Yeast Pgc1p (YPL206c) Controls the Amount of Phosphatidylglycerol via a Phospholipase C-type Degradation Mechanism*

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Mária Šimočková1, Roman Holíč, Dana Tahotná, Jana Patton-Vogt, and Peter Griač

From the 1Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Moyzesova 61, Ivanka pri Dunaji, 900 28, Slovakia and the 2Department of Biological Sciences, Duquesne University, Pittsburgh, Pennsylvania 15282

The product of the open reading frame YPL206c, Pgc1p, of the yeast Saccharomyces cerevisiae displays homology to bacterial and mammalian glycerophosphodiester phosphodiesterases. Deletion of PGCI causes an accumulation of the anionic phospholipid, phosphatidylglycerol (PG), especially under conditions of inositol limitation. This PG accumulation was not caused by increased production of phosphatidylglycerol phosphate or by decreased consumption of PG in the formation of cardiolipin, the end product of the pathway. PG accumulation in the pgc1Δ strain was caused rather by inactivation of the PG degradation pathway. Our data demonstrate an existence of a novel regulatory mechanism in the cardiolipin biosynthetic pathway in which Pgc1p is required for the removal of excess PG via a phospholipase C-type degradation mechanism.

Cardiolipin (CL) is a major mitochondrial anionic phospholipid with important functions in promoting cell growth, anaerobic metabolism, mitochondrial function, and biogenesis (1, 2). Phosphatidylglycerol is not only a metabolic precursor in the biosynthetic pathway leading to the formation of cardiolipin but itself is an important phospholipid; for example it is the sole phospholipid of thylakoid membranes of prokaryotic and eukaryotic oxygenic photosynthetic organisms (3). In mammals, PG is especially important in pulmonary surfactant, an essential fluid produced by alveolar type II cells that covers the entire surface of the lung (4). Considering the importance of this anionic phospholipid, little is known about the mechanisms by which eukaryotic cells control PG membrane composition.

In yeast cells, PG is a low abundance phospholipid, present at best as a few tenths of a percent of total cellular phospholipids, even under the conditions of respiratory growth (5). Therefore, PG is considered to be mainly a metabolic precursor to CL. Biosynthesis of CL starts with common intermediates of phospholipid biosynthesis, phosphatidic acid and CDP-diacylglycerol (DAG) (Fig. 1). Phosphatidylglycerol phosphate (PGP) is formed from CDP-DAG and glycerol-3-phosphate. This step is catalyzed by PGP synthase, product of the PGS1/PEL1 gene (6, 7). PGP is subsequently dephosphorylated to form PG. Finally, CL is synthesized in yeast and other eukaryotic organisms from CDP-DAG and PG via a reaction catalyzed by cardiolipin synthase (CRD1) (5, 8, 9). It is important to note that both CL and PG are subject to intense remodeling subsequent to their de novo synthesis (10).

Mitochondrial phospholipid biosynthetic activities in general respond to factors affecting mitochondrial development and function, such as carbon source, growth phase, availability of oxygen, and mutations affecting mitochondrial development and function (11, 12). In fact, both enzymatic activities of the CL biosynthetic pathway, PGP synthase and CL synthase, respond to mitochondrial development factors in such a way as to increase the production of CL when cells switch from fermentative to respiratory growth (13–15). A typical feature of yeast phospholipid biosynthesis is its regulation by soluble precursors of phospholipid biosynthesis, inositol and choline. Much of this regulation occurs at the transcriptional level (for review see Refs. 16–18). Many yeast phospholipid biosynthetic genes contain in their promoters inositol-sensitive upstream-activated sequence and are derepressed in the absence of inositol and repressed in the presence of inositol. This gene activation and repression are facilitated by two transcription factors, Ino2p and Ino4p (19), and a negative regulator, Opi1p (20). The CL branch of the yeast phospholipid biosynthetic pathway is also repressed by the presence of exogenous inositol. This repression occurs mostly at the formation of PGP, a rate-limiting reaction of the pathway catalyzed by PGP synthase (15, 21). This repressive effect of inositol on the PGP synthase activity is not altered in mutants with defective or missing transcriptional regulators Ino2p, Ino4p, or Opi1p, indicating a mechanism other than transcriptional repression (15). Post-translational regulation of PGP synthase activity via phosphorylation was recently found to be responsible for rapid decrease in PGP synthase activity upon addition of inositol (22). The PGP phosphatase and CL synthase activities appear not to be regulated by inositol (23), but a novel regulation of the CRD1 gene by Ino4p has been recently reported (24).
**Phosphatidylglycerol Control in Yeast**

