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Sphingolipid signaling in yeast: potential implications for understanding disease

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1. ABSTRACT

Sphingolipids are essential components of membranes and important for cellular integrity. The main focus of research in the past years has been to demonstrate their role as second messengers. The yeast *Saccharomyces cerevisiae* is an excellent model for the study of sphingolipids, because the first steps of this metabolic pathway are highly conserved among fungal, plant and the animal kingdoms. The yeast model is a valuable system for the understanding of pathways and development of tools that will help to better understand and intervene into the molecular mechanisms controlling health and disease. Different classes of sphingolipids have been shown to act in different pathways. Sphingoid bases were shown to be involved in protection against a series of stresses such as heat shock, osmotic stress and low pH. Ceramides have been shown to be involved in G1 arrest, heat shock response and more recently as a target of the TORC 2. Complex sphingolipids are essential for cell wall integrity and proper localization of GPI anchored proteins.

2. INTRODUCTION

The structural role of lipids in the function of cellular membranes has been appreciated for a long time. However, it was not until the late 1990's and early 2000's that sphingolipids were also recognized to have a role as signaling molecules in different physiological pathways (1, 2). Since then much evidence, both in yeast and mammals, has been obtained to demonstrate roles as second messengers for different classes of sphingolipids. The yeast *Saccharomyces cerevisiae* is an excellent model for the study of sphingolipids, because the first steps of this metabolic pathway are highly conserved across fungal, plant and the animal kingdoms (3). The enzymes involved in the early part of this pathway were all identified with the help of yeast genetics. Up to the stage of synthesis of ceramide there are only minor differences with relation to chain length, hydroxylation and saturation of the chains in the synthesis of sphingolipids. Whereas in mammalian cells the steps after the synthesis of ceramide lead to the formation of an enormous variety of complex

