The Phosphatidylglycerol/Cardiolipin Biosynthetic Pathway Is Required for the Activation of Inositol Phosphosphingolipid Phospholipase C, Isc1p, during Growth of Saccharomyces cerevisiae*

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function of Isc1p were investigated. Deletion of ISC1

resulted in markedly lower growth in non-fermentable

carbon sources. Interestingly, the growth defect of isc1Δ

strains resembled that of pgs1Δ strains, lacking the com-

mitted step in the synthesis of phosphatidylglycerol

(PG) and cardiolipin (CL), which were shown to activate

Isc1p in vitro. Therefore, the role of Pgs1p in activation

of Isc1p in vivo was investigated. The results showed

that in the pgs1Δ strain, the growth-dependent activa-

tion of Isc1p was impaired as was the ISC1-dependent

increase in the levels of phytoceramide during the post-

diauxic phase, demonstrating that the activation of

Isc1p in vivo is dependent on PGS1 and on the mitochon-
drial phospholipids PG/CL. Mechanistically, loss of

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(2001) J. Biol. Chem. 276, 25262–25272), thus suggesting

that Isc1p mediates at least some functions downstream

of PG/CL. This study provides the first evidence for the

mechanism of in vivo activation and function of Isc1p. A

model with endogenous PG/CL as the in vivo activator of

Isc1p is proposed.

Ceramide is a bioactive lipid that in eukaryotic cells func-
tions as a mediator of a variety of extracellular signals through the regulation of several downstream effectors which in turn control basic cellular functions such as cell growth, cell cycle arrest, apoptosis, and senescence (1–6). Sphingomyelinases, which hydrolyze the phosphodiester linkage of sphingomyelin (SM)¹ to produce ceramide and phosphorylcholine, function as key regulators of the intracellular levels of ceramide in mammalian cells.

Sphingolipids are also important regulatory molecules in yeast where they are known to be required for viability (7), optimal life span (8), cell cycle regulation (9), endocytosis (10), and regulation of responses of yeast cells to stress (1, 11). Although Saccharomyces cerevisiae do not contain SM, they do contain inositol phosphoceramides, and recently we identified a homologue of neutral sphingomyelinase in S. cerevisiae, Isc1p, which acts on phosphoceramides to generate ceramide. This enzyme displays ~30% identity to neutral sphingomyelinase 2 and shares with it several common features (12) including hydrolytic activity on SM, the requirement of Mg²⁺ for optimal activity, optimal neutral pH, and the presence of a newly discovered domain that is conserved in the entire family of sphingo-

zymelinas, the P-loop-like domain (13), which appears to be important for substrate binding and/or catalysis.

In addition, both enzymes demonstrate an absolute require-
ment for anionic phospholipids for in vitro activation. Thus, Isc1p is activated selectively in vitro by cardiolipin (CL), phosphatidylglycerol (PG), or phosphatidylserine. In a recent study we demonstrated that the enzyme binds these anionic phospholipids, and we identified the second transmembrane domain and the C terminus of Isc1p as required for this binding, and it was proposed that this interaction plays a critical role in en-

zyme function through a novel tethering mechanism of enzyme activation by lipid cofactors (14).

Given these specific requirements for anionic phospholipids in vitro, it became important to determine whether the enzyme requires phospholipids for activation in vivo. Our attention was particularly directed to the possible roles of PG and CL as the deletion of PGS1, the gene that encodes the enzyme responsible for the synthesis of PG phosphatase, and subsequent synthesis of PG and CL was shown to cause a late defect during growth in glucose media similar to that of ISC1. Moreover, these lipids are almost exclusively present in mitochondria, and we have shown recently that Isc1p localizes preferentially to mitochondria in the post-diauxic phase of growth.

Synthesis of CL in yeast requires three sequential reactions.

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1 The abbreviations used are: SM, sphingomyelin; ISC1, inositol phosphosphingolipid phospholipase C; PGS1, phosphatidylglycerophosphophate synthase; CLS1, cardiolipin synthase; CL, cardiolipin; PG, phosphatidylglycerol; cox, cytochrome c oxidase; NFCs, non-fermentable carbon sources; MUSC, Medical University of South Carolina; NBD, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)) styryl; wt, wild type; CLs1p, cardiolipin synthase; GFP, green fluorescent protein; SD, synthetic minimal medium; MES, 4-morpholineethanesulfonic acid.

