Isolation and Characterization of the Gene (CLS1) Encoding Cardiolipin Synthase in Saccharomyces cerevisiae*

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In eukaryotic cells, cardiolipin (CL) synthase catalyzes the final step in the synthesis of CL from phosphatidylglycerol and CDP-diacylglycerol. CL and its synthesis are localized predominantly to the mitochondrial inner membrane, and CL is generally thought to be an essential component of many mitochondrial processes. By using homology searches for genes potentially encoding phospholipid biosynthetic enzymes, we have cloned the gene (CLS1) encoding CL synthase in Saccharomyces cerevisiae. Overexpression of the CLS1 gene under its endogenous promoter or the inducible GAL1 promoter in yeast and expression of CLS1 in baculovirus-infected insect cells resulted in elevated CL synthase activity. Disruption of the CLS1 gene in a haploid yeast strain resulted in the loss of CL synthase activity, no detectable CL, a 5-fold elevation in phosphatidylglycerol levels, and lack of staining of mitochondria by a dye with high affinity for CL. The cls1::TRP1 null mutant grew on both fermentable and non-fermentable carbon sources but more poorly on the latter. The level and activity of cytochrome c oxidase was normal, and a dye whose accumulation is dependent on membrane proton electrochemical potential effectively stained the mitochondria. These results definitively identify the gene encoding the CL synthase of yeast.

In eukaryotic cells cardiolipin (CL) and its synthesis are predominantly localized to the mitochondrial inner membrane (1–3). Due to its cellular distribution, CL is thought to be involved in critical cellular functions such as cytochrome c oxidase function (4), binding of matrix Ca2+ (5), maintenance of mitochondrial membrane permeability (6, 7), and mitochondrial protein import (8–12). In addition to basic cell functions, CL has been postulated to play a role in disease processes such as lipid peroxidation observed in reperfusion after ischemic injury and in cellular aging (13, 14). A vast amount of literature details biochemical approaches to determine the role of CL in cell processes (2, 15); however, it has been difficult to correlate the in vitro experimental findings with in vivo cellular processes due to the absence of genetic evidence supporting CL involvement in these processes. The focus of the work in our laboratory is to characterize the genes and enzymes necessary for the biosynthesis of CL and the role this anionic lipid plays in mitochondrial function.

CL is synthesized by three sequential reactions (Fig. 1) in all organisms and requires the biosynthetic intermediate, CDP-diacylglycerol (CDP-DAG). The committed and rate-limiting step in CL biosynthesis in Saccharomyces cerevisiae is catalyzed by phosphatidylglycerophosphate (PG-P) synthase (step 1) yielding PG-P which is rapidly dephosphorylated (16). In eukaryotic cells phosphatidylglycerol (PG) reacts in the final step (step 3) with another moleucle of CDP-DAG to yield CL catalyzed by CL synthase (17–19). In Escherichia coli the synthesis of PG proceeds by an analogous set of reactions (20) except CL is synthesized by the condensation of two PG molecules with the release of glyceral (21).

We recently isolated from S. cerevisiae and characterized the gene (PGS1) encoding the first enzyme in this pathway (22). In this paper we report the isolation and characterization of the gene (CLS1) encoding the last enzyme in the pathway for CL biosynthesis. The gene was cloned and expressed in both yeast and baculovirus-infected insect cells and shown to encode CL synthase activity. Interruption of the CLS1 gene resulted in no detectable CL or CL synthase activity and elevated levels of PG. Mitochondrial function was not grossly perturbed as evidenced by growth on non-fermentable carbon sources and maintenance of mitochondrial membrane potential in vivo. This is the first detailed report definitively establishing the isolation of a eukaryotic gene encoding CL synthase activity. A preliminary report of this work has appeared (23).

EXPERIMENTAL PROCEDURES

Materials—All chemicals were reagent grade or better. Restriction endonucleases and DNA-modifying enzymes were from Promega Corp., New England Biolabs, Stratagene, and Boehringer Mannheim. DASPMI (2-(4-dimethylamino styryl)-1-methylpyridinium iodide), DAPI (4′, 6-diamidino-2-phenylindole), and 10-Nonyl acridine orange were purchased from Molecular Probes. Thin layer chromatography Silica Gel 60 and HPTLC Silica Gel 60 plates were from EM Science. The polymerase chain reaction (PCR) was performed using PCR SuperMIX from Life Technologies, Inc. Oligonucleotides were prepared commercially by Genosys. The T7 and T3 primers used for automated DNA sequencing were provided by the Molecular Genetics Core Facility, University of Texas Houston Medical School, Houston. QIAEX™ II gel extraction kit was from Qiagen. The Genius™ 1 Kit (DNA Labeling and Detection Kit, Nonradioactive), digoxigenin-labeled DNA molecular weight markers, positively charged nylon membranes, and Lumi-
Cardiolipin Synthesis of *S. cerevisiae*

