Roles for Sphingolipids in *Saccharomyces cerevisiae*

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Abstract

Studies using *Saccharomyces cerevisiae*, the common baker's or brewer's yeast, have progressed over the past twenty years from knowing which sphingolipids are present in cells and a basic outline of how they are made to a complete or nearly complete directory of the genes that catalyze their anabolism and catabolism. In addition, cellular processes that depend upon sphingolipids have been identified including protein trafficking/exocytosis, endocytosis and actin cytoskeleton dynamics, membrane microdomains, calcium signaling, regulation of transcription and translation, cell cycle control, stress resistance, nutrient uptake and aging. These will be summarized here along with new data not previously reviewed. Advances in our knowledge of sphingolipids and their roles in yeast are impressive but molecular mechanisms remain elusive and are a primary challenge for further progress in understanding the specific functions of sphingolipids.

Introduction

Besides providing many satiating bakery and brewery products for human pleasures, *Saccharomyces cerevisiae*, has been a remarkably informative host for discovering how sphingolipids are made and degraded and the cellular functions they perform. Carter's laboratory identified the long-chain bases (LCBs) dihydrosphingosine (DHS, sphinganine) and phytosphingosine (PHS) in the 1950s and early 1960s.[1] In the 1970s and 1980s the Lester laboratory identified the three classes of inositol phosphate-containing complex sphingolipids in yeast and their mode of synthesis and in doing so devised excellent methods for their extraction and analysis.[2,3] These advances and others enabled yeast genes to be identified and manipulated in every imaginable way and have permitted the elucidation of most, perhaps all, genes for making and degrading sphingolipids in *S. cerevisiae*, the first organism for which this has been achieved (reviewed in ref. 4-6). Many of these genes made possible the identification of homologs in organisms ranging from bacteria to plants to man. In this chapter I try to summarize our knowledge of sphingolipids in *S. cerevisiae* and suggest where future research is needed.

Sphingolipid Metabolism in *S. cerevisiae*

Sphingolipids are abundant in *S. cerevisiae* representing about 7% of the mass of the plasma membrane or 30% of phospholipids.[7] *S. cerevisiae* sphingolipid metabolism including metabolites, enzymes and their cognate genes are diagramed in Figure 1. Synthesis begins with condensation of serine and a fattyacyl-CoA, typically palmitoyl-CoA, in every organism that has been examined and generates the short-lived intermediate 3-ketodihydrosphingosine,

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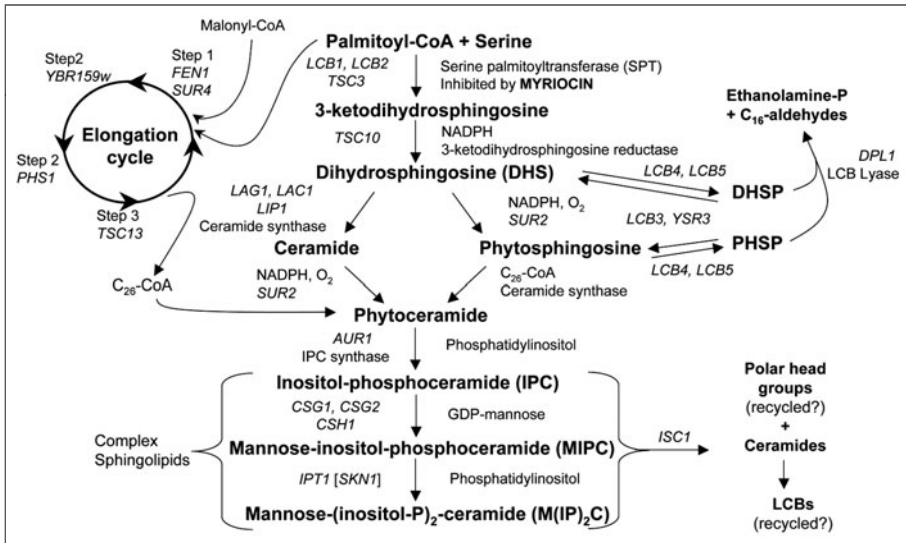
Figure 1. Pathways of sphingolipid metabolism in *Saccharomyces cerevisiae*. Metabolic intermediates and complex sphingolipids are shown in bold, genes are indicated by italics and enzyme names are in regular lettering. When grown aerobically the fatty acid in complex sphingolipids is often hydroxylated at C_2 and sometimes at C_3 (not shown), a reaction that requires Scs7 (not shown).[107] Ceramides can be hydrolyzed by two ceramidases, Ydc1 and Ypc1 (not shown), to yield a fatty acid and an LCB.[35,36] Structures of the indicated compounds are presented in previous publications.[4,8] Adapted from reference 6.

which is reduced to yield DHS, the first LCB in the pathway. DHS can either be N-acylated with a fatty acid to give ceramide or hydroxylated on C4 to give PHS which is N-acylated to yield phytoceramide. The C1 hydroxyl of phytoceramide is decorated with polar head groups by the three sequential reactions diagramed in Figure 1 which yield three species of complex sphingolipids including inositol phosphoceramide (IPC), mannose inositol phosphoceramide (MIPC) and mannose-(inositol-P)$_2$-ceramide M(IP)$_2$C. The genes and enzymes for the steps in sphingolipid metabolism have been discussed thoroughly in previous reviews.[2-5,8-10]