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The first step is the synthesis of phosphatidylglycerophosphate from CDP diacylglycerol and glycerol 3-phosphate. This reaction, catalyzed by phosphatidylglycerophosphate synthase (Pgs1p), is the committed and rate-limiting step. Then PG phosphate is rapidly dephosphorylated to PG. Finally, PG is converted to CL by cardiolipin synthase (Cls1p) with CDP diacylglycerol as co-substrate.

CL has been postulated to be important for the function of mitochondria (15–17), e.g. to stabilize and maintain the proper orientation of cox4p (18), a nuclear-encoded subunit of cytochrome c oxidase (cox). On the other hand, the absence of PG (and CL) because of lack of PGS1 was shown to cause severe mitochondrial dysfunction (19–21) and to lead to a severe growth defect on non-fermentable carbon sources, suggesting a key role for PG (and/or CL) in mitochondrial function (19, 22, 23). Recently, a role for Pgs1p in the translation of cox proteins was proposed based on the finding that the pgs1Δ strain exhibited a serious deficiency in translation of cox proteins (24).

Recently, Isc1p was found to become activated and to localize to the mitochondria during the diauxic shift, resulting in an increase in the levels of phytoceramide during growth of yeast cells (12). However, the molecular basis for enzyme activation remains unknown. In the present study the in vivo mechanism of Isc1p activation was investigated.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-porin, anti-cox3p, and anti-cox4p were obtained from Molecular Probes. Monoclonal mouse anti-FLAG M2 antibody was obtained from Sigma. Goat anti-mouse and donkey anti-goat horseradish peroxidase-conjugated antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Anti-GFP antibody was obtained from Clontech. [Choline-methyl-14C]SM was synthesized in the Lipidomics Core Facility at the Medical University of South Carolina (MUSC) as described (25). Spingomylins and phosphatidylethanolamine were purchased from Avanti Polar lipids Inc. (Alabaster, AL). Ceramides and phytoceramides (d-erythro-C12:NBD-ceramide, d-erythro-C16-ceramide, d-ribo-C12:NBD-phytoceramide, and d-ribo-C16-phytoceramide) were obtained from the Lipidomics Core at MUSC. All other reagents were purchased from Sigma.

**Yeast Strains, Media, and Culture Condition**—The yeast wild type strain JN9–3da (wt) and the deletion mutant strain JN9–3da/JISC1 (Jisc1 cells) (MATa trp1 leu2-3 his4 ura3 ade2 rme1 JSC1:G418) (26) were used in this study, and other strains were derived from them. In addition, the wild type parental and mutant strains for genes of the cardiolipin pathway used in this study were previously reported: YPH499 [Par (pG)] (ade2-101, his3A200, leu2A1, lys2-801, trp1Δ63, ura3-52, MATa), YZD1 (pgs1Δ cells) (ade2-101, his3A200, leu2A1, lys2-801, ura3-52, pgs1Δ:TRP1, MATa), DL1 (Par cl) (his3-11,15, leu2-3,112, ura3-251,328,372, MATa) (24), and YZD5 (crd1Δ cells) (ura3-251,328,372, crd1Δ::HIS3, MATa) (23). The expression of GFP-tagged JSC1 and FLAG-tagged JSC1 in pYES2 under the control of the GAL1 promoter was described previously (14). Cells were grown in yeast extract/peptone/dextrose or yeast extract/peptone containing non-fermentable carbohydrates as the only carbon source (NFCS) was evaluated. Compared with the wild type strain, isc1Δ showed a severe defect in growth when glycerol was used as the carbon source (Fig. 1a), and this defect in growth was significantly more pronounced compared with the growth defect in glucose (maximal ~88% versus ~30%). In addition, growth of isc1Δ was markedly affected when cells were grown in other NFCS such as lactate, ethanol, or acetate as opposed to the fermentable sources glucose or galactose (Fig. 2). Although the defects in ethanol or lactate were less pro-