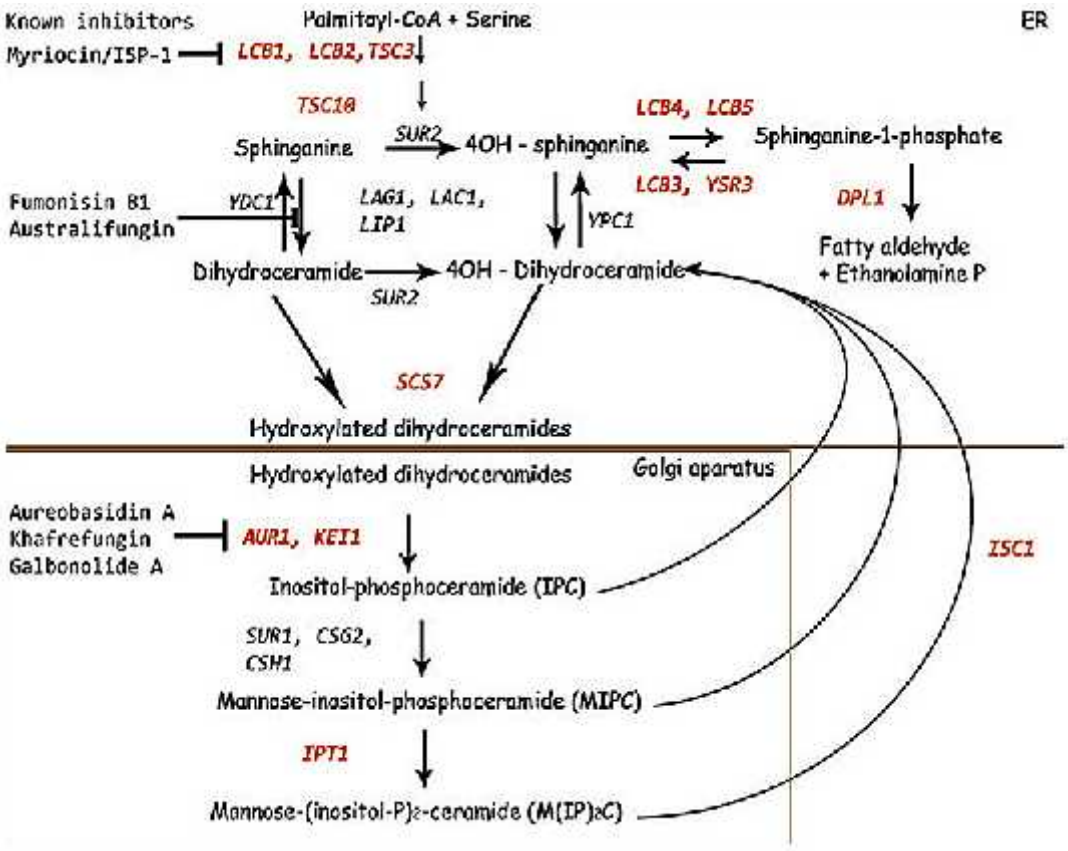

Figure 1. Sphingolipid biosynthetic pathway in yeast and its known inhibitors. In red are some of the enzymes that are potential targets for drug discovery. The sphingolipid synthesis pathway is not linear with respect to hydroxylation and the different hydroxylation states can exist for all of the ceramides and complex sphingolipids.

sphingolipids, with a multitude of different head groups, in *S. cerevisiae* only three types of complex sphingolipids are produced: IPC, MIPC and $M(IP)_2C$. This diminished complexity, although it cannot perfectly mimic the mammalian diversity, provides a much easier model system to manipulate, that can, nonetheless, give us important insights into the function of complex sphingolipids. For a more complex fungal model one could turn to *Pichia pastoris* which has both inositolphosphorylceramides and glucosylceramides(4).

The sphingolipid metabolic route in yeast starts with the serine palmitoyltransferase, composed of three subunits, Lcb1p, Lcb2p and Tsc3p (Figure1), which synthesizes 3-ketosphinganine and which have mammalian homologs. This enzyme is essential for cell viability in yeast, but a deletion mutant can be grown by supplementation with sphinganine or 4-hydroxysphinganine. A temperature sensitive allele has been isolated (5) that has allowed the study of the role of sphingolipid biosynthesis in many processes. This allele has allowed researchers to conditionally inactivate Lcb1p, which stops the *de novo* synthesis of sphingolipids and modulates its pathway. Many studies in signaling link the sphingoid bases produced after this step and before the synthesis of ceramide to diverse physiological processes leading to the hypothesis that the two yeast sphingoid bases (PHS and DHS) and their phosphorylated forms are crucial as second messengers, as will be discussed bellow.

The next crucial step in sphingolipid biosynthesis is the condensation of sphingoid bases and fatty acids to form dihydroceramide. The dihydroceramide synthase genes were first discovered in yeast as two redundant homologs called Lag1p and Lac1p(6, 7). They were soon proved to be required for dihydroceramide synthase activity and orthologs were found in most species(8). The deletion of either of the dihydroceramide synthases alone is not enough to suppress ceramide production, has no clear growth phenotype, but expands life span of yeast cells up to 50%(9). The deletion of both genes can be lethal in certain yeast strain backgrounds(6, 10), but in others the strain is viable and makes some still not fully identified lipids. The Lac1p and Lag1p proteins were purified together with another essential subunit, Lip1p, and the complex showed dihydroceramide synthase activity *in vitro* demonstrating that the genes encode a *bona fide* ceramide synthase (11). No clear homologs of the *LIP1* subunit have been found in higher eukaryotes and some of these mammalian homologs of *LAG1* do not require *LIP1* for activity when expressed in yeast(12).

In yeast, the endogenous dihydroceramide synthases enzymes are only capable of utilizing very long chain fatty acids (with a strong preference for C26, and to a much smaller extent C24) and this has an effect on downstream complex sphingolipids that all have very long chains. Mammalian cells have six orthologs that have been named CerS 1 through 6. Various studies have shown that each of CerS enyzmes has different fatty acid specificity and the variations in the length of the fatty acyl chains have different physiological consequences(12-17). One of these proteins has been purified and shown to be a *bona fide* dihydroceramide synthase that, unlike its yeast counterpart, does not require any other subunits for functionality(13). The difference in chain length can therefore be studied in yeast by the replacement of the endogenous dihydroceramide synthases by the expression of the mammalian homologs. This has been done successfully for all 6 genes(18), but little has been shown in terms of physiological effects for the moment.