Sphingolipid synthesis begins in the endoplasmic reticulum and generates ceramides which are transported by both vesicular and nonvesicular transport to the Golgi apparatus for addition of the polar head groups.[11] Most enzymes involved in sphingolipid synthesis have been localized to these two compartments although there are exceptions.[12] Movement of sphingolipids between these compartments has been examined in detail although much remains to be elucidated (reviewed in refs. 8,9,13,14). Most complex sphingolipids are then transported to the plasma membrane but small amounts are found in other membrane compartments[15,16] including mitochondria.[17] The function of sphingolipids in these other cellular compartments is not understood. About three-fourths of the mass of the sphingolipids in *S. cerevisiae* cells is M(IP)$_2$C with the rest being equal parts of IPC and MIPC.[18] It is not known how these ratios are determined, but they are probably important in ways that remain to be identified. The shear mass of sphingolipids and their negative charge are likely to affect processes dependent upon the plasma membrane. Many types of sphingolipids in mammals are localized to one or the other leaflet of the plasma membrane, but this has not been determined for yeast sphingolipids.

A unique and distinguishing feature of *S. cerevisiae* sphingolipids is a C_{26} fatty acid, although a small percentage of C22 and C24 fatty are also present.[16,19] These long-chain fatty acids are synthesized by an elongation system[19-21] whose components have now been identified and this has led to a model for how the length of the fatty acid is determined.[22] The functions of such a long-chain

fatty acid in sphingolipids are not well defined, although one function may have something to do with the fact that they span both leaflets of a membrane bilayer. They have been suggested to play roles in nuclear membrane pores.[23] Others have argued that sphingolipids do not need them but that they are necessary to perform some function that remains to be identified.[24] While it is true that cells making sphingolipids with shorter acyl chains can survive in laboratory situations, it is highly unlikely that such strains would survive in the wild, thus arguing that the C_{26} fatty acid is an essential component of yeast sphingolipids under natural conditions.

While *S. cerevisiae* only makes inositolphosphoceramides and no other type of sphingolipids such as glycosylceramides, many other fungi make both classes of sphingolipids[3] as do plants.[25,26] Phosphoinositol-containing sphingolipids have not been identified in mammals. It probably is no accident that *S. cerevisiae* and other fungi have sphingolipids that are more like plants than mammals given that the natural habitat of *S. cerevisiae* appears to be forests, particularly oaks and other broadleaf trees.[27]

Like most other membrane components, sphingolipids are broken down as a normal part of membrane remodeling. The need for turnover is apparent in mammals where defects in enzymes that catalyze turnover lead to debilitating diseases termed sphingolipidoses.[28] Such a need has been much less apparent in yeast but this notion is changing. One type of sphingolipidoses is Niemann Pick type C, a fatal neurodegenerative disorder caused by a defect in the human *NPC1* gene. The yeast homolog of this gene, *NCR1*, has been suggested to play a role in recycling of sphingolipids.[29] The yeast sequence similarity between a bacterial neutral sphingomyelinase and the Isc1 protein of *S. cerevisiae* lead to the demonstration that Isc1 has phospholipase C-type activity and cleaves the polar head group from yeast sphingolipids, much like mammalian sphingomyelinases cleave the polar head group from sphingomyelin.[30,31]

Unraveling the physiological importance of sphingolipid turnover in *S. cerevisiae* has been challenging and much remains to be learned. At least in some strains backgrounds, Isc1 enzyme activity is necessary for growth on nonfermentable carbon sources, implying a role in mitochondrial respiration[32] and several types of experiments support this mitochondrial connection as discussed in detail in a previous review.[6] Another role for Isc1 is to regulate the concentration of IPC which is toxic above its physiological concentration.[33,34] In response to heat stress and perhaps other stress that perturb the plasma membrane, Isc1 appears to be activated to breakdown complex sphingo-lipids (we don't know their location) by a pathway involving synthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2) as described below along.

A good deal of effort has gone into trying to determine the physiological functions of the other enzymes that degrade sphingolipids including the ceramidases Ypc1[35] and Ydc1,[36] the LCB kinases Lcb4 and Lcb5, the LCB-phosphate phosphatases Lcb2 and Ysr3 and the LCB lyase Dpl1. Some may disagree, but I think it is fair to say that other than their basic catalytic function, we do not know the real physiological roles of these enzymes and their precursors and products in *S. cerevisiae* (reviewed in refs. 2-5,8,9). They must be important because most are conserved in fungi and other organisms.

Both natural and synthetic inhibitors of several enzymes in the sphingolipid biosynthesis pathway have been identified and have been very useful in a range of experiments (reviewed in refs.[3,37]). These include myriocin, sphingofungins, lipoxamycin and viridiofungins that inhibit serine palmitoyltransferase (SPT), the first enzyme in sphingolipid biosynthesis (Fig. 1), australi-fungin and the fumonisins that inhibit ceramide synthase and aureobasidin and khafrefungin that inhibit IPC synthase.