**RESULTS**

The yeast wild type strain JN9–3da (wt) and the deletion mutant strain JN9–3da/JISC1 (Jisc1 cells) (MATa trp1 leu2-3 his4 ura3 ade2 rme1 JSC1:G418) (26) were used in this study, and other strains were derived from them. In addition, the wild type parental and mutant strains for genes of the cardiolipin pathway used in this study were previously reported: YPH499 [Par (pG)] (ade2-101, his3A200, leu2A1, lys2-801, trp1Δ63, ura3-52, MATa), YZD1 (pgs1Δ cells) (ade2-101, his3A200, leu2A1, lys2-801, ura3-52, pgs1Δ:TRP1, MATa), DL1 (Par cl) (his3-11,15, leu2-3,112, ura3-251,328,372, MATa) (24), and YZD5 (crd1Δ cells) (ura3-251,328,372, crd1Δ::HIS3, MATa) (23). The expression of GFP-tagged JSC1 and FLAG-tagged JSC1 in pYES2 under the control of the GAL1 promoter was described previously (14). Cells were grown in yeast extract/peptone/dextrose or yeast extract/peptone containing other carbon sources (3% glycerc, 2% lactate, 2% ethanol, or 3% acetate) as stated. Plasmids were transfected into cells from the strains mentioned above as described (27). Strains carrying pYES2 constructs were grown in SD, 2% glucose and ura dropout supplement. Single colonies were inoculated in SD-ura media and incubated at 30 °C in a shaker incubator at 250 rpm/min. Exponentially growing cultures were induced in galactose media for 24 h before inoculating new cultures in galactose overnight to reach the exponential phase, and then cells were washed before experiments. For experiments with strains that did not grow in galactose, media containing glucose and galactose was employed. Growth experiments were started by dilution of the culture with 5 ml of fresh media to a density of 0.1 A590 units and incubating the cultures at 30 °C. Sphingolipids resuspended in ethanol or ethanol as vehicle were added to cells, and the final concentration of ethanol was 0.05%, which was not inhibitory for the growth of wild type or mutant cells. Growth curves were determined by measuring the A590 at different time points.

**Assay of Isc1p Activity**—Because untransformed isc1Δ cells contain no sphingomyelinase activity and because the activity of partially purified enzyme from JSC1-overexpressing cells was unstable, as described previously (14), unless otherwise stated cell lysates were used to determine Isc1p activity as described with modifications (14). Briefly, cell lysates were incubated in 100 μl of buffer containing 100 mM Tris (pH 7.5), 5 mM MgCl2, 5 mM dithiothreitol, 0.1% Triton X-100, 10 nmol (6.7 mol %) of phosphatidyleserine, 10 nmol (6.7 mol %) of unlabeled SM, and 100,000 dpm of [choline-methyl-14C]SM at 30 °C for 30 min. After the incubation, 1.0 ml of chloroform, 0.5 ml of methanol, and 0.2 ml of water were added according to the method of Folkh et al. (28), and the radioactivity in a portion (400 μl) of the upper phase was mixed with Safety Solve (Research Products International) for liquid scintillation counting.

**Protein Determination, SDS-PAGE, and Immunoblotting**—Five micrograms of total protein (for detection using anti-FLAG antibody in overexpressor extracts), 40 μg of total protein (for anti-cox detection in mitochondrial extracts), or 10 μg of total protein unless otherwise specified were resuspended in reducing buffer, resolved in a 10% SDS-PAGE gel and transferred to nitrocellulose. Membranes were blocked, and immunoblotting was performed as described previously (12). Protein concentration was determined using Bio-Rad protein assay reagent.

**Measurements of Mass Levels of Phytoceramide**—Cells were harvested and washed with water, and lipids were extracted following the method of Bligh and Dyer (29). The chloroform organic phase was divided into aliquots, dried down, and processed for inorganic phosphorus determination or phytoceramide measurements using the Schere rica coli diacylglycerol kinase assay. Phytoceramide was quantitated using external standards and normalized for phosphorous content (30).

**Analysis of Phosphoglycerolipids**—Yeast cells were cultured as described above and inoculated in 5 ml of complete synthetic media with low phosphate and glucose as a carbon source. Phospholipids were labeled to steady state by adding 50 μCi of [14C]phosphatidylcholine to glycerol or uracil, and cells were incubated for 24 h. Phospholipids were extracted by chloroform and analyzed by TLC using a 68:28:8 chloroform:methanol:acetic acid solvent system. TLC plates were exposed to phosphorimaging screens, and PG, CL, and phosphatidylethanolamine were quantified.

