Biochemistry, Cell Biology and Molecular Biology of Lipids of Saccharomyces cerevisiae

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The yeast Saccharomyces cerevisiae is a powerful experimental system to study biochemical, cell biological and molecular biological aspects of lipid synthesis. Most but not all genes encoding enzymes involved in fatty acid, phospholipid, sterol or sphingolipid biosynthesis of this unicellular eukaryote have been cloned, and many gene products have been functionally characterized. Less information is available about genes and gene products governing the transport of lipids between organelles and within membranes, turnover and degradation of complex lipids, regulation of lipid biosynthesis, and linkage of lipid metabolism to other cellular processes. Here we summarize current knowledge about lipid biosynthetic pathways in S. cerevisiae and describe the characteristic features of the gene products involved. We focus on recent discoveries in these fields and address questions on the regulation of lipid synthesis, subcellular localization of lipid biosynthetic steps, cross-talk between organelles during lipid synthesis and subcellular distribution of lipids. Finally, we discuss distinct functions of certain key lipids and their possible roles in cellular processes. © 1998 John Wiley & Sons, Ltd.

KEY WORDS — yeast; S. cerevisiae; lipids; phospholipids; sterols; sphingolipids; fatty acids

CONTENTS

Introduction ........................................ 1471
Phospholipids .................................... 1472
Phospholipid synthesis .......................... 1473
A brief survey on the biosynthesis of fatty acids in yeast ............... 1480
Subcellular location of phospholipids, sites of synthesis and general aspects of phospholipid transport .......................... 1481
Sterols ............................................. 1482
Sterol biosynthesis: acetate to farnesyl pyrophosphate ............... 1482
Sterol biosynthesis in yeast: farnesyl pyrophosphate to ergosterol .......... 1485
Intracellular transport and location of sterol in yeast .................... 1489
Sphingolipids .................................... 1490
Sphingolipid biosynthesis pathway: genes, enzymes and phenotypes . . . . 1490
Cellular location, sites of synthesis and functions of sphingolipids .......... 1494
General conclusions and future perspectives .......................... 1495
Acknowledgements ................................ 1497
References ...................................... 1497

INTRODUCTION

Membranes of eukaryotic cells have several important functions. First, they serve as a diffusion barrier between the interior of the cell and its environment, and between the lumen of organelles and the cytosol. Second, membranes harbour proteins that catalyse selective transport of molecules or act as enzymes in metabolic and regulatory pathways. Third, membranes harbour receptors which contribute to recognition processes. Major lipid components of eukaryotic membranes are phospholipids, sterols, sphingolipids and glycerolipids. Some lipids are recognized as

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PHOSPHOLIPIDS

Phospholipids, which are regarded as a primary structural element of the biological membranes, consist of a glycerol backbone esterified with fatty acids in the sn-1 and sn-2 positions, and a phosphate group in the sn-3 position. One hydroxyl group of the phosphate is linked to a polar head group, which is relevant for classifying the various phospholipids and for the physical properties of these molecules. Fatty acids in the sn-1 position are mostly saturated whereas those in the sn-2 position are unsaturated. The fatty acid composition of S. cerevisiae phospholipids is fairly simple: the most abundant fatty acids are C-16:0 (palmitic acid), C-16:1 (palmitoleic acid), C-18:0 (stearic acid) and C-18:1 (oleic acid). Minor amounts of
C-14:0 (myristic acid) can be detected, and most recently C-26 fatty acids were recognized as typical minor components.226,227 Other fatty acids, however, may be present in minor quantities but still be of physiological importance. As an example, phospholipids containing C-26 fatty acid appear to be essential for nuclear function.226,227 Although phospholipids are indispensable as bulk components of yeast organelle membranes, it is not clear whether or not all individual classes of phospholipids are essential. One exception is phosphatidylinositol (PtdIns). Mutations in the PISI gene encoding PtdIns synthase (Figure 1) result in lethality.190 Defects in structural genes encoding enzymes involved in the biosynthesis of the aminoglycerophospholipids, phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho), cause phenotypic changes but are not lethal under laboratory conditions.

**Phospholipid synthesis**

The pathway of glycerophospholipid formation in yeast is shown in Figure 1. This scheme is divided into two sections representing the extra-
mitochondrial and the mitochondrial spaces. It is well accepted that the endoplasmic reticulum and mitochondria are the subcellular compartments that contribute most to yeast phospholipid biosynthesis (for a review, see ref. 54). Recently, it was recognized that other subcellular compartments, such as the Golgi and lipid particles contribute to phospholipid synthesis as well.\(^{137,268,308}\) Biosynthesis and intracellular migration of phospholipids are highly interconnected because extensive transfer of intermediates and cross-compartment coordination of partial reactions are required. The most prominent example in this respect is the sequence of steps in aminoglycerophospholipid synthesis.\(^{54,267,282,283}\) PtdSer, which is synthesized in the endoplasmic reticulum, has to be transferred to the mitochondria and/or a Golgi/vacuolar compartment to be decarboxylated to phosphatidylethanolamine (PtdEtn).\(^{268,269,270}\) Some of this PtdEtn migrates to the endoplasmic reticulum where methylation to phosphatidylcholine (PtdCho) occurs. Another requirement for balanced phospholipid biosynthesis is the supply of phosphatic acid (PA) to its sites of metabolic conversion. PA formed in the endoplasmic reticulum and lipid particles\(^{9}\) is required as a precursor for the synthesis of CDP-DAG, which appears to occur in the endoplasmic reticulum and mitochondria.\(^{129}\) Thus, the biosynthesis of phospholipids and their inter-organelle transport have to be linked and coordinated processes.

A general precursor of all glycerolipids is phosphatic acid (PA). The first step of PA synthesis in yeast is acylation of glycerol-3-phosphate (G-3-P) or dihydroxyacetone phosphate (DHAP), respectively.\(^{172}\) 1-Acyl-DHAP is then reduced to form 1-acylglycerol-3-phosphate (lysophosphatic acid; LPA),\(^{210}\) which is also the direct product of G-3-P acylation. In a second acyltransferase reaction, LPA is converted to PA. While much is known about further conversion of PA to more complex phospholipids, biosynthesis of PA itself is not as well understood. In S. cerevisiae, the highest specific activity of the enzyme(s) that catalyses the acylation of G-3-P was found in the lipid particle fraction.\(^{9,42,308}\) a compartment consisting of a hydrophobic core of triacylglycerols and steryl esters that is enveloped by a phospholipid monolayer with a small amount of embedded proteins.\(^{140}\) Recently, it was shown by subcellular analysis of acyltransferase mutants\(^{185,260}\) that lipid particles harbour two distinct acyltransferases.\(^{9}\) The first enzyme, a putative Gat1p that has neither been isolated nor characterized on the gene level, acylates G-3-P and forms LPA as an intermediate. The second enzyme encoded by the SLC1 gene (sphingolipid compensation)\(^{185}\) is a 1-acylglycerol-3-phosphate acyltransferase (AGAT) which completes the biosynthesis of PA. In an slc1 deletion strain, LPA accumulates. Both enzymes, however, were detected not only in lipid particles, but also in the endoplasmic reticulum.\(^{7}\) In addition to Gat1p and Slc1p, the microsomal fraction seems to contain another system of PA synthesis with at least one additional GAT and AGAT, respectively. This observation explains why mutations of GAT1 or SLC1 are not lethal. Redundancy of acylation systems may also be the reason for experimental difficulties in isolating acyltransferases and identifying their respective genes.

A second important aspect of PA synthesis in yeast is the use of G-3-P and/or DHAP as substrate for the first step of acylation. In animal cells, the DHAP pathway of PA biosynthesis is obligatory for ether lipid biosynthesis.\(^{278}\) The possible occurrence of ether lipids in yeast has not been unambiguously proven. Johnston and Paltauf\(^{110}\) demonstrated that yeast cells incorporate DHAP into PA. DHAPAT activity was detected in S. carlsbergensis\(^{110}\) and S. cerevisiae.\(^{222}\) Controversial results were presented regarding the question of whether glycerol-3-phosphate acetyltransferase (GAT) and dihydroxyacetone phosphate acyltransferase (AGAT) are different enzymes or whether one enzyme can utilize both substrates. Tillman and Bell\(^{260}\) suggested that acylation of the precursors G-3-P and DHAP is catalysed by the same protein. In contrast, Racenis et al.\(^{210}\) argued that GAT and DHAPAT activities might be attributed to different enzymes because of different pH optima and different degrees of sensitivity to inhibition by N-maleimide of the two enzyme activities. The latter findings, however, are also consistent with a single enzyme containing two active sites. Both GAT and DHAPAT activities were increased during respiratory growth, but the degree of increase in the levels of the two activities were different.\(^{172,210}\) Employing elaborate cell fractionation techniques,\(^{306}\) GAT and DHAPAT activities were measured in highly purified organelles (K. Athenaestad, personal communication). Using a gat1 mutant strain, it was shown that Gat1p present in lipid particles and the endoplasmic reticulum could accept both G-3-P and DHAP as substrates. Similarly, the additional acyltransferase(s) present in the endoplasmic

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reticulum\(^9\) can acylate both precursors. More specifically, however, yeast mitochondria harbour enzyme(s) that appear to prefer DHAP as a substrate for acylation (see Figure 1), suggesting that at least one additional independent DHAPAT is present in this organelle. Enzymatic activity of 1-acyl-DHAP reductase, which is required for the conversion of 1-acyl-DHAP to lysophosphatidic acid, was only detectable in lipid particles and the endoplasmic reticulum, but not in mitochondria. The subcellular distribution of enzymes involved in PA synthesis requires migration of acylation intermediates between organelles. Such migration may occur by free exchange between subcellular compartments, since PA, LPA and 1-acyl-DHAP are largely water-soluble.

A central metabolite in phospholipid biosynthesis is CDP-diacylglycerol (CDP-DAG). The yeast gene encoding CDP-diacylglycerol synthase, \(CDS1\), was identified by its homology to the corresponding \textit{Escherichia coli} and \textit{Drosophila} genes.\(^{58,236}\) Consistent with its central role in \textit{de novo} glycerolipid synthesis, deletion of \(CDS1\) is lethal, suggesting that \(CDS1\) encodes the only CDP-DAG synthase in yeast or supplies an essential subcellular pool of CDP-DAG. Cds1p, which has been purified to near homogeneity from yeast,\(^{115}\) converts PA to CDP-DAG in a CTP-dependent reaction (see Figure 1). A high level of CDP-DAG synthase activity favours synthesis of PtdIns over PtdSer.\(^{234}\) Cells expressing low levels of Cds1p excrete inositol into the medium.\(^{233}\) CDP-DAG synthase levels, and hence CDP-DAG levels, affect the transcription of the \(INO1\) and \(CHO1/\text{PSSI}\) genes independent of the expression of the regulator genes \(INO2\) and \(INO4\) or the inositol/choline levels in the medium. Reduction of the level of CDP-DAG synthase activity results in an increase of PtdIns, because the \(K_m\) of Pis1p for inositol is much higher than the intracellular inositol concentration.\(^{51}\) The increase of PtdIns occurs at the expense of PtdSer because both PtdIns synthase (Pis1p) and PtdSer synthase (Ps1p/Cho1p) compete for CDP-DAG, the common precursor.

Phosphatidylinositol (PtdIns) is a phospholipid that is essential for yeast. It is probably not the structural requirement for membrane assembly that makes PtdIns essential but rather its roles in cellular signalling and as a membrane sensor. PtdIns is synthesized from CDP-DAG and inositol (Figure 1). Inositol required for this enzymatic reaction can either be synthesized endogenously or taken up from the medium.\(^{232}\) Free endogenous inositol is derived from inositol-1-phosphate produced from inositol-1-phosphate synthase (Ino1p), which utilizes glucose-6-phosphate as a precursor (for a review, see ref. 152). Inositol uptake from the medium is actively catalysed by two transporters, Itr1p and Itr2p.\(^{152}\) Transcription of the \(ITR1\) gene depends on repression by inositol.\(^{131,191}\) When inositol is present in the medium the activity of Itr1p is reduced. The transcriptional regulation of \(ITR1\) depends on the \(INO2\), \(INO4\) and \(OPI3\) genes. In addition, repression of \(ITR1\) is regulated at the posttranscriptional level,\(^{132}\) namely by internalization of Itr1p through the endocytic pathway and degradation in the vacuole. In the stationary phase, degradation of Itr1p occurs also in inositol-free medium, indicating that at least two distinct mechanisms contribute to the posttranslational regulation of the level of the transporter.\(^{215}\) It is not known whether the \(ITR2\) gene is subject to the same control. Regulation of phospholipid biosynthetic gene expression requires inositol transport, since an \(itr1\) \(itr2\) double mutant is defective in repression of the \(INO1\) gene.\(^{131}\)

PtdIns synthase (Pis1p) has been purified from \textit{S. cerevisiae}.\(^{70}\) and the structural gene \(PIS1\) has been cloned.\(^{189,190}\) In yeast, PtdIns is essential since disruption of \(PIS1\) is lethal.\(^{190}\) Transcription of \(PIS1\) is not affected by the presence of inositol in the medium.\(^{71}\) Nevertheless, supplementation of yeast cells with inositol increases the cellular level of PtdIns, because the \(K_m\) of Pis1p for inositol is much higher than the intracellular inositol concentration.\(^{51}\) The increase of PtdIns occurs at the expense of PtdSer because both PtdIns synthase (Pis1p) and PtdSer synthase (Ps1p/Cho1p) compete for CDP-DAG, the common precursor.