** Saccharomyces cerevisiae**  
| CDP-Diaclyglycerol | Escherichia coli |
|---------------------|-----------------|
| 1 PGSI  | CDP-Diaclyglycerol |
| 3 PGSI  | CDP-Diaclyglycerol |
| Phosphatidylglycerophosphate | Phosphatidylglycerophosphate |
| 3 CLSI  | CDP-Diaclyglycerol |

**Cardiolipin**

**FIG. 1.** CL biosynthetic pathway of *S. cerevisiae* and *E. coli*. The indicated reactions are catalyzed by the following enzymes encoded by the genes indicated: 1, PG-S synthase; 2, PG-P phosphatase; and 3, CL synthase.

Phos™ 530 were purchased from Boehringer Mannheim. All media for yeast growth and selection were from Bio 101 and Difco, and all media for bacterial growth were from Difco. Radiochemicals were obtained from Amersham Pharmacia Biotech and American Radiolabels. The BCA kit for protein determination was from Pierce. The lambda phage clone APM-5829 was purchased from American Type Culture Collection. PG derived from *E. coli* was purchased from Matreya Inc. CDPCDAG (dipeol) was synthesized (24) and generously provided by Dr. George Carman (Rutgers University). All other phospholipids were from Sigma.

**Strains, Plasmids, and Growth Conditions—** A list of strains and plasmids used in this work is given in Table I. Methods for *E. coli* growth and selection were described previously (25). Strain ADC90/pDD72 was used to make radiolabeled PG. Strain DH5α was used to propagate lambda phage (25). Methods of media were supplemented with 2% agar (yeast) or 1.5% agar (bacterial). Strain LE392 was used to propagate lambda phage (25). Methods of growth and selection were described previously (25). Strain DH5α/pDD72 which is deficient in phosphatidylethanolamine synthase when grown on plates. Yeast strains were grown at 30 or 37 °C, and bacterial strains were grown at 30, 37, or 42 °C.

**DNA Manipulations—** Methods for plasmid and genomic DNA preparation, restriction enzyme digestion, DNA ligation, and sequence analysis were carried out as described previously (22). For both analytical and preparative purposes, PCR was performed after optimizing conditions (31). Amplification of the CLS1 gene from yeast chromosomal DNA employed primers based on the open reading frame YDL142w as follows: primer 1, 5'-CGCAAAGCTTACAATGTTCAATGTTCACTGC-3' (the added HindIII site is underlined) and the start codon for the open reading frame is in boldface, and primer 2, 5'-CGTGTGTTGAGGAGGAGGACG-3' (trimming beginning 500 bp 3' of the stop codon). For Southern hybridization analysis DNA samples were digested with restriction endonuclease and prepared for hybridization with a digoxigenin-labeled probe as described previously (22). Template DNA for labeling by random priming using the Genius™1 Kit and use in the above procedure was generated by HindIII and BamHI digestion of plasmid pYCLS10-1 which produced a fragment (982 kb) containing the CLS1 gene.

**Preparation of Mitochondria-enriched Membrane Fraction—** All cell fractionation procedures were performed at 4 °C. Mitochondria-enriched membrane fractions were prepared as described previously (22). Briefly, yeast cells were grown to late log phase in CSM with auxotrophic selection when appropriate and either 2% glucose or 2% galactose as carbon source. Cells were pelleted and washed with homogenization buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.3 mM sucrose, 10 mM 2-mercaptoethanol) and frozen at −80 °C for later use. Cells were disrupted at 4 °C by mechanical shearing using a Mini-BeadBeater™

2 J. M. Cherry, C. Adler, C. Ball, S. Dwight, S. Chervitz, Y. Jia, G. Juvik, S. Weng, and D. Bostein, *Saccharomyces* genome data base [http://genome-www.stanford.edu/Saccharomyces](http://genome-www.stanford.edu/Saccharomyces) SGID: 000358, now named CRDI.
alized by iodine staining and ninhydrin spray) as well as comparison with previous reports (35). The results were visualized by exposure to x-ray film, and for quantitative results the radioactivity was counted using a Packard Instant Imager. Alternatively, one-dimensional thin layer chromatography was used (34). Phospholipid content is expressed as mol % of total phospholipid based on the radiolabel in each spot and its respective molar phospholipid content.

