Cardiolipin is a key determinant for mtDNA stability and segregation during mitochondrial stress

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ABSTRACT

Mitochondria play a key role in adaptation during stressing situations. Cardiolipin, the main anionic phospholipid in mitochondrial membranes, is expected to be a determinant in this adaptive mechanism since it modulates the activity of most membrane proteins. Here, we used *Saccharomyces cerevisiae* subjected to conditions that affect mitochondrial metabolism as a model to determine the possible role of cardiolipin in stress adaptation. Interestingly, we found that thermal stress promotes a 30% increase in the cardiolipin content and modifies the physical state of mitochondrial membranes. These changes have effects on mtDNA stability, adapting cells to thermal stress. Conversely, this effect is cardiolipin-dependent since a cardiolipin synthase-null mutant strain is unable to adapt to thermal stress as observed by a 60% increase of cells lacking mtDNA (ρ^0). Interestingly, we found that the loss of cardiolipin specifically affects the segregation of mtDNA to daughter cells, leading to a respiratory deficient phenotype after replication. We also provide evidence that mtDNA physically interacts with cardiolipin both in *S. cerevisiae* and in mammalian mitochondria. Overall, our results demonstrate that the mitochondrial lipid cardiolipin is a key determinant in the maintenance of mtDNA stability and segregation.

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1. Introduction

Phospholipids are components of all cellular structures. Most, including PC, are synthesized in the lumen of the endoplasmic reticulum and subsequently distributed intracellularly [1]. Non-bilayer phospholipids such as CL and PE are synthesized mainly in mitochondria, where they also exert their functions [2]. After synthesis, PE is distributed through all cellular membranes, where it exerts diverse functions such as autophagy [3] or signal transduction [4].

CL is the main anionic phospholipid in mitochondrial membranes, where it constitutes about 15% of the total phospholipid content. It is synthesized in the inner leaflet of the inner membrane and remains within mitochondria, and distributed throughout the inner membrane [5], where it confers the negative surface charge necessary to maintain a localized proton motive force, used to synthesize ATP [6]. Furthermore, CL participates in the biogenesis and assembly of respiratory chain components and several metabolite transporters [7]. Functionally, CL activates the adenine nucleotide translocator (ANT) and uncoupling protein 1 (UCP1; [8,9]).

Additionally, CL stabilizes F_1F_O-ATP synthase dimers [10] and respiratory chain supercomplexes [11], affecting mitochondrial morphology [12]. CL normally adopts a bilayer conformation, but when calcium is present in the interface, it assumes a conical inverted shape due to the low hydration of the small polar headgroup [13]. Interestingly, it has been recently demonstrated that CL is able to respond to the chemical component of the proton motive force, promoting the formation of cristae-like structures in the inner membrane [14], an effect that resembles that of calcium on CL-containing membranes.

In bacteria, both CL and its precursor, PG, are key elements in the response which confers resistance to osmotic and thermal stresses [15]. Moreover, anionic phospholipids are necessary to attract and stabilize the translational machinery during the synthesis of hydrophobic proteins [16]. Since mitochondria are ancestrally related to bacteria, these mechanisms may have been conserved throughout evolution. Indeed, studies have shown that the loss of cardiolipin results in elevated sensitivity to thermal stress, including loss of viability, mitochondrial DNA, changes in mitochondrial bioenergetics, mitochondrial protein import,

and vacuolar acidification [17–19]. However, little is known to date regarding the mechanisms involved in the stabilization of mitochondrial function by CL during these non-permissive conditions.

A puzzling characteristic of CL is that, despite its described importance in several pivotal aspects of mitochondrial physiology, several model organisms tolerate the absence of this phospholipid. It has been suggested that PG and/or PE could substitute for CL under optimal growth conditions [20]. This hypothesis is confirmed by the synthetic lethality of yeast lacking both PE and CL [21] and the *petite* (respiratory-incompetent) phenotype present in mutants of the PGS1 gene, which codes for phosphatidylglycerol phosphate synthase, the rate limiting step in anionic phospholipid biosynthesis [22]. Other reports, however, have indicated that CL is required under stressful conditions, and cannot be completely replaced by these phospholipids [23–25].

Here, we directly investigate the role of CL in the cellular response to mitochondrial stress using a genetic model in which CL biosynthesis was disrupted by deleting the cardiolipin synthase gene (CRD1). This strain has been previously characterized [17,18] and accumulates PG, which is believed to replace CL in certain functions, since the cells are respiratory-competent. Interestingly, we found that while CL is not essential for respiratory function under optimal growth conditions, the biophysical properties it confers to mitochondrial membranes are essential for mtDNA stability and segregation under stress.

2. Materials and methods

2.1. Yeast strains and culture

The *Saccharomyces cerevisiae* BY4741 strain *MAT***a** *ura3Δ0 leuΔ0 met15Δ0*, its isogenic cardiolipin synthase-null strain *MAT***a** *ura3Δ0 leu2Δ0 met15Δ0 crd1::G418* and BY4742 *MAT***α** *ura3Δ0 leu2Δ0 lys2Δ0* strains were used in this study.