One reason why PtdIns may be essential is because it is required for synthesis of GPI anchors. \textit{S. cerevisiae} contains numerous GPI-anchored

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proteins, but only two of them, Gas1p and α-agglutinin, have been studied in detail. In contrast to mammalian cells, GPI anchoring seems to be essential for yeast.145 Another reason for the essential role of PtdIns is that it serves as a substrate for the synthesis of inositol-containing sphingolipids (see below in this review).

A small portion of PtdIns is converted to polyphosphoinositides (Figure 1). Two yeast phosphatidylinositol 4-kinases, gene products of \( \text{PIK1} \) and \( \text{STT4} \), have been characterized by function. Pik1p is associated with the yeast nucleus and is essential for growth.72,78 Conditional mutants have a cytokinesis defect, and it was suggested that Pik1p might control cytokinesis through the actin cytoskeleton. \( \text{STT4} \) was originally discovered in a screen for sensitivity to staurosporin.302 Stt4p shows marked homology to protein kinase C (Pkc1p), and an interaction of the two gene products was proposed. Deletion of \( \text{STT4} \) is lethal under normal growth conditions, but Yoshida et al.302 reported that an \( \text{stt4} \) deletion defect can be rescued by high osmotic strength (1 M sorbitol). This effect, however, may be strain-dependent because Trotter et al.271 were not able to reproduce this result in a different genetic background. Whereas Yoshida et al.302 concluded that Stt4p may be involved in the Pkc1 protein kinase pathway, Trotter et al.271 demonstrated that, in an \( \text{stt4} \) mutant strain, the transport of PtdSer from the endoplasmic reticulum to a Golgi/vacuole fraction is disturbed (see below). Thus, the PtdIns branch of phospholipid biosynthesis indirectly affects the biosynthetic pathway of aminoglycerophospholipids.

The example of Stt4p, however, is not the only one that demonstrates the effect of PtdIns kinases on the intracellular trafficking of macromolecules. The \( \text{VPS34} \) gene, which is required for sorting of vacuolar proteins in yeast,95,352 encodes a phosphatidylinositol 3-kinase (Figure 1). The role of phosphatidylinositol 3-phosphate (PtdIns-3-P) formed by Vps34p in vesicle-mediated trafficking is still a matter of dispute. It was hypothesized that phosphorylated PtdIns may be important to maintain the appropriate curvature of transport vesicles.36 Another possible role for PtdIns-3-P is as a signal or a tag for vesicle docking on a target membrane.

### Table 1. Genes for \( S. \) cerevisiae phospholipid metabolism.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function of gene product</th>
<th>Key references</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{CCT1/PCT1} )</td>
<td>Cholinephosphate:CTP cytidyltransferase</td>
<td>164, 273</td>
</tr>
<tr>
<td>( \text{CDS1} )</td>
<td>CDP-diacylglycerol synthase (CTP:phosphatidic acid cytidyltransferase)</td>
<td>115, 236</td>
</tr>
<tr>
<td>( \text{CKI1} )</td>
<td>Choline kinase</td>
<td>101, 120, 164</td>
</tr>
<tr>
<td>( \text{CPT1} )</td>
<td>Cholinephosphate:CTP cytidyltransferase</td>
<td>164, 166</td>
</tr>
<tr>
<td>( \text{CRD1} )</td>
<td>Cardiolipin synthase</td>
<td>39, 109, 276</td>
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<tr>
<td>( \text{DPP1} )</td>
<td>Diacylglycerol pyrophosphatase</td>
<td>262</td>
</tr>
<tr>
<td>( \text{ECT1/MUQ1} )</td>
<td>Ethanolamine phosphate:CTP cytidyltransferase</td>
<td>170, 184</td>
</tr>
<tr>
<td>( \text{EPT1} )</td>
<td>CDP-ethanolamine.1,2-diacylglycerol ethanolamine phosphotransferase</td>
<td>97, 165, 167</td>
</tr>
<tr>
<td>( \text{GAT1*} )</td>
<td>Glycerol-3-phosphate acyltransferase</td>
<td>9, 260</td>
</tr>
<tr>
<td>( \text{GAT2*} )</td>
<td>Glycerol-3-phosphate acyltransferase</td>
<td>9</td>
</tr>
<tr>
<td>( \text{LPP1} )</td>
<td>Phosphatidate phosphatase</td>
<td>263</td>
</tr>
<tr>
<td>( \text{MSS4} )</td>
<td>Phosphatidylinositol-4-phosphate 5-kinase</td>
<td>51, 58, 98</td>
</tr>
<tr>
<td>( \text{PEMI/CHO2} )</td>
<td>Phosphatidylethanolamine ( N )-methyltransferase</td>
<td>124, 255</td>
</tr>
<tr>
<td>( \text{PEM2/OP13} )</td>
<td>Phospholipid ( N )-methyltransferase</td>
<td>162, 255</td>
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<tr>
<td>( \text{PGS1/PEL1} )</td>
<td>Phosphatidyglycerophosphate synthase</td>
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<tr>
<td>( \text{PIK1} )</td>
<td>Phosphatidylinositol-4-kinase</td>
<td>72, 78</td>
</tr>
<tr>
<td>( \text{PIS1} )</td>
<td>Phosphatidylinositol synthase</td>
<td>70, 190</td>
</tr>
<tr>
<td>( \text{PSD1} )</td>
<td>Phosphatidylerine decarboxylase (mitochondrial)</td>
<td>43, 269</td>
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<tr>
<td>( \text{PSD2} )</td>
<td>Phosphatidylerine decarboxylase (extramitochondrial)</td>
<td>268, 270</td>
</tr>
<tr>
<td>( \text{PSS1/CHO1} )</td>
<td>Phosphatidylerine synthase</td>
<td>12, 121, 248</td>
</tr>
<tr>
<td>( \text{SLC1} )</td>
<td>1-Acylglycerol-3-phosphate acyltransfer</td>
<td>9, 183</td>
</tr>
<tr>
<td>( \text{STT4} )</td>
<td>Phosphatidylinositol 4-kinase</td>
<td>271, 302</td>
</tr>
<tr>
<td>( \text{VPS34} )</td>
<td>Phosphatidylinositol-3-kinase</td>
<td>95, 252</td>
</tr>
</tbody>
</table>

*Uncharacterized genes.
Recently, Mss4p was identified as a phosphatidylinositol-4-phosphate 5-kinase \(^{51,58,98}\) by homology to the human enzyme. Mss4p is localized on the plasma membrane of \(S.\ cerevisiae\). The \(MS\)S4 gene is essential, and a temperature-sensitive \(ms\)s4 mutant was found to be defective in the organization of the actin cytoskeleton.

Besides the above-mentioned kinases, whose enzymatic activities have been functionally proven, a number of putative PtdIns kinases were identified by homology. The most promising candidate is Tor2p. \(^{223}\) Similar to Mss4p, Tor2p appears to be required for the organization of the actin cytoskeleton. Furthermore, biochemical evidence for the formation of phosphatidylinositol 3,5-bisphosphate in yeast has been presented, \(^{57}\) but the respective kinase has not yet been identified.

Phosphatidylserine (PtdSer) is only a minor component of total cell phospholipids, but an important intermediate in the de novo synthesis of the two main yeast phospholipids, PtdEtn and PtdCho. In contrast to mammalian PtdSer synthases, \(^{130}\) which form PtdSer through a base-exchange mechanism, yeast phosphatidylserine synthase, which is encoded by the \(PSS1/CHO1\) gene (for a review, see ref. 297), forms PtdSer from CDP-DAG and serine (Figure 1). PtdSer, by itself, appears to be non-essential and disruption of the \(PSS1/CHO1\) structural gene is not lethal when cells are grown in the presence of ethanolamine/choline. \(^{12}\) In \(pss1/cho1\) mutants, PtdEtn and PtdCho can be formed via the so-called Kennedy or CDP-ethanolaminecholine pathway (see below). A \(pss1/cho1\) null mutant has no detectable PtdSer, which suggests that there is only one copy of the \(PSS1/CHO1\) gene in yeast. \(^{21,248}\) Expression of the \(PSS1/CHO1\) gene is maximal in the absence of inositol and choline and is repressed several-fold by the presence of these two substances in the growth medium. \(^{13}\) Moreover, derepression of the \(PSS1/CHO1\) gene was found to be sensitive to mutations in the \(INO2\) and \(INO4\) activator genes and the \(OPI1\) repressor gene. \(^{14}\) Thus, \(PSS1/CHO1\) expression underlies the same response to inositol and choline supplementation as \(INO1\).

The \(Pss1p\) of yeast is located in the endoplasmic reticulum. Recently, it was recognized that a specific subfraction of the yeast endoplasmic reticulum called MAM (mitochondria-associated membrane) is highly enriched in \(Pss1p\). \(^{97}\) This finding appears to be important because the majority of cellular PtdSer is converted to PtdEtn, and the major site of PtdSer decarboxylation is the mitochondrion. \(^{129}\) Thus, juxtaposition of sites of synthesis and metabolic conversion of PtdSer may pre-determine its route of intracellular migration. Membrane association was suggested as a prerequisite for PtdSer transport from the endoplasmic reticulum to mitochondria. \(^{1,77,275}\) Most likely, surface proteins of the organelle(s) are involved in this process, although they have not yet been characterized at a molecular level. A second PtdSer decarboxylase present in the Golgi/vacuolar compartment \(^{268,270}\) also requires PtdSer from the endoplasmic reticulum. Recently, the first component involved in this translocation process was identified. This component is a PtdIns 4-kinase (see above), the \(STT4\) gene product. \(^{271}\) It is not clear at present if PtdIns-4-P formed by Stt4p acts as an intracellular signal required for the exit of PtdSer from the endoplasmic reticulum or if it is involved in formation of active transport vesicles as has been suggested for PtdIns-3-P formed by Vps34p.

The major route of phosphatidylethanolamine (PtdEtn) synthesis in yeast is the de novo pathway through decarboxylation of PtdSer (Figure 1). The majority of PtdSer decarboxylase (PSD) activity has been localized to the mitochondrial fraction. \(^{129}\) It came as a surprise that the function of the mitochondrial PSD, Psd1p, is dispensable for the cell\(^{13,269}\) even in the absence of ethanolamine, which is substrate for PtdEtn biosynthesis through the Kennedy pathway (see Figure 1). Inactivation of the \(PSD1\) structural gene had no effect on cell viability and little impact on the cellular lipid composition. Residual PSD activity in \(psd1\) deletion strains was attributed to Psd2p, which accounts for 5–10% of the cellular PSD activity in vitro. \(^{268,270}\) The intracellular location of Psd2p is not completely clear. A Golgi/vacuole compartment has been suggested to harbour Psd2p, and a Golgi targeting/retention signal similar to that found in the Golgi located Kex2p protease has been identified in Psd2p. Inactivation of both PSD enzymes results in a complete loss of cellular PSD activity and renders cells auxotrophic for ethanolamine. The relative roles of the two forms of PSD are not known. Whereas transcription of \(PSD1\) is under control of inositol and choline, \(^{43}\) no such regulation has been observed for \(PSD2.\) \(^{270}\) The response of the major cellular PSD activity attributable to \(PSD1\) is dependent on wild-type alleles of the \(INO2, INO4\) and \(OPI1\) regulatory genes, demonstrating that it is co-regulated with the other enzymes in the pathway. \(^{124}\)
An alternative route for the synthesis of PtdEtn in yeast is the Kennedy pathway originally described by Kennedy and Weis. This route is a salvage pathway for yeast cells which are either unable to synthesize PtdSer or lack PSD activity (see above), provided that sufficient ethanolamine/choline is present in the growth medium. Alternatively, ethanolamine can be provided by the action of phospholipases type D or by degradation of long-chain sphingoid base phosphates. Some of the genes involved in the ethanolamine branch of the Kennedy pathway (Figure 1) also catalyse reactions involved in the utilization of choline through the choline branch of this pathway. The first step of the CDP-ethanolamine pathway, phosphorylation of ethanolamine by choline kinase (Cki1p), is catalysed by such an enzyme with dual substrate specificity. Choline kinase, which exists as an oligomer in its native form, was recently purified to homogeneity from the cytosol. It is not known, at present, whether or not additional enzymes may catalyse phosphorylation of ethanolamine. Ethanolaminephosphate is then converted to CDP-ethanolamine by the ethanolaminephosphate cytidylyltransferase (Ect1p/Muq1p). In an ect1null deletion strain, conversion of ethanolamine phosphate to CDP-choline appears to be unaffected. Finally, PtdEtn is formed in the endoplasmic reticulum by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (Ept1p). Although Ept1p can utilize CDP-ethanolamine, CDP-monomethylethanolamine, CDP-dimethylethanolamine, and CDP-choline as substrates in vitro, the almost exclusive substrate in vivo is CDP-ethanolamine.