It is believed that PG can substitute for CL in most cellular functions in yeast, as demonstrated by the absence of any gross mitochondrial dysfunction in *crd1A* mutant (5, 8, 9). However, not all CL functions can be substituted for by PG, because a CL-deficient mutant has decreased mitochondrial membrane potential, decreased maximum respiratory rate, and decreased ATPase and cytochrome c activities (25). Absence of both PG and CL, as occurs in a *PGS1/PEL1* deletion mutant, causes more severe phenotypes: dependence on a fermentable carbon source for growth, temperature sensitivity, and "petite lethality" (7, 26). However, the use of osmotic stabilizers in the growth medium alleviated reported inability of *pgs1Δ* mutant to grow in the presence of ethidium bromide (27), demonstrating that mitochondrial anionic phospholipids and functional mitochondria are required for cell wall biogenesis (28). Furthermore, simultaneous deletion of *PGS1/PEL1* and *CHO1* (encoding for phosphatidylserine synthase) is synthetically lethal even for phosphatidylserine synthase, as occurs in a *clp5ΔΔ* strain (5, 8, 9). However, the use of osmotic stabilizers in the growth medium alleviated reported inability of *pgs1Δ* mutant to grow in the presence of ethidium bromide (27), demonstrating that mitochondrial anionic phospholipids and functional mitochondria are required for cell wall biogenesis (28). Furthermore, simultaneous deletion of *PGS1/PEL1* and *CHO1* (encoding for phosphatidylserine synthase) is synthetically lethal even for phosphatidylserine synthase, as occurs in a *clp5ΔΔ* strain (5, 8, 9). However, the use of osmotic stabilizers in the growth medium alleviated reported inability of *pgs1Δ* mutant to grow in the presence of ethidium bromide (27), demonstrating that mitochondrial anionic phospholipids and functional mitochondria are required for cell wall biogenesis (28). Furthermore, simultaneous deletion of *PGS1/PEL1* and *CHO1* (encoding for phosphatidylserine synthase) is synthetically lethal even for phosphatidylserine synthase, as occurs in a *clp5ΔΔ* strain (5, 8, 9).

**EXPERIMENTAL PROCEDURES**

**Materials**—Yeast extract, peptone, and bacteriological agar were purchased from Difco. Radiochemicals: l-[^14C]glycerol-3-phosphate (50 Ci/mol) was supplied by American Radiolabeled Chemicals, [^32P]orthophosphoric acid and [^14C]palmitate were purchased from MP Biomedicals. CDP-DAG and 1-palmitoyl-2-[6-[^14C]-nitro-2-1,3-benzoxadiazol-4-yl)aminophenyl]-sn-glycerol-3-phospho-rac-(1-14C)-glycerol (NBD- PG) were supplied by Avanti Polar Lipids. *Bacillus cereus* phospholipase C was obtained from Calbiochem. Thin-layer Silica Gel 60 plates were purchased from Merck. Zymolyase 20T was supplied by Seikagaku Corp. All other chemicals were reagent grade or better.

**Strains and Culture Conditions**—*S. cerevisiae* strains used in this study are listed in Table 1. *Pichia pastoris* strain GS115 (his4) was obtained from Invitrogen. Cultures were maintained on YPD (2% yeast extract, 1% peptone, 2% glucose) medium. Cells were grown aerobically at 30 °C in chemically defined synthetic medium prepared as described previously (33), unless otherwise stated. Synthetic medium either lacked inositol (I−) or were supplemented with 75 μM inositol (I+) or 75 μM inositol (I+). Synthetic I−-URA medium contained the same ingredients as I− medium with the omission of uracil.

**Plasmid and Strain Construction**—Standard genetic methods were used throughout the work (34). Yeast transformation was performed by the lithium acetate method (35). Yeast strains PGY223 and PGY224 were obtained from Research Genetics. Multicopy plasmid B95 containing *YPDL206c* ORF (*PGC1*) was prepared as follows: cosmid 70982 (ATCC) was digested with XbaI and EcoRI to release a 4827 bp fragment containing *PGC1* together with two neighboring ORFs. This DNA fragment was ligated into XbaI and EcoRI sites of plasmid YEp195 (36), creating plasmid B91. Plasmid B91 was digested with Xhol and Stul to release only *PGC1* and its flanking sequences (497 bp upstream and 982 bp downstream). The released fragment was ligated into Sall and Smal sites of YEp195 to create plasmid B95.