Membrane-Associated Functions and Processes

A screen for mutants defective in endocytosis gave the first indication that sphingolipids are necessary for this process.[38] The screen identified *end8-1*, later shown to be allelic with *LCB1* and renamed *lcb1-100*.[39] The *lcb1-100* mutation causes serine palmitoyltransferase (Fig. 1) activity to be labile even in cells grown at a permissive temperature[40] and sphingolipid synthesis becomes limiting for growth at the restrictive temperature of 37˚C,[41] most likely because LCB levels drop

quickly and limit sphingolipid synthesis.[42] The *lcb1-100* mutation has been extremely useful for studying sphingolipid metabolism and functions. Sphingolipids affect multiple aspects of endocytosis including the actin cytoskeleton and these will be described below in the section on signal transduction. Other experiments implicating sphingolipids in endocytosis used strains defective in *SNC1* and/or *SNC2* that encode v-SNARE proteins.[43] Sphingolipids have been shown to play several roles in endocytosis of the uracil transporter Fur4 (reviewed in ref. 9) as have the Slm proteins[44] (see below).

Sphingolipids along with sterols (cholesterol in mammals and ergosterol in fungi and plants) are critical for formation of microdomains within membranes that have been referred to as lipid rafts and are typically isolated by treating a yeast extract with detergent at low temperature to form what are referred to as detergent-insoluble complexes or detergent-resistant membranes.[45,46] In *S. cerevisiae* rafts are vital for sorting and delivering membrane-bound proteins to their proper cellular address and they are also necessary for fusion of cells during mating.[45,47,48] Such proteins include Pma1, which exports protons across the plasma membrane to maintain intracellular pH and also to generate a proton gradient necessary for cells to take up nutrients from their surroundings.[49-54] Other membrane proteins requiring rafts/sphingolipids include Gas1, a Beta-1,3-glucanosyltransferase and Nce2,[49] Fus2, Fig1, Sho1, Ste1 and Prm1,[55] Fur4[42,56] and Can1, an arginine transporter.[57] The general amino acid permease Gap1 depends upon sphingolipids for its transport to the plasma membrane in an active conformation capable of amino acid transport and that can resist degradation.[58]

Roles for sphingolipids in exocytosis based upon suppressor mutant analyses were reviewed previously.[4,9] In wild-type cells the C_{26}-fatty acid in sphingolipids is required for raft association and stable surface transport of newly made Pma1 to the plasma membrane.[59] Recently, a large-scale visual screen for genes that play roles in the sorting of proteins in the trans-Golgi network for delivery by the exocytosis pathway to the cell surface identified genes in sphingolipid metabolism (*SUR2*, *SUR4* and *YPC1*).[48] It is not clear why the Sur2, Sur3 and Ypc1 proteins are required for protein sorting. Ypc1 is particularly interesting because it degrades phytoceramides.[35]

In mammals, sphingosine-1-phosphate is involved in intracellular calcium signaling through an unknown mechanism.[60] Likewise, sphingolipids may regulate calcium fluxes and signaling pathways in yeast, but the mechanisms are unknown (reviewed in ref. 2) and see also ref. [61] Cells defective in *CSG1* or *CSG2* (Fig. 1) are sensitive to 100 mM calcium and this phenotype has played a key role in identifying genes in sphingolipid metabolism.[33,34,62,63] Why mutants defective in *CSG1* or *CSG2* are sensitive to calcium is unclear, but it indicates a connection of some type between sphingolipids and calcium metabolism or signal transduction and these potential connections are presented below in the section on Signal Transduction Pathways.

Nutrient transport is affected by LCBs as first suggested by the extreme sensitivity of auxotrophic strains to LCBs in the culture medium.[64] PHS, but not other LCBs, was found to block uptake of tryptophan, leucine, histidine and uracil.[65] It is not entirely clear how LCBs are regulating nutrient transport, except in the case of uracil where PHS has been shown to be important for heat-induced, ubiquitin-mediated breakdown of Fur4, the uracil transporter.[40] Other data also support the idea that heat-induced LCBs promote ubiquitination of proteins[66] and that the immunosuppressant drug, FYT720, a synthetic sphingolipid-like molecule, acts in ways very similar to PHS to inhibit yeast growth.[29] As described below in Signal Transduction Pathways, LCBs maybe regulating nutrient transporters via activation of the Pkh1/2 kinases (Fig. 2) or their downstream kinases such as Ypk1 or Sch9, which play roles in nutrient sensing.[67-69]