Similar to the utilization of free ethanolamine, yeast cells can incorporate choline into PtdCho through the Kennedy pathway (see Figure 1). This pathway is not only active when exogenous choline is present but also functions continuously to recycle degradation products of PtdCho. The enzymes involved in the choline branch of this salvage pathway are choline kinase (Cki1p), cholinephosphate cytidylyltransferase (Pct1p/Cct1p) and CDP-choline:1,2-diacylglycerol cholinephosphotransferase (Cpt1p). As mentioned above, Cki1p can phosphorylate both choline and ethanolamine. At high choline concentrations, the conversion of choline to cholinephosphate becomes the rate-limiting step in this pathway. Cholinephosphate cytidylyltransferase (Cct1p/Pct1p) can utilize cholinephosphate and N,N-dimethylethanolaminephosphate in vitro, but N-methylethanolaminephosphate and ethanolaminephosphate are poor substrates. Most of the activity of Cct1p/Pct1p in yeast cells is associated with membranes, suggesting that yeast cells possess a mechanism that promotes membrane association of the cytidylyltransferase, perhaps similar to that in mammalian cells. Under choline limitation, cholinephosphate cytidylyltransferase activity becomes rate-limiting for the biosynthesis of PtdCho through the Kennedy pathway. Expression of CCT1/PCT1 is not dramatically affected by inositol and choline. The ultimate enzymatic step in the Kennedy pathway for the genesis of de novo synthesized PtdCho is catalysed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase. Genetic approaches revealed that this enzyme activity can be attributed to two proteins, namely to the gene product of CPT1 and to CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase. Ept1p. Whereas cells disrupted either in CPT1 or EPT1 retain approximately 50% of measurable cholinephosphotransferase activity in vitro, Cpt1p contributes 95% of PtdCho synthesis through the Kennedy pathway in vivo. Expression of CPT1 underlies an inositol-induced mechanism of transcriptional regulation.

As mentioned above, expression of the CKII and CPT1 genes is regulated at the transcriptional level by inositol, whereas expression of CCT1 is only affected moderately by choline or a combination of choline and inositol. Another regulatory mechanism of the Kennedy pathway appears to occur through the action of Sec14p, which is the yeast PtdIns/PtdCho transfer protein. SEC14 was originally discovered as a gene whose product is involved in protein secretion and acts at the level of Golgi function. The lethality of a defect in the SEC14 gene is suppressed in second-site mutations that block PtdCho biosynthesis through the Kennedy pathway. This observation, and the fact that Sec14p inhibits cholinephosphate cytidylyltransferase activity in vitro, suggested that Sec14p might regulate the PtdIns/PtdCho ratio of Golgi membranes. Strains carrying mutations in the CDP-choline pathway, such as cki1, in combination with a temperature-sensitive sec14 mutation, exhibit a dramatically increased choline secretion phenotype. Thus, Sec14p appears to be involved in formation of phosphatidic acid (PA) through an interplay with phospholipase D1 (Pld1p)-catalysed degradation of PtdCho.

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Alternatively, it was proposed that Sec14p may be required to maintain a sufficient pool of diacylglycerol in the Golgi to support the production of secretory vesicles.14

In the absence of exogenous choline, PtdCho is synthesized primarily by a three-step methylation of PtdEtn (Figure 1). These reactions are catalysed by two independent methyltransferases, PtdEtn methyltransferase (Pem1p/Cho2p) and phospholipid methyltransferase (Pem2p/Opi3p), which are localized to the endoplasmic reticulum.112,123 Mutations in the PEL1 gene cause a dramatically decreased level of PtdCho and lead to an accumulation of PtdEtn.124,255 In a pem1 cho2 deletion strain, the activity of PtdEtn methyltransferase activity is greatly reduced but the ability of Pem2p/Opi2p to catalyse the first methylation of PtdEtn allows formation of some PtdCho, albeit somewhat inefficiently.255 When the PEM2/OPI3 locus was deleted, phospholipid methyltransferase activity was completely lost and phosphatidylmonomethylethanolamine accumulated. Since Pem2p/Opi3p can replace Pem1p/Cho2p to a certain extent, and mono- and dimethylated PtdEtn appear to replace PtdCho sufficiently, single mutations of pem1 cho2 and pem2 oip3 were found not to be auxotrophic for choline.162,255 Opposite results regarding the choline auxotrophy124,298 may have been the result of different strain backgrounds. The double mutant pem1 cho2 pem2 oip3, however, is clearly auxotrophic for choline.255 Both PEM1/CHO2 and PEM3/OPI3 are members of the group of genes regulated by inositol.125

Under standard growth conditions, phosphatidylglycerol (PtdGro) is a minor phospholipid component of yeast subcellular membranes. Cardiolipin (diphosphatidylglycerol; CL) comprises 10–15% of mitochondrial phospholipids, especially when mitochondria are fully developed in the presence of non-fermentable carbon sources. PtdGro and CL are closely related biosynthetically (Figure 1). First, phosphatidylglycerophosphate (PtdGro-P) is synthesized from CDP-DAG and glycerol-3-phosphate by the action of phosphatidylglycerophosphate synthase (for a review, see ref. 171). The PGS1 gene encoding this enzyme has only recently been identified.38 Surprisingly, the same gene (named PEL1 at that time) has been studied before and was thought to encode a minor PtdSer synthase activity in yeast.104,105,106 Strains mutated in PEL1 were isolated as petite lethals; they exhibited a temperature-sensitive growth phenotype on glucose, were not able to grow on synthetic medium with a non-fermentable carbon source, and had very low levels of cytochrome c oxidase. These properties were confirmed recently with well-defined pgs1 deletion strains that were shown to lack PtdGro and CL.38 These results demonstrated that the PGS1/PEL1 gene encodes the major, or even the only, PtdGro-P synthase of yeast, and that neither PtdGro nor CL is absolutely essential for cell viability. PtdGro-P synthase activity is stimulated by factors affecting mitochondrial development19 and regulated through cross-pathway control by inositol.23,25

The next reaction in CL synthesis is dephosphorylation of PtdGro-P yielding PtdGro. The gene encoding PtdGro-P phosphatase has not yet been cloned, neither has the gene product been characterized. As the ultimate step in this biosynthetic sequence, CL is formed by reaction of PtdGro with a second molecule of CDP-DAG through catalysis performed by CL synthase.221 The gene encoding CL synthase, CRD1 (formerly named CLS1), was recently identified.39,276 Crd1p was shown to form an oligomeric complex whose biogenesis and/or activity is influenced by the assembly of cytochrome c oxidase.304 Strains deleted of CRD1 are able to grow on all carbon sources, although the growth rate on non-fermentable carbon sources is decreased. The lack of CL synthase activity in crd1 mutants results in a lack of detectable CL and accumulation of PtdGro when cells are cultivated aerobically on non-fermentable carbon sources. Levels and activities of cytochromes are normal in crd139,276 and the mitochondrial membrane potential is not grossly compromised. Thus, CL is not essential for mitochondrial function and can, at least in part, be replaced by PtdGro-P as an oxidase.304

CL and PtdGro are typical mitochondrial phospholipids, although yeast peroxisomes were shown to contain amounts of CL that cannot be attributed to cross-contamination with mitochondria.306,308 CL synthase and PtdGro-P synthase activity are mainly present in the mitochondrial inner membrane but some of the latter enzymatic activity was also localized to microsomal membranes.129,308

Although it is beyond the scope of this review to discuss degradation and turnover of glycerophospholipids in detail, the role of diacylglycerol (DAG) has to be briefly addressed. In principle, DAG may be derived from degradation of phospholipids by catalysis of phospholipases type C.
One such protein, Plc1p, was characterized as a phosphatidylinositol-specific phospholipase C. Alternatively, phosphatidate phosphatase (Lpp1p) and diacylglycerol pyrophosphatase (Dpp1p) appear to be involved in DAG formation. Diacylglycerol pyrophosphate (DGPP) was recently detected in minor quantities in yeast. It was suggested that this lipid is involved in a lipid signalling pathway. Dpp1p catalyses both the diphosphorylation of DGPP, yielding phosphatidic acid (PA), and the dephosphorylation of PA, yielding DAG. The PA phosphatase activity of Dpp1p is not the only one in yeast, since a mutant with a \textit{DPP1} deletion contains residual PA phosphatase activity but lacks DGPP phosphatase activity. Recently, the \textit{LPP1} gene was shown to encode another PA phosphatase distinct from Dpp1p. Lpp1p also exhibits lyso-PA and DGPP phosphatase activities. Strains with deletions in \textit{DPP1}, \textit{LPP1} and the \textit{dpp1 lpp1} double mutant are viable and do not exhibit any obvious growth defects.

DAG is also the substrate for the synthesis of triacylglycerol (TAG), which is a storage form of fatty acids in lipid particles of \textit{S. cerevisiae}. TAG can be used as a source of fatty acids when endogenous fatty acid synthesis is blocked, e.g. in the presence of the inhibitor cerulenin. The fatty acids derived from TAG are not used as a primary energy source in yeast, unlike in mammalian cells, because \textit{S. cerevisiae} has no mitochondrial \(\beta\)-oxidation, and peroxisomal \(\beta\)-oxidation induced by growth of cells on oleic acid is not very active, although it is sufficient to maintain growth. In contrast, fatty acids set free from TAG are incorporated into phospholipids that are required for membrane formation. Little is known about TAG synthase in yeast. Some activity of this enzyme has been attributed to lipid particles, but more recently the microsomal fraction was shown to contain the highest TAG synthase activity (K. Athenstaedt, personal communication). The gene encoding TAG synthase has not yet been identified, neither has the enzyme been isolated.

\textbf{A brief survey on the biosynthesis of fatty acids in yeast}

The fatty acid composition can greatly influence the physical state of phospholipids and thereby affect membrane fluidity and permeability. The fatty acid pattern of yeast phospholipids appears to be fairly simple. The major species are C-16 and C-18 fatty acids without or with one double bond. Other fatty acids, present in minor amounts in yeast, may be of physiological importance but they have not been systematically analysed. Genes involved in the formation of fatty acids in yeast (Table 2) are \textit{ACCI}, encoding acetyl-CoA carboxylase; \textit{FAH1/SCS7}, encoding the \(\beta\)- and \(\alpha\)-subunits of the fatty acid synthase complex; and \textit{FAS1} and \textit{FAS2}, encoding the \(\beta\)- and \(\alpha\)-subunits of the fatty acid synthase complex.

\begin{table}
\centering
\caption{Genes for \textit{S. cerevisiae} fatty acid metabolism.}
\begin{tabular}{lll}
\hline
\textbf{Gene} & \textbf{Function of gene product} & \textbf{Key references} \\
\hline
\textit{ACBI} & Acyl-CoA binding protein & 220 \\
\textit{ACCI} & Acetyl-CoA carboxylase & 2, 91 \\
\textit{ACP1} & Mitochondrial acyl carrier protein & 31, 225 \\
\textit{CEMI} & Mitochondrial beta-ketoacyl-[acyl-carrier protein] synthase & 89, 224 \\
\textit{ELOI} & Fatty acid elongase (long-chain fatty acids) & 64, 261 \\
\textit{ELO2/FEN1} & Fatty acid elongase (synthesis of C-24 fatty acids) & 194 \\
\textit{ELO3/SUR4} & Fatty acid elongase (conversion of C-24 to C-26 fatty acids) & 194 \\
\textit{FAH1/SCS7} & Hydroxylation of the C-26 fatty acid in ceramide & 85, 174 \\
\textit{FAS1} & Fatty acid synthase (beta subunit) & 41, 229 \\
\textit{FAS2} & Fatty acid synthase (alpha subunit) & 176, 229 \\
\textit{MCTI} & Malonyl CoA:acyl carrier protein transferase & 224 \\
\textit{OARI} & Mitochondrial 3-oxoacyl-[acyl-carrier-protein] reductase & 224 \\
\textit{OLE1/MDM2} & Fatty acid desaturase & 173, 253 \\
\textit{SUR2/SYR2} & Hydroxylation of sphinganine or sphinganine-containing dihydroceramides at the C-4 position to yield phytoceramide & 84, 85 \\
\hline
\end{tabular}
\end{table}

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Mutations of \textit{FAS1} and \textit{FAS2} are lethal unless mutants are supplemented with fatty acids.\textsuperscript{194} \textit{ACCI} mutations are lethal even in the presence of fatty acids in the culture medium.\textsuperscript{291} The existence of a second, mitochondrial, fatty acid-synthesizing system has been reported recently. Four genes possibly encoding for components of this machinery, \textit{ACP1, CEM1, OAR1} and \textit{MCT1}, have been described.\textsuperscript{31,89,90,224,225} Deletion of each gene causes a dramatic decrease in the lipoic acid content of cells and a respiratory-deficient phenotype.

