Brave little yeast, please guide us to Thebes: sphingolipid function in S. cerevisiae

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Summary
Sphingolipids typically cover the exoplasmic leaflet of the plasma membrane of eukaryotic cells. They differ from the more abundant glycerophospholipids in that they contain ceramide instead of diacylglycerol as a hydrophobic anchor. Why did nature choose to invent this complex class of lipids, and why do eukaryotic cells follow elaborate remodelling pathways in order to generate dozens to hundreds of different molecular species of sphingolipid, depending on cell type? Yeast may, once again, serve as a model to dissect sphingolipid function at various levels. Almost the complete pathway for sphingolipid synthesis in yeast has been uncovered during the past two decades. More recently, key enzymes in sphingo-lipid degradation and signalling have been identified. Together with a wealth of genetic data obtained from the characterization of various suppressor mutants, this information now allows for an unprecedented analysis of sphingolipid function in this organism. This overview summarizes recent data on sphingolipid function in cell signalling, their role in the heat-stress response and Ca\(^{2+}\) homeostasis, and addresses their function in transport of glycosylphosphatidylinositol-anchored proteins. BioEssays 21:1004–1010, 1999. © 1999 John Wiley & Sons, Inc.

Nomen est omen
The ancient Greek Sphinx, pictured as a winged lion with a female head, was a demon of death, destruction, and bad luck. She sat on a high rock near Thebes and posed the following riddle to all who passed: “What has four feet, two feet and three feet, but one voice?” Those who could not solve it, she strangled. Finally, Oedipus approached and answered the riddle: It is Man, who first crawls on all fours, then walks upright on two legs, and, when old uses a staff as a third leg. After hearing the correct answer, the Sphinx threw herself headlong from the rock to her death. The name “sphinx” is derived from the Greek “sphingo,” which means “to strangle,” and in the context of these pages, refers to a lipid, whose presence in all eukaryotic cells, and some prokaryotes, such as the gram-negative bacteria of the genus Sphingomonas, poses a riddle as to what its function might be. While still incomplete, our appreciation of the different roles that these lipids play in a number of processes has improved considerably in the last 3 years. Progress in dissecting the synthetic and catabolic pathways of the various sphingolipids allows us to ask specific questions about the function of the different classes of sphingolipids and about the functions of different parts of the lipid molecule.

Want to build a sphingolipid?
A sphingolipid is essentially composed of three parts: the sphingoid backbone or long chain base that is amide-bound to a saturated fatty acid to form ceramide, and a polar head group, linked to ceramide at carbon atom C-1 (see Fig. 1). The head group substituent in higher eukaryotic cells is glucose, galactose, or phosphocholine yielding glucosylceramide, galactosylceramide, and sphingomyelin, respec-
Fungal sphingolipids, on the other hand, have inositol-phosphate attached to C-1 of ceramide (for review see Refs. 1–5).

The synthesis of the sphingoid backbone is the first and commitment step in sphingolipid biosynthesis in all organisms. Palmitoyl-CoA and serine condense to form 3-ketodihydrosphingosine (3-ketosphinganine). This reaction is catalyzed by serine palmitoyltransferase, an enzyme composed of at least two subunits, Lcb1p and Lcb2p in yeast.16–19 In higher eukaryotic cells, and probably also in yeast, this takes place at the cytosolic surface of the endoplasmic reticulum (ER).10 Yeast cells defective in Lcb were isolated in pioneering screens for mutants that cannot grow without long chain base supplementation.11 This indicated that at least one of the pathways downstream of long chain base synthesis was essential. Today, we know that two ceramide-dependent processes, the synthesis of glycosylphosphatidylinositol (GPI)-anchored proteins and sphingolipid synthesis, are essential (see below). Subsequent to long chain base synthesis, 3-ketohydrosphingosine is reduced to dihydrosphingosine (DHS, sphinganine), the precursor of both mammalian sphingosine, which has a trans-double bond between carbon atoms 4 and 5, and phytosphingosine (PHS) of plants and fungi, which contains a hydroxyl at position 4 but lacks the double bond (Fig. 2).12–14