**Isolation of Mitochondria**—Cell lysates were centrifuged at 13,000 × g for 20 min to obtain the P13 pellets. Isolation of mitochondria was modified from the protocol of Glick and colleagues (33) which removes the mitochondrial surface membrane. The mitochondria were isolated in buffer containing 100 mM Tris (pH 7.9) or 0.2 M sucrose, 50 mm potassium phosphate, 0.1% bovine serum albumin, and resuspended in buffer containing 100 mM K+ -MES (pH 6.0), 0.6 M sorbitol, 0.5 mM EDTA. After the addition of 5 mM phenylmethylsulfonyl fluoride, the cellular homogenate was obtained, and crude mitochondrial preparations were isolated as described by Glick and Pon (31).

**Fluorescence**—Cells were loaded on a glass slide precoated with poly-l-lysine, and the cells were covered with a coverslip for microscopic visualization. The excitation wavelength for GFP was 488 nm, and images were captured using LSM 510 META software version 3.2.
found, these were still more pronounced than with the fermentable sources. Therefore, these results confirm the role for Isc1p in growth, and they seem to indicate that the reduced growth of isc1Δ at the diauxic shift (when most glucose is consumed and only NFCS remains) is due to a defect of this mutant to grow in NFCS.

The above results coupled with previous results demonstrating activation of Isc1p in mitochondria during the diauxic shift (12) led us to investigate the molecular mechanisms underlying the regulation of Isc1p in growth and, in particular, the roles of CL and PG, the main anionic phospholipids in mitochondria. Initially, the growth of isc1Δ was compared with that of a strain null for PG synthase (pgs1Δ), the enzyme responsible for synthesis of PG and CL. The behavior of isc1Δ was very similar to that of pgs1Δ (Fig. 1). Indeed, both strains showed growth similar to wild type strains when grown in glucose up to 8 h, and from 12 h to 72 h a reduction in the A600 values for isc1Δ and pgs1Δ was readily detected and persisted between 24 and 72 h. In glycerol, growth of isc1Δ and pgs1Δ was profoundly impaired, remaining low for the entire duration up to 72 h (Fig. 1a).

The similarity between the defects observed with the pgs1Δ and the isc1Δ strains suggested a link between the in vivo role of Pgs1p/Clp1p and the role of Isc1p during growth such that Isc1p and Pgs1p and/or Clp1p might either work (a) sequentially (upstream or downstream in the same pathway), (b) in separate branches of the same pathway, or (c) in different signaling pathways regulating cell growth. To evaluate these possibilities two approaches were carried out. First, the activation of Isc1p during growth of the mutant strains was determined as described previously (12). Isc1p specific activity was, therefore, assayed in 4- and 24-h (pre- and post-diauxic, respectively) extracts from the wild type, pgs1Δ, or cls1Δ strains. Isc1p showed the predicted higher specific activity at the diauxic shift versus the early log phase with ~3-fold activation at 24 versus 4 h. Interestingly and in contrast, the growth-dependent activation of Isc1p was markedly lost in the pgs1Δ strain with only a ~40% increase in the activity of Isc1p observed at the post-diauxic phase (Fig. 3a). These results reveal that lack of PGS1 and production of PG/CL impairs the activation of Isc1p during growth. On the other hand, cls1Δ showed Isc1p activation very similar to the parental strain, indicating that the lack of CLS1 and of only CL does not impair the activation of Isc1p. Because it has been shown that Isc1p changes its localization from endoplasmic reticulum to mitochondria after the post-diauxic shift (12), it became of interest to determine whether Pgs1p exerts a role on the translocation of Isc1p. Cells from wild type, pgs1Δ, or cls1Δ strains were transformed with GFP-Isc1 and analyzed by confocal microscopy at 4 and 24 h (Fig. 4a). All strains showed similar localization of GFP-Isc1, the curved endoplasmic reticulum structure during the pre-diauxic phase of growth (at 4 h), and the mitochondrial shape of a tubular branched network after the post-diauxic shift (at 24 h), suggesting that the localization of GFP-Isc1 is not altered in these strains. Although this distinct pattern for mitochondria is widely accepted, a biochemical approach was employed to further test the localization of Isc1p during the diauxic shift in cells that are mutant for the PG/CL pathway. Thus, P13 (13,000 × g pellet) and crude mitochondrial fractions were prepared as described previously (31) from wild type or pgs1Δ cells overexpressing FLAG-Isc1 cultured for 24 h and then subjected to immunoblotting for Isc1p, Dpm1p, and porin as endoplasmic reticulum and mitochondrial marker proteins, respectively. These three proteins were detected in the P13 fraction, but only porin and Isc1p were enriched in the further-purified mitochondrial fraction from the pgs1Δ cells (Fig. 4b, right panels), which was similar to the wild type fractions (Fig. 4b, left panels), indicating that the mitochondrial localization of Isc1p is not altered in the pgs1Δ strain. These results argue against a role for Pgs1p in the regulation of Isc1p translocation to the mitochondria during growth.