Interactions of some sort or cross-talk between sphingolipids and both ergosterol and glycerophospholipids has been observed. For example, defects in ergosterol synthesis are suppressed by mutations in *SUR4*[70] and a decrease in ergosterol content is compensated by an increase in sphingolipids.[71] Other data show that defects in ergosterol synthesis affect hydroxylation of yeast sphingolipids.[72] Yeast cells also have mechanisms for maintaining asymmetry in the distribution of sphingolipids and glycerophospholipids in the two leaflets of the plasma membrane so that a change in one class of lipids is compensated by a change in another class.[73]
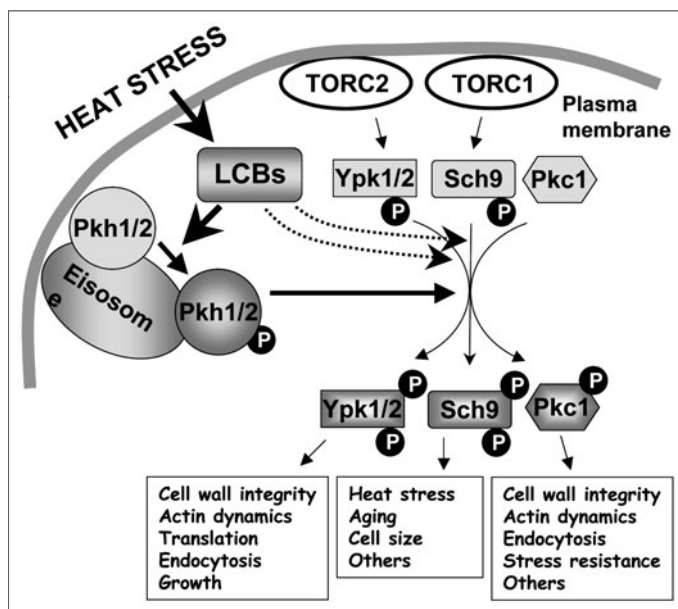
Figure 2. *S. cerevisiae* signal transduction pathways regulated by LCBs. LCBs transiently increase during a heat stress and are hypothesized to activate Pkh1 and Pkh2. As discussed in the text, Pkh2 is probably more dependent upon LCBs than is Pkh1 although this has not been defined for each cellular response. Kinase assays with purified proteins suggest that LCBs can directly trigger a small increase in Ypk1, Ypk2 and Sch9 activity (indicated by a dotted line).[97] Pkh1/2 phosphorylate Ypk1, Ypk2, Sch9 and Pkc1 in their activation loop (PDK1 site) but the proteins are not enzymatically active. To become active they also need to be phosphorylated in a hydrophobic region (PDK2 site) and in a turn motif in their C-terminus. Phosphorylation of these sites in Ypk2 is mediated by TORC2[124] and for Sch9 phosphorylation is mediated by TORC1.[94] Ypk1/2 and Pkc1 are shown working in parallel pathways to control cell wall integrity, but data also support an alternative pathway in which Ypk1/2 work upstream of Pkc1.[90,91] Adapted from reference 6.

While the complex yeast sphingolipids, IPCs, MIPCs and $M(IP)_2Cs$, are abundant, progress in identifying unique functions for them has been slow. The antifungal action of the plant defensin DmAMP1, a peptide defensin produced by *Dahlia merckii*, requires $M(IP)_2C$, which serves as a high affinity receptor.[74] DmAMP1 is postulated to bind or interact with $M(IP)_2C$-containing lipid rafts making the plasma membrane more permeable thereby disrupting essential cellular processes.[75] Syringomycin E is an antifungal cyclic lipodepsinonapeptide that interacts with the plasma membrane and inhibits growth of *S. cerevisiae* cells by forming ion channels. Yeast mutants defective in *ipt1*, *fen1* or *sur4*, *scs7* and *sur2* are drug resistant, showing that $M(IP)_2C$ with a C_{26}-fatty acid and PHS but not DHS is essential for the antifungal action of syringomycin E.[76-78] While interesting, these results do not represent normal functions of the complex sphingolipids beyond their role as structural components of the plasma membrane and essential elements of lipid rafts. One would imagine that they interact in physiologically essential ways with plasma membrane proteins, but evidence is lacking and will require advances in three dimensional structure of membrane proteins bound to natural lipids.

Sphingolipids play roles in exocytosis of glycosylphosphatidylinositol-anchored proteins (reviewed in ref. 79). For example, transport of Gas1p from the endoplasmic reticulum to the Golgi apparatus requires ceramide or a related sphingolipid for transport.[39,80,81] Furthermore, the diacylglycerol moiety in glycosylphosphatidylinositol anchors is often replaced by ceramide.[79]

Sphingolipids and the Slm protein (see below) are required for exocytosis of arginine transporter Can1[82] and references therein). Sphingolipids have also been shown to play roles in generating a functional V1 component of the vacuolar ATPase.[83]

Signal Transduction Pathways That Require Sphingolipids

One of the most exciting and extensively explored functions of sphingolipids is the regulation of signal transduction pathways and ceramides and sphingosine-1-phosphate in mammals are the most well documented signaling species.[84-86] There is no firm evidence that ceramides and long-chain base phosphates play similar roles in yeast. Instead, LCBs appear to regulate signaling pathways. The one piece of evidence that would firmly establish LCBs as regulators of signaling pathways is to show that they bind to specific proteins such as the protein kinases discussed below that are implicated to be regulated by LCBs (Fig. 2).