**pGAL-GST-PGC1** allele was prepared as follows: fragment containing regulatable GAL1 promoter, the glutathione S-transferase (GST) and 40-bp homology to the target sequence was obtained using pFA6a-kanMX-PGAL-GST as a PCR template (37) using forward primer 5′-AGT GTC GAA CTT TTC TTC TCT CTT GTG TAG TAC AAC CAC GTT CCA GAA TTC GAG GTC GTT TAA AC-3′ and reverse primer 5′-GGA AAC TCC CTT GAG CTC GCT TCC ACC ATG CTA GTT CCA GAA TTC GAG GTC GTT TAA AC-3′.
5′-ATA TCT TGC TTT AAA AGC TCT GTG GCC CAC AAT TTC AAC CAT ACG CGG AAG CAG ATC CGA TT-3′. The underlined sequences are homologous to the PGC1 gene region. Obtained PCR product was transformed into BY4741 strain using lithium acetate procedure (35), and G418-resistant colonies were selected. To verify integration at the correct location, genomic DNA was isolated and used as a template in PCR reaction using forward primer 5′-CAA CGA CTT GTA GCC AAC ATA-3′ and reverse primer 5′-AGG TTT AAA GAT GCT ACT AAC-3′. Integration of the PCR module resulted in the following changes in the genomic DNA: (i) deletion of ~500 bp immediately upstream of the native PGC1 start codon and replacement with the GAL1 promoter and (ii) fusion of the PGC1 gene to the 5′-end of PGC1. The resulting strain was named pGAL-GST-PGC1 (PGY241).

Plasmid pPIC3.5K + PGC1 to express Pgc1p in P. pastoris was prepared as follows: PGC1 gene was amplified from plasmid B95 as PCR template using primers BamHI- and EcoRI sites of pPIC3.5K plasmid (Invitrogen). The resulting plasmid pPIC3.5K + PGC1 was transformed into P. pastoris strain GS115 using electroporation protocol according to Wu and Letchworth (38).

Preparation of Mitochondria—Yeast mitochondria were isolated as described previously (39). Briefly, spheroplasts were prepared using Zymolyase 20T from cells grown in 1–URA medium to late logarithmic phase of growth. The spheroplasts were then homogenized in a tight-fitting Dounce homogenizer in 0.6 M mannitol, 10 mM Tris-Cl, pH 7.4, 0.1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged for 5 min at 5,000 g to remove residual cell debris. The supernatant was saved and centrifuged for 20 min at 13,000 g. The final mitochondrial pellet was washed twice in 0.6 M mannitol, 10 mM Tris-Cl, pH 7.4, and resuspended in washing buffer at the approximate final concentration of 10 mg of protein/ml. Highly purified mitochondria for localization studies were prepared accordingly to the procedure of Zinser et al. (40) using a linear sucrose gradient of 30 to 60%.

Isolation of Yeast Lipid Particles—Lipid particles of yeast were isolated according to Leber et al. (41) using a linear sucrose gradient of 30 to 60%.

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Isolation of Yeast Lipid Particles—Lipid particles of yeast were isolated according to Leber et al. (41) from cells grown to early stationary phase of growth.

Western Blot Analysis—Immunological characterization of subcellular fractions was carried out after separating proteins on 12% SDS-polyacrylamide gels and transferring to nitrocellulose filters (42). Proteins were detected using rabbit antibodies (Sigma) against the respective antigens; anti-GST for GST-Pgc1p (Sigma), anti-Erg7p for lanosterol synthase (generous gift of G. Daum, Technical University of Graz, Austria), and anti-Aac2p for mitochondrial ADP/ATP carrier protein (generous gift of I. Hapala, Institute of Animal Biochemistry and Genetics, Ivanca pri Dunaji, Slovakia).

Steady-state Phospholipid Analysis—To determine steady-state phospholipid composition, strains were grown in 1+ or 1– synthetic media (5 ml) containing 5 μCi of [32P]orthophosphoric acid/ml. The cultures were harvested in mid to late logarithmic phase of growth after five to six generations of aerobic growth at 30 °C. Lipids were extracted by using a modified Folch extraction (43), and individual phospholipids were resolved by two-dimensional paper chromatography (44), visualized by phosphorimaging, and quantified by Quantity One software (Bio-Rad).