**Determination of Cytochrome c Oxidase Levels—Activity of cytochrome c oxidase was measured at 22 °C with an oxygen electrode in 0.25 M sucrose, 50 mM Tris-HCl buffer, pH 7.4, 50 mM KCl, 1 mM MgCl2, 5 mM ascorbate, 0.5 mM NADH, 0.1 mM DCCD, and 5 mM Na2S2O4. A peak at 605 nm (minus the differential absorbance at 630 nm) was used to calculate the cytochrome aa3 content.**

**Fluorescence Microscopy—** Exponentially growing strains DL1 and YCD4 in YPD or strains YPH98 and YCD2 in the CSM with 2% isothiocyanate filter set, and a 100 µm DASPMI for 20 min (38) at room temperature; in the latter case addition of 50 µM FCCP (carbonyl cyanide-(trifluoromethoxy)phenylhydrazone), 2 mM N,N,N',N'-tetramethyl-p-phenylenediamine, 5 µM FCCP (carbonyl cyanide-p-trifluoromethoxy)phenyldihydrazine), 2 µg of antimycin A, and mitochondria (0.05 mg of protein per ml) was added as described above. To determine cytochrome aa3 content, optical spectroscopy was performed with Hitachi U-3000 spectrophotometer at 22 °C using mitochondria (0.1 to 0.15 mg of protein per ml) in 0.25 M sucrose, 50 mM Tris-HCl buffer, pH 7.4, 50 mM KCl, 1 mM MgCl2, 5 mM ascorbate, and 10 µM ferricyanide was added to either reduce or oxidize the cytochromes, respectively, and the visible difference spectrum (reduced versus oxidized) was determined. A Δεred-ox = 227 mm-1 cm-1 (36) for the peak at 605 nm (minus the differential absorbance at 630 nm) was used to calculate the cytochrome aa3 content.

**Isolation of the CLS1 Gene—** Based on the deduced amino acid sequence of the products of *pgsA* genes (encoding PG-P synthases from *E. coli* (59) and *Rhodobacter sphaeroides* (40)), a computer-based homology search of the *S. cerevisiae* genome data base yielded open reading frame YDL142c on Chromosome IV which has a derived amino acid sequence showing 26 and 29% identity and 53 and 54% similarity, respectively, to the bacterial enzymes (Fig. 2). The CLS1 gene is defined by an 849-bp open reading frame encoding a protein of 283 amino acids with a predicted molecular mass of 31,999 Da. Although sequence comparison suggested the gene encodes a PG-P synthase, subsequent biochemical and genetic analyses established the gene to encode CL synthase (see below). The CLS1 gene product also showed weaker homology (20% or less identity and less than 45% similarity) with other phosphatidyltransferases from bacteria and yeast. The characteristic CDP-alcohol/phosphatidyltransferase signature (41) is present between positions 106 and 136 of the CL synthase. This motif is present in the PG-P synthases shown in Fig. 2 and is also found in phosphatidylserine synthases from *Bacillus subtilis* and *S. cerevisiae*, in phosphatidylinositol synthase from *S. cerevisiae*, and in numerous other bacterial PG-P synthases (40); this motif is not found in either the phosphatidylserine synthase (42) or CL synthase (43) of *E. coli*.

No sequence in the 5′-untranslated region matched the UASING (upstream activation sequence responsive to repression by inositol), 5′-CATGTGAAAT-3′ (44, 45). This is consistent with absence of regulation of the level of CL synthase activity by inositol in the growth media (19, 46). Analysis of the predicted amino acid sequence by PSORT (47) indicated targeting of this protein to the inner mitochondrial membrane with a certainty of 0.76 versus the 0.56 for peroxisomes and 0.44 for plasma membranes. A M, of 28,000 based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis has been reported for the CL synthase (48) which would be consistent with a mitochondrial targeting sequence of about 36 amino acids and place the beginning of the putative mature protein very close to the beginning of its homology with bacterial PG-P synthases.

The CLS1 gene was obtained from DNA isolated from strain DL1 using PCR and primers which added a 5′ HindIII site. The PCR product was digested with HindIII and NsiI (110 bp 3′ of the stop codon) and cloned into the HindIII and PstI sites of the plasmid pUC19. Subsequently, the PCR-generated CLS1 gene was released from plasmid pUC19 by digestion with HindIII and BamHI (vector site) and transferred to the HindIII and BamHI sites of plasmid pYES2 generating plasmid pYCLS10-1 which placed the CL5 gene under the regulation of the GALI promoter (pGALI-CLS1). The PCR product was transferred to plasmid Bluescript II KS in the same manner, and its DNA sequence was confirmed by automated DNA sequencing. The resulting DNA sequence matched that of open reading frame YDL142c at all nucleotides with the exception of the third position of codon 198 of the CLS1 gene which was not