The first clue that LCBs might be intracellular signaling molecules or second messengers was the observation that they rapidly but transiently increase following heat stress typically executed in the laboratory by shifting cells from 25˚C to 37˚C or 39˚C[87,88] and reviewed in detail in ref.[4,9,10]

Insight into LCB signaling pathways was first found during a screen to identify genes whose overexpression bypassed growth inhibition by myriocin,[89] an inhibitor of serine palmitoyltransferase (Fig. 1). The *YPK1* gene bypassed the myriocin block. The Ypk1 protein kinase has a role in cell wall maintenance and actin cytoskeleton dynamics,[90,91] endocytosis[92] and translation during nitrogen starvation and nutrient sensing.[67] Ypk1 and its paralog Ypk2 are structural and functional homologs of mammalian serum and glucocorticoid-inducible kinase (SGK).[93] Ypk1 is phosphorylated and activated by Pkh1[93] and multiple copies of *PKH1* were found to also bypass the myriocin block.[89] One explanation for these results is that a sphingolipid acts upstream of and activates the Pkh1/Ypk1-Ypk2 the signaling pathway. Myriocin-treated cells lacked a phosphorylated and presumably active form of Ypk1 but this form reappeared in vivo following PHS-treatment of cells. The original and still accepted view of these experiments is that PHS activates Pkh1 or its homolog Pkh2, which then phosphorylate and activate Ypk1 and probably Ypk2. Many laboratories (reviewed in ref. 9,10) have contributed data supporting this hypothesis and expanded it to include activation of other kinases including Sch9[94] and Pkc1 which are activated by the LCB-Pkh1/2 pathway (Fig.2). To be active, Sch9 and Ypk1/2 must be phosphorylated at other residues by the Target of Rapamycin (TOR) pathways as indicated in Figure 2.

The diagram shown in Figure 2 suggests that Pkh1/2 and the kinases and cellular processes downstream of them are equally regulated by LCBs, but current data suggest that this is probably an oversimplification. First, initial analysis in vitro showed that purified Pkh2 was more strongly activated by LCBs than was Pkh1.[95] Second, as discussed below, the Slm2 protein is more responsive to sphingolipids (likely to be LCBs) than is Slm1, consistent with the finding that *slm1*Δ cells are more sensitive to growth inhibition by myriocin than are *slm2*Δ cells.[44] Likewise, homozygous diploid *pkh1*Δ cells were 1000-fold more sensitive to myriocin than *pkh2*Δ cells, consistent with LCBs having a stronger affect on Pkh2 than on Pkh1. Additionally, this screen found both heterozygous and homozygous diploid *ypk1*Δ cells to be extremely sensitive to myriocin while *ypk2*Δ cells were not sensitive, implying that LCBs regulate Ypk2 but not Ypk1 activity. These myriocin data only apply to a process or processes that are necessary for growth: nonessential processes may depend upon LCBs to regulate Pkh1 and Ypk1, as suggested by the finding that in vitro the activity of Pkh1 and both Ypk1 and Ypk2 is stimulated by LCBs.[97] As mentioned above, demonstrating which proteins bind LCBs would help to clarify their physiological roles.

Pkh1/2 are primarily found on eisosomes,[98] newly described very large structures, estimated to contain about 2000 copies each of the Pil1 and Lsp1 proteins, which bind to the cytoplasmic face of the plasma membrane.[99] Pil1 and Lsp1 are highly conserved but seem to be found only in fungi. Eisosomes play roles in endocytosis of lipids and some proteins and localize at cites of endocytosis where they may physically interact with the actin cytoskeleton based on confocal fluorescent microscopy and genetic interaction studies.[99] Pil[98,100] and Lsp1 (Dickson, et al unpublished data) are highly phosphorylated by Pkh1/2 and phosphorylation plays roles in assembly

and disassembly of eisosomes. Eisosomes are also thought to be involved in sensing changes in the plasma membrane caused by stresses such as heat and then signaling the cell to adjust the lipid and protein composition of the membrane by endocytosis and exocytosis in order for cells to be more stress tolerant.[98,100] Earlier data[95] had indicated that Pil1 and Lsp1 indeed play roles in resisting heat stress and they may do so in several ways including serving as binding sites for Pkh1/2 to enable them to properly control downstream kinases with known roles in heat and other stress responses (Fig. 2). It is not known if some fraction of Pkh1 or Pkh2 also resides in other cellular locations or whether they cycle on and off of eisosomes. Clearly there is much to learn about eisosomes and the role they play in regulating cellular processes.

Previous results from genetic suppression experiments[34,101] suggested that sphingolipids interacted in some manner with phosphoinositides, probably phosphatidylinositol-5,4-bisphosphate (PIP$_2$) and with the TOR pathways. These novel interactions along with the calcineurin signaling pathway have now been shown by genetic and biochemical assays to control phosphorylation and dephosphorylation of the Slm1 and Slm2 proteins during heat stress thereby modulating actin polarization, endocytosis and sphingolipid metabolism.[44,82,102]