To provide further evidence for a function of Pgs1p upstream

![Fig. 1](image_url)

**Fig. 1.** A isc1Δ growth defect in glucose or glycerol carbon sources. wt or isc1 null mutant (isc1Δ) cells (a) and pgs1 mutant (pgs1Δ) or the wild type parental (Par-pg) cells (b) were grown for the indicated number of hours in media containing either glucose (upper) or glycerol (lower) as the carbon source, and the A600 was determined. The results are averages of triplicate experiments.
of Isc1p in vivo, the levels of phytoceramide, the lipid product of Isc1p, were evaluated. We have previously shown that phytoceramide levels increase during growth, and this increase was dependent on ISC1 (12). Therefore, the levels of phytoceramide were measured in extracts prepared from parental, pgs1/H9004, or isc1/H9004 cells grown for 4 or 24 h, and the levels of phytoceramide were analyzed by the diacylglycerol kinase assay. The levels of phytoceramide increased significantly in the wild type strain in post-diauxic versus early log phase cells (Fig. 5). As previously noted (12), the levels of phytoceramide not only failed to increase in the isc1/H9004 strain compared with a wild type strain but also significantly decreased. Importantly, the levels of phytoceramide in the cls1/H9004 were comparable with the wild type (data not shown); however, the levels of phytoceramide were markedly lower in pgs1/H9004 cells at the diauxic shift when compared with wild type or to the early log phase cells (Fig. 5). These results provide evidence that in vivo the levels of phytoceramide are reduced in yeast lacking PGS1, reinforcing the conclusion that the lipid products of Pgs1p, PG, and CL exert a role upstream of Isc1p and that PG and CL are required for the Isc1-dependent differential accumulation of phytoceramide during growth (diauxic versus early).

Next, we explored the possibility that ISC1 may function upstream of Pgs1p. Because Pgs1p is responsible for the synthesis of both PG and CL, we evaluated the levels of these two lipids in wild type cells or cells lacking Isc1p using 32P labeling as described previously (34). The autoradiograph showed very similar levels of PG and of CL in wild type and isc1/H9004 extracts.

Fig. 2. Effect of carbon sources on growth of isc1Δ. wt or isc1 null mutant (isc1Δ) cells were grown for the indicated number of hours in media containing galactose (a), ethanol (b), acetate (c), or lactate (d) as the carbon source, and the A600 was determined. The results are the averages of triplicate experiments. The results shown are representative of two independent experiments.

Fig. 3. Growth-dependent activation of endogenous Isc1p in mutants of the cardiolipin pathway. pgs1 mutant (pgs1/H9004) or the wild type parental (Par-pg) cells (a) and cls1 mutant (cls1/H9004) or the wild type parental (Par-cl) (b) cells were grown for 4 and 24 h, and cell lysates were prepared as described under “Experimental Procedures.” The activity of Isc1p was determined using 30 μg of total protein. Results are the averages ± S.D. of three different experiments.
Requirement of Cardiolipin Pathway for Activation of Isc1p

(Fig. 6, first and second lanes, respectively), and this was confirmed by increasing the amount of radioactivity spotted in the thin layer chromatography (third versus fourth lanes). PG and CL were quantified, and the levels were normalized to phosphatidylethanolamine. The molar ratio of CL/phosphatidylethanolamine was 0.05 versus 0.052 (wild type versus isclΔ), and PG/phosphatidylethanolamine was 0.017 versus 0.016 (wild type versus isclΔ), confirming that these strains have similar levels of CL and PG. Therefore, it is concluded that the lack of Isc1p does not alter the levels of the lipid products of Pgs1p, and thus, Isc1p does not act upstream of Pgs1p.