Pulse Labeling of Phospholipids—To determine the rate of phospholipid synthesis yeast cells were pulse-labeled for 45 or 90 min in 1– medium (0.5% glucose) with 50 μCi of [32P]orthophosphoric acid/ml, followed by lipid extraction as described (45). Harvested cells were spheroplasted for 15 min in 1 ml of digestion mixture (1.2 mM glycerol, 100 mM sodium thioglycolate, 50 mM Tris-SO₄, pH 7.5, and 2.5 mg/ml Zymolase 20T). Spheroplasts were subsequently collected by centrifugation, and lipids were extracted with 5 ml of chloroform–methanol (2:1) for 1 h at room temperature by occasional vortexing. 1 ml of water was added, and the phases were separated by centrifugation. The organic phase was dried under a stream of nitrogen. Phospholipids were separated and quantified as described above for steady state analysis.

PGP Synthase Activity—PGP synthase activity was determined by quantification of the incorporation of radiolabeled substrate [14C]glycerol-3-phosphate into chloroform-soluble product PGP (46). Cells were grown in 1–URA medium to late-logarithmic phase of growth. The PGP synthase assay was performed in the presence of 50 mM MES-Cl, pH 7.0, 0.1 mM MnCl₂, 0.2 mM CDP-DAG, 1 mM Triton X-100, 0.02 mM [14C]glycerol-3-phosphate (100,000 cpm/nmol) and mitochondrial fraction corresponding to 100 μg of mitochondrial protein in a total volume of 100 μl. The reaction was stopped after 20 min by the addition of 0.5 ml of 0.1 N HCl in methanol, and the lipids were extracted with 1 ml of chloroform and 1.5 ml of 1 M MgCl₂. Aliquots of 0.5 ml of the chloroform layer were transferred into scintillation vials and evaporated under the stream of nitrogen. Scintillation mixture (3 ml) was added, and the radioactivity of each sample was determined with a scintillation counter. A unit of enzymatic activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol of product/min under the assay conditions described above. The specific activity is defined as units/mg of protein.

In Vitro Assay for Lipid Degradation Activity—Enzymatic PG degradation activities of mitochondrial fractions were assayed by measuring the conversion of fluorescently labeled substrate NBD-PG or 14C-labeled PG. The assay mix contained 20 μl of 0.3 M MES, pH 6.5, 20 μl of NBD-PG (1 μg) in 0.1% Triton X-100, and 80 μl of mitochondrial protein (100 μg) in a total volume of 120 μl. The typical reaction was allowed to proceed for 40 min at 30 °C. After this incubation time the reaction was stopped by the addition of 2 ml of chloroform–methanol–HCl (100:100:0.6), followed by the addition of 1 ml of water to produce two phases. The samples were vortexed, and aliquots of 0.9 ml of the organic layer were dried under a stream of nitrogen. NBD species were resolved by one-dimensional TLC chromatography on silica plates for 30 min using chloroform–methanol–28% ammonium hydroxide (65:35:5) as mobile phase (47). NBD-containing molecules were visualized by using a PhosphorImager Typhoon (Amersham Biosciences) and quantified by Quantity One software (Bio-Rad). The major NBD-PG degradation product under these assay con-
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Results

Ypl206cp (Pgc1p) Displays Homology to Glycerophosphodiester Phosphodiesterases—Two proteins encoded by S. cerevisiae ORFs YPL206c and YPL110c, share significant sequence homology to bacterial and mammalian glycerophospholipid phosphodiesterases (for sequence alignment and phylogenetic analysis see Ref. 30). The product of YPL110c was recently identified as a glycerophosphocholine-specific phosphodiesterase (31, 32), and the coding gene was named GDE1. The role of the product of ORF YPL206c (PGC1) in homeostasis of yeast phospholipids is the subject of this report. PGC1 is located on the left arm of chromosome 16 and encodes a protein of 321 amino acids with a predicted molecular mass of 37 kDa. The NCBI CDART tool (49, 50) predicts the GPDE motif to be present at the N terminus of the Pgc1p (Fig. 2A). Importantly for our study, the Superfamily HMM library and genome assignments server (51, 52) assigns Pgc1p to the superfamily of PLC-like phosphodiesterases. In addition, the C terminus of the protein contains a potential single pass membrane anchor localized at amino acids 297–315 (SwissProt data base) (Fig. 2B).