At this stage two hydroxylations can be added to modify the ceramides. *SUR2* is the gene responsible for the hydroxylation of the sphingoid base and *SCS7* for the fatty acid(19). Another important gene is *ISC1* that encodes a yeast homolog of the mammalian sphingomyelinase(20), which. hydolyzes complex sphingolipids producing ceramide and releasing the head group, thereby recycling complex lipids. After the synthesis of dihydroceramide, the pathways in yeast and mammalian cells diverge. While mammals produce a wide variety of complex sphingolipids, starting with sphingomyelin and glucosylceramide, yeast has only three complex sphingolipids formed in a sequential manner. The first step is the addition of an inositol phosphate using phosphatidylinositol as a donor by an enzyme called Aur1p, forming an inositol phosphoryceramide (IPC). Deletion of *AUR1* is lethal(21) and its activity can be blocked by the antifungal Aureobasidin A. Currently there is much discussion in the literature if this lethality is the consequence of accumulation of dihydroceramide or the lack of complex sphingolipids. Some mutants allow cells to grow in the presence of AbA, notably strains defective in dihydroceramide synthase (8) and the elongation of fatty acids, which also reduces dihydroceramide synthesis, suggesting that the very long chain ceramide is toxic(22). There is also evidence that complex sphingolipids can be required for growth because another group found that even the *lag1 lac1* double mutant, which is resistant to AbA since it does not accumulate ceramide, became inviable after being grown consecutive times in the presence of AbA(23). Recently another subunit essential for IPC synthesis has been discovered, Kei1p. It is thought that this protein is essential for Aur1p transport from the ER to Golgi and without it cells show decreased levels of IPC(24).

The next step in sphingolipid biosynthesis in yeast is the addition of a mannose from GDP-mannose, forming mannose inositol phosphorylceramide (MIPC). This reaction is catalyzed by either Sur1p (Csg1p) or Csh1p, the catalytic subunits, that together with Csg2p, the regulatory subunit, (25, 26), produces the reaction. Deletion of *SUR1* and *CSH1* render the cells unable to produce MIPC and have reduced levels of $M(IP)_2C$, but this is not lethal, indicating that complex mannosylated sphingolipids are not essential for the survival of yeast cells(25).

The last step in sphingolipid biosynthesis in yeast is the addition of a second inositol phosphate from phophatidylinositol to MIPC forming $M(IP)_2C$(27) and is carried out by Ipt1p. A deletion mutant of *IPT1* is viable indicating that the final inositol phosphorylation is not required for viability.

3. SPHINGOID BASE, THE UPSTREAM PHYSIOLOGICAL REGULATORS

In yeast there are two main forms of sphingoid bases, sphinganine (called dihydrosphingosine, DHS) and its hydroxylated form 4-hydroxysphinganine(called phytosphingosine, PHS). Both are present in very low amounts in yeast cells and can either be converted into dihydroceramide or phosphorylated to form DHS1-P and PHS1-P respectively. The first experiments with these molecules were done in mammalian cells but the discovery of the related genes in yeast provided a simpler and more effective way to study the intracellular roles of sphingoid bases. The first clues in yeast for a signaling role for sphingoid bases was the discovery that cells defective in their synthesis were viable but unable to survive a series of stresses such as heat shock, osmotic stress and low pH (28).

Two genes encoding sphingoid base kinases, Lcb4p and Lcb5p, have been identified in yeast and the corresponding mutants have been useful to probe the function of sphingoid bases and their phosphorylated derivatives(29). These, together with *dpl1* (sphinganine 1 phosphate lyase) and other mutants in the pathway were used to probe the role of long chain bases. The accumulation of sphingoid base phosphates proved to inhibit cell growth(30). The levels of accumulation in this study were however much higher (>70 fold) than the ones observed under heat shock conditions (5 to 8 fold). This indicates that total amounts and the duration of their elevation are essential to define if sphingoid bases accumulation will lead to cell proliferation or arrest(30).

Heat shock stress provided the first experiments where sphingoid bases were shown to be second messengers. PHS1-P and DHS1-P were shown to exist in very small amounts that increased transiently during heat shock, in a mechanism essential to help the cells survive the temperature change(31). The deletion of the sphinganine phosphate lyase (*DPL1*) or of one of the phosphatases (*LCB3*), thus impeding the catabolism of the phosphorylated lipids, led to an increase in heat shock resistance and a better survival rate than the WT strain, while the down regulation of Lcb1p (serine palmitoyl tranferase) led to cell death via the inability of the cells to transiently increase their levels of PHS1-P and DHS1-P(31). One of the mechanisms of action of resistance involves the induction of heat shock proteins (HSPs) which help to disaggregate and refold misfolded or aggregated proteins. When the HSPs are unsuccessful another process

increased by heat shock, ubiquitin-dependent degradation of misfolded proteins takes over. The *lcb1-100* mutant fails to induce heat shock proteins upon heat shock, but the strain was able to survive the heat shock if ubiquitin was overexpressed (32) suggesting that it is not the loss of activity of proteins through heat shock that is most critical under these conditions, but the accumulation of misfolded or aggregated proteins. These findings could be relevant to diseases associated with protein misfolding and aggregation. Another role of sphingoid bases during heat shock is in protein translation control. The increase in sphingoid bases seems to be required for translation initiation of the heat shock proteins(33), which are translated during heat shock while other mRNAs are not. Furthermore, the deletion of the lyase gene, *DPL1*, leads to the accumulation of sphinganine-1-phosphate, which under certain conditions has been demonstrated to cause a block of cell division and a failure to recruit cells to G1 phase (34).