Because a role for PG and CL in regulating the translation of the cox subunits was recently reported (24), it became of great interest to determine whether Isc1p functioned as a downstream mediator of the actions of PG/CL on the level of these mitochondrial proteins. Therefore, the levels of mitochondrial- and nuclear-encoded cox subunits were determined by immunoblotting mitochondrial fractions obtained from wild type or isclΔ cells as described. When probing for cox3p, a mitochondrial-encoded subunit III are reduced in isclΔ. wt, iscl1 mutant (isclΔ), or iscl1Δ cells expressing ISC1 under the control of galactose promoter (isclΔ + ISC1) were grown for 24 h. Spheroplasts were prepared at 4 and 24 h, and the cellular lysates were obtained and subjected to fractionation to further isolate mitochondrial fractions as described under "Experimental Procedures." Immunoblotting of the mitochondrial fractions was performed using anti-subunit 3 of cytochrome c oxidase (cox3p) or anti-porin (porin) antibodies. The results shown are from one experiment representative of three experiments.

FIG. 6. Phospholipid analysis of the wild type and isclΔ null strain. wt and iscl deletion mutant (isclΔ) strains were grown to late log phase in the presence of [32P]orthophosphate. Total membrane phospholipids were extracted and separated on thin layer chromatography plates as described under "Experimental Procedures." First and third lanes, wt; second and fourth lanes, isclΔ; first and second lanes, 50,000 cpm were loaded onto the TLC; third and fourth lanes, 100,000 cpm were loaded onto the TLC. Standard phospholipids were used to assign the identity of each spot. PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine.
observed when the blots were stripped and re-probed for the nuclear-encoded cox4p subunit; in this case a specific band of 15 kDa was detected in the wild type, and a significant reduction of the signal was observed in the lane corresponding to \textit{isc1} extract, which was reverted by expression of plasmid-borned \textit{ISC1} (data not shown). Thus, deletion of \textit{ISC1} mimics the effects of deleting \textit{PGS1} in the regulation of the protein levels of cox subunits.

Because the above results indicate a role for Isc1p downstream of Pgs1p, we next investigated if we could bypass the phenotype of \textit{PGS1} deletion by providing phytoceramide to yeast cells, thus bypassing the inability of the \textit{PGS1} deletion to cause the activation of Isc1p. It should be noted that overexpression of \textit{ISC1} in \textit{pgs1} did not rescue the defect of this mutant to grow in NFCS (data not shown), which was an expected result since the products of Pgs1p (PG and CL) appear to be absolutely required for activation of Isc1p. Yeast endogenous phytoceramide contains primarily very long chain fatty acids; however, short chain ceramides are taken up by yeast cells and are recognized by enzymes of sphingolipid metabolism (1). Wild type or mutant strains were cultured for 24 h in glucose-containing media as described under “Experimental Procedures” in the presence of 5 μM C12-phytoceramide, 5 μM C12-ceramide, or vehicle, and the \textit{A}_{600} was determined. Therefore, we next investigated if we could bypass the phenotype of \textit{PGS1} deletion by providing phytoceramide to yeast cells, thus bypassing the inability of the \textit{PGS1} deletion to cause the activation of Isc1p.