Deletion of PGC1 Leads to Accumulation of Phosphatidylglycerol—In our attempt to understand the role of Pgc1p in lipid homeostasis, steady-state phospholipid analysis was performed with wild-type and pgc1Δ strains. These experiments were performed in yeast synthetic media with different combinations of the phospholipid biosynthesis precursors, inositol and choline, that are known to regulate lipid metabolism (Table 2). The most striking difference in steady-state phospholipid composition between wild-type and pgc1Δ strains is accumulation of PG in the pgc1Δ strain. This accumulation is more evident in media without inositol, where PG amounts to ∼3% of total cellular phospholipids compared with a few tenths of a percent in wild-type cells. Addition of choline does not change the pattern of PG accumulation (data not shown). Accumulation of PG is evident also in media with inositol, however, the amounts of PG in both wild-type and pgc1Δ strains are much lower. Other phospholipids, including CL, were either unchanged or display only minor changes upon PGC1 deletion.

CL Formation Is Unchanged upon Deletion of PGC1—Accumulation of PG in the pgc1Δ strain indicated a possible role for Pgc1p in the cardiolipin branch of phospholipid biosynthetic pathways. We considered three reasons for accumulation of PG in the pgc1Δ strain: (i) a biosynthetic step downstream from PG, e.g. formation of CL from PG and CDP-DAG (Fig. 1) is defective or slowed down upon deletion of PGC1; (ii) a biosynthetic step leading to formation of PG is up-regulated in the pgc1Δ strain; or (iii) the product of the PGC1 gene is involved in turnover of PG.

To test the first possibility, the rate of phospholipid biosynthesis in wild-type and pgc1Δ strains was determined by pulse-labeling of phospholipids using [32P]orthophosphoric acid for 45 or 90 min in synthetic medium without inositol (Table 3). These data confirm that pgc1Δ cells accumulate PG and indicate that the pgc1Δ strain has no defect in CL synthesis. Formation of other phospholipids is either unchanged or displays only minor changes upon PGC1 deletion.

PGP Synthase Activity Is Not Significantly Affected by Deletion of PGC1—PGP synthase catalyzes the rate-limiting step in CL biosynthesis. Enhanced PGP synthase activity could lead to increased production of PGP and subsequently, upon dephosphorylation, to increased production of PG. Thus PG could be accumulated in pgc1Δ strain as a result of increased PGP synthase activity. PGP is very rapidly dephosphorylated to form PG and PGP levels are too low to measure. Therefore, to determine the rate of PGP formation, an established in vitro assay for PGP synthase activity was performed (see “Experimental Proce-

A Ypl206cp (Pgc1p)

B

FIGURE 2. In silico analysis of Pgc1p. A, the NCBI CDART tool (49, 50) predicts the GPDE motif to be present at the N terminus of the Pgc1p. B, C terminus of the protein contains a potential single pass membrane anchor localized at amino acids 297–315, indicated in boldface.

| TABLE 2 | Steady-state phospholipid composition of the pgc1Δ mutant compared to wild-type strain |
| --- | --- |
| Yeast cells were grown in 1–C– or 1+ C– synthetic media in the presence of [32P]orthophosphoric acid for five or six generations. Data are expressed as percentage of radiolabel [32P]orthophosphoric acid incorporated into total phospholipids ± S.D. Data represent the average of two independent experiments. |
| Medium | Strain | PG | CL | PA* | PC | PE | PI | PS | Other |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 1–C– | WT | 0.21 ± 0.24 | 1.91 ± 0.85 | 2.86 ± 0.44 | 45.50 ± 0.96 | 19.63 ± 1.11 | 9.36 ± 0.21 | 10.58 ± 0.70 | 9.95 ± 2.85 |
| pgc1Δ | WT | ND | 2.75 ± 0.43 | 1.70 ± 0.82 | 2.85 ± 0.22 | 44.75 ± 4.27 | 19.94 ± 2.78 | 8.48 ± 2.13 | 8.80 ± 1.62 | 10.17 ± 3.66 |
| 1+C– | WT | ND | 2.84 ± 2.18 | 1.78 ± 0.39 | 27.88 ± 3.61 | 21.11 ± 2.52 | 28.96 ± 1.51 | 7.76 ± 0.16 | 9.67 ± 1.39 |
| pgc1Δ | WT | ND | 0.36 ± 0.51 | 2.55 ± 1.67 | 1.79 ± 0.06 | 27.55 ± 1.43 | 21.34 ± 1.57 | 29.46 ± 0.89 | 7.21 ± 0.60 | 9.73 ± 1.68 |

*PA, phosphatidic acid; PS, phosphatidylserine; PI, phosphatidylserinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; WT, wild type.

