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Specific degradation of phosphatidylglycerol is necessary for proper mitochondrial morphology and function

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ABSTRACT

In yeast, phosphatidylglycerol (PG) is a minor phospholipid under standard conditions; it can be utilized for cardiolipin (CL) biosynthesis by CL synthase, Crd1p, or alternatively degraded by the phospholipase Pgc1p. The *Saccharomyces cerevisiae* deletion mutants *crd1Δ* and *pgc1Δ* both accumulate PG. Based on analyses of the phospholipid content of *pgc1Δ* and *crd1Δ* yeast, we revealed that in yeast mitochondria, two separate pools of PG are present, which differ in their fatty acid composition and accessibility for Pgc1p-catalyzed degradation. In contrast to CL-deficient *crd1Δ* yeast, the *pgc1Δ* mutant contains normal levels of CL. This makes the *pgc1Δ* strain a suitable model to study the effect of accumulation of PG *per se*. Using fluorescence microscopy, we show that accumulation of PG with normal levels of CL resulted in increased fragmentation of mitochondria, while in the absence of CL, accumulation of PG led to the formation of large mitochondrial sheets. We also show that *pgc1Δ* mitochondria exhibited increased respiration rates due to increased activity of cytochrome *c* oxidase. Taken together, our results indicate that not only a lack of anionic phospholipids, but also excess PG, or unbalanced ratios of anionic phospholipids in mitochondrial membranes, have harmful consequences on mitochondrial morphology and function.

1. Introduction

Mitochondrial membranes represent a highly specialized functional unit comprised of two different lipid bilayers. The composition of the inner mitochondrial membrane (IMM) is quite unusual, exhibiting a low phospholipid:protein (mg/mg) ratio of 0.15, whereas in the outer mitochondrial membrane (OMM), this ratio is up to 0.91 [1,2,3]. The IMM also contains two specific anionic phospholipids: phosphatidylglycerol (PG) and cardiolipin (CL).

CL, a unique dimeric phospholipid exclusive to mitochondrial membranes, is synthesized in yeasts in a reaction of PG with cytidine diphosphate-diacylglycerol (CDP-DAG), catalyzed by the CL synthase, Crd1p [4,5,6]. Under standard conditions, PG in yeasts is a minor phospholipid that is mainly used for CL biosynthesis. Alternatively, PG can be degraded to DAG and glycerol-3-phosphate (G3P) by the PG specific phospholipase Pgc1p [7]. Accordingly, yeast lacking either *CRD1* or *PGC1* accumulate PG. In the *crd1Δ* strain, accumulation of PG is accompanied by the absence of CL [4,5,6,8,9]; in *pgc1Δ* cells, PG accumulates but does not cause significant changes in other phospholipids, including CL [7]. Thus, the *pgc1Δ* strain provides an excellent model in which to study the effects of changes in PG levels independent of changes in CL or other phospholipids.

Diverse biological functions of these two anionic phospholipids have been described. All proteins of the oxidative phosphorylation (OXPHOS) system have high affinity binding sites for CL. CL thus represents a major phospholipid necessary for the proper function of respiratory complexes and for the stability of OXPHOS supercomplexes [10,11]. CL also acts as a proton trap and provides a source of charge during OXPHOS [12]. Furthermore, CL is required for protein import [9], formation of cristae morphology [13,14], mitochondrial fusion [15,16], cellular iron homeostasis [17] and apoptosis [18,19], reviewed in [20]. PG, aside from its essential role in CL biosynthesis, also fulfills various functions in specific membranes of some eukaryotic organisms. For example, PG is the sole phospholipid of thylakoid membranes in eukaryotic oxygenic

Abbreviations: CCCP, carbonyl cyanide m-chlorophenyl hydrazine; CL, cardiolipin; DAG, diacylglycerol; ER, endoplasmic reticulum; ETS, electron transport system; G3P, glycerol-3-phosphate; I, inositol; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; OXPHOS, oxidative phosphorylation; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PI, phosphatidylinositol; RCI, respiration control index; SMD, glucose synthetic minimal medium; SMDGE, glucose, glycerol and ethanol synthetic minimal medium; WT, wild type.