### Table I

**Strains and plasmids**

| Strains or plasmids | Relevant characteristics | Reference or source |
|---------------------|--------------------------|---------------------|
| **E. coli** DH5α | *endA1, hisD17(rpsL20), supE44, λ-, gyrA96, relA1, (f80d lacZΔM15), ΔlacZYA-argF U169* | 25 |
| LE392 | *psi33:kan, cslc tet/psA3*, Camr | 26 |
| ADC90/pDD72 | *psi33:kan, cslc tet/psA3*, Camr | Xi and Dowan (University of Texas Medical School) |
| **S. cerevisiae** YPH98 | *ura3-52, lys2-801, ade2-101, leu2-Δ1, trp1-Δ1* | 27 |
| YCD2 | *cslc:TRP1, derivative of YPH98* | This work |
| DL1 | *his3-Δ11, his3-15, leu2-3, leu2-12, ura3-251, ura3-372, ura3-328* | 25 |
| YCD4 | *pgs1::HIS3, derivative of DL1* | This work |
| Plasmids | | |
| pBluescript II KS | Cloning vector, *ampR* | Stratagene |
| pRS304 | pBluescript, TRP1, *ampR* | 27 |
| pUC19 | *lacOPZ, *ampR*, ColE1 origin | Life Technologies, Inc. |
| pYES2 | Pgal-UAS3, *ampR*2, 2-µm circle, ColE1 origin | This work |
| pYCLS1 | CLS1 (genomic), derivative of pYES2 | Invitrogen |
| pYCLS10-1 | Pgal-CLS1 (PCR product), derivative of pYES2 | This work |
| pVL1392 | Very late Ac/MMPV polyhedrin promoter | Invitrogen |
| pVCLS1B | Pgal-CLS1, derivative of pVL1392 | This work |

* Partial list of genetic markers.

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**RESULTS**

**Isolation of the CLS1 Gene—** Based on the deduced amino acid sequence of the products of *pgsA* genes (encoding PG-P synthases from *E. coli* (59) and *Rhodobacter sphaeroides* (40)), a computer-based homology search of the *S. cerevisiae* genome data base yielded open reading frame YDL142c on Chromosome IV with a derived amino acid sequence showing 26 and 29% identity and 53 and 54% similarity, respectively, to the bacterial enzymes (Fig. 2). The CLS1 gene is defined by an 849-bp open reading frame encoding a protein of 283 amino acids with a predicted molecular mass of 31,999 Da. Although sequence comparison suggested the gene encodes a PG-P synthase, subsequent biochemical and genetic analyses established the gene to encode CL synthase (see below). The CLS1 gene product also showed weaker homology (20% or less identity and less than 45% similarity) with other phosphatidyltransferases from bacteria and yeast. The characteristic CDP-alcohol/phosphatidyltransferase signature (41) is present between positions 106 and 136 of the CL synthase. This motif is present in the PG-P synthases shown in Fig. 2 and is also found in phosphatidylserine synthases from *Bacillus subtilis* and *S. cerevisiae*, in phosphatidylinositol synthase from *S. cerevisiae*, and in numerous other bacterial PG-P synthases (40); this motif is not found in either the phosphatidylserine synthase (42) or CL synthase (43) of *E. coli*.

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definitively assigned; the nucleotide at this position does not affect the encoded amino acid arginine. A genomic copy of the CLS1 gene with its endogenous promoter elements was obtained by subcloning the HindIII-BamHI fragment of λPM-5829 containing yeast genomic DNA into plasmid pYES2 generating plasmid pYCLS-1; this fragment contains 667 nucleotides 5' to the start codon and 2446 nucleotides 3' of the stop codon of the open reading frame and therefore should contain all upstream and downstream factors necessary for normal expression of CLS1.

Disruption of the Genomic CLS1 Gene—This disruption was accomplished by the replacement of 323 bp in the N-terminal half of the open reading frame with the TRP1 gene from vector pRS304 (27). The N-terminal HindIII-EcoRI fragment (~667 bp to ~574 bp) from plasmid pYCLS-1 was subcloned into plasmid pUC19. Next, the SnaBI-BamHI fragment (1,608 bp) of plasmid pRS304 containing the TRP1 gene was ligated between the HpaI (~89 bp) and BclI (~421 bp) sites, respectively, within the N-terminal half of the CLS1 gene. The cls1::TRP1 gene was released from the above plasmid with HindIII and EcoRI (both outside of the CLS1 gene) digestion and transformed into the haploid yeast strain YPH98. Transformants were selected on CSM-Trp plus 2% glucose medium resulting in haploid null mutant strain YCD2 (cls1::TRP1).

Genomic DNA from wild type strain YPH98 and cls1 null mutant YCD2 was isolated and either subjected to PCR amplification using primers 1 and 2 or digested with NcoI and BamHI for Southern hybridization analysis. The products of PCR amplification (Fig. 3A) showed the predicted size for the wild type CLS1 gene-containing fragment (1384 bp) of strain YPH98 and the predicted size (2992 bp) for the interrupted gene-containing fragment of strain YCD2. A labeled probe specific for the CLS1 gene was generated using the HindIII-BamHI insert of the plasmid pYCLS-1 as template. In strain YPH98 (Fig. 3B), a band consistent with the predicted 3,903-bp fragment for the wild type allele hybridized with the probe, and in strain YCD2, a band consistent with the predicted 5,188-bp fragment for the disrupted allele hybridized with the probe.