Pre-existing fatty acid in yeast can be modified by desaturation, elongation and hydroxylation. Mono-unsaturation of fatty acids is catalysed by a single essential desaturase, Ole1p.\textsuperscript{173,253} The encoding gene, \textit{OLE1/MDM2}, has been demonstrated to be also involved in partitioning of mitochondria between mother and daughter cells. The \textit{ole1/mdm2} mutants are viable when fed unsaturated fatty acids. Elongation of fatty acids is accomplished by introducing malonyl-CoA as a C-2 unit into pre-existing fatty acids.\textsuperscript{64} This reaction is catalysed by the product of the \textit{ELO1} gene.\textsuperscript{64,261} Strains deleted of \textit{ELO1} cannot grow on media containing C-14:0 as the only fatty acid, but are rescued by adding C-16:0 and C-18:0 fatty acids to the media. Subsequently, two genes structurally related to \textit{ELO1}, namely \textit{ELO2} and \textit{ELO3}, were identified which encode components of the very long chain (C-24 and C-26) fatty acid elongation system.\textsuperscript{194} The major hydroxylated fatty acid in \textit{S. cerevisiae} is the C-26 species, which is required for sphingolipid biosynthesis (see this review article). Alpha-hydroxylation of fatty acids is catalysed by Scs7p,\textsuperscript{85,174} and long chain base hydroxylation at C-4 by Sur2p.\textsuperscript{84,85}

\textit{Subcellular location of phospholipids, sites of synthesis and general aspects of phospholipid transport}

The major phospholipids, PtdCho, PtdEtn and PtdIns, are present in all subcellular membranes of \textit{S. cerevisiae}, although in variable amounts. Two exceptions are PtdSer and CL, which are present only at low concentrations in most membranes, but are major phospholipid components of the plasma membrane and the inner mitochondrial membrane, respectively (for a review see ref. 306).

Classically, the endoplasmic reticulum has been thought of as the major site of lipid biosynthesis, with minor contributions from the mitochondria. This view needs revision. Besides these two compartments, the Golgi and lipid particles are now recognized to harbour certain enzymes for yeast phospholipid formation (see above). The question remains, however, how phospholipids are transported from their site(s) of synthesis to their proper subcellular location. It is not only the supply of the quantitatively predominant phospholipids (PtdCho, PtdEtn and PtdIns) to all organelles which necessitates efficient mechanisms of intracellular lipid transport, but also the migration of precursor phospholipids to their site of metabolic conversion that is essential for the balanced synthesis of cellular phospholipids. The example of PtdSer and PtdEtn translocation between organelles has already been mentioned above.

Phospholipids appear to be transported within a cell by vesicle flow, membrane contact and monomer transport, with the aid of so-called lipid transfer proteins (for recent reviews, see refs 267, 280, 282). Whereas there is no proof at present for the contribution of lipid-transfer proteins to the subcellular distribution of lipids in \textit{vivo}, experimental efforts have tried to distinguish between vesicle flow and membrane contact as mechanisms of lipid translocation. In the past, most of these studies have focused on the intracellular movement of aminoglycerophospholipids (see ref. 54), because translocation of PtdSer and PtdEtn between organelles (endoplasmic reticulum and mitochondria) can be studied by metabolic conversion assays. Decarboxylation of PtdSer to PtdEtn by mitochondrial Ptd1p provides such a means of following the import of PtdSer into mitochondria \textit{in vivo} or \textit{in vitro}. Most experimental evidence favours membrane contact as the mechanism of transport of PtdSer from MAM, a specialized subfraction of the endoplasmic reticulum and mitochondria.\textsuperscript{1,54,77} As was shown for mammalian MAM,\textsuperscript{238} proteolytic treatment of yeast mitochondria negatively affected translocation of PtdSer.\textsuperscript{275} Fusogenic proteins similar to those described for mammalian cells by Corazzi’s group\textsuperscript{73,211} and which are partially inactivated by exogenous proteases, may be involved in this process. None of the putative components, however, has been characterized on a molecular level, neither have the respective genes been identified. The first gene product involved in PtdSer transport from the endoplasmic reticulum to a Golgi/vacuole compartment (the PtdIns-4-kinase, Stt4p) has been described recently.\textsuperscript{271} The mechanism of PtdSer translocation between these two compartments, however, may not involve membrane contact.
Transport of phospholipids to the plasma membrane of yeast is even less well understood. While it has become clear that sphingolipids utilize the protein secretory machinery\(^9\) to reach their destination at the cell periphery, no such evidence has been presented for phospholipids. Rather, preliminary evidence\(^80\) has indicated that, in temperature-sensitive secretory mutants (sec mutants), PtdCho and PtdIns are transported to the plasma membrane at a normal rate. This result is in line with the hypothesis of Atkinson\(^10\) that different types of vesicles are responsible for protein export and membrane growth. The existence of specific phospholipid transporting vesicles, however, still needs to be verified experimentally.

**STEROLS**

Sterols are essential lipid components of eukaryotic membranes and have been shown to be responsible for a number of important physical characteristics of membranes. The specific sterol present in eukaryotic species can vary and there have been many studies using a variety of biochemical and biophysical techniques to demonstrate that sterols are important regulators of membrane permeability and fluidity. Although other membrane lipids also play a role in defining these properties, eukaryotic cells are unable to maintain viability without sterol. More recently, preliminary studies have described some physiological properties and effects of sterols on aerobic metabolism,\(^244,245\) completion of the cell cycle,\(^72\) sterol uptake\(^130\) and sterol transport.\(^274\)

The structural features of sterols derived from eukaryotes vary in plants, animals and fungi, as shown in Figure 2. The fungal sterol, ergosterol, differs from the animal sterol, cholesterol, by the presence of unsaturations at C-7,8 in the ring structure and at C-22 in the side chain and by the presence of a methyl group at C-24 on the side chain. The major plant sterols, sitosterol and stigmasterol, have ring structures identical to that found in cholesterol but have slight modifications of the side chain. Common to all sterols is the saturation at C-5,6 and the presence of the hydroxyl group at C-3. The latter provides the only hydrophilic component of the molecule and allows for the proper orientation of the sterol molecule in the membrane.

Much of the work in defining the role of sterol in eukaryotic membranes has been done using the yeast model system. Ergosterol mutants have been used to document the effects of sterol substitution on membrane fluidity\(^142\) and membrane permeability.\(^20,122\) Membrane sterol modification in yeast has also been shown to affect energy source utilization\(^144\) and the activity of membrane-bound ATPase.\(^76\) In addition to providing these bulk functions for membranes, sterols have been implicated in providing a ‘sparking’ function\(^52\) involved in the completion of the cell cycle. Unlike the bulk functions, which can be provided by a number of sterols, the sparking function is conferred by the presence of a specific sterol structure and is required in nanomolar quantities. Recent reports\(^244,245\) have indicated that the sparking function may be related to the presence or absence of heme. In addition, cloning and gene disruption procedures have contributed significantly in defining essential features of fungal membrane sterol.\(^43\)

*S. cerevisiae* has also served as a model system for defining aspects of sterol biosynthesis which have application for the development and evaluation of antifungal compounds. The sterol biosynthetic pathways or its end product are the targets for most of the antifungal compounds currently in use in agricultural settings and in combating human infections. The polyene antifungals, such as nystatin and amphotericin B, function by binding to membrane ergosterol and inducing cellular leakage, resulting in cell death.\(^30\) Other groups of antifungals, including the allylamines,\(^217\) morpholines\(^15\) and azoles,\(^279\) inhibit specific steps in ergosterol biosynthesis and will be discussed in more detail later in this review. All of these compounds have limitations as to their application to human infection or have seen the appearance of significant levels of resistance in several pathogenic fungi.\(^57,92,205,219,281,291\) A recent review has outlined factors which have led to this increased resistance.\(^293\) In order to identify new possible target sites in the ergosterol pathway, molecular studies of ergosterol biosynthesis have been employed to identify essential steps in the pathway against which antifungals might be developed.

**Sterol biosynthesis: acetate to farnesyl pyrophosphate**

Sterol biosynthesis is a major metabolic commitment on the part of the cell and involves over 20 distinct reactions. The pathway is initiated with acetyl-CoA, as depicted in Figure 3, which
presents an abbreviated version of what is commonly called the mevalonate or isoprenoid pathway. This portion of the pathway ends with the formation of farnesyl pyrophosphate, a pivotal intermediate which is the starting point for several essential pathways. The syntheses of heme, quinones, and dolichols involve the participation of farnesyl components derived from this pathway. In addition, the farnesyl units and the related geranyl and geranylgeranyl species are important elements for the posttranslational modification of proteins that require hydrophobic membrane

![Ergosterol and Cholesterol](image1)

![Sitosterol and Stigmasterol](image2)

Figure 2. The principal membrane sterols in fungi (ergosterol), animal cells (cholesterol), and higher plants (sitosterol and stigmasterol).
anchors for proper placement and function. Among such proteins are the ras proteins. Thus, farnesyl pyrophosphate leads to the synthesis of several critical end products, including sterols. For this reason, mutations in the pathway to this point are lethal, since multiple essential metabolic products cannot be synthesized.

The first step in the pathway, leading ultimately to the production of ergosterol, is the synthesis of acetoacetyl-CoA from two molecules of acetyl-CoA, a reaction catalysed by acetoacetyl-CoA thiolase, encoded by the ERG10 gene. Yeast contains two forms of the enzyme, one of which is found in the mitochondrion, while the second is located in the cytosol. This step in yeast, unlike that in animal cells, is subject to regulation. Early studies described decreased enzyme activity upon the addition of exogenous sterol and increased activity resulting from ergosterol starvation. These studies, however, could not clearly distinguish between transcriptional and translational mechanisms to explain the regulatory phenomena. Subsequent work showed that ERG10 was induced by low levels of sterol and that specific promoter regions regulate gene expression. In a more recent report using the ERG10 promoter fused to a reporter, additional evidence was presented which suggests that regulation occurs at the transcriptional level and is mediated by pathway intermediates.

The conversion of acetoacetyl-CoA to hydroxymethylglutaryl-CoA (HMGCoA) by the ERG13 gene product, HMGCoA synthase in yeast, deviates from that described in animal systems in that the enzyme appears to be subject to regulatory control. The enzyme is a multimer possibly comprised of two gene products, although only one gene is found in the yeast database for the production of this enzyme. The specifics of the regulatory mechanism involved remain uncharacterized.

The third enzyme in the pathway, HMGCoA reductase, is the most studied step in the mevalonate pathway. It is the major site of regulation in cholesterol synthesis and the target for several compounds that are employed clinically as cholesterol-lowering agents. The yeast enzyme is also subject to regulation by a number of factors and conditions. Unlike humans, yeast has two copies of the gene encoding HMGCoA reductase. This was discovered upon the disruption of one gene, HMG1, which resulted in a viable cell. This unanticipated result was explained by the identification of a second gene, HMG2. Disruption of both genes renders the cell non-viable, as would be predicted. Yeast HMGCoA reductase activity shows feedback inhibition in the presence of ergosterol and is subject to catabolite repression. In addition, HMGCoA reductase activity was found to be induced by unsaturated fatty acids and glucose. However, these studies were conducted before the demonstration of two isoforms of the enzyme. Thorsness et al. have found that HMG1 expression was stimulated by heme while HMG2 expression was inhibited, again indicating a relationship between heme and sterol biosynthesis.