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photosynthetic organisms [21,22] and it is an important component of pulmonary surfactant, an essential fluid produced by alveolar type II cells that covers the entire surface of the lung [23]. In yeast cells, PG is a low abundance phospholipid, even under the conditions of aerobic growth [6]. Therefore, PG is mainly considered to be a metabolic precursor of CL in yeast.

The absence of CL in mitochondrial membranes is associated with pleiotropic defects. The *crd1Δ* yeast strain displays growth defects and decreased viability on both fermentable and nonfermentable carbon sources at elevated temperatures, decreased mitochondrial membrane potential, and decreased respiratory rates [8,9,24]. Respiratory chain supercomplexes are destabilized in *crd1Δ* cells [14,25,26] and the lack of CL leads to mitochondrial DNA instability [8,9,27].

The absence of both PG and CL, which occurs upon deletion of *PGS1*/*PEL1*, the gene encoding phosphatidylglycerolphosphate (PGP) synthase (Pgs1p), causes even more severe phenotypes: its growth is strictly dependent on the presence of a fermentable carbon source in the media and its temperature sensitivity is higher compared to *crd1Δ* yeast [28,29,30,31,32,33]. This remarkable difference between the *crd1Δ* and *pgs1Δ* strains, which both lack CL but contain different amounts of PG, indicates that either PG can partially substitute for some essential functions of CL or some functions ascribed to CL could in fact be mediated by PG [9,34]. This interpretation is supported by other observations. For example, the defective translation of mRNA coding for Cox4p, an essential subunit of cytochrome *c* oxidase, detected in *pgs1Δ* cells is directly caused by the lack of PG and CL in mitochondrial membranes. In the absence of these lipids, binding of a protein factor(s) specifically to 5′ *cis* element of *COX4* mRNA is observed, which inhibits translation and lowers the Cox4p levels in *pgs1Δ* cells [35]. In contrast, the *crd1Δ* mutant, which contains PG, has normal Cox4p protein levels [14]. Similarly, activation of Isc1p, the inositol sphingolipid phospholipase C, after the diauxic shift is dependent on *PGS1* but not on *CRD1* [36]. These results suggest that PG could play roles beyond just being a biochemical precursor of CL or a structural part of mitochondrial membranes, and instead is itself an important player in various cell functions.

Most defects in *crd1Δ* cells are attributed to the absence of CL. Nevertheless, these cells also accumulate PG. In this study, we asked whether the accumulation of PG alone could generate some of the defects observed in *crd1Δ* yeast. We used the *pgc1Δ* mutant, which contains normal levels of CL but elevated PG, to characterize the specific effects of PG accumulation on mitochondrial respiration and morphology. Comparison of *pgc1Δ* and CL-deficient (*crd1Δ* and *pgc1Δcrd1Δ*) yeast allowed us to distinguish the effects of the absence of CL from the accumulation of PG. Based on our data, we report that PG accumulation induces defects in mitochondrial morphology. Further, accumulation of PG caused respiratory defects, mainly increased respiration rates and uncoupling, associated with activation of cytochrome *c* oxidase.

2. Materials and methods

2.1. Yeast strains and growth conditions

All yeast strains used in this study are listed in Table 1. Cultures were maintained on complex YPD media (2% yeast extract, 1% peptone, 2% glucose). For experiments, yeast were grown aerobically at 28 °C in defined synthetic medium prepared as described previously [37] with various carbon sources: SMD (2% glucose) or SMDGE (0.2% glucose, 3% glycerol, 1% ethanol). Synthetic medium was either supplemented with 75 μM inositol (I+) or lacked inositol (I-). Transformants were selected on synthetic medium without uracil.