Gross phenotypic characterization of strain YCD2 showed only small differences in growth properties compared with wild
type cells. Overnight cultures of strains YCD2 and YPH98 were inoculated into rich YP medium, CSM or CSM-Trp with either 2% glucose or 2% glucose plus 0.2% glycerol or 0.2% galactose as carbon source. To induce the appropriate metabolic enzymes, the cells were allowed to grow for 4 h and subsequently with serial dilutions plated on the following agar plates: YPG; YPGal; YPGly; CSM or CSM-Trp with either 2% glucose, 2% glycerol, or 2% galactose as carbon source. The cells were grown at both 30 and 37 °C. Strain YCD2 grew well on all carbon sources at all temperatures tested and therefore was not respiratory-deficient. In order to test whether the null cls1 mutant exhibited the petite lethal phenotype (49) (i.e. lack of growth in the presence of ethidium bromide which generates a high frequency of deletions in mitochondrial DNA), a duplicate set of plates (YPD and CSM or CSM-Trp with 2% glucose) containing 25 μM ethidium bromide was seeded. A high level of colony formation by both the mutant and wild type parental strain in the presence of 25 μM ethidium bromide ruled out incompatibility with mutations in mitochondrial DNA (petite lethal phenotype); pgs1 mutants which lack both PG and CL display the petite lethal phenotype (22, 49). In liquid culture strain YCD2 grew slower and reached stationary phase at consistently lower cell densities when grown on a non-fermentable carbon source as compared with strain YPH98 but did not show other difference in growth properties compared with its parental strain YPH98.

CLS1 Synthase Activity—Mitochondria-enriched fractions were prepared as described under “Experimental Procedures” of late log phase cultures of the strains YPH98 and YCD2 grown in CSM or strain YCD2 transformed with either plasmid pYCLS-1 or pYCLS10-1 grown in CSM-uracl; either glucose or galactose was used as carbon source. Expression of the cls1 gene from plasmid pYCLS10–1 should be induced by galactose and repressed by glucose due to transcription under its native promoter. Expression of CLS1 under GAL1 regulation from a multicopy plasmid (pYCLS10-1) showed the expected overexpression of CL synthase activity (82 ± 3 units/mg) when cells were grown in galactose as compared with cells grown in glucose (1.0 ± 0.1 units/mg). These results are consistent with the CLS1 gene encoding over 90% if not all of the CL synthase of yeast.

To rule out the possibility that the mutation resulted in a general lack of anionic phospholipid biosynthetic capability, the level of PG-P synthase activity was measured. The specific activity of PG-P synthase in glucose grown cells was reduced in the cls1 null strain (0.17 ± 0.02 units/mg) by about 35% relative to the wild type parent strain (0.26 ± 0.02 units/mg). This difference is well within the range of specific activities for this enzyme as a function of growth conditions and stage of growth on glucose (22).

Expression of the Yeast CLS1 Gene Using Baculovirus—Plasmid pVL1392 (P_{PH-CLS1}) was introduced into insect cell line Sf9 by transfection as described under “Experimental Procedures.” The transfected cell lysates were greatly enriched in CL synthase activity (13 ± 2 units/mg) as compared with the uninfected cell lysates (0.8 ± 0.1 units/mg). This level of over-expressed synthase activity compares favorably with the specific activity of the synthase in the enriched mitochondrial fraction from cells in which CL synthase was amplified by multiple copies of the CLS1 gene (see above). This amplified activity in Sf9 cells is definitive proof that the CLS1 gene encodes a CL synthase and does not encode a regulator of either gene expression or mitochondrial biogenesis.

Phospholipid Composition—Strains YPH98 and YCD2 were labeled with [32P]orthophosphate and analyzed for phospholipid composition as described under “Experimental Procedures” (Fig. 4 and Table II). The wild type strain showed the expected complement of phospholipids including easily detectable CL with trace amounts of PG (Fig. 4, A, C, and E). The cls1 null mutant lacked detectable CL and had elevated levels (about 5-fold over wild type) of PG (Fig. 4, B, D, and E). In addition the mutant showed a new radiolabeled component near the front of all solvent systems (arrow in Fig. 4, D and E). This material which constituted about 0.2% of the total radiolabeled material may be a higher order acylated derivative of PG which only becomes detectable when PG accumulates to abnormal levels. These results further confirm the identity of the gene product of open reading YDL142w as the CL synthase.