Recently two studies have shown that two proteins from the ORMDL family are involved in the regulation of Lcb1/2, namely Orm1 and Orm2(35, 36). Deletion of both genes leads to accumulation of sphingoid bases and activation of UPR(36). This is important for the understanding of the relationship between ER stress and lipid homeostasis and could lead to better understanding of the mechanism of UPR regulation and diabetes. Also, as pointed by the authors, this proteins are related to the human ORMDL3 protein, which has recently been shown to be an asthma susceptibility gene(37). The ORM genes in yeast have been shown to be regulated by phosphorylation and to form the SPOT complex that includes the products of three sphingolipid synthesis genes (*LCB1*, *LCB2* and *TSC3*) and their regulator *SAC1*, besides *ORM1* and *ORM2*. A similar situation has also been shown in mammalian cells, were the ORMDL3 gene was found to form a complex with SPTLC1(35).

The above cases are proposed functions of phosphorylated forms of sphingoid bases, but several physiological functions in yeast seem to depend upon sphingoid bases. The absence of sphingoid bases leads to a defect in the internalization step of endocytosis and proper actin organization, which can be rescued by the addition of external bases(38). The blockage of sphingoid base phosphorylation does not seem to inhibit this process, and this study in yeast was the first to propose a role for sphingoid bases as active molecules instead of their phosphorylated forms. The mechanism of action was later found to be mediated by the Pkh kinases, homologs of the PDK kinases in animals, and the overexpression of the Pkh kinases led to a rescue of the endocytic defect(39). *In vitro* experiments showed that even nanomolar amounts of sphingoid bases were capable of activating the kinases and downstream effectors were identified(39).

Phosphorylated sphingoid bases have also been linked to calcium influx in both yeast and mammalian cells. In yeast the role of the two kinases (Lcb4p and Lcb5p) was demonstrated to be essential when exogenous sphingosine was added to stimulate the calcium signaling pathway. The absence of the kinases rendered the addition of the sphingosine innocuous, while the activity of the lyase and phosphatase (Dpl1p and Lcb3p) inhibited the activation of the pathway(40).

Very recently yeast has been used to investigate Parkinson disease associated toxicity of alpha-synuclein. This protein has been linked to Parkinson's and is thought to be involved in other neurodegenerative diseases. They expressed this protein and a subset of its mutants in yeast defective in sphingolipid metabolism and looked for increased toxicity. The study was done only on single mutants which probably prevented the finding of additional interactions with the sphingolipid pathway, but one subset of genes that did emerge from the screens were the *ELO1*, *FEN1* and *SUR4*. These 3 genes are fatty acid elongases that work in sequence to produce the very long chain fatty acids used for dihydroceramide synthesis. All three mutants showed increased sensitivity to the wt alpha-sync as well as to two of the three variants tested, as well as less viability of old cells(41). The cause of this sensitivity remains elusive. The *elo* mutants lower amounts of sphingolipids, due to the specificity of the dihydroceramide synthases, which have a low affinity towards shorter (less then 26 carbons) fatty acyl CoAs. The approach using yeast is a way to simplify the study of a very complex problem and the insights gained in yeast might be applicable to neuronal cells, which would make it useful for the development of drugs and markers for neurodegenerative malignancies.

Yeast has also been used as a model system to investigate the metabolism and possible targets of the immunosuppressant FTY720, a sphingolipid analog (42) that has recently been approved to treat multiple sclerosis(43). In vertebrates the mechanism of action that seems to be important for FTY720 involve its phosphorylation by sphingosine kinase 2 and action as a sphingosine-1P mimic(44). It has also been shown to inhibit sphingosine kinase 1(45). In yeast the effects do not seem to depend upon FTY720 phosphorylation suggesting that the molecule might have other effects, mimicking sphingoid bases, that need to be understood, including effects on the ubiquitin pathway, trafficking of amino acid permeases, and on transcriptional profiles(41, 46).