Ceramide (phytoceramide) is then formed by N-acylation of the sphingoid backbone with a saturated fatty acid, catalyzed by an as yet unidentified ceramide synthase. For sphingomyelin, the abundant mammalian ceramide with a phosphorylcholine head group, this fatty acid substituent is generally a saturated chain of 16/18 carbon atoms. Yeast sphingolipids, however, more closely resemble mammalian glycolipids in both their head group structure and the fact that they incorporate a α-hydroxylated very long chain saturated fatty acid (C24, C26).15,16 α-Hydroxylation of the very long chain fatty acid is catalyzed by Scs7p, presumably after the formation of ceramide since scs7 mutant cells accumulate a non-hydroxylated sphingolipid species (IPC-B, see Fig. 2).12,17,18

Ceramide is now able to receive head group substituents at C-1. While mammalian cells make dozens to hundreds of sphingolipid species that differ in their precise head group modification, there are only three major classes of sphingolipids in yeast. These are formed by the sequential addition of two head group substituents: inositolphosphate and mannose. The simplest yeast sphingolipid, inositolphosphorylcercamide (IPC) is made by transferring inositolphosphate from the glycerolipid phosphatidylinositol (PI) onto ceramide, thereby generating diacylglycerol (DAG), a potential signaling molecule. This reaction is catalyzed by Aur1p,19,20 AUR1 and hence IPC synthesis is essential. The topology of that reaction is not clear, but it is presumed to occur either in the lumen or the cytosolic face of the ER or the Golgi apparatus.

Subsequent maturation of IPC requires vesicle transport between ER and Golgi and probably takes place in the lumen of the Golgi apparatus where IPC is mannosylated to form mannosylinositolphosphorylcercamide (MIPC), with GDP-mannose serving as the mannoside donor.21–23 This reaction requires CSG1 and CSG2.24–26 Based on the sequence homology of CSG1 to mannosyltransferases, Csg1p has been proposed to catalyze the mannosylation of IPC, while Csg2p has been suggested to catalyze transmembrane flip-flop of IPC.25 Remarkably, CSG1 and CSG2 are nonessential indicating that yeast is viable with IPC alone.

Finally, MIPC is converted to mannosyl-dinositolphosphorylcercamide (M(IP)2C), the most complex sphingolipid in

**Figure 1.** Structure of the three classes of yeast sphingolipids: IPC, MIPC, and M(IP)2C. The three parts of the lipid are colour coded as follows: head group substituent, red; long chain base sphingoid backbone, blue; very long chain fatty acid, yellow. Potential hydroxylation sites are indicated.
yeast.\textsuperscript{(27)} PI is again the inositolphosphate donor of this reaction and the synthesis of one molecule of M(IP)\textsubscript{2}C thus generates two molecules of DAG, the first in the ER and the second in the Golgi apparatus.

The three main classes of sphingolipids, IPC, MIPC and M(IP)\textsubscript{2}C, together with variations in the hydroxylation pattern of the sphingoid base, its precise chain length (C18 DHS/PHS versus the much less abundant C20 DHS/PHS), and variations in the chain length (C24, C26) and hydroxylation pattern of the very long chain fatty acid make up a variety of more than 30 different sphingolipid molecular species. These localize to the plasma membrane where they constitute 20–30\% of all the lipids.\textsuperscript{(28)}

Break it down again
In mammalian cells, the catabolic pathway of complex glyco- and sphingolipids proceeds stepwise. Individual units of the head group are removed by as yet unidentified hydrolases to yield ceramide. Sphingosine, formed by deacylation of ceramide, is then phosphorylated to sphingosine-1-phosphate, a potential signalling molecule.\textsuperscript{(5,28–31)} Sphingosine-1-phosphate is either broken down to ethanolamine phosphate and palmitaldehyde,\textsuperscript{(32)} or phosphorylated and recycled for sphingolipid synthesis\textsuperscript{(33,34)} (Fig. 2). Importantly, the ethanolamine phosphate generated by sphingosine-1-phosphate lyase can be utilized for the synthesis of phosphatidylethanolamine via the Kennedy pathway. This appears to be a one-way pathway that interconnects sphingolipid turnover to glycerophospholipid synthesis.\textsuperscript{(35)} Moreover, treating yeast cells with fumonisnin \(\text{B}_1\), an inhibitor of ceramide synthase, decreases the synthesis of sphingolipids, phospholipids, and neutral lipids and dramatically affects the overall lipid composition of the cell, which further suggests that the synthesis of the major lipid classes is coordinately regulated.\textsuperscript{(36)}

The signalling function
The two antagonistic signalling lipids, ceramide and DAG, are critical intermediates in sphingolipid and glycerophospholipid turnover, which makes the enzymes of sphingolipid synthesis prime candidates for sensors of the relative levels of these two signals.