**ND, not detectable.
pgc1Δ strains. The data are shown in Fig. 3; only a very small, insignificant difference can be seen between wild type, pgc1Δ, and the strains with overexpression of the PGC1 gene, indicating that the synthesis of PGP is not the metabolic step influenced by the Pgc1p.

Degradation of PG to DAG and Glycerophosphate Is Decreased upon Deletion of PGC1—Because deletion of PGC1 had no effect upon CL formation or *in vitro* measured PGP synthase activity, we explored the possibility that the PGC1 gene product is involved in PG turnover. This possibility is especially appealing considering the homology of the PGC1 gene product to bacterial and mammalian enzymes that cleave phosphodiester bonds (30). To test the role of Pgc1p in degradation of PG, an *in vitro* assay to analyze degradation products of PG by the yeast extract was developed. The assay is based on the quantification of degradation products of fluorescently labeled substrate, NBD-PG, upon exposure of the substrate to mitochondrial fractions. The major NBD-PG degradation product upon the incubation of NBD-PG with mitochondrial fractions in these assay conditions (see “Experimental Procedures”) was identified as NBD-DAG. The identification is based on comparison of the TLC mobility of the NBD-PG degradation products formed upon exposure to yeast mitochondrial extracts to the NBD-PG degradation product of commercially available *B. cereus* phospholipase C, NBD-DAG. Both products have the same mobility using two different TLC mobile phases, alkali and acidic (Fig. 4, A and B). *In vitro* NBD-DAG production using the above described assay displays time and mitochondrial protein amount dependence (Fig. 5). Mitochondrial fractions from four different strains were compared in the assay, wild-type strain, *pgc1Δ* strain, wild-type strain with overexpressed PGC1, and *pgc1Δ* mutant strain with overexpressed PGC1. After TLC chromatography and visualization by UV light (Fig. 4A), individual degradation products containing NBD label were quantified using fluorescence imaging (Fig. 6A). This quantification clearly shows that the amount of NBD-DAG produced from NBD-PG is much lower using the mitochondrial fraction from *pgc1Δ* strain compared with wild-type strain. Upon overexpression of PGC1 from multicopy plasmid B95, a large increase in NBD-DAG production from NBD-PG could be seen. In addition to NBD-labeled PG substrate, radioactive PG was used in this assay. Results depicted on Fig. 6B show that the use of authentic (radiolabeled) PG leads to the same outcome as the use of NBD-labeled PG validating the use of NBD-PG. The specificity of Pgc1p was tested using various lipid substrates in the above described *in vitro* lipid degradation assay. NBD-PS, NBD-PE, NBD-PC, and [3H]phosphatidylinositol degradation was independent on the absence or increased presence of Pgc1p in the assay (data not shown). All these data support the specific role of Pgc1p in PG degradation via a phospholipase C-type mechanism.

*S. cerevisiae* Pgc1p was also expressed in the methylotrophic yeast *P. pastoris* GS115 from the plasmid pPIC3.5K+PGC1. Cell extracts from *P. pastoris* cells overexpressing Pgc1p were about three times enriched in PG degradation activity *in vitro* as compared with cell extract from the untransformed wild-type culture (Fig. 7).