Step 4 in the pathway is catalysed by mevalonate kinase, which is encoded by the ERG12 gene. The yeast enzyme is subject to inhibition by certain downstream intermediates. Farnesyl and geranyl pyrophosphates exert an inhibitory influence on the enzyme, while isopentenyl and dimethylallyl pyrophosphates do not. Step 5 also involves phosphorylation. The ERG8 gene encodes...
phosphomevalonate kinase, an enzyme which is not subject to feedback inhibition by ergosterol.

Steps 6 and 7 of the pathway are largely uncharacterized in terms of the properties of the enzymes involved. The ERG19 gene encodes the mevalonate pyrophosphate decarboxylase, which converts mevalonate pyrophosphate to isopentenyl pyrophosphate (IPP). Yeast erg19 mutants were first isolated in a broad screen which selected for temperature-sensitive, nystatin-resistant isolates. Among the many possibilities were erg19 mutants, which were identified based on sterol intermediates that accumulated and enzyme assays specific for each possible step. The yeast ERG19 gene has been cloned using sequence data obtained from the human version of the gene. Overexpression of ERG19 has been shown to result in a reduction of sterol accumulation, suggesting that the enzyme is rate-limiting and that downstream intermediates may accumulate and function as feedback inhibitors at an early pathway step. The subsequent step converts IPP to dimethylallyl pyrophosphate (DMAPP) through the action of isopentenyl diphosphate isomerase, a product of the IDI1 gene. Although the IDI1 gene has been cloned, little is known about the properties of the enzyme and its regulation.

The final step in the early portion of the pathway is the conversion of DMAPP to farnesyl and geranyl pyrophosphates. This step is accomplished by the action of farnesyl (geranyl) pyrophosphate synthase, a product of the ERG20 gene. The enzyme combines DMAPP and IPP to form geranyl pyrophosphate (GPP). The same enzyme is then able to extend geranyl pyrophosphate by combining it with a second IPP to form farnesyl pyrophosphate (FPP). This brings the pathway to the pivotal molecule, which is the substrate for several enzymes which initiate several metabolically important pathways. Overexpression of the ERG20 gene has been shown to result in increased levels of enzyme activity and ergosterol production, indicating that this step may be rate-limiting.

Sterol biosynthesis in yeast: farnesyl pyrophosphate to ergosterol

The conversion of farnesyl pyrophosphate to the end-product, sterol, represents an 11-step pathway dedicated to ergosterol biosynthesis (Figure 4). Mutations in any step up to this point are lethal because of a failure to make multiple essential end products. Mutations in the last eight steps of this pathway segment are lethal only if the intermediate formed cannot substitute functionally for ergosterol. Since yeasts require sterol, one would predict that the first three steps (formation of squalene to lanosterol) would be essential, since no sterol molecule is synthesized to this point. This turns out to be the case. In addition, one would predict that regulatory phenomena specific to sterol levels would be found in this portion of the pathway.
since no other products are formed from the intermediates. There are a few well-characterized points of regulation, although the pathway remains largely unexplored in this regard.

Squalene synthase, a product of the ERG9 gene, combines two FPP molecules to form squalene. As the first enzyme in a linear pathway, this step would be expected to be an ideal place for regulation.\(^{108}\) The enzyme competes with other enzymes for the FPP substrate and has been shown to respond to cellular sterol content in a manner similar to that shown by HMGCoA reductase.\(^{214}\) Studies of this type present specific limitations, since yeast synthesize sterol only under aerobic conditions and are unable to import sterol under these conditions. Thus, sterol depletion in aerobic cells is possible only with the use of inhibitors or by using mutant strains. Using the latter approach, M’Baya et al.\(^{151}\) showed that early (pre-mevalonate) pathway erg mutants induced ERG9 activity by 1.5 to 5-fold, suggesting that mevalonate may play a key role in the regulatory process. In the same study, excess sterol added exogenously to anaerobically growing cells resulted in the depression of squalene synthase activity. These preliminary studies indicate that this branch enzyme is subject to regulation, but much remains to be done to define the control mechanisms at the molecular level.

The ERG1 gene encodes squalene epoxidase (monooxygenase), which converts squalene to 2,3-oxidosqualene. This oxygen-requiring step\(^{102}\) precludes sterol synthesis in anaerobic conditions, thus requiring the addition of exogenous sterol for growth. This step is inhibited by the allylamine class\(^{217}\) of antifungals and by thiocarbamates.\(^{178}\) In fact, the ERG1 gene in yeast was cloned by using a yeast library constructed from a strain that was resistant to the allylamine, terbinafine.\(^{103}\) Employing the same conditions as described above for squalene synthase, squalene epoxidase activity increased in all erg mutants grown aerobically, except erg11.\(^{151}\) The effects of excess sterol could not be determined under anaerobic conditions, since the enzyme requires oxygen.

The third step in the conversion of FPP to ergosterol represents an impressive single-step conversion of 2,3-oxidosqualene to lanosterol, the first sterol molecule of the pathway. 2,3-Oxidosqualene cyclase or lanosterol synthase, the product of the

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**Table 3. Genes involved of the biosynthesis of ergosterol in yeast.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function of gene product</th>
<th>Key references</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERG1</td>
<td>Squalene epoxidase</td>
<td>102, 103</td>
</tr>
<tr>
<td>ERG2</td>
<td>Sterol C-8 isomerase</td>
<td>6, 8</td>
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<tr>
<td>ERG3</td>
<td>Sterol C-5 desaturase</td>
<td>7, 149, 216</td>
</tr>
<tr>
<td>ERG4</td>
<td>Sterol C-24 reductase</td>
<td>40, 133</td>
</tr>
<tr>
<td>ERG5</td>
<td>Sterol C-22 desaturase</td>
<td>241</td>
</tr>
<tr>
<td>ERG6</td>
<td>Sterol C-24 methyltransferase</td>
<td>17, 75, 177</td>
</tr>
<tr>
<td>ERG7</td>
<td>Lanosterol synthase</td>
<td>50, 237</td>
</tr>
<tr>
<td>ERG8</td>
<td>Phosphomevalonate kinase</td>
<td>272</td>
</tr>
<tr>
<td>ERG9</td>
<td>Squalene synthase</td>
<td>108</td>
</tr>
<tr>
<td>ERG10</td>
<td>Acetoacetyl-CoA thiolase</td>
<td>127, 128</td>
</tr>
<tr>
<td>ERG11</td>
<td>Lanosterol C-14 demethylase</td>
<td>111, 258, 266</td>
</tr>
<tr>
<td>ERG12</td>
<td>Mevalonate kinase</td>
<td>66</td>
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<tr>
<td>ERG13</td>
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<td>113, 231</td>
</tr>
<tr>
<td>ERG20</td>
<td>Farnesyl pyrophosphate synthase</td>
<td>4, 37</td>
</tr>
<tr>
<td>ERG27</td>
<td>Geranyl pyrophosphate synthase</td>
<td>4, 37</td>
</tr>
<tr>
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<td>Sterol C-14 reductase</td>
<td>133, 148, 158</td>
</tr>
<tr>
<td>ERG25</td>
<td>Sterol C-4 methyloxidase</td>
<td>19, 74</td>
</tr>
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<td>ERG26</td>
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<td>Gachotte et al. (pers. comm.)</td>
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<td>ERG(?)*</td>
<td>Sterol C-3 keto reductase</td>
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<tr>
<td>IDI1</td>
<td>Isopentenyl pyrophosphate isomerase</td>
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</table>

*The gene for the sterol C-3 keto reductase has yet to be isolated and characterized.
ERG7 gene, performs several ring closures and bond cleavages in this very complex reaction.50 The ERG7 gene from yeast has been cloned by two laboratories50,237 which should allow for characterization of this gene and the encoded enzyme.

The pathway has now reached the point where a sterol molecule is present and the question to be asked from here to the end of the pathway is whether the intermediate produced at each step is able to meet both the bulk and sparking requirements and thus permit growth. One of the characteristics of this part of the pathway is that blocks in most steps do not prevent subsequent enzymes from altering the improperly modified substrates. Thus, blocks lead to the accumulation of several sterol structures that are not normal intermediates of the pathway.

Mutants blocked in the terminal five steps of the pathway have been available for many years.17,177 They were isolated based on resistance to the polyene nystatin, which shows less affinity for sterol intermediates than for ergosterol. The viability of these mutants indicated that the sterol intermediates accumulated satisfied the sparking function. However, as these were point mutations, trace and undetectable amounts of ergosterol might be produced. Subsequent cloning and disruption of each of the genes provided definitive evidence that these genes are, indeed, non-essential for viability under normal growth conditions.

Lanosterol, or other sterols containing a C-14 methyl group, cannot support growth of S. cerevisiae except under very unusual circumstances.76 Thus, lanosterol C-14 demethylase, the product of the ERG11 gene, is an essential enzyme in sterol biosynthesis. The enzyme is a cytochrome P-450 enzyme and is the target of theazole antifungals, the most commonly employed family of drugs in human fungal infections.279 Yeast erg11 mutants have been described126,266 but were found to be leaky and to contain a second leaky mutation in the gene encoding the sterol C-5 desaturase (ERG3). Subsequent work231 using disrupted versions of the ERG35 and ERG11111 genes confirmed the essential nature of the ERG11 gene and the ability of the accompanying ERG3 mutation to suppress lethality of the erg11 phenotype. This downstream suppression has been explained286 by the accumulation of 14α-methyl-ergosta-8,24(28)-diene-3β,6α-diol in erg11 mutants. This unusual sterol is toxic and results from downstream metabolism of C-14 methyl sterol intermediates that cannot be completely desaturated at C-5. The diol is not formed in cells that are missing a functional desaturase (erg3).

The lanosterol C-14 demethylase is one of the few pathway reactions where regulation at the molecular level has been explored. Protein levels of the enzyme have been shown to respond to oxygen concentration, carbon source and other growth conditions. Levels of ERG11 mRNA have been demonstrated to increase during growth on glucose, in the presence of heme, and under conditions where oxygen is limited.277 In addition, promoter analysis identified two activating sequences, one of which responds to the HAPI gene product and one definite repressor element which responds to the ROX1 gene product. In a subsequent report15 it has been shown that ERG11 is repressed by the HAPI gene product under low heme conditions. Thus, the Hap1p transcription factor has both positive and negative effects on ERG11 expression, depending on the physiological conditions in the cell.

The ERG24 gene encodes the sterol C-14 reductase, which converts 4,4-dimethylcholesta-8,14,24-trienol to 4,4-dimethylcholesta-8,24-dienol. This enzyme and the sterol C-8 desaturase (ERG2) are the targets of the morpholine antifungals.15 Morpholine treatment leads to the production of ergosta-8,14-dienol (ignosterol), which cannot support growth under normal conditions. Cloning and disruption133,148,158 of the ERG24 gene indicated that it was essential, but conflicting evidence was provided as to whether growth inhibition was due to the accumulation of non-utilizable 8,14-dien sterols148 or the lack of ergosterol.158

Non-viability resulting from the elimination of the ERG24 gene product is also subject to suppression in yeast. A mutant resistant to fenpropimorph, a commonly employed morpholine, was found to produce ignosterol in the presence of the drug.148 The mutation responsible for resistance, fen1, was able to allow growth with ignosterol. Recent reports have led to the identification of the fen1 mutation. SR 31747, an immunosuppressive agent that also blocks yeast growth by inhibiting the Δ8-Δ7 isomerase (ERG2p), has been used to select for SR 31747-resistant mutants.239 The mutants fall into two categories and have been localized to FEN1 and SUR4. Mutations in FEN1 and SUR4 have pleiotropic effects in yeast and affect phospholipid synthesis, budding and other cell functions. Oh et al.194 have recently described two genes, ELO2 and ELO3, that are involved in fatty
acid elongation and sphingolipid synthesis. Sequencing of ELO2 and ELO3 has indicated identity to FEN1 and SUR4, respectively. A mutation in either of these genes was able to suppress the erg24 phenotype and permit growth. In addition, fen1 and sur4239 were shown to suppress the lethal effects of an erg2 mutation which previously had been reported to be non-lethal.8 These findings indicate that sterol requirements for yeast membranes can vary depending on the presence or absence of other membrane lipids.