The yeast ceramide pathway is remarkably similar to that of mammalian cells. Incubating exponentially growing cells with low micro molar concentrations of a cell-permeable

Figure 2. Sphingolipid biosynthetic and catabolic pathways. Reaction products are labelled in black, biosynthetic steps and enzymes are indicated in blue, the catabolic pathway is indicated by yellow arrows. See text for details. A more comprehensive description of the gene products involved may be found at http://www.proteome.com/databases/index.html
ceramide (C2-ceramide) or C2-phytoceramide, but not C2-dihydroceramide, produces a dose-dependent inhibition of proliferation. In both yeast and mammalian cells, this antiproliferative effect is mediated through a ceramide-activated protein phosphatase (CAPP), which belongs to the heterotrimeric subfamily of the protein phosphatase 2A group, and is inhibited by okadaic acid. Cells deficient in various subunits of CAPP (Sit4p, Tpd3p, and Cdc55p) are resistant to the effects of ceramide. The relevant downstream targets of CAPP are not known. However, activation of the RAS pathway is synthetically lethal with reduced CAPP function, suggesting that the most downstream target of the RAS pathway, i.e., activation of cAMP-dependent protein kinase, is antagonized by CAPP.\(^{37–39}\)

While the ceramide signal is growth inhibitory, DAG signalling is stimulatory. A first glimpse at how refined DAG function may be is indicated by the role of the yeast phosphatidylinositol/phosphatidylincholine transfer protein, Sec14p, in controlling membrane flux through the Golgi apparatus. The requirement for Sec14p function is relieved by inactivation of the Kennedy pathway for phosphatidylincholine biosynthesis, indicating that Sec14p is an essential component of a regulatory pathway that links phospholipid metabolism to vesicle trafficking/membrane biogenesis.\(^{40}\) These sec14 bypass mutants require a dramatically increased phospholipase D-mediated turnover of phosphatidylincholine, which generates phosphatidic acid, a precursor of DAG.\(^{41,42}\) A more direct connection between Sec14p function and Golgi levels of DAG is suggested by the observation that mutations in Sac1p, an integral membrane protein related to inositol-5-phosphatases, relieve the Sec14p requirement.\(^{43}\) Topologically, DAG produced in the lumen of the Golgi flip-flops at much higher rates between membrane leaflets, than for example, phosphatidylglycerol.\(^{44}\) This high rate of translocalization may be required for fast and efficient remodelling of the membranes lipid composition in preparation of vesicle budding.\(^{45}\)

**The heat stress response**

A possible role of sphingolipids in the heat stress response is suggested by the phenotype of sphingolipid compensation (SLC) suppressor mutants that are viable without synthesizing the sphingoid base and hence lack ceramide.\(^{46}\) SLC1 encodes a putative sn-2 specific acyltransferase.\(^{47}\) SLC1 mutant cells synthesize novel PIs substituted with a C26 fatty acid in position sn-2 of the glycerol. These C26-substituted PIs are subject to head group modifications typical of sphingolipids, i.e., they contain mannosyl inositol and diphosphoryl inositol mannoside. Thus, the SLC1 suppressor strain appears to overcome the essential function of sphingolipids by synthesizing novel inositol glycerophospholipids that structurally mimic sphingolipids\(^{48}\) and indicates that ceramide synthesis and signalling is not essential for viability. The mutant fails to grow, however, under condition of cellular stress such as high osmolarity or increased temperature.\(^{49}\) The heat-sensitive phenotype of SLC1 is reversed by long chain base supplementation and hence depends on ceramide synthase.\(^{50}\)