The addition of C12-phytoceramide to the culture media of \textit{isc1} cells provoked a reproducible (35%) increase in growth at 24 h (Fig. 8a). This effect was specific for phytoceramide since no stimulation of growth was found when cells were supplemented with 5 μM C12-ceramide for the same time (Fig. 8a). On the other hand, when wild type cells were treated with phytoceramide, no stimulatory effect was observed. Notably, \textit{pgs1} cells also responded to the addition of C12-phytoceramide, revealing an increase in growth at the 24 h (Fig. 8b). The effect of phytoceramide on the growth of \textit{isc1} in glucose-containing media demonstrated dose (Fig. 8c) and time dependence (data not shown). Importantly, the temporal increase in growth due to phytoceramide was more dramatic (~2-fold versus ~1.4-fold at 24 h of growth) in ethanol-containing media (Fig. 8d). No increase in growth due to C12-phytoceramide was observed up to 4 h in either media, a small effect was seen
between 8 and 12 h, and the maximal increases (−1.4- and −2.5-fold) were visualized at 24 h in glucose media (data not shown) and at 48 h in ethanol media (Fig. 8d), respectively. The pgs1Δ strain also showed a growth-dependent increase after addition of C12-phytoceramide in non-fermentable carbon source media (Fig. 8e), indicating that C12-phytoceramide caused a partial recovery of the growth defect of this strain in NFCs; however, this rescue was lower than in the isc1Δ strain (−1.7-versus −2.7-fold at 48 h, respectively). It is worth noting that the late addition (at 22 h) of C12-phytoceramide to cells that were vehicle-treated caused a stimulation of growth within 2 h as opposed to a lag until 24 h when C12-phytoceramide was supplied at time 0. These results suggest that the C12-phytoceramide is required more specifically at or after the diauxic shift but not earlier. Therefore, these results indicate that the growth defect due to lack of isc1Δ or pgs1Δ can be bypassed by providing phytoceramide, the lipid product of Isc1p.

**DISCUSSION**

The results from this study shed light on the *in vivo* mechanisms of activation of Isc1p, its role (and the role of its product phytoceramide) in regulation of growth, and its mechanistic relationship to the Pgs1p/Cls1p pathway of synthesis of the anionic phospholipids PG and CL. The results also provide evidence for the function of PG/CL as a bioactive lipid.

Disruption of the *ISC1* gene in *S. cerevisiae* resulted in a close-to-absolute dependence on fermentable carbon sources for growth. Thus, Isc1p must play a critical role in allowing yeast to execute mitochondrial metabolism that is required in the post-diauxic shift when cells are grown on glucose. Interestingly, the growth phenotype in both fermentable and non-fermentable carbon sources paralleled that of the *pgs1Δ* strain but not of the *cls1Δ* strain, whereby the *pgs1Δ* strain showed a more critical dependence on fermentable carbon sources than the *cls1Δ*, consistent with previous reports on the growth of *pgs1Δ* and *cls1Δ* (22–24).

This similarity of growth phenotype as well as the previous results demonstrating a requirement for *in vitro* activation of Isc1p by PG/CL, the products of Pgs1p, suggested the hypothesis that endogenous PG (or PG/CL) may be required for activation of Isc1p *in vivo*. Multiple lines of evidence are provided to support such a role for Pgs1p and PG (or PG/CL). First, loss of function by disruption of *PGS1* resulted in the loss of the growth phase-dependent activation of Isc1p that is observed in wild type cells between early logarithmic and the post-diauxic shift phases of growth. The effects of Pgs1p were specific for the activation of Isc1p as the translocation of Isc1p to the mitochondria seemed to be unaffected. Second, the accumulation of phytoceramide during the post-diauxic phase of growth was prevented by the lack of *PGS1*, demonstrating that the regulation of Isc1p by the function of the gene responsible for the synthesis of PG/CL occurs *in vivo*. Third, the addition of exogenous phytoceramide was able to partly restore the growth defects of both *isc1Δ* and *pgs1Δ* strains. Reciprocally, Isc1p did not function upstream of Pgs1p since PG/CL reached its normal levels at the diauxic shift in an Isc1p-independent manner.

The results from this study also suggest an important role for yeast ceramides, the products of Isc1p, in mediating the effects of Isc1p on growth. Thus, adding exogenous NBD-C12-phytoceramide overcame partially the growth defect of the *isc1Δ* in both glucose (during the post-diauxic shift) and ethanol (or glycerol) media (Fig. 8). Because of the dynamic and highly interconnected nature of sphingolipid metabolism, it was not possible to firmly conclude that growth stimulation was caused by phytoceramide itself. However, the effects of phytoceramide were specific in that C12-ceramide did not enhance cell growth, and the stimulatory effects of phytoceramide were not observed for the wild type strains, which exhibit an increase in endogenous ceramide during the post diauxic phase. Importantly, C12-phytoceramide exerted a stimulatory effect on the growth of *pgs1Δ*, partially correcting the growth phenotype of this strain. These results together with the requirement of Isc1p for yeast growth in non-fermentable carbon sources support the hypothesis that the Isc1p/phytoceramide is a key regulator of growth on non-fermentable carbon sources.