The removal of the two methyl groups from the C-4 position remains the final step in yeast sterol biosynthesis for which all the genes responsible have not been cloned. The process of demethylation is very complex and has been described enzymatically in cholesterol biosynthesis.74 The first step employs the C-4 sterol methyloxidase (encoded by the ERG25 gene) and results in the oxidation of the C-4α methyl group to a carboxylic acid. A second enzyme then removes the carboxylic acid, which results in the formation of a keto function at C-3. A third enzyme would reduce the C-3 keto function to the desired alcohol. The ERG25 gene, encoding C-4 sterol methyloxidase, has been isolated and cloned.19 As was the case for the ERG11 and ERG24, the ERG25 gene was shown to be essential for growth.

Subsequent studies indicated that the erg25 phenotype was also subject to suppression, but by a mechanism distinct from those observed in ERG11 and ERG24 suppression.76 In this case, suppression was due to the accumulation of two mutations. One was a mutation in the upstream ERG11 gene, while the second was a leaky mutation in either HEM2 or HEM4, genes in the heme biosynthetic pathway. A curious observation in the triple mutants (erg25, erg11, and hem2 or hem4) was that downstream conversions of lanosterol were blocked and that the cell was now capable of growing exclusively on lanosterol. This is the only report of such a capability and has implications regarding the structural features of sterol that can be tolerated in yeast.

The second gene in the C-4 demethylation reaction, ERG26, encoding a C-3 sterol dehydrogenase (decarboxylase), required to remove the C-4 carboxylic acid groups, has recently been cloned and disrupted (Gachotte et al., manuscript submitted). The ERG26 gene was reported to be essential and erg26 disruptants required both exogenous sterol and a mutation in the heme pathway for viability. These results suggested that either the accumulation of carboxylic acid sterol intermediates was toxic to growing heme-competent yeast cells, or that the heme mutation was required for sufficient uptake of exogenous sterol even under anaerobic conditions.

Zymosterol, the product of the C-4 demethylation step, serves as the substrate for the sterol C-24 methyltransferase. Yeast strains with mutations in the encoding ERG6 gene have been available24 for a considerable period of time. This step has been of particular interest, since it represents a step not found in cholesterol biosynthesis and might be projected to provide some unique function in yeast. The yeast ERG6 gene has been cloned, and analysis of a strain carrying an erg6 deletion disruption indicated that it was not essential for aerobic growth. Mutants of erg6 were found to have several deficiencies, including altered permeability characteristics,10,122 the inability to utilize respiratory energy sources,145 reduced mating capability75 and the inability to import tryptophan.75 Although survival is possible without a functional ERG6 gene, the yeast is at a considerable disadvantage, and the essentiality of a given gene may vary depending on changes in environmental conditions.197

As a target for the morpholine antifungals,15 the sterol C-8 isomerase was at one time postulated to provide the sparking function. However, disruption of the encoding ERG2 gene showed that it was not essential in yeast.6,8 Recent findings239 have indicated that erg2 mutants are non-viable in an aerobic environment and that erg2 mutants can be suppressed by fen1elo2 and sur4elo3 mutations. Apparently, the strain in which the ERG2 disruption was made6 carried an undetected fen1 or sur4 mutation.

The next step in sterol biosynthesis is a desaturation which adds the C-5,6 double bond. This is a highly conserved reaction in sterol biosynthesis and sterols from plant, animal and fungal sources have this feature. For this reason, the sterol C-5 desaturase was hypothesized to be essential in providing sparking sterol, and sterol-feeding experiments seemed to support the essential nature of this reaction.149,216 Subsequently, the encoding yeast ERG3 gene was cloned and disruption of the gene resulted in a strain that retained viability under aerobic conditions.5 The resolution of these contradictory conclusions lies in consideration of other markers in the strains used.244 ERG3 disruptions were done in a heme-competent strain, while feeding experiments were accomplished in a

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heme-deficient background. The addition of heme precursor, allowing for heme synthesis in the latter system, permitted growth without ERG3 function. Therefore, ERG3 is essential only in heme-deficient cells and is thus required for respiratory growth. Further analysis has shown that heme, and not heme precursors prior to the heme biosynthesis block, exhibits this characteristic, suggesting that ergosterol, or sparking sterol and heme play important and interrelated roles in aerobic and anaerobic metabolism in yeast.

ERG3 is one of the few pathway genes in which regulatory phenomena have been explored at the molecular level. Since the ERG3 gene has been shown to be essential in some backgrounds and has been implicated in the regulation of ergosterol biosynthesis in the yeast strain GL7, it represents a potentially important regulatory point in the pathway. Two research groups have recently reported on regulation of ERG3. Using the ERG3 promoter fused to the bacterial lacZ reporter gene, both groups explored the activity of the promoter under a variety of conditions that effect sterol biosynthesis. One study showed 30–40 fold increases in ERG3 activity in strains that contained additional mutations in the ergosterol pathway (erg5, erg6, and erg24) and were unable to synthesize ergosterol. In addition, ERG3 mRNA levels were significantly elevated in these mutant strains. Increased mRNA levels were also observed when sterol biosynthesis inhibitors were used in place of the erg mutations. Deletion analysis of the ERG3 promoter identified a 22-bp region as playing a major role in the sterol regulation of transcription. The second study utilized a similar ERG3 promoter-lacZ fusion to examine regulation of ERG3. Promoter activity was increased in several erg mutants (erg2, erg3, erg4, erg5 and erg6) as well as in cells that contain a null mutation in HMG1, the major yeast gene encoding HMG-CoA reductase. In addition, null mutations in both genes (ARE1 and ARE2) responsible for sterol esterification resulted in decreased levels of ERG3 expression as well as a decrease in total cell sterol.

The final two steps of the pathway involve the introduction of the double bond at C-22 and the reduction of the double bond at C-24(28). In both cases, mutants produce sterol intermediates that are close to the final structure and, thus, the cells have characteristics very similar to those exhibited by the wild type. The reduction at C-22 is accomplished by the sterol C-22 desaturase, a product of the ERG5 gene. This gene has been cloned, sequenced and disrupted and found to be non-essential for viability. The production of ergosterol results from the action of sterol C-24 reductase, an enzyme encoded by the ERG4 gene. The ERG4 gene in yeast was cloned as YGL022 and was shown to be non-essential for viability.

**Intracellular transport and location of sterol in yeast**

The ultimate fate of sterol synthesized or imported by yeast is critical for the proper function of the membranes of eukaryotes. Ergosterol is not present in equal amounts in all membranes and specific preference is shown for the incorporation of ergosterol rather than sterol intermediates. Ergosterol is found in the highest concentrations in the plasma membrane and in the secretory vesicles. In contrast, the membrane ergosterol content in the microsomes, mitochondria and other intracellular membranes is significantly lower and the relative concentration of sterol intermediates is increased. Yeast differ from most eukaryotes in that the mitochondrial ergosterol is concentrated in the inner membrane rather than in the outer membrane.

The highest concentration of sterol found in yeast is in lipid particles which are comprised primarily of triacylglycerols and steryl esters. Of the latter, about half are ergosteryl esters, while half are esters of sterol intermediates. Esterification of sterol takes place in the microsomes and, thus, the esters must be moved to the lipid particles for storage. Lipid particles were once thought to be storage depots for sterols and fatty acids which could be retrieved during times when endogenous sterol synthesis does not occur. However, recent studies have detected a significant level of sterol biosynthetic enzymes in these structures. The C-24 sterol methyltransferase (Erg6p) has been found in lipid particles at levels which suggest that most of the methylation activity occurs at this location. More recently, squalene epoxidase (Erg1p) has been detected in lipid particles and accounts for 38% of the cellular squalene epoxidase protein, as contrasted to the 62% of the enzyme found in the endoplasmic reticulum. Interestingly, the lipid particle enzyme is not active but partial activity can be generated when lipid particles are mixed with microsomes devoid of enzyme, indicating that there is a factor in the endoplasmic reticulum that
is necessary for squalene epoxidase activity. Thus, lipid particles play a more important role in what is generally referred to as ‘sterol trafficking’ than was formerly assumed.

Sterol esterification in yeast has recently been shown to be accomplished by the products of two distinct genes.299 The enzymes are similar to the esterification enzymes found in human (acyl-coenzyme A: cholesterol acyl transferase or ACAT). Deletions or disruptions of ARE1 and ARE2 (ACAT-related enzymes) in yeast, individually or together, do not result in a loss of viability. Deletion of ARE2 results in a 25% loss of sterol ester levels, while elimination of ARE1 function has no effect on ester levels. The double deletion results in a cell that forms no ester and exhibits an overall decrease in sterol biosynthesis.299 The latter observation suggests a regulatory role in sterol biosynthesis for yeast ACATs.

The inverse of the ACAT function in yeast is the steryl ester hydrolase function, which is necessary for the recycling of esterified sterol to free sterol and is sensitive to energy-supply disruption and conditions have shown that movement of esters from the lipid particles does not involve microtubules and is sensitive to energy-supply disruption and the inhibition of protein synthesis.139 The specifics of the mechanism involved remain undetermined.

SPHINGOLIPIDS

Our knowledge of S. cerevisiae genes necessary for sphingolipid metabolism has increased many-fold since the genome sequence was released in April 1996. There have also been many advances in identifying potential functions for sphingolipids in yeast (reviewed in ref. 60). Yet in spite of these advances, our understanding of sphingolipid metabolism and function in yeast is in its infancy, and our level of understanding pales in comparison to what is known about fungal glycerophospholipids and sterols. In this section of the review, we summarize current information about the genes that govern sphingolipid metabolism and the little that is known about the location of the gene products. We will also attempt to identify areas that seem ripe for future research.

Sphingolipids are characterized by the presence of a sphingoid long-chain base that is amide-linked to a fatty acid to form a ceramide. The type of long-chain base and fatty acid differ considerably between organisms but are generally fairly uniform within an organism. For example, the long-chain base in S. cerevisiae is phytosphingosine (N-erythro-2-amino-octadecane-1,3,4-triol). Phytosphingosine is also the primary long-chain base found in most plant ceramides, with only trace amounts found in animal sphingolipids (reviewed in ref. 146). Phytosphingosine lacks the 4,5-double bond found in sphingosine (N-erythro-2-amino-trans-4-octadecene-1,3-diol), the predominant long-chain base seen in mammalian sphingolipids, and has instead the hexadecyl on C-4 (Figure 5).

Ceramides in S. cerevisiae contain a long fatty acid of 26 carbons while most, but not all, the ceramides in animals contain shorter fatty acids.168 Ceramides are modified on the C-1 hydroxyl to give complex sphingolipids. Fungal sphingolipids, including those in S. cerevisiae, have inositol-phosphate attached to C-1 (Figure 5, see ref. 146). Animals are not known to make this modification but, instead, add glucose, galactose or phosphocholine to yield glucosylerceramide, galactosylceramide and sphingomyelin, respectively. Glucosylerceramide is further modified by addition of carbohydrates to give many hundreds of different types of complex sphingolipids.168 Plants are unique because they contain complex sphingo- lipids formed either from glucosylerceramide or inositol phosphorylerceramide.60

Sphingolipid biosynthetic pathway: genes, enzymes and phenotypes

Sphingolipid synthesis begins with the condensation of serine and palmitoyl-CoA to yield the long-chain base 3-ketodihydrophosphinosine (Figure 5). This reaction is catalysed by serine palmitoyltransferase (SPT) (for a review, see ref. 169), a pyridoxal phosphate-containing enzyme. Two essential genes, LCB1 and LCB2, are necessary for SPT activity and are thought to encode subunits of the enzyme.87,180,288 The growth defect produced by mutation of either LCB1 or LCB2 can be circumvented by adding any one of several
long-chain bases to the culture medium. In the absence of a long-chain base, the cells stop making sphingolipids and die within a few hours, even though protein, DNA and other lipid synthesis continues. It is not known how cells take up the relatively hydrophobic long-chain bases from the culture medium. Other sphingolipid metabolites, such as ceramides or long-chain base phosphates and the sphingolipids containing inositol, are not taken up by cells and this property has prevented many potentially interesting experiments from being performed. SPT is the target of several potent natural inhibitors, including sphingofungins, lipoxamycin, myriocin and viridofungins.

In the second step in sphingolipid synthesis, 3-ketodihydrosphingosine is reduced in a reaction utilizing NADPH to produce the long-chain base dihydrosphingosine (sphinganine). The 3-ketosphinganine reductase is encoded by TSC10 (YBR265w), as demonstrated by expressing the gene in E. coli.

In the next reaction, a fatty acid (generally 26 carbons long, but sometimes 24) is linked by an amide bond to dihydrosphingosine to yield dihydroceramide. This reaction, catalysed by dihydroceramide (sphinganine) N-acyltransferase (ceramide synthase), is poorly characterized since the enzyme has not been purified from any organism, neither has any gene necessary for enzyme activity been identified.