Addition of DHS to wild type cells promotes trehalose accumulation at 24°C via gene activation, thus implying sphingolipids in a well known stress response pathway of yeast. Moreover, upon shifting cells to 37°C, a large and transient increase in the low abundant C20 DHS/PHS long chain base is detected.\(^{50}\) Although a phospholipase C-type enzyme activity that generates ceramide from sphingolipids is present in yeast, the heat-induced increase in ceramide is a result of de novo synthesis rather than breakdown of existing sphingolipid. It has been suggested that heat-induced accumulation of ceramide is due to temperature-dependent activation of the enzymes that generate ceramide, such as ceramide synthase itself.\(^{51}\) Remarkably, survival of cells with either reduced levels or a complete lack of sphingoid base-phosphate phosphatase activity (dpl1, lcb3, and lcb3/lbp2 double mutants, respectively) is dramatically enhanced after severe heat shock.\(^{35,52}\) The relative level of the two sphingolipid metabolites, ceramide and sphingosine-1-phosphate, has thus been proposed to function as an evolutionary conserved rheostat that determines cell fate.\(^{53}\)

Addition of PHS, furthermore, inhibits cellular uptake of a number of amino acids, such as tryptophan, leucine, proline, and histidine, and induces the general control response, explaining why different strains display different sensitivity to growth arrest by PHS\(^{54}\) (see Table 1 for summary).

**TABLE 1. Functions of Yeast Sphingolipids and Their Intermediates**

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td>Long chain base</td>
<td>Essential intermediate in ceramide synthesis,(^{5,55}) inhibited by myriocin and sphingogentin B, PHS addition inhibits uptake of amino acids,(^{5,54}) accumulate under heat stress,(^{55,51}) growth inhibitory(^{44})</td>
</tr>
<tr>
<td>Long chain base phosphate</td>
<td>Growth promoting second messenger,(^{19,20}) may act as signal for resistance to heat(^{35,52})</td>
</tr>
<tr>
<td>Ceramide</td>
<td>Growth inhibitory second messenger,(^{37–39}) induced by heat stress,(^{11,51}) inhibited by australafungin(^{55}) and fumonisin B(_1)(^{56,58})</td>
</tr>
<tr>
<td>IPC</td>
<td>Essential class of mature sphingolipids, inhibited by aureobasidin(^{19,20}) and kahfufungin,(^{57}) inhibition affects cell morphology(^{40})</td>
</tr>
<tr>
<td>MIpc</td>
<td>Non essential class of mature sphingolipids,(^{5,14,26,38,58}) affects cell morphology(^{25}) and lipid composition(^{26})</td>
</tr>
<tr>
<td>M[IP](_2)C</td>
<td>Non essential class of mature sphingolipids,(^{27}) mutants are more resistant to nystatin(^{28})</td>
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Sphingolipids and transport of GPI-anchored proteins

GPI-anchored proteins are attached to the membrane via a lipid anchor that contains either DAG or ceramide. The biosynthesis of these two types of lipid anchors employs a number of highly specific lipid remodelling reactions that serve to introduce a C26 fatty acid, in the form of either a sn-2 C26-subsitituted DAG, or ceramide. This exchange of DAG for ceramide occurs in both the ER and the Golgi and requires ceramide rather than IPC as substrate.

Ceramide/sphingolipid synthesis in the ER is required for efficient transport of GPI-anchored proteins from the ER to the Golgi apparatus, but not for transport of other soluble or transmembrane proteins, as indicated by two lines of evidence. First, inhibition of ceramide synthesis leads to a rapid and specific reduction in the rate of transport of Gas1p, a well characterized GPI-anchored protein that contains a DAG-based lipid anchor. Second, in the absence of ceramide synthesis, survival of the SLC suppressor at low pH is enhanced by overexpression of a GPI-anchored cell wall protein, Cwp2p. Consistent with the observed delay of Gas1p maturation in the SLC suppressor strain, it has been suggested that the rate of transport of Cwp2p is limiting for cell survival at low pH.

Under ceramide-limiting conditions, Gas1p accumulates in the ER in its GPI-anchored form, indicating that sphingolipids are not necessary for anchor attachment. Packaging of Gas1p, into COPII coated vesicles, however, requires prior GPI-attachment. More recent in vitro data are consistent with a requirement for ceramide after the exit from the ER, possibly in the fusion of GPI-containing vesicles with the Golgi or a pre-Golgi compartment.