2.2. RNA isolation and RT-qPCR analysis

The yeast cells were grown in SMD medium with or without inositol to the indicated growth phase. Total RNA was isolated from $3x10^8$ cells. Briefly, cells were broken by vortexing with 150 μl of glass beads in 100 μl of 10 mM TrisHCl, pH 8.0; 0.1 mM EDTA pH 8.0 for 3 × 45 s with 1 min cooling on ice in between. RNA was purified using the GeneJet RNA kit (ThermoScientific). RNA was eluted with 100 μl of water. 10–20 μg of isolated RNA was treated with DNase I to eliminate DNA contamination. DNase was removed using the RapidOut DNA removal kit (ThermoScientific). Purified RNA was reverse transcribed to cDNA using the RevertAid Premium First Strand cDNA Synthesis Kit (ThermoScientific). Final cDNA was diluted 10 times, and a 5 μl aliquot was used for qPCR analysis with the following primers: *PGC1* (sense, 5′-AGCGATGGTATGGTGGTGG-3′; antisense, 5′-GGAACCATCCTCTTTG CAGC-3′), *ACT1* (sense, 5′-ACCGCTGCTCAATCTTCTTC-3′; antisense, 5′-GGTCAATACCGGCAGATTCC-3′), *IPP1* (sense, 5′-ATGAAGGTGAGACCGA TTGG-3′; antisense, 5′-CTGGCTTACCATCTGGGATT-3′), and FastStart Essential DNA Green master (Roche) according to the manufacturer's instructions. *PGC1*, *ACT1* and *IPP1* transcripts were analyzed on the LightCycler 96 (Roche). PCR products were confirmed by melting curve analysis. Standard curves were generated from PCR amplification of template dilutions. Final data were normalized to *ACT1* and *IPP1* mRNA levels, and mRNA levels in wild type (WT) cells were set to 1.

2.3. Analysis of fatty acids

Extraction of phospholipids was performed by addition of 4.5 ml of chloroform–methanol–HCl (60:30:0.26) to mitochondrial extracts corresponding to 1 mg of proteins, followed by 30 min incubation at RT. Subsequently, 4.5 ml of 0.1 M $MgCl_2$ was added and following a brief vortex, incubated for another 30 min at RT. Phases were separated by centrifugation. The organic phase was dried under a stream of nitrogen. Phospholipids were separated by one-dimensional thin layer chromatography on silica plates using chloroform–methanol–acetic acid (65:25:8) [38].

Table 1
Yeast strains. All strains were in BY4741 or BY4742 background.

Strain	Genotyp	Source
pgc1Δ	*MATα his3, leu2, ura3, lys2, pgc1::KanMX*	Research Genetics
pgc1Δ + EV	*MATα his3, leu2, ura3, lys2, pgc 1::KanMX*, YEplac195 (URA3)	[7]
pgc1Δ + *PGC1*	*MATα his3, leu2, ura3, lys2, pgc 1::KanMX*, YEplac195-*PGC1*	[7]
WT	*MATa his3, leu2, met15, ura3, lys2*	Research Genetics
WT + EV	*MATa his3, leu2, met15, ura3, lys2*, YEplac195 (URA3)	[7]
WT + *PGC1*	*MATa his3, leu2, met15, ura3, lys2*, YEplac195-*PGC1*	[7]
crd1Δ	*MATa his3, leu2, met15, ura3, crd1::KanMX*	Research Genetics
crd1Δ + EV	*MATa his3, leu2, met15, ura3, crd1::KanMX*, YEplac195 (URA3)	This study
crd1Δ + *PGC1*	*MATa his3, leu2, met15, ura,3 crd1::KanMX*, YEplac195-*PGC1*	This study
pgc1Δcrd1Δ	*MATa leu2, ura3, met15, crd1::KanMX, pgc1::HIS3*	[7]
pgc1Δcrd1Δ + EV	*MATa leu2, ura3, met15, crd1::KanMX, pgc1::HIS3*, YEplac195 (URA3)	This study
pgc1Δcrd1Δ + *PGC1*	*MATa leu2, ura3, met15, crd1::KanMX, pgc1::HIS3*, YEplac195-*PGC1*	This study
pgc1Δcrd1Δ + *PDA1-GFP*	*MATa leu2, ura3, met15, crd1::KanMX, pgc1::HIS3*, pUG35-*PDA1-GFP* (URA3)	This study
pgc1Δcrd1Δ + *ss-GFP-HDEL*	*MATa leu2, ura3, met15, crd1::KanMX, pgc1::HIS3*, Ylp211-*TRP1-TKC-GFP-HDEL* (URA3)	This study