Carboxylic acidine dye staining procedures were used to detect the presence of CL in mitochondrial fractions. 10-N-Nonyl acridine orange is a lipophilic dye that has been shown to have an affinity for CL about 50-fold higher and a resulting red fluorescence emission yield about 5-10-fold higher than other anionic phospholipids such as phosphatidylserine or phosphatidylinositol (37). It has been used to specifically visualize mitochondria in living cells using fluorescence microscopy and for quantification of CL in mitochondria of whole cells (1). Wild type cells when treated with this dye clearly show fluorescent structures (Fig. 5, B and F) which have the morphology of mitochondria. Similar structures were barely detectable in the cls1 null mutant (Fig. 5D) consistent with the lack of CL in the mitochondria. Cells grown in either glucose (not shown) or galactose showed similar results. In the pgs1 null mutant (YCD4), which lacks both PG and CL (22), no distinct struc-
tures could be seen (Fig. 5H), suggesting that the faint fluorescence seen in the cls1 null mutant was due to the high PG content of the mitochondria. These results are consistent with the predicted specificity of this dye for CL over PG and provide the first in vivo evidence that this dye can be used to identify the distribution of CL in vivo with a high level of confidence.

**Cytochrome c Oxidase Activity and Mitochondrial Function**—Null mutants in pgs1 lacking both PG and CL cannot grow on non-fermentable carbon sources (22) and have only trace levels of cytochrome c oxidase content or activity but appear to be normal in the b cytochromes (50). In contrast, both cytochrome c oxidase activity (1120 ± 79 and 1120 ± 5 nanogram atoms of oxygen per min per mg of protein, respectively) or cytochrome aa₃ content (0.37 ± 0.04 and 0.33 ± 0.03 nmol per mg of protein, respectively) were the same in wild type and cls1 null mutant strains (grown in YPGal) consistent with the ability of the mutant to grow on non-fermentable carbon sources. Mitochondria have a high proton electrochemical potential (positive outward) and therefore accumulate the fluorescent dye DASPMI that has been used as a specific marker for mitochondria in living cells (38). Both wild type cells (Fig. 6, B and C) and the cls1 null mutant (Fig. 6, E and F) accumulate this dye in a similar manner suggesting that the potential across the mitochondrial membrane of the mutant is not grossly compromised; no fluorescence was observed with either strain if the uncoupler FCCP was added to cells prior to addition of DASPMI. With this dye the transfer of filamentous mitochondrial structures from mother to daughter cells could also be observed (Fig. 7) suggesting normal mitochondrial segregation during cell division.

On the other hand no distinct structures were seen with the

| TABLE II | Phospholipid composition of the yeast strains |
|-----------------------------------------------|-----------------------------------------------|
| Cultures of the indicated strains were labeled with [³²P]orthophosphate, and the phospholipids were analyzed as described under “Experimental Procedures” for either two-dimensional (A–D) or one-dimensional (E) thin layer chromatography. A and B were short exposures (2 h) and C and D were long exposures (12 h) of the same plates. The arrows in C–E denote a unique spot in strain YCD2 that is absent in the wild type strain YPH98. Standard phospholipids were used to assign the identity of each spot: CL, cardiolipin; PA, phosphatidic acid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine. |
| YPH98 (CLS1) | YCD2 (cls1::TRP1) |
|-----------------------------------------------|-----------------------------------------------|
| Phosphatidylcholine | 35 ± 0.1 | 34 ± 0.7 |
| Phosphatidyl ethanolamine | 10 ± 0.2 | 9.1 ± 0.3 |
| Phosphatidylethanolamine | 21 ± 0.3 | 24 ± 0.4 |
| Phosphatidylserine | 8.4 ± 0.6 | 6.6 ± 0.3 |
| Phosphatidic acid | 5.8 ± 0.3 | 6.4 ± 0.3 |
| Phosphatidic acid | 0.7 ± 0.2 | 3.9 ± 0.3 |
| CL | 2.1 ± 0.5 | UDᵇ |
| Othersᵃ | 15 ± 0.8 | 16 ± 1.4 |

ᵃ Combination of several minor radioactive spots, includes lysophosphatidylcholine and sphingolipids.
ᵇ CL was undetectable.

**Cytochrome c Oxidase Activity and Mitochondrial Function**—Null mutants in pgs1 lacking both PG and CL cannot grow on non-fermentable carbon sources (22) and have only trace levels of cytochrome c oxidase content or activity but appear to be normal in the b cytochromes (50). In contrast, both cytochrome c oxidase activity (1120 ± 79 and 1120 ± 5 nanogram atoms of oxygen per min per mg of protein, respectively) or cytochrome aa₃ content (0.37 ± 0.04 and 0.33 ± 0.03 nmol per mg of protein, respectively) were the same in wild type and cls1 null mutant strains (grown in YPGal) consistent with the ability of the mutant to grow on non-fermentable carbon sources. Mitochondria have a high proton electrochemical potential (positive outward) and therefore accumulate the fluorescent dye DASPMI that has been used as a specific marker for mitochondria in living cells (38). Both wild type cells (Fig. 6, B and C) and the cls1 null mutant (Fig. 6, E and F) accumulate this dye in a similar manner suggesting that the potential across the mitochondrial membrane of the mutant is not grossly compromised; no fluorescence was observed with either strain if the uncoupler FCCP was added to cells prior to addition of DASPMI. With this dye the transfer of filamentous mitochondrial structures from mother to daughter cells could also be observed (Fig. 7) suggesting normal mitochondrial segregation during cell division.