While inhibition of ceramide synthesis affects the transport of GPI-anchored proteins, the converse holds also true. In cells defective in GPI anchoring, the synthesis of IPC and MIPC is reduced significantly and the activity of serine palmitoyltransferase is strongly inhibited. Thus, inhibition of GPI-anchored protein transport out of the ER might cause a concomitant block in export of IPC, resulting in the accumulation of IPC in the ER, which might then feedback inhibit its own synthesis.

Hydroxylated sphingolipid species and Ca^{2+} homeostasis

A screen to identify genes important for the regulation of the cytosolic Ca^{2+} concentration isolated two mutants that fail to grow in medium containing high Ca^{2+} (100 mM). Both mutants, csg1 and csg2 (calcium sensitive growth) turned out to be defective in the mannosylation of IPC. These cells grow normally in YPD, indicating that the synthesis of MIPC and M(IP)3C is not essential. Remarkably, suppressor mutants that reverse the Ca^{2+}-sensitivity of csg2 (SCS) decrease the synthesis of IPC-C or alter its structure; suggesting that it is the accumulation of a specific subclass of IPC, IPC-C, rather than the absence of mannosylated IPCs that renders cells susceptible to high Ca^{2+}.

Complex genetic interactions between suppressors of the Ca^{2+}-sensitivity of csg2 (SCS) and suppressors of rvs161 (SUR) suggests a role for sphingolipids in some Rvs161p-dependent processes. Rvs161p is required for endocytosis, correct actin localization, and viability upon nitrogen, carbon, or sulphur starvation. It is similar to amphiphysin, a neuronal protein found in synaptic vesicles that is the autoantigen in stiff-man syndrome. The molecular function of Rvs161p and the basis of suppression by mutations in the SUR genes is not well understood. Three genes (SUR1, SUR2, and SUR4) that mutate to suppress rvs161 are related to CSG1 and CSG2 or to genes that mutate to suppress the Ca^{2+}-sensitive phenotype of csg1 and csg2 mutants.

Future directions

A new class of temperature-sensitive suppressors of the Ca^{2+}-sensitivity of csg2 (tsc) mutants has recently been isolated. tsc mutants fall into 15 complementation groups and uncover novel essential genes in sphingolipid synthesis and regulation such as the 3-ketosphinganine reductase, TSC10, and additional ORFs of poorly characterized function such as YDL015c and YER093c. The fact that two of the tsc mutants, tsc14 (tor2) and tsc15 (mss4) encode PI 4- and PI(4)P 5-kinase, respectively, may provide a link to understand how sphingolipid maturation is connected to glycerophospholipid turnover in the Golgi. Furthermore, the future characterization of these tsc mutants is likely to fill the remaining gaps in the biosynthetic and catabolic pathway and identify the genes for the still elusive ceramide synthase and ceramidase.

Another productive area of future research is likely to be the determination of the subcellular localization of enzymes of the pathways described previously. The question, for example, of how sphingolipid synthesis is coupled to vesicular transport may be addressed by determining the site of synthesis of key intermediates of the biosynthetic pathway, e.g., ceramide versus IPC. Similarly, determining the localization of the long chain base phosphatases and kinases may help to understand how long chain base transport is coupled to its phosphorylation and dephosphorylation.

Little is known about the regulation of synthesis of ceramide and the mature sphingolipids. What determines the ratio of IPC to MIPC and M(IP)3C at the plasma membrane? Is the ratio of dihydroceramide to phytyoceramide containing species regulated, and if so, how? What are the roles of the various hydroxylated species and how is their synthesis controlled? Yeast is viable with IPC alone. So why are there so many sphingolipid molecular species?

Major aspects of sphingolipid function, i.e., the rheostat function of the relative level of the two sphingolipid metabolites, ceramide and sphingosine-1-phosphate, appear to
be conserved between yeast and mammals.\(^{(3,31)}\) The fact that these molecules have such diverse and frequently pleiotropic roles in controlling the welfare of a cell makes it difficult, however, to assign specific functions to them. Using yeast genetics combined with advanced analytical methods\(^{(72)}\) should help to increase our understanding of this fascinating class of evolutionary more recent lipids.

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