The dihydroceramide component of dihydroceramide is hydroxylated at C-4 to yield a ceramide (sometimes referred to as phytoceramide) containing the long-chain base generally referred to as phytosphingosine.

Although the pathway diagrammed in Figure 5 shows dihydroceramide as the substrate for C-4 hydroxylation by the Sur2p/Syr2p, it is still not clear if dihydroceramide or both compounds are substrates for hydroxylation at C-4. A rapid accumulation of free phytosphingosine following heat shock, which precedes an increase in the level of ceramide, suggests that free dihydroceramide...
can be hydroxylated prior to incorporation into dihydroceramide, at least under these experimental conditions.\textsuperscript{61} What we do know is that the \textit{SUR2}/\textit{SYR2} gene is necessary for hydroxylation of the long-chain base at C-4.\textsuperscript{84,85}

The predicted Surf2 protein shows similarity to desaturase/hydroxylase enzymes characterized by a motif containing eight histidines grouped into three clusters. The clusters may be part of the catalytic site, known to contain oxo-diiron, Fe-O-Fe.\textsuperscript{23} The \textit{SUR2/SYR2} gene is not essential for vegetative growth but a deletion mutant has the interesting property of increased resistance to the antifungal compound syringomycin E.\textsuperscript{84} It is unclear why hydroxylation of the long-chain base component of sphingolipids sensitizes cells to syringomycin E. Sensitivity would not seem to provide a selective advantage to cells in the wild, so there must be some other reason why most fungi hydroxylate C-4 of their long-chain bases.

Ceramide is converted to inositol phosphorylceramide (IPC), the first of three so-called complex sphingolipids, each of which contains inositol phosphate. The inositol phosphate is transferred to C-1 of ceramide in fungi and is catalysed by phosphatidylinositol:ceramide phosphoinositol transferase (IPC synthase), a membrane-bound enzyme.\textsuperscript{23} The \textit{SUR1} gene encodes IPC synthase or a subunit of the enzyme.\textsuperscript{181} IPC synthase activity is inhibited by the antifungal agents aureobasidin A\textsuperscript{181} and khafrefungin,\textsuperscript{155} which are potent inhibitors with an IC\textsubscript{50} of less than 1 nM.\textsuperscript{155,181} IPC synthase is a promising target for development of antifungal drugs because this enzyme is not found in mammals and its inhibition leads to fungal cell death. More efficacious antifungal drugs are needed for treatment of patients taking immunosuppressive drugs and in those with AIDS.

IPC is mannosylated to yield mannosine-inositol-P-ceramide (MIPC). This reaction requires two genes, \textit{SUR1}\textsuperscript{24} and \textit{CSG2}.\textsuperscript{203} IPC accumulates in the absence of either gene. The Surf1 protein is probably a mannosyltransferase since it contains a region of 93 amino acids, which is similar to two yeast \textalpha{}-1,6-mannosyltransferases, Ochlp\textsuperscript{185} and Hoclp.\textsuperscript{186} The function of the Surf2 protein is not obvious. It has an EF-Ca\textsuperscript{2+}-binding domain and 9–10 predicted transmembrane domains.\textsuperscript{26,257} As discussed below, it is likely that IPC is delivered to the Golgi apparatus with the inositol–phosphate head group facing the cytosol. It is then flipped across the membrane so that it can be mannosylated in the lumen of the Golgi. Perhaps the Surf2 protein does the flipping.

Strains mutated in \textit{CSG2} have proved useful in identifying genes involved in sphingolipid metabolism. Such strains do not grow in the presence of 100 mM Ca\textsuperscript{2+} and make only one species of IPC and no MIPC or M(IPP)\textsubscript{2}C.\textsuperscript{203} Seven classes of suppressors of calcium-sensitive growth, referred to as \textit{SCS} genes, have been identified including \textit{SCS1/LCB2} and \textit{SCS7/FAH1} (Table 4). The SCS suppressor genes seem to partially restore synthesis of some complex sphingolipids or adjust the concentration of different species of IPC so that cell growth is restored. A recent variation of the suppressor selection procedure has uncovered over a dozen new \textit{SCS} complementation groups (T. Dunn and T. Beeler, personal communication). Identifying the function of all of the \textit{SCS} genes should provide new insights into sphingolipid metabolism and function.

The final and most abundant sphingolipid, M(IPP)\textsubscript{2}C, is formed by transfer of inositol phosphate from phosphatidylinositol onto MIPC (Figure 5), a reaction very similar to the one requiring the Surf1 protein. This similarity suggested that open-reading frame \textit{YDR072c}, renamed \textit{IPT1},\textsuperscript{62} whose predicted protein was similar to the Surf1 protein, might be necessary for synthesis of M(IPP)\textsubscript{2}C. This prediction proved correct, since an \textit{ipt1} deletion mutant lacked M(IPP)\textsubscript{2}C synthase activity and made no detectable M(IPP)\textsubscript{2}C. The deletion mutant had an increased level of MIPC.\textsuperscript{62} M(IPP)\textsubscript{2}C normally accounts for about 75% of the sphingolipids in wild-type \textit{S. cerevisiae} cells, with the other 25% being divided between IPC and MIPC.\textsuperscript{247} It appears that \textit{S. cerevisiae} is able to sense its total sphingolipid content and adjust the level of MIPC to compensate for the absence of M(IPP)\textsubscript{2}C.\textsuperscript{62,136}

Under laboratory conditions, the \textit{ipt1} deletion mutant grows at a normal rate in complex or defined medium. Phenotypically it shows a slight sensitivity to a high concentration of calcium.\textsuperscript{62} It seems unlikely that \textit{S. cerevisiae} cells would retain M(IPP)\textsubscript{2}C if it were not providing some selective advantage. A clue about M(IPP)\textsubscript{2}C function comes from analysis of a genetically uncharacterized mutant strain that is unable to make M(IPP)\textsubscript{2}C. It showed increased resistance to the polyene antibiotic nystatin.\textsuperscript{136} Nystatin is thought to kill cells by interacting with sterols, with the complexes...
forming pores in the plasma membrane. Leber et al. suggest that M(IP)2C, in addition to ergosterol, is necessary for nystatin action on membranes and that the two types of lipids may exist as microdomains within the plasma membrane. Experimentally, it needs to be shown that M(IP)2C lipids have a propensity to interact with ergosterol and to do so better than MIPC and IPC. The complex sphingolipids of S. cerevisiae, IPC, MIPC and M(IP)2C, are mixtures, not unique molecular species, which differ in chain length and the extent of hydroxylation of both the long-chain base and the fatty acid. These differences are likely to be physiologically important but supporting data are meagre. It is known that cells grown at 25°C primarily contain C-18 phytosphingosine, whereas when grown at 37°C they contain an increased amount of C-20 phytosphingosine. The mechanism underlying this change is not entirely clear, but it may be partly a consequence of increased C-18 fatty acid synthesis over C-16 synthesis, known to occur during a heat shock. In addition, cells grown anaerobically have no hydroxyl groups on the fatty acid component of ceramide (R. L. Lester, unpublished data). Finally, one area of sphingolipid metabolism that has recently received attention is the formation and breakdown of phosphorylated long-chain bases, dihydrosphingosine-1-phosphate (DHS-1-P) and phytosphingosine-1-phosphate (PHS-1-P). Interest in these compounds stems from work on tissue-culture cells suggesting that the mammalian long-chain base phosphate, sphingosine-1-phosphate (SPP), is a signalling molecule. Depending on the cell type, it is thought to regulate growth, motility or programmed cell death (reviewed in refs 207, 249, 250, 309). Until recently, the existence of DHS-1-P and PHS-1-P in S. cerevisiae has been indirect and based upon the existence of mutations that suppress the choline or ethanolamine requirement of a strain mutated in cho1 and lacking phosphatidylserine synthase activity. Presumably the suppressor enabled cells to derive ethanolamine from a metabolite, the most likely one being the phosphoethanolamine produced from breakdown of DHS-1-P and PHS-1-P (Figure 5). Direct evidence for the existence of both DHS-1-P and PHS-1-P in S. cerevisiae cells has been presented.

Progress in identifying the genes for synthesis and degradation of long-chain base phosphates has been rapid. DPS-1-P and PHS-1-P are degraded in S. cerevisiae by a long-chain base phosphate lyase activity encoded by DPL1. It was

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function of gene product</th>
<th>Key references</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUR1</td>
<td>Synthesis of IPC, possible IPC synthase or a subunit of the enzyme</td>
<td>181</td>
</tr>
<tr>
<td>CSG2</td>
<td>Addition of mannose to IPC, function unknown</td>
<td>303</td>
</tr>
<tr>
<td>DPL1</td>
<td>Breakdown of long-chain base phosphates, long-chain base-phosphate lyase</td>
<td>218</td>
</tr>
<tr>
<td>ELO2/FEN1</td>
<td>Fatty acid elongase (synthesis of C-24 fatty acids)</td>
<td>194</td>
</tr>
<tr>
<td>ELO3/SUR4</td>
<td>Fatty acid elongase (conversion of C-24 to C-26 fatty acids)</td>
<td>194</td>
</tr>
<tr>
<td>FAH1/SCS7</td>
<td>Hydroxylation of the C-26 fatty acid in ceramide</td>
<td>85, 174</td>
</tr>
<tr>
<td>IPT1/SYR4</td>
<td>Synthesis of M(IP)C, possible M(IP)C synthase</td>
<td>62, 136</td>
</tr>
<tr>
<td>LCB1</td>
<td>Synthesis of long-chain bases, subunit of serine palmitoyltransferase</td>
<td>32</td>
</tr>
<tr>
<td>LCB2/SCS1</td>
<td>Synthesis of long-chain bases, catalytic subunit of serine palmitoyltransferase</td>
<td>180, 303</td>
</tr>
<tr>
<td>LCB3/YSR2</td>
<td>Diphosphorylation of long-chain base phosphates, long-chain base-1-phosphate phosphatase</td>
<td>156, 157, 208</td>
</tr>
<tr>
<td>LCB4</td>
<td>Long-chain base kinase</td>
<td>182</td>
</tr>
<tr>
<td>LCB5</td>
<td>Long-chain base kinase</td>
<td>182</td>
</tr>
<tr>
<td>SUR1/CSG1</td>
<td>Necessary for α-mannosylation of IPC</td>
<td>24</td>
</tr>
<tr>
<td>SUR2/SYR2</td>
<td>Hydroxylation of sphinganine or sphinganine-containing dihydroceramides at the C-4 position to yield phytoceramide</td>
<td>84, 85</td>
</tr>
<tr>
<td>TSC10</td>
<td>3-Ketosphinganine reductase</td>
<td>25</td>
</tr>
<tr>
<td>YSR3/LBP2</td>
<td>Long-chain base-1-phosphate phosphatase</td>
<td>156, 157</td>
</tr>
</tbody>
</table>

not clear if DHS-1-P and PHS-1-P could also be degraded by dephosphorylation until two phosphatase genes, LCB3/YSR2,156,157 and YSR3/LBP2,156,157 were uncovered. It now seems clear that either the lyase or phosphatase pathway can degrade long-chain base phosphates: the physiological function and regulation of each pathway remain to be determined. Mammals have a homologue of the DPL1 lyase,305 but no homologue of the phosphatases has been identified yet. Finally, two S. cerevisiae genes, LCB4 and LCB5, encoding all of the detectable long-chain base kinase activity in log phase cells, have been identified.192 They appear to have mammalian homologues based upon searches of expressed sequence tag databases.

The genes known to be necessary for metabolism of sphingolipids are summarized in Table 4. A major task is to characterize the enzymes biochemically. The challenge of purifying and biochemically characterizing these enzymes cannot be over-emphasized, considering that no enzyme in yeast sphingolipid metabolism has been purified. Such biochemical studies are essential if we are even to begin to understand how sphingolipid metabolism is regulated and how it is coordinated with other lipid metabolism and with cellular physiology. While at least one gene has been identified for nearly all steps in yeast sphingolipid metabolism (Figure 5 and Table 4), there are probably more genes to be identified, either ones encoding subunits of multimeric enzymes or ones encoding regulatory molecules.

