostasis via the SREBP pathway, in which a decrease in cholesterol concentration in the ER releases a membrane-bound transcription factor into the nucleus to activate genes involved in cholesterol metabolism (Brown and Goldstein, 1999). This pathway is activated not only by proteins containing sterol-sensing domains (SSD), but also by the sterol transfer proteins OSBP and the related ORPs (Olkkonen et al., 2006), which also act as sterol sensors. By its sterol-dependent activation of CERT, OSBP forms a regulatory link between sterol and sphingolipid metabolism. Elevated levels of 25-hydroxy cholesterol lead to increased synthesis of sphingomyelin (Perry and Ridgway, 2006).

For sphingolipids, little is known about sensing mechanisms. It is an interesting notion that CerS, apart from CerS1, contain Hox domains (Pewzner-Jung et al., 2006). Hox domains are related to homeobox proteins, transcription factors involved in developmental regulation, and in CerS they are not essential for enzyme activity (Mesika et al., 2007). This system exhibits marked similarities to the SREBP system, suggesting that CerS could be sphingolipid sensors, but experimental evidence to support this is still lacking.

To gain further insight into sphingolipid biology and to fully understand the phenotypes related to mutations in proteins involved in sphingolipid metabolism and transport, it is important to identify their interaction partners *in vivo*. Unraveling of the dynamic structural and functional interactions of lipid transfer proteins and their embedding in cellular signaling networks will greatly contribute to an understanding of how eukaryotic cells use sphingolipids for their vital functions.

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