FTY720 has also been reported to inhibit the sphingosine phosphate lyase(47). It is not known if this inhibition plays a physiological role in the mechanism of action of FTY720. Structure-function relationships in the yeast homolog of the enzyme, Dpl1p, have been published(48)and provide information about the localization and function of the enzyme. Furthermore, recent studies have determined the 3-D structure of a related enzyme from bacteria by X-ray crystallography (49), which has allowed the modeling of the structure of the eukaryotic enzyme. Information about the active site of the enzyme and its structure should allow the design of novel inhibitors and perhaps other modulators of the lyase activity. A specific inhibitor should allow the dissection of the role of this inhibition in physiological processes.

Not all regulatory functions of sphingoid bases found in mammalian cells can be found in yeast. This possibly arises from the fact that the major sphingoid bases

in yeast are hydroxylated in the 4 position whereas a desaturation occurs in mammalian cells. An example of this specific regulation is the E3 ubiquitin ligase TRAF2, which has been shown to require sphingosine-1-phosphate but not sphinganine-1-phosphate, to enhance branching of ubiquitin chains on lysine 63(50). No homologous regulation has yet been found in yeast. Another example of the action of sphingosine-1-P not yet found in yeast is its ability to bind to histone deacetylase enzymes and inhibit their action(51).

4. DIHYDROCERAMIDES

Sphingoid bases and their phosphorylated forms are not the only intermediates in the sphingolipid biosynthesis pathway with signaling functions. Dihydroceramides themselves have been shown to be involved in G1 arrest, heat shock response and more recently as a target of the TORC 2 (target of rapamycin complex 2) pathway(52-55).

The first insight into how ceramides could act as second messenger in yeast came in 1993 (56) where the investigators showed that small amounts of soluble ceramides inhibited cell growth in yeast. The treated cells had an activated phosphatase, that could be inhibited by okadaic acid, making it a class 2A ceramide activated phosphatase (CAPP)(56). Nickels and Broach extended this study showing that ceramide can activate CAPP, whose catalytic subunit is encoded by *SIT4* and regulatory subunits by *CDC55* and *TPD3*. Activation of CAPP leads to G1 arrest(54) . It was also shown that this pathway could be counteracting a cAMP-dependent protein kinase of the RAS pathway.

Ceramide has also been implicated in heat stress response. Although much of the research in this area has focused on the transient increase in sphingoid bases (see above), the heat stress also generates a more durable elevation of ceramides. This elevation is the result of *de novo* synthesis, because the addition of australifungin, a dihydroceramide synthase inhibitor, was shown to block the increase(55). This finding differed from what was postulated previously about ceramide generation under stress conditions in vertebrates, where most of the ceramides produced come from the degradation of complex sphingolipids. This finding illustrated the importance of the *de novo* pathway and encouraged the study of dihydroceramide synthesis as a possible candidate source for signaling molecules.

More recently the interaction between sphingolipid metabolism and the TOR pathway has been demonstrated. The TOR kinase, which was first identified as the target of rapamycin(57) and has been shown to regulate cell growth and metabolism. It forms two complexes, TORC1 and TORC2, of which only the former is sensitive to rapamycin(58). The kinase gene is conserved through evolution in eukaryotes and its study in mammalian cells has associated it with several diseases such as cancer, cardiovascular, autoimmunity and metabolic disorders. Many excellent reviews exist on the subject(59-61), one of which discusses the relationship of TORC with lipid synthesis, specifically its control of lipogenesis. In yeast the TORC2 complex clearly has an influence on sphingolipid metabolism, however, the precise mechanism is still unclear. The most direct experiments involve the investigation of the function of *AVO3*, which encodes a subunit of the TORC2 complex. It was shown that incubation of a temperature sensitive *avo3-30* mutant at nonpermissive temperature led to a reduction in ceramide levels and an increase in phosphorylated sphingoid bases. This mutant has a slow growth phenotype suggesting that the lack of ceramide and complex sphingolipids led to cell cycle arrest or cell death(52). The precise mechanism of this regulation is unclear, however, they showed a genetic interaction with the calcineurin pathway. The calcineurin pathway has previously been shown to interact with TORC2 (62) and with another set of homologous proteins, Slm1p and Slm2p(63). Furthermore, the Slm proteins have been implicated in regulation of sphingolipids(64) and the actin cytoskeleton, another function of TORC2(65). More recently, the plekstrin homology domain of the Slm proteins, which are required for actin organization and bind phosphoinositides, has been shown also to bind sphingolipids(66). It will be very interesting to see to what extent this regulation can be reproduced in vertebrates as there are no obvious Slm1p homologs.