A minor difference between growth of *pgs1Δ* and *isc1Δ* was also observed such that the *isc1Δ* strain seemed to partially recover and grow in glycerol by 72 h, whereas the *pgs1Δ* strain did not. This difference suggests that Isc1p does not mediate all functions of Pgs1p; therefore, Pgs1p and its lipid products may regulate other downstream targets/effectsors in addition to Isc1p.

Mechanistically, the results demonstrate at least one role for Isc1p in regulating mitochondrial function through the regulation of levels of components of the mitochondrial respiratory system. Analysis of the levels of mitochondrial (cox3p)- or nuclear (cox4p)-encoded subunits of cox in mitochondrial extracts from cells in which Isc1p is disrupted showed a reduction of the levels of these proteins compared with the wild type extracts. These results indicate that Isc1p regulates the level of these mitochondrial proteins. Because the level of cox subunits in yeast showed regulation at the level of translation (24, 35), it has been demonstrated that the translation of cox subunits is depressed in *pgs1Δ* strains (24), and knowing that Isc1p is regulated by Pgs1p *in vivo*, it is tempting to speculate that the

**FIG. 9.** A schematic view for the involvement of Isc1p in growth during the diauxic shift. Dashed arrows indicate that other pathways may coexist.
translation of cox3p and cox4p is regulated by Isc1p. As such, re-insertion of the ISCI gene restored the defect in levels of cox subunits.

Taken together, the results therefore place Isc1p between Pgs1p and the regulation of cox levels and optimal growth. Thus, a novel pathway (Fig. 9) (Pgs1p → PG → Isc1p → phytoceramide → cox → respiration → normal growth) is proposed that begins to tie in the roles of Pgs1p and Isc1p in mitochondrial respiration and function.

Although the results demonstrating a strict requirement for PGS1 in activation of Isc1p in vivo suggest a direct role for endogenous PG as the activator of Isc1p, a role for CL cannot be ruled out at this point. Both lipids are capable of activating Isc1p in vitro, actually with a slight preference for CL. Moreover, it is worth noting that PG levels were reported to be ∼5-fold higher when CLS1 is deleted; therefore, the elevated PG may substitute for some functions of CL. Thus, the inability of the PGS1 deletion to support activation of Isc1p may be due to loss of either PG or CL, whereas the ability of the CLS1 deletion (in which the levels of cox subunits are not affected) to maintain Isc1p activation may be due either to the lack of a role for CL in vivo activation of Isc1p or to the compensation for the loss of CL by the increased PG. It should be noted, however, that PG does not substitute for all of CL functions; for example, the formation of supercomplexes of the mitochondrial electron transport chain (between homodimers of complex III and complex IV or cox complexes) (36, 37) is not found in clsΔ despite the higher levels of PG.

Based on the results presented in this study, a possible role for phytoceramide as a new bioactive lipid is emerging since; (a) the exogenous addition of phytoceramide elicited a cellular response (stimulation of growth), and this action of phytoceramide demonstrates lipid specificity; (b) the levels of phytoceramide are regulated (diauxic shift); (c) the generation of phytoceramide at this phase of growth is regulated by Isc1p; and (d) the generation of phytoceramide and of activation of Isc1p are controlled by Pgs1p; and (e) Isc1p and phytoceramide regulate downstream targets (levels of subunits of cytochrome c oxidase).

Equally as important, these results coupled with previous studies define a role for PG (and possibly CL) as a bioactive molecule since it satisfies criteria for a bioactive lipid; (a) the levels of PG increase during the diauxic phase, (b) PG directly activates Isc1p in vitro, (c) PG is required for Isc1p/phytoceramide action in vivo, and (d) loss of PG synthesis results in a growth defect on non-fermentable sources. Thus, changes in PG levels act on a direct target that, as previously shown, is required for important physiologic functions. In addition, previous studies have suggested functional roles for PG and CL in protein folding and apoptosis.

In conclusion, the results from this study provide novel insight into mechanisms of action of PG in cells involving bioactive sphingolipids, mechanisms of regulation of Isc1p in vivo, and the role of Isc1p in regulating growth of yeast in non-fermentable carbon sources and during the post-diauxic phase of growth.

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