The Slm proteins have overlapping functions and at least one is required for viability.[103] They also have a PH domain enabling binding to PIP$_2$, known to arise transiently during stresses such as during heat shock on the inner leaflet of the plasma membrane.[103,104] Slm binding to the membrane is further strengthened by interactions with the Avo2 and Bit61 subunits of the Target Of Rapamycin Complex 2 (TORC2)[103,105] and these protein-protein interactions promote phosphorylation of Slm1 and Slm2 by TORC2.[103] The phosphorylated Slm proteins then mediate downstream effects of PIP$_2$ and TORC2 that control roles of the actin cytoskeleton essential for growth, cell wall integrity and receptor-mediated endocytosis (Fig. 3). A recent study suggests that TORC2 is located on eisosomes, if true, then eisosomes may play a role in Slm protein function.[106]

Data supporting a role for sphingolipids in regulating the Slm proteins and how they in turn regulate sphingolipid synthesis and turnover were recently reviewed in ref.[6] and are summarized diagrammatically in Figure 3. The model depicts events during unstressed growth and during heat stress: whether similar events are active during other stresses is unknown.[44,82,102] Central to this model are the Slm proteins functioning downstream of the PIP$_2$, TORC2 and LCB-Pkh1/2 signaling pathways. Heat stress causes a transient increase in PIP$_2$ thereby recruiting the Slm proteins to the plasma membrane where they are phosphorylated by TORC2 and also by Pkh1/2, which are activated also by the transient burst of LCBs induced by heat. Activated Slm proteins control movement of the actin cytoskeleton via the Rho1/Pkc1 pathway. Unexpectedly, the Slm proteins also control sphingolipid metabolism by down-regulating Isc1 activity, thus slowing breakdown of complex sphingolipids, especially the very abundant IPC-C (IPC with one hydroxyl group on the C$_{26}$ fatty acid.[107] The Slm proteins also down-regulate calcineurin phosphatase activity, which interacts with Csg2, in an unknown manner, to regulate conversion of IPC-C to MIPC. Hence, during heat stress the Slm proteins are thought to interface between phosphoinositides and sphingolipids and orchestrate changes in membrane lipid composition to promote survival.

Once cells adjust to a heat stress they down-regulate activated Slm proteins by calcineurin-mediated dephosphorylation in what appears to be a negative feedback loop (Fig. 3). It is also likely that IPC, possibly a specific pool of IPC-C, regulates actin organization and viability.[102,108] Identifying this pool of IPC-C could provide important clues for understanding how such regulation occurs.

Recognition that the LCB-Pkh1/2, PIP$_2$ and TORC2 pathways use the Slm proteins and calcineurin to regulate sphingolipid metabolism provides a framework for deciphering the molecular basis for maintaining sphingolipid levels that are optimal for growth in the absence of stress and for surviving a heat stress. Many questions remain unanswered. Do Pkh1/2 directly phosphorylate Slm1 and Slm2 or does one of the protein kinases regulated by Pkh1/2 (Fig. 2)? Available evidence suggests that Pkh1/2 do not phosphorylate the Slm proteins nor do Ypk1/2, but the data do not exclude these possibilities and more work is needed.[82] How do the Slm proteins
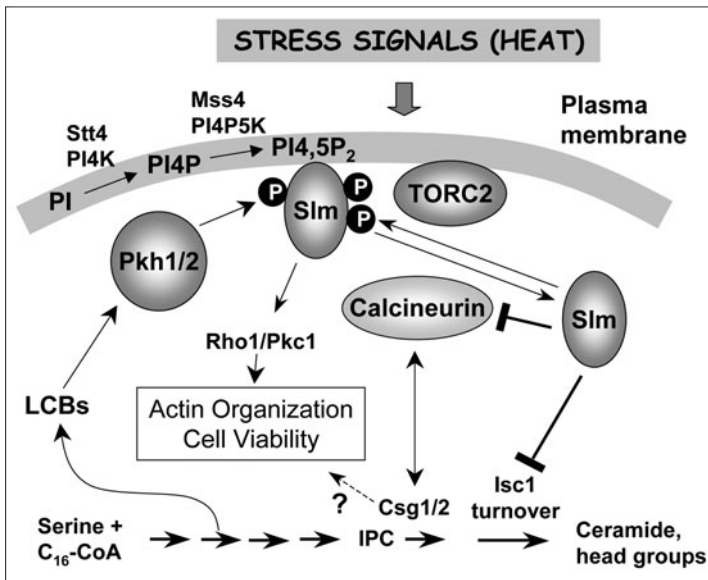
Figure 3. Synthesis and turnover of complex sphingolipids along with actin dynamics are regulated by PI4,5P$_2$ and the Slm1 and Slm2 proteins. PI4,5P$_2$, synthesized by Stt4 and Mss4 on the plasma membrane, in cooperation with the TORC2 and the Pkh1/2 protein kinases are proposed to activate Slm1 and Slm2. The Slm proteins then impair turnover of complex sphingolipids, particularly IPC, by inhibiting Isc1 and they also regulate the calcium/calmodulin-regulated protein phosphatase calcineurin which dephosphorylates and inactivates the Slm proteins and also interacts with Csg1/2 in an unknown manner to regulate conversion of IPC to MIPC by the Csg1/2 enzymes. Regulation of the Rho1/Pkc1 pathway by Slm1/2 works independently from regulation of sphingolipid metabolism. Genetic interactions suggest that IPC plays a role in actin organization, but the mechanism is unknown. Pkh1/2 are probably attached to eisosomes[98] (not shown) as depicted in Figure 2. Adapted from reference 6.

regulate Isc1 activity? Does Isc1 play a direct role in regulating the actin cytoskeleton and how do Csg1\2 interact with calcineurin?