On the other hand no distinct structures were seen with the
null mutant (not shown) but only a dispersed DASPMI fluorescence that was not stable and disappeared rapidly. When cells were stained with DAPI for DNA (Fig. 8), wild type cells showed a characteristic focused fluorescence for the nucleus and strings of beads for the mitochondria. The pgs1 mutant strain only exhibited what appears to be a single condensed structure and no evidence of elongated mitochondria. Clearly this mutant still has mitochondrial DNA because its mutant characteristics can be complemented by introduction of a plasmid-borne copy of the PGS1 gene under the control of its own promoter (49). These results would indicate a very low mitochondrial membrane potential and/or highly irregular mitochondrial structure in cells lacking both PG and CL consistent with gross defects in mitochondrial function in this mutant.

DISCUSSION

CL is an anionic phospholipid thought to be involved in numerous cellular processes. To gain further insight into its role in the cell, we undertook the isolation and characterization of genes involved in CL biosynthesis in S. cerevisiae. We have presented definitive evidence that open reading frame YDL142c (CLS1) encodes CL synthase. Overexpression of the gene in yeast resulted in the expected increase in CL synthase activity as did expression of the gene in a heterologous insect cell system. The null mutant had no detectable CL or CL synthase activity and elevated levels of PG.

The yeast CLS1 gene product shows significant homology with the PG-P synthases of prokaryotic organisms (40) and little or no homology with the CL synthase of E. coli (43). Although initially surprising, this result is still consistent with the enzymatic reactions catalyzed by PG-P and CL synthases (51–54) which are both CDP-DAG-dependent phosphatidyltransferases, whereas the prokaryotic CL synthase does not utilize this liponucleotide. The PGS1 gene encoding the yeast PG-P synthase has been identified (22), and its product shows little homology with either bacterial PG-P synthases or any CL synthase. The PGS1 gene was originally named PEL1 based on

FIG. 5. Staining of living cells with 10-N-nonyl acridine orange. Strains DL1 and YCD4 were grown in YPD and strains YPH98 and YCD2 were grown in CSM with 2% galactose, and all were treated with 10-N-nonyl acridine orange as described under “Experimental Procedures.” A–H are arranged in pairs with phase-contrast photomicrographs on the right and fluorescence photomicrographs on the left. Panels show YPH98 (A and B), YCD2 (C and D), DL1 (E and F), and YCD4 (G and H). Exposure times were either ¼ s (B and F) or 2 s (D and H). Bar, 5 μm.

FIG. 6. Energy-dependent accumulation of DASPMI. Strains YPH98 and YCD2 were grown in CSM with 2% galactose and treated with DASPMI as described under “Experimental Procedures.” A–C show strain YPH98, and D–F show strain YCD2. A and D are phase-contrast photomicrographs, and B, C, E, and F are fluorescence photomicrographs. Exposure times were either 1 s (B and E) or 2 s (C and F). Bar, 5 μm.

FIG. 7. Mitochondrial transfer during cell division. Strain YCD2 was grown in CSM with 2% glucose and treated with DASPMI as described under “Experimental Procedures.” Exposure time was 1 s. Bar, 5 μm.

FIG. 8. Staining of cells for DNA. Strains DL1 (A and B) and YCD4 (C and D) grown in YPD and treated with DAPI as described under “Experimental Procedures.” A and C are phase-contrast photomicrographs and B and D are fluorescence photomicrographs. Bar, 5 μm.

pgs1 null mutant (not shown) but only a dispersed DASPMI fluorescence that was not stable and disappeared rapidly. When cells were stained with DAPI for DNA (Fig. 8), wild type cells showed a characteristic focused fluorescence for the nucleus and strings of beads for the mitochondria. The pgs1 mutant strain only exhibited what appears to be a single condensed structure and no evidence of elongated mitochondria. Clearly this mutant still has mitochondrial DNA because its mutant characteristics can be complemented by introduction of a plasmid-borne copy of the PGS1 gene under the control of its own promoter (49). These results would indicate a very low mitochondrial membrane potential and/or highly irregular mitochondrial structure in cells lacking both PG and CL consistent with gross defects in mitochondrial function in this mutant.

DISCUSSION

CL is an anionic phospholipid thought to be involved in numerous cellular processes. To gain further insight into its role in the cell, we undertook the isolation and characterization of genes involved in CL biosynthesis in S. cerevisiae. We have presented definitive evidence that open reading frame YDL142c (CLS1) encodes CL synthase. Overexpression of the gene in yeast resulted in the expected increase in CL synthase activity as did expression of the gene in a heterologous insect cell system. The null mutant had no detectable CL or CL synthase activity and elevated levels of PG.