In a systematic synthetic interaction screen with a thermosensitive mutant in the phosphatidylinositol transfer protein (Sec14p) implicated in the secretory pathway and Golgi function, a strong interactor was a mutant in the snare protein Tlg2p(53), which functions in membrane trafficking associated with the Golgi complex and endosomes(67-68). The combination of the *sec14* and *tlg2* mutations affected the TOR signaling pathway, the unfolded protein response (UPR) pathway in the endoplasmic reticulum and caused the accumulation of ceramides perhaps the cause of the UPR. The proposed mechanism is that the double defect in trafficking around the Golgi compartment causes an increased catabolism of complex sphingolipids (IPC, MIPC and MIP_2C) that would elevate the pools of ceramide(53). This elevation would in turn affect a ceramide activated phosphatase in a similar mechanism to that proposed above.

Apart from signaling functions ceramides are also important in the intracellular trafficking of GPI-anchored proteins. The anchors of most GPI(glycosylphosphatidylinositol)-anchored protein in *S. cerevisiae* are remodeled from a diacylglycerol structure to a ceramide structure in the endoplasmic reticulum, with some contribution from later compartments(69). In a screen for inhibitors of GPI-anchored protein biogenesis, a potent inhibitor of serine-palmitoyltransferase, myriocin, was found(70). The synthesis of ceramide is critical for GPI-anchored protein transport because only stereoisomers of sphinganine that can be incorporated into ceramide can restore transport when serine palmitoyltransferase is blocked(71). GPI-anchor remodeling is required for ER exit(72) acting at the step of concentration into ER exit sites(73). In mammalian cells ceramide synthesis is not required for GPI-anchored protein transport(74), however,

the process of remodeling is required for ER exit(75), and the mechanism of transport seems to be conserved although the organization of the pathway is somewhat different with respect to the sites and nature of the latter remodeling steps. Defects in GPI biosynthesis can lead to diseases, such as paroxysmal nocturnal hemoglobinuria(76).

Ceramides can also be modified by hydroxylation of either the sphingoid base or the fatty acid moiety. *SCS7*, the gene that introduces a hydroxyl group to position 2 of fatty acids has been shown to be important for resistance to the drug PM02734, a novel synthetic antitumor drug. Its mode of action is the induction of rapid necrotic cell death in yeast. The deletion of *SCS7* renders cells more resistant to necrosis and these results have been validated in mammalian cells, where *SCS7* has a homolog, FA2H(77).

Another pathway for the formation of ceramide is the degradation of complex sphingolipids. In mammalian cells this function is carried out by sphingomyelinases and in yeast by a single gene, *ISC1*, which is capable of cleaving the headgroups of different complex sphingolipids. It has been shown that the mammalian sphingomyelinase 2 is capable of rescuing the yeast *ISC1* deletion (20). This deletion strain also showed cell cycle defects, being blocked at the G2/M phase, when treated with methyl methanasulfonate or hydroxyurea(78) and having a lower life span with death by apoptosis when treated with hydrogen peroxidase(79). Curiously, the protein encoded by *ISC1*, which is normally localized in the endoplasmic reticulum has been located in the mitochondria following glucose depletion treatment or late in the growth phase (78). These results suggest that the higher levels of ceramide seen in apoptotic cells might come from the degradation of complex sphingolipids rather then from the *de novo* synthesis and this mechanism is conserved in yeast. Furthermore *ISC1* has been shown to be involved in other stress response pathways like the halotolerance against Na+ and Li+ ions(80).