Cellular location, sites of synthesis and functions of sphingolipids

Unlike proteins, which can usually be localized in cells by immunomicroscopy, localizing lipids can be a challenge, particularly since antibodies specific for individual types of yeast lipids, among them sphingolipids, are not available. Generally, the location of sphingolipids has been accomplished by isolating subcellular fractions and analysing them biochemically. Using these approaches, it appears that IPC, MIPC and M(IP)2C are located primarily in the plasma membrane of S. cerevisiae.194,199 It remains to be determined whether they are present in both the inner and outer leaflet of the plasma membrane or if there is an asymmetric distribution, as there is for many types of lipids present in the plasma membrane of higher eukaryotes. Sphingolipids are detected in other locations in S. cerevisiae cells, especially IPC, which is found in the Golgi as well as in the vacuole. Trace amounts of sphingolipids are found in mitochondria but none has been detected in lipid particles, and nuclei have not been examined.34

Some yeast proteins are anchored to membranes by ceramide in an uncharacterized process that involves replacing a glycosylphosphatidylinositol anchor with a ceramide anchor. This remodelling seems to be done in the Golgi apparatus.49,212,240 Recently, C-26 fatty acids have been detected in the nucleus, although it is not known if they are free or incorporated into sphingolipids, glycolipids or some novel lipid(s) species.225 These C-26 fatty acids have been hypothesized to play a role in stabilizing the sharp curvature in the membrane occurring at nuclear pore complexes. C-26 fatty acids have also been found in the sn-2 position of the glycerol moiety of glycosylphosphatidylinositol-anchored proteins. Other potential roles for very long-chain fatty acids in yeasts and other organisms have been suggested.226

The endoplasmic reticulum (ER) is the site for de novo long-chain base synthesis in all organisms that have been examined and also where all the steps up to, and including, ceramide formation occur. Some ceramide is converted to IPC in S. cerevisiae before transport to the Golgi apparatus, followed by further synthesis of IPC and the other complex sphingolipids (Figure 5).206 In S. cerevisiae, most of the ceramide and IPC are believed to be transported to the Golgi apparatus and then to the plasma membrane by secretory vesicles.100,206 However, it would not be surprising if alternative forms of transport are found.

Nothing is known about the site(s) of synthesis of DHS-1-P and PHS-1-P by the Lcb4 and Lcb5 kinases. A portion of both lipid kinases is found in the soluble, and in the 100 000 × g membrane fraction, of cells.182 It is presumed that at least some fraction of one of them is in the endoplasmic reticulum, since this is where DHS and PHS are synthesized and where substrates are most likely to be located. It is not known whether DHS or PHS exist in other organelles, where they could serve as substrates for LCB kinase, or whether some long-chain bases are in the cytoplasm and thus available for phosphorylation. Localizing the enzymes for DHS-1-P and PHS-1-P metabolism, Lcb3, Lcb4p, Lcb5p, Lhp2p and Dpl1p will be important for determining whether DHS-1-P and PHS-1-P are regulatory molecules, as is currently
suspected or if they are simply intermediates in the pathway that directs sphingolipid metabolites into aminoglycerophospholipid synthesis (Figure 5).

Long-chain base phosphates are found in all eukaryotes that have been examined, making it likely that they provide a function(s) with survival value. This function is not obvious, however, since mutants lacking Lcb4 and Lcb5 kinase activity, and unable to make any detectable DHS-1-P or PHS-1-P, grow normally in complex or synthetic medium. Other data suggest that long-chain base phosphates are important for resistance to heat shock in stationary phase cells. What needs to be done to verify the role of DHS-1-P and PHS-1-P in mediating resistance to heat stress in stationary phase is to demonstrate that the level of these compounds changes during heat treatment.

Finally, complex mammalian sphingolipids, such as sphingomyelin, are turned over in most types of cells, either as a part of normal cellular physiology or in response to extracellular signals. The breakdown products (such as ceramide, phytosphingosine, and a stable increase in dihydrosphingosine and sphingosine and SPP) are believed to regulate multiple physiological or in response to extracellular signals.

In contrast to mammals, it appears that the majority of S. cerevisiae sphingolipids are stable and are not turned over in haploid cells during vegetative growth. Thus, the production of potential sphingolipid signalling molecules in S. cerevisiae, including DHS, PHS, DHS-1-P, PHS-1-P and ceramide, are most likely derived from de novo synthesis, not from the turnover of preformed sphingolipids.

Sphingolipids are involved in other cellular processes in S. cerevisiae (reviewed in ref. 60). They have been shown to be necessary for resistance to 37°C, low pH and osmotic stress, and for survival in stationary phase (R. Dickson, unpublished results). The exact role of sphingolipids in these stress responses remains to be elucidated. Heat shock has been shown to cause a rapid and transient increase in dihydrosphingosine and phytosphingosine, and a stable increase in ceramide, which suggests that one or all of these molecules are second messengers during the heat stress response. Potential processes signalled by one or more of these compounds are trehalose accumulation and induction of transcription of TPS2, encoding a subunit of trehalose synthase. Calcium homeostasis or components in calcium-mediated signalling pathways may be regulated by sphingolipids or vice versa, but the mechanisms await characterization. It has been suggested that ceramide regulates cell cycle arrest at G1 via a pathway that utilizes a ceramide-activated protein phosphatase encoded by SIT4, TPD3 and CYC55. However, these experiments are based upon the use of ceramide-containing N-linked acetate (C-2 ceramide) and it is questionable if this compound really mimics the behaviour of authentic ceramide, with its very hydrophobic C-26 fatty acid. In fact, one published report found that C-2 ceramide did not arrest growth. If ceramide is regulating the cell cycle, its concentration should change in a cell-cycle-dependent manner.

Finally, ceramide synthesis is known to be necessary for trafficking of secretory vesicles from the endoplasmic reticulum to the Golgi apparatus. It remains to be determined what ceramide is doing in vesicle trafficking.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Sequencing of the yeast genome has opened new avenues of research for yeast lipidologists. Homology searches and the development of rapid techniques to construct deletion mutants have facilitated analysis of a large number of genes for their possible involvement in yeast lipid metabolism. As a consequence of these developments, a number of new genes involved in lipid metabolism have been discovered during the last few years. It has become evident that some of the lipid synthetic enzymes exist as isoenzymes, such as Psd1p and Psd2p, Are1p and Are2p, or acyltransferases involved in PA biosynthesis. Minor enzymatic activities that were ignored in the past were thus pinpointed and the responsible genes and gene products identified.

Great progress has been made in identifying S. cerevisiae genes necessary for sphingolipid metabolism. At the current rate of progress, it appears likely that S. cerevisiae will be the first eukaryotic organism for which all genes necessary for sphingolipid metabolism are identified. This information will provide a unique opportunity to biochemically characterize sphingolipid metabolic enzymes and to identify, by both luck and design, new and unimagined functions for sphingolipids. Besides the discovery of structural genes, some mechanisms for regulating lipid biosynthetic
pathways have been unravelled. The most prominent regulatory pathway for yeast phospholipid formation is transcriptional control by inositol. This regulatory circuit forms the basis for cross-pathway regulation (for reviews, see refs 35, 82, 198).

Other levels of regulating lipid metabolism and lipid assembly into yeast membranes also deserve mention. Since lipid synthesis occurs in specific organelles, the physiological state of the yeast cell can greatly influence lipid synthesis. The most prominent example of such phenomena is the control of mitochondrial development by oxygen. Not only are CL synthesis and the heme required for certain steps of sterol biosynthesis dependent upon mitochondrial development, but so is the energy state of a yeast cell, since it can change dramatically during anaerobiosis or as a consequence of mitochondrial dysfunction. Since most steps of lipid biosynthesis are directly or indirectly dependent upon the availability of energy, such changes affect lipid metabolism. As another example, sphingolipid synthesis is linked to protein secretion, because steps of sphingolipid formation occur in organelles along the secretory pathway, and intermediates and products are transported to their destination(s) by the protein secretory machinery. Thus, lipid transport is not only required for lipid targeting and lipid assembly into cellular membranes, but also to link lipid biosynthetic pathways, in which steps are distributed among various organelles. Finally, the interplay of the endoplasmic reticulum and mitochondria during synthesis of aminolipids and phospholipids, and the interplay of the endoplasmic reticulum with lipid particles during sterol synthesis or the deposition of triacylglycerols and sterol esters, are also examples of processes that regulate lipid metabolism.

Future research efforts need to be directed towards understanding how lipid synthesis and turnover are integrated with other metabolic pathways and cellular processes during all aspects of S. cerevisiae cell biology. In the case of phospholipids, understanding mechanisms of targeting among organelles will be a significant focus area. Identification of components involved in these processes will open new aspects of organelle biogenesis from the viewpoint of the lipidologist. Improved analytical methods will allow us to detect less abundant species and phospholipids that may play important roles in signalling processes or membrane integrity. New levels of regulation of phospholipid biosynthesis and turnover, like the one described for SEC14, will be of great interest.

Remodelling and degradation of lipids may play an important role in the maintenance of a specific membrane lipid composition and in intracellular signalling. Distinct phospholipid molecules are produced, for example, by phospholipase A-catalysed deacylation in the sn-2 position of existing phospholipids, followed by reacylation of the lysophospholipid with a coenzyme-A-activated fatty acid. Deacylation and reacylation of glycerophospholipids appear to be very efficient, but cellular functions that depend on this energetically expensive process have yet to be identified.

Phospholipases of S. cerevisiae are only poorly characterized. Phospholipase B (PLB) activities catalysing the complete deacylation of glycerophospholipids have been identified in the plasma membrane and the periplasmic space of yeast. The physiological function and relevance of the two forms of the enzyme are not clear. Interestingly, disruption of the PLB1 gene completely abolishes PLB activity in vitro, but has no effect on cell viability and lipid composition and only slightly effects release of deacylation products into the culture medium. Thus, S. cerevisiae is likely to have additional genes encoding PLB activities that have escaped detection.

The S. cerevisiae PLC gene, encoding a phospholipase C (PLC), is not essential. Cells lacking Plc1p, however, grow poorly on fermentable carbon sources, are unable to grow on non-fermentable carbon sources or at elevated temperatures, and are extremely sensitive to hyperosmotic stress. In yeast, PLC-dependent inositolphospholipid turnover functions to maintain cell morphology, chromosome segregation, cytokinesis and the integrity of the plasma membrane, and may be required at several or all stages of the cell cycle.

Phospholipase D (PLD) cleavage releases the second messenger phosphatidic acid. Only recently was the yeast gene encoding Pld1p identified. Surprisingly, PLD1 is identical to SPO14, which encodes a factor essential for sporulation. The role of PLD activity in spore formation, its site of action and the nature of the target lipid(s) have not yet been established.

Similar to phospholipid metabolism, the sterol biosynthetic pathway contains several steps that are subject to regulation. Most of these are unexplored and the availability of the genes for most steps makes this an imperative area for research in
the near future. In addition, we are beginning to unravel some of the elements of the overall scheme for targeting sterol to specific cellular membrane systems, selection of sterol for esterification or utilization, storage and recovery of sterol, and movement of various sterol forms throughout the cell. All of these trafficking phenomena have implications for the regulation of sterol biosynthesis as well. Finally, there remains the interest in yeast sterol metabolism and physiology as a model for the same events that occur in mammalian systems. For example, the yeast Ncr1 protein has been found to be 42% identical to the human Niemann-Pick product, which seems to function in sterol transport or sensing. These would seem to be areas of great future interest, as we now are reaching the end of the discovery and characterization of sterol biosynthetic genes and related aspects concerned with essentiality and suppression.

In the field of yeast sphingolipid research, the challenge for the future will be to purify and biochemically characterize the enzymes, most of which are membrane-bound, and establish their cellular location(s) in intact, not disrupted, cells. Compared to glycerophospholipids and sterols, we know very little about the function of sphingolipids and even less about how their synthesis is controlled. Identifying new functions for the polar and hydrophobic components of sphingolipids and understanding how de novo synthesis is regulated are two areas that could greatly benefit from insightful hypotheses and clever experimental designs. Unlike mammalian cells, where complex sphingolipids are normally turned over, there is no detectable turnover of complex sphingolipids (IPC, MIPC and M(IP)2C) in haploid yeast cells. This may represent a fundamental difference in sphingolipid metabolism between fungi and complex eukaryotes. It is possible that small amounts of complex sphingolipids are turned over in S. cerevisiae cells, but improved analytical techniques are needed to identify and to quantify such changes. Sphingolipid metabolites produced by turnover have the potential to be important regulatory molecules, as seems to be the case in higher eukaryotes.

In conclusion, using a combination of cell biology, molecular biology and biochemical techniques, future experiments in yeast are likely to reveal new roles for lipids as structural elements in membranes. They should also identify new roles of lipids as modulators of enzymes and mediators of cellular processes, such as sporation, mating, stress resistance and protein assembly into membranes. This experimental background will also be the basis for extended comparative studies of lipids and lipid metabolism in yeast and higher eukaryotes.

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