Subcellular Localization of Pgc1p—Already published localization data of Pgc1p are conflicting. Global analysis of protein localization in budding yeast (53), accomplished by attaching the green fluorescent protein tag (GFP) to the C terminus of proteins, indicated a cytosolic localization of Pgc1p. In our experiments we also observed cytosolic localization of Pgc1p upon tagging of the C terminus with yEGFP. In these experiments we either tagged Pgc1p at its native chromosomal location (54), or we used C-terminal yEGFP-tagged Pgc1p cloned into the yeast episomal plasmid pRS425 (55). The observed cytosolic localization, however, probably does not reflect the true localization of the native Pgc1p, because overexpression of the C-terminally tagged Pgc1p in the *pgc1Δ* strain does not complement the *pgc1Δ* phenotype (accumulation of PG in I−C− medium) (data not shown). A possible explanation could be the presence of a hydrophobic tail anchor at the C terminus of the protein that is probably masked by attachment of GFP to this end (56). Tail-anchored proteins fold co-translationally, and the single hydrophobic segment at their C terminus allows for post-translational insertion into membranes (57). Following identification of Pgc1p as a tail-anchored membrane protein in a genome wide analysis study, Pgc1p was cloned behind the modified GFP (GFP-S65T). Subcellular localization of this
N-terminally tagged Pgc1p was described as lipid particles (56). Interestingly, in our hands this N-terminal construct of GFP-S65T-Pgc1p was able to revert the accumulation of PG in pgc1Δ/H9004 strain to normal, wild-type levels (data not shown). In a study designed to determine the proteome of yeast mitochondria, Pgc1p was identified as a mitochondrial protein (58). In this study highly purified yeast mitochondria were prepared, and all mitochondrial proteins were identified using mass spectrometry. A clear advantage of this method is the absence of any tag that can obscure the localization signal or change in some other way the subcellular localization of the native protein. A disadvantage of mass spectrometry is, ironically, its high sensitivity. Some proteins that are contaminants of the purified mitochondria could be thus detected as mitochondrial proteins. To make the situation more complicated, even in silico prediction of subcellular localization is not clear. PSORTII analysis (Prediction of Protein Sorting Signals) predicts cytoplasmic localization for Pgc1p. In contrast, the Yale Protein Localization Server (59) predicts mitochondrial localization for Pgc1p. Clearly, there was a good reason to determine the correct subcellular localization of Pgc1p.

Subcellular localization of Pgc1p was studied using the GST-Pgc1p hybrid construct. GST was attached to the N terminus of Pgc1p as described under “Experimental Procedures” and expressed in wild-type yeast under the control of galactose promoter. Standard techniques of yeast organelle isolation (60) and subsequent Western blot analysis were used to identify subcellular localization of GST-Pgc1p hybrid (Fig. 8A). Purity of individual subcellular fractions was determined using antibodies to the lipid particle lanosterol synthase protein Erg7 (61) and to the inner mitochondrial ADP/ATP carrier protein Aac2 (62). Western blot analysis of subcellular fractions revealed the presence of GST-Pgc1p in both mitochondria and lipid particles. Subsequent analysis of purified mitochondria, lipid particles, and cytosol showed that only the mitochondrial fraction displayed significant in vitro phospholipase C type activity against PG (Fig. 8B) indicating that active Pgc1p localized to the mitochondria.

DISCUSSION

In this study we present evidence for a novel mechanism for the regulation of PG levels in a eukaryotic cell. We report here that in S. cerevisiae, PG levels are regulated via a phospholipase C-type degradation mechanism that involves the product of the yeast PGC1 gene (Fig. 1). Thus, our study represents a substantial advancement in an understanding of the regulatory mechanisms operational in the CL branch of phospholipid biosynthetic pathway.

Steady-state phospholipid composition revealed accumulation of PG in the yeast strain lacking the product of the PGC1 gene.
gene, especially in media without the important regulatory pre-cursor of phospholipids biosynthesis, inositol (Table 2). The data indicate that the observed accumulation of PG is due to the absence of a degradation pathway that removes excess PG which is not immediately used for cardiolipin formation. Pgc1p plays an essential role in this PG “removal pathway” that cleaves PG to DAG and glycerophosphate. DAG is formed from phospholipids by the action of phospholipases of the C-type (63). Thus our data support a model in which excess PG in yeast is removed via a phospholipase C-type mechanism involving the product of the PGC1 gene.