5. DOWNSTREAM OF DIHYDROCERAMIDES: COMPLEX SPHINGOLIPIDS

Although there is much data on the role of the products of earlier steps of sphingolipid biosynthesis in physiological functions, much less is known about the roles played by complex sphingolipids. The synthesis of complex sphingolipids is simpler in *S. cerevisiae* than in higher eukaryotes. Mammalian cells produce sphingomyelin and glucosylceramides, the latter being transformed into a series of different glycolipids. *S. cerevisiae* makes only three complex sphingolipids, IPC, MIPCs and $M(IP)_2Cs$, which are synthesized by reactions that are similar to that of sphingomyelin. One function suggested for sphingolipids is the stable insertion and folding of proteins in the membrane. GPI anchored proteins have been shown to depend on either ceramide and/or complex sphingolipids for their proper insertion or retention in the endoplasmic reticulum membrane. In the absence of *de novo* ceramide synthesis GPI proteins have their traffic impaired and can no longer be tightly associated with the ER membrane(81). As mentioned above, ceramide synthesis is required for proper function, but the responsible lipid might be a complex sphingolipid. Other data also show a dependence on sphingolipids for cell wall integrity. Since GPI anchored proteins are essential components of cell walls their improper placement and transport leads to cell wall defects(82). The intracellular transport of the yeast general amino acid permease, Gap1p, has also been suggested to be coupled to *de novo* sphingolipid synthesis(83). The idea is that sphingolipids would be required for proper folding of the permease. The cell wall integrity pathway has been proposed to be an excellent target for antifungal drugs since it is unique to fungi and would not affect host cells(84).

In a recent study it has been shown that the proper GPI-anchor is necessary not only for the proper transport of the proteins attached to it but also for the transport of ceramides from the ER to Golgi compartment therefore affecting the downstream steps of sphingolipid synthesis(85). The *ARV1* gene was identified as being essential to cells that lack the ability to esterify sterols (86). The human *ARV1* gene can complement the yeast mutation so its function is likely to be conserved (87). The precise function of Arv1p is unknown, but is likely to be involved at some step of GPI biosynthesis(85).

The deletion of two genes that synthesize MIPC, *SUR1* and *CSH1*, render cells hypersensitive to calcium ions. As complex sphingolipids have not been shown to act as signaling molecules, one explanation for the sensitivity would be the degradation of IPCs into ceramides or sphingoid bases that would in turn be the active signaling molecules. Another would be the direct interaction of Ca^{++} with IPC or its role as a signaling molecule in a yet undiscovered pathway. The overexpression of *HOR7* was able to counteract the calcium sensitivity of IPC accumulating cells by depolarizing the plasma membrane(88). IPCs have also been implicated as regulators of cell cycle. During the formation of IPC, the substrates, phosphatidylinositol and dihydoceramide are converted to IPC and diacylglycerol (DAG). When this process is interrupted it seems to cause yeast cells to arrest in G1. The ratio between dihydroceramide and DAG seems to be essential for the G1 to S transition and the block of IPC synthesis by the addition of aureobasidin A (inhibitor of Aur1p) arrests the cells(89). Again it is unclear whether IPCs act as signaling molecules or if the principle problem results from regulation of the dihydroceramide/DAG ratio.

MIP_2C has an important role in plasma membrane function. It has been shown that cells lacking this lipid are resistant to a toxin called zymocin as well as to the antibiotics hygromycin B and nystatin(90). The first hypothesis suggested by these findings was that since MIP_2C is the major sphingolipid in plasma membranes, its absence would lead to the mislocalization of ion transporters, like Pma1p, the proton ATPase of the plasma membrane. This turned out to be false. This leaves two theories (1) that MIP_2C itself is essential for the docking and internalization of different toxins or (2) that this lipid is important for membrane properties that affect the function of the membrane proteins either by affecting function directly or by affecting interaction with other proteins(90).

It has also been suggested that the production of IPC by Aur1p is the step through which phosphatidyilinositol-4-phosphate regulates sphingolipid levels. This pathway would require the Sac1p phosphatase to act upon PtIns4P to provide phosphoinositol head groups for the formation of IPCs and in this way decrease the levels of dihydroceramides and sphingoid bases(91).

Sphingolipids have also been shown to interact with other lipids in response to different stress conditions. An unbiased study has shown that mutations in ergosterol biosynthesis, which lead to the accumulation of different intermediates, lead to changes in the overall sphingolipid composition(92). This adaptation could allow cells in nature to regulate their overall lipid composition in response to environment cues or aggressions. Although not many transcriptional differences were found in the enzymes of the sphingolipid metabolic pathways, lipidomic analysis showed substantial differences in headgroups and hydroxylation of sphingolipids. A genetic approach creating double mutants in ergosterol and sphingolipid biosynthesis showed that sphingolipids and sterols act together to carry out a variety of biological processes. Interestingly, one of these functions seem to be for function of the TOR signaling pathway as evidenced by the fact that cells were more sensitive to caffeine and rapamycin(92). Surprisingly, while the TORC1 complex is clearly the direct target of caffeine and rapamycin(93) the *erg2 scs7* double mutant showed reduced TORC2, but not TORC1 activity, confirming previous genetic evidence for an interaction between the two TORC signaling pathways(94). These results illustrate a very important point. Changes in sphingolipid and sterol content in cells can affect how they respond to drugs in unsuspected ways. Perhaps changes in lipid composition are part of explanation for the variability in response to and secondary effects of medications.