Slm2 seems more dependent upon sphingolipids for function than does Slm1. First, haploid *slm1Δ* cells are more sensitive to growth inhibition by myriocin than *slm2Δ* cells. Second, heat-induced and sphingolipid-dependent endocytosis of the uracil transporter requires Slm2 but not Slm1.[44] Lastly, a recent large-scale screen of deletion mutants found that *slm1Δ* heterozygous and homozygous diploid cells were extremely sensitive to myriocin compared to all other gene deletion mutants including *slm2Δ* cells.[96]

Longevity and Cellular Aging

Long before it was realized that *LAG1* (Longevity-Assurance Gene[109]) encodes a ceramide synthase,[110,111] it had been found to affect replicative lifespan, measured by determining how many times a cell can bud. Deletion of *LAG1* increases replicative lifespan by 50 percent,[109] but the mechanism remains unknown.

Sch9 (Fig. 2) plays roles in both replicative and chronological lifespan, measured by how long cells survive in stationary phase when division has ceased. Deletion of *SCH9* increases chronological lifespan by 300%[112] and increases both the mean and maximal replicative lifespan.[113] No one has yet determined if LCBs play a role in lifespan by regulating Pkh1/2 which in turn phosphorylate Sch9 on threonine 570 in its activation domain.[94,97] To be active, Sch9 must also be phosphorylated by TORC1 at several serine and threonine residues located in its C-terminus.[94]

Isc1, which cleaves polar head groups from complex sphingolipids to generate ceramides, has been shown to play a role in oxidative stress resistance (hydrogen peroxide) and chronological lifespan.[114] For lifespan measurements, cells were grown to log phase or early stationary phase in YP medium containing glycerol as the carbon source and then transferred to water, a severe form of calorie restriction. Both log and stationary phase *isc1Δ* cells died extremely rapidly compared to *ISC1* cells, indicating that viability under these experimental conditions depends on Isc1 function. The oxidative stress theory of aging argues that cells die because of oxidative stress and several but not all measures of oxidative stress were elevated in *isc1Δ* cells. Apoptosis is induced by oxidative stress and during aging[115-118] and *isc1Δ* cells showed classic signs of apoptosis including DNA fragmentation and activation of Yca1 metacaspase activity. The rapid loss of viability in calorie-restricted stationary phase *isc1Δ* cells was completely prevented by deletion of *YCA1*, arguing that Isc1 is involved in a pathway that regulates Yca1 and apoptosis.

To begin to elucidate the role of Isc1 in oxidative stress resistance and chronological lifespan, a global analysis of mRNAs was performed using microarray technology.[114] Expression of 72 genes was found to increase and sorting these into biological processes showed that six were involved in iron uptake. The authors reasoned that induction of these genes during iron abundance could cause iron overloading and enhance oxidative stress. They found iron increased in both log and stationary phase *isc1Δ* cells and data from several types of experiments supported the notion that such accumulation contributed to but was not entirely responsible for increased oxidative stress and cell death. At this time it is not clear how Isc1 protects cells against oxidative stress and apoptosis. A basic question is whether the ceramide generated by Isc1 activity is necessary for stress protection or whether accumulation of one or more complex sphingolipids such as IPC1-C in *isc1Δ* cells causes cell death. Others have argued that the increased level of $M(IP_2)C$ found in *isc1Δ* cells[30,114] is the cause of cell death. It would be interesting and novel if ceramide production by Isc1 protected against oxidative stress, since ceramide plays an opposite role in mammals during stresses and promotes cell death.[84,119,120] Other roles for Isc1 were recently reviewed and readers are encouraged to examine previous work on this interesting enzyme.[6,9]

Finally, recent studies showed that overexpression of the Ydc1 ceramidase (Fig. 1) shortened chronological lifespan, but this shortening probably results from fragmentation of mitochondria and vacuoles and increased apoptosis and it is not clear how these studies relate to normal physiological functions of Ydc1 and sphingolipids.[121] Analysis of ceramides in Ydc1 overproducing cells showed that both dihydroceramides and phytoceramides were reduced, suggesting that the enzyme does not specifically hydrolyze dihydroceramides in vivo. These studies are supportive of a role for ceramide as an inducer of apoptosis in yeast similar to what transpires in mammals.