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3 M. Rho and W. Dowhan, unpublished observations.
the petite lethal phenotype of point and null mutants in this gene (49, 50). Subsequently, it also was named PSS2 based on the sequence homology of its gene product with the phosphatidylserine synthase (pssA gene product) of E. coli (55, 56). The definitive identification of the PGS1 gene product as a PG-P synthase was established based on biochemical characterization of overexpression of the isolated gene in both yeast and E. coli and a more detailed biochemical characterization of mutants null in PGS1 (22). A cautionary note resulting from these unexpected homology relationships is the unreliability of assigning gene product function solely on the basis of sequence homology or even the phenotype of mutants without biochemical verification of a direct gene product relationship. The lack of homology at the protein level and at the mechanistic level between prokaryotic and eukaryotic organisms in the PG/CL biosynthetic pathway might make this required pathway in bacteria an important target for development of antibiotics.

During the preparation of this manuscript two reports (57, 58) were published that putatively assigned CL synthase as the product of the CLS1 gene based solely on characterization of cls1 null mutants. This assignment is consistent with lack of detectable CL synthase activity (57, 58), and accumulation of PG in mutants null in CLS1 (58). However, no definitive evidence was presented to rule out a regulatory role rather than a biosynthetic role for the CLS1 gene product in CL synthesis or the possibility that mutants in CLS1 are defective in mitochondrial processes necessary to support CL synthase function and/or synthesis. For instance mutants in cytochrome c oxidase subunits show up to a 60% reduction in CL synthase activity (48). Fortunately, the gene locus assigned by these papers as encoding CL synthase was correct as established by the results reported here.

Disruption of the CLS1 gene resulted in an absence of CL and a 5-fold increase in total PG content. This is the expected result based on the pathway for CL synthesis. The more interesting result was that the absence of CL was neither lethal to the cell nor did it result in gross mitochondrial dysfunction particularly in oxygen-dependent energy metabolism, although qualitatively cells grew poorer on non-fermentable carbon sources as was previously reported for this mutant (57, 58). In addition gross mitochondrial morphology and the migration of mitochondria to daughter cells were not perturbed as indicated by staining with fluorescent dyes. Previous results had shown that cytochrome c, aa3, and b levels were normal in the null mutant (58) consistent with our findings which also showed that in vivo mitochondrial membrane potential was not significantly perturbed based on accumulation of DASPMI by mitochondria. Cells lacking mitochondrial DNA and hence completely devoid of functions necessary for oxidative phosphorylation can also tolerate the cls1 null allele, and therefore this mutant does not exhibit a petite lethal phenotype. These properties are in marked contrast to those of pgs1 null mutants that lack both PG and CL (22). These latter mutants cannot grow on non-fermentable carbon sources (22, 50) and have very low levels of cytochrome c oxidase (50). These properties are consistent with the lack of staining in this mutant by both DASPMI and 10-N-nonyl acridine orange. In addition pgs1 null mutants exhibit the petite lethal phenotype (22, 49) that would indicate a more extensive mitochondrial dysfunction than simply loss of the ability to carry out oxidative phosphorylation. The lack of detection of filamentous mitochondrial structures by DAPI also suggests a disruption of mitochondrial structure. Chinese hamster ovary cell mutants with reduced levels of both PG and CL are similar in their properties to the yeast pgs1 mutants and display significant alterations in mitochondrial morphology (59, 60). These results taken together strongly suggest that PG can substitute for CL in all essential functions in the mitochondria, but complete loss of both of these anionic phospholipids results in severe mitochondrial dysfunction.

Yeast cls1 mutants are somewhat compromised metabolically when grown on non-fermentable carbon sources, and in some genetic backgrounds have been reported to be temperature-sensitive for growth (57) as are pgs1 null mutants (22). It is likely that CL does not play an important role under well supported conditions, but in a nutrient-poor environment or under conditions of cell stress, CL may indeed be necessary for mitochondrial function or cell vitality. This is similar to the finding in E. coli where the absence of PG but not CL is lethal (32, 61). However, E. coli lacking CL are reduced in their ability to survive under harsh conditions such as cycles of freeze-thaw or in long term arrest in stationary phase (62).

The availability of mutants in PG and CL biosynthesis in E. coli and construction of strains in which the levels of these lipids can be regulated have been effectively used to define specific roles for anionic lipids in membrane structure, DNA replication, and translocation of proteins across membranes (20). PG and/or CL may also be involved in protein import into the mitochondria (8–12). In particular cytochrome c oxidase, which is very low in pgs1 mutants, may require either PG or CL for assembly and/or stability. Now that the genes encoding the enzymes for the synthesis of these two lipids in yeast have been identified, the requirements for PG and CL in mitochondrial function at the molecular level can be more precisely defined.

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