6. SYSTEMS APPROACH

Genetics screens, a technique in which different mutants have been presented with either drugs or stress conditions, in order to find pathway interactions has been a favorite tool of yeast researchers for many decades. This approach has only recently been developed for mammalian cells with the advent of siRNA, but the technical difficulties still remain much larger than for yeast genetic screens. Genetic screens in yeast have led to the discovery of most of the genes involved in sphingolipid metabolism (95). In the last few decades the advances made on more complex screening techniques and, especially, using the bioinformatics necessary for the analysis of large data sets have led to the appearance of new types of screens and systematic approaches. Again, the development of such techniques and their initial usage has the budding yeast as its first and most apt model organism. The complexity of networks makes it a challenge to work with system wide information in a single cell organism and it provides the possibility of transferring the discoveries to other organisms. Sphingolipids are primary candidates for such screens as much is already known about their interactions with proteins and different pathways, but the exact relationship of such pathways and the exact lipids that affect each one remain incompletely understood.

One such example is the systematic screen for lipid–protein interactions made by the Gavin lab (66). By using a series of lipid targets to bind proteins with putative lipid binding domains, they were able to uncover 530 lipid-protein associations, many of which had not been known before (66). A particular example has been discussed above concerning the Slm proteins. Of course the great challenge in a screen like this is separating real results that can be validated by traditional methods from the false positives. The fact that most of the interacting proteins have homologs in mammalian cells, allows researchers to use this material as a starting point to novel investigations on dysfunctional pathways in diseased cells.

Another way to evaluate the role of lipids at a genome scale is by looking at metabolites and their flux within the network. Mathematical modeling of the sphingolipid pathway is a promising approach (96). A few groups have started to address the issue and build more complex network maps that, with the help of computer power, allow for the prediction of disruption on the network. *S. cerevisiae* is again the primary target for the development of the technique before their optimization for multicellular organisms (97, 98).

The integration of genomic, metabolomic and lipomics data should allow for a broad view of the integration of such systems. The disruption of specific pathways have effects both downstream and upstream of the targets and localizing the precise metabolite that leads to a physiological change can be challenging. To address this problem one solution is the integration of data from genomics, metabolomics, lipidomics and functional annotation data(99). One example of a novel relationship that came from such analysis is the involvement of PHS-1P in the expression of the HAP genes, a family of cellular respiration genes (100). The validation of the informatics predicted interconnection validated the process that can know be used to uncover novel, unsuspected functions for sphingolipids species that were not thought to play a specific biological role.

7. CONCLUSION

The sphingolipid pathway is one of the best characterized pathways in yeast and its initial steps are very similar to the ones found in mammalian cells. This presents a unique opportunity for the understanding of lipid synthesis and for the search of potential inhibitors of specific enzymes. Selection of compounds that allow for drug therapy and also for the understanding the mechanism of action of different drugs can be useful to development treatments for a variety of diseases. For instance the tumor cell invasion inhibitor dihydromotuparimine C has been shown in yeast to act through the inhibition of the sphingolipid pathway(101). Another example is the off target effects of psychoactive drugs revealed by genome wide assays. Pimozide, an antipsychotic drug, was shown

to affect genes in the sphingolipid pathway and its signalling associated proteins, for example Ypk1p(102).

As in higher eucaryotes, in yeast complex signal transduction cascades are critical for sensing environmental changes and mediating appropriate cellular responses. Some of these signalling pathways are well conserved among eucaryotes and yeast offers a powerful system to study these pathways. Another part of these cascades are unique to fungi and could be used to combat fungal infections. As an example of the latter is the discovery of azaphilones, which are molecules that inhibit fatty acid synthases in yeast but not mammalian cells(103). All this leads to the belief that the yeast model is a valuable system for the understanding and development of pathways and tools that will help to better understand and intervene into the molecular mechanisms controlling health and disease.

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Abbreviations: GPI: glycosylphosphatidylinositol, IPC: inositol phosphorylceramide, MIPC: mannose inositol phosphorylceramide, $M(IP)_2C$: mannose (inositol phosphoryl)$_2$-ceramide, TORC: target of rapamycin complex, DHS: dihydroceramide, PHS: phytoceramide.

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