The existence of a PG degradation pathway generates a number of questions. First, what is the role of this PG “removal pathway” in overall cell physiology and in mitochondrial bioenergetics in partial? Our results (data not shown) indicate no growth phenotype of the pgc1Δ/H9004 strain compared with the wild-type on I−/H11002, I+/H11001, or I+/H11001 media. Similarly, there was no growth difference between the pgc1Δ/H9004 strain and the wild-type strain on YP media with various non-fermentable carbon sources, measured at 16 °C, 28 °C, or 37 °C. Thus, it seems that this degradation pathway either serves to “fine tune” the cardiolipin biosynthetic pathway or generates molecules, DAG and glycerophosphate, needed in mitochondria under some special conditions. Another question is why PG accumulation in the pgc1Δ/H9004 strain is much more evident in media without inositol? At least two explanations for the observed inositol effect exist. First, it had been shown that inositol negatively regulates the activity of PGP synthase (15), thus in inositol-containing media much less PGP and consequently PG is formed. This smaller amount of PG formed could then be all used for the synthesis of CL. In media without inositol, activity of PGP synthase is up-regulated (15), and this enhanced PGP synthase activity may create a surplus of PG that is removed via a degradation mechanism in the wild-type strain, but not in the pgc1Δ strain. Another explanation for the inositol dependence of PG accumulation in the pgc1Δ strain is simply the fact that multiple biosynthetic pathways leading to different phospholipids compete for the common precursor of phospholipid biosynthesis, CDP-DAG (17). Upon addition of inositol, the PI biosynthetic pathway increases consumption of the phospholipid biosynthesis precursors, phosphatidic acid and CDP-DAG.
(64). As a consequence of this increased competition for the common precursor, CDP-DAG, less PG could be formed in the presence of inositol than in the absence of inositol. Both of these mechanisms could contribute to the enhanced production of PG in the absence of inositol. Thus, in the absence of inositol and in the absence of a functional PG “removal pathway” involving Pgc1p, PG accumulation would be expected to be at its greatest.

And finally, what is the role of Pgc1p in the described PG turnover pathway? Our data suggest that Pgc1 protein is the actual phospholipase C specific for PG. First, in an in vitro assay to monitor PG degradation products (Figs. 4–6), overproduction of Pgc1p caused massive production of DAG from the fluorescein labeled substrate NBD-PG or from authentic (radio-labeled) PG, whereas deletion of PGC1 causes a decrease in DAG production. This DAG formation is consistent with Pgc1p having a phospholipase C activity. Second, enzymes of the glycerophosphodiester phosphodiesterase family display weak homology to mammalian phospholipase C pfam00388 (using the NCBI CDD tool (49)), suggesting that an enzyme with a glycerophosphodiester phosphodiesterase signature could actually be a phospholipase C. Importantly, in silico analysis (51, 52) assigns Pgc1p to the family of PLC-like family of phosphodiesterases. In addition, available evidence indicates that Pgc1p is not glycerophosphocholine or glycerophosphoinositol phosphodiesterase (31, 32). PG-specific phospholipase C should be localized where the cardiolipin biosynthetic pathway is present, e.g. in mitochondria (65). Available literature data localized Pgc1p to three different subcellular locations based on the method used: cytosol (53), lipid particles (56), and mitochondria (58). Our results (Fig. 8A) indicated dual localization of Pgc1p to mitochondria and lipid particles. However, only protein extracts prepared from the mitochondrial fraction showed significant PLC-type degradation activity toward PG (Fig. 8B). It remains to be seen if the lipid particle localization of Pgc1p is an artifact of the protein tag (GFP in published databases (56), GST in our experiments) or if this is an authentic localization of Pgc1p in addition to its localization in mitochondria. To address more precisely the role of Pgc1p in PG turnover we attempted to purify the tagged version of Pgc1p. Unfortunately, all our attempts to purify functional Pgc1 protein failed. None of our fusion constructs, GST on the N terminus, or His tag on both C and N termini, yielded functional protein after purification. As the most likely possibility to explain our failure to detect in vitro degradation activity of purified Pgc1p we consider the disruption of potentially essential protein-protein or protein-lipid interactions in the course of Pgc1p purification. An additional support for the PLC-type activity of Pgc1p represents data from the heterologous expression of Pgc1p in the yeast P. pastoris. Upon overexpression of Pgc1p in this methylo trophic yeast significantly increased in vitro PLC-like degradation activity toward PG was observed (Fig. 7).

In conclusion, our data demonstrate the existence of a novel regulatory pathway in the CL branch of phospholipid biosynthesis. This pathway removes excess PG via a phospholipase C type degradation of PG. The product of the PGC1 gene is an essential part of this degradation pathway, most likely by acting as a phospholipase C. Our findings demonstrate, for the first time, the existence of a degradation pathway for PG, an intermediate in the biosynthesis of CL, and an important component of lung surfactant and thylakoid membranes. Future studies will focus on purification of the active enzyme, characterization of its enzyme activity, and analysis of its role in mitochondrial bioenergetics.

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