Regulation of Sphingolipid Biosynthesis

I think it is safe to say that we do not really understand how de novo sphingolipid synthesis is regulated in any organism. Readers should consult previous reviews describing what we do know about the regulation of sphingolipid biosynthesis including regulation of gene transcription as only the latest studies will be discussed herein.[4-6,8-10]

Recently it was shown that TORC2 controls ceramide synthase activity.[122] The TOR protein kinases sense nutrients and stresses and coordinate metabolism both temporally and spatially to regulate cell growth.[123] All eukaryotes that have been examined contain two TOR protein complexes, TORC1, which is inhibited by rapamycin, a bacterial macrocyclic lactone and TORC2, which is rapamycin-insensitive. A genetic screen had implied a link between sphingolipids, the TOR proteins and calcium homeostasis in yeast. This screen used the sensitivity of a *csg2* mutant to 100 mM Ca^{++} to identify temperature-sensitive mutations that bypassed the calcium-sensitivity.[62] Bypass mutations occurred in several genes including *TOR2* and *AVO3/TSC11* that encode components of TORC2.[34] TORC2 specifically controls the organization of the actin cytoskeleton in yeast and mammals.[123]

A critical advance in understanding the connection between TOR signaling and sphingolipids relied upon the isolation of a temperature-sensitive allele of *AVO3* (*avo3-30*) that diminished growth even at 30°C. Three hours after shifting *avo3-30* cells from 25°C to 30°C the major yeast

ceramide species containing PHS and a C_{26}-fatty acid was 5-fold lower in concentration compared to wild-type cells and minor ceramide species having shorter fatty acid chains were reduced about 10-fold.[122] Even when grown at 25°C, the concentration of the major ceramides was reduced 2-fold in *avo3-30* cells. These results implied a reduction in ceramide synthase activity in *avo3-30* cells and this prediction was confirmed by measuring enzyme activity in cells grown at 30°C.

How might TORC2 influence ceramide synthase activity? The Ypk2 protein kinase was known to be activated by TORC2[124] and mutant *ypk2* and *avo3-30* cells showed similar defects in cell wall integrity and actin polarization, suggesting that Ypk2 working downstream of TORC2 and Pkh1/2 (Fig. 2) might regulate ceramide synthase activity. This possibility was supported by data showing that a constitutively active allele of *YPK2* reversed all *avo3-30* phenotypes including the ceramide deficiency.[122] These results argue that Ypk2 acts downstream of TORC2 to activate ceramide synthase activity. The molecular details of how Ypk2 governs ceramide synthase activity remains to be determined. Further analysis implicated calcineurin as a regulator of ceramide levels. Deletion of the *CNB1* gene, which encodes the calcineurin regulatory subunit B, restored ceramide levels in *avo3-30* cells and promoted growth at 30°C, suggesting that calcineurin down-regulates ceramide synthase activity. Perhaps calcineurin dephosphorylates ceramide synthase and one wonders if the Slm proteins (Fig. 3) are somehow involved is such regulation. Surprisingly, deletion of *CNB1* did not restore actin polarization to *avo3-30* cells, implying that it is controlled by TORC2 in a distinctly different manner from ceramide synthesis activity.

A reduction in ceramide synthase activity predicts an increase in DHS and PHS based upon previous work (reviewed in ref. 6) and *avo3-30* cells did indeed have elevated DHS and PHS levels.[122] These elevated LCBs could act in a feed-forward manner to activate ceramide synthase activity and promote synthesis of complex sphingolipids. This may occur because DHS and PHS activate Pkh1 and Pkh2 which then phosphorylate Ypk2 in its activation domain while TORC2 phosphorylate residues in the C-terminus (Fig. 2). To be active, Ypk2 must be phosphorylated in both domains. The possibility of similar types of regulation of ceramide synthase in mammals has been discussed.[125]

These studies form a basis for understanding how yeast cells coordinate ceramide and sphingolipid synthesis with nutrient availability and cell growth and how synthesis is reduced during stress when cells need to shift from growth to survival mode. But there is likely to be a lot more to this story than is apparent now and there are likely to be many more layers of regulation including regulation of Isc1 as depicted in Figure 3.

Conclusion and Future Developments

Since sphingolipids are abundant in the plasma membrane and also present in smaller amounts in other cellular membranes and compartments it is not surprising that they have been found to play roles in many processes occurring in or on membranes (Figs. 2 and 3 and see also Fig. 3 in ref. 9). Most of what we know about sphingolipid involvement in these processes has come from directed, small-scale experiments involving one or a few proteins or cellular processes as readouts and such studies will continue to be required to elucidate molecular mechanisms. Larger-scale, genome or proteome-wide experiments have started to identify new roles for sphingolipids and proteins that require sphingolipids for function.[12,48,96,126-130] More large-scale experiments that interrogate all yeast proteins need to be devised to determine if there are general rules for why proteins depend upon sphingolipids for function or whether such functional dependence is unique for each protein. The ability to analyze and quantify ("profile") of nearly all species of sphingolipids in a single sample by mass spectrometry is a great advance in methodology that will likely become the standard way to measure sphingolipids in yeast[16,24,131,132] much as it has become the standard way to analyze sphingolipids in mammals.[131,133] One of the most challenging problems will be to advance our understanding of the roles that sphingolipids play in cells from phenomenology and associations to molecular mechanisms. This will require techniques to measure sphingolipid binding to proteins either in vivo or in vitro and to demonstrate that binding promotes or inhibits a cellular process. For example, do LCBs directly interact with Pkh1/2 to stimulate their activity or do they act indirectly such as by binding to eisosomes which then activate Pkh1/2?

Acknowledgements

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