Mitochondrial DNA loss (ρ^0) was induced in the BY4742 background by culturing in YPD broth (2% glucose, 2% peptone and 1% yeast extract) supplemented with 25 μg/mL ethidium bromide and growing until saturation. Cells were then plated in solid YPD and replica-plated on glycerol media to select for *petite* colonies. Cells were pre-cultured in respiratory media (YP broth supplemented with 2% glycerol and 3% ethanol) at 30 °C under vigorous shaking (300 rpm) to positively select against mtDNA loss. After six generations [approximately 15 h], the cells were washed and YPD (2% glucose) or YPGal (2% galactose) culture broth was inoculated at an initial optical density of 0.01 at 30 °C or 37 °C, to induce thermal stress. Growth was followed under both conditions by measuring optical density at 600 nm.

To verify the effect of intermediate levels of CL on mtDNA stability, a tet-off system coupled to the promoter region of the phosphatidylglycerol phosphate synthase (PGS1) gene was used (Dharmacon, GE Healthcare). Cells were routinely grown in YPGal until saturation and subsequently diluted to an optical density of 0.01 in fresh YPGal media incubated with the indicated concentrations of doxycycline (Dox; 2.5–7.5 μg/mL) for 2 generations to repress the expression of *PGS1*. Cellular growth was not affected by doxycycline supplementation (not shown).

2.2. Mitochondrial DNA stability and hypersuppressiveness test

Aliquots of YPEG cultures at the stationary phase were diluted to a final optical density of 0.2 in distilled water and serially diluted to a final optical density of 0.0002. This last dilution was spread on solid YPD to quantify *petite* formation frequency and on solid YPEG supplemented with 50 μg/mL erythromycin to select for mtDNA mutation frequency. Under both conditions, approximately 80–100 colony forming units (CFU) of the erythromycin-resistant petite ρ^0 and ρ^- colonies were spread on YPDG medium (similar to YPD, in which both 0.5% glucose and 2% glycerol are present) and counted manually. A solution of 2% agar supplemented with 5 mg/mL phenyl tetrazolium was layered onto these plates and incubated for 3 h. Red (respiratory competent) and white/pink (respiratory incompetent) colonies were counted manually. Results are expressed as percentage of respiratory competent cells [26]. Additionally, the previous serial dilutions were spotted on YPD and YPEG plates supplemented with 2 and 4 μg/mL ethidium bromide. All plates were incubated at 30 °C or 37 °C.

The hypersuppressive respiratory phenotype was analyzed by crossing *MAT***a**− ρ^+ strains (wild type BY4741 and *crd1Δ*) with BY4742 ρ^0 (*MAT***α**) cells. Briefly, strains were inoculated in YPD broth until the stationary phase. Each strain was washed and mixed at a 1/1 ratio with fresh YPD broth [27]. Mating mixtures were incubated overnight at 30 °C or 37 °C. Samples were plated on glucose minimum mineral media lacking methionine and lysine to select for diploid cells. Afterward, the plates were layered with tetrazolium solution to count for respiratory suppressiveness in colonies.

2.3. mtDNA copy number detection

A 147 bp size fragment within the *COXI* mtDNA gene was amplified using the primer sequences: forward 5′-CTACA GATACAGCATTTCCAA GA-3′ and reverse 5′-TGCCTGAATAGATGATAATGGT-3′. Mitochondrial DNA copy number was normalized to the amplification of the nuclear single copy gene *ACT1*, which was amplified using the following primers: forward primer 5′-GTATGTGTAAA GCCGGTTTTG-3′, and reverse primer 5′-CATGATACCTTGGTGTCTTGG-3′. Primer design was performed using Primer Express 2.0 software (Applied Biosystems). Real-time PCR amplification was performed in 25 μL containing 1× TaqMan Universal PCR Master Mix with SYBR green (Applied Biosystems), 100 nM of each primer, and 1–10 μL DNA extract. The thermal cycling conditions were 95 °C for 10 min, 45 cycles at 95 °C for 15 s and 60 °C for 1 min. The correct amplification of the desired products was confirmed by sequencing. At least three "no-template-controls" were included in each experiment. The comparative Ct method was applied for quantification of the mitochondrial DNA copy number [28].

2.4. Isolation of yeast mitochondria

Mitochondria were isolated from galactose-grown cultures after enzymatic treatment of cell cultures [29]. Briefly, cells from 500 mL cultures were washed in pre-chilled distilled water and diluted to a final concentration of 0.25 mg/mL in zymolyase buffer (1.3 M sorbitol, 10 mM EDTA, 20 mM HEPES pH 7, 10 mM β-mercaptoethanol) supplemented with 2 mg/g$_{cell}$ zymolyase and incubated for 40 min at 30 °C with intermittent shaking. After incubation, spheroplasts were washed in isolation buffer (0.6 M mannitol, 10 mM EDTA, 20 mM HEPES pH 7, 0.2% BSA). Pelleted spheroplasts were diluted in 10 mL of the same buffer and broken with 20 strokes of a Dounce homogenizer with a tight pestle. Mitochondria were then isolated by differential centrifugation [30]. Isolated mitochondria were assayed for coupling and integrity by following oxygen consumption and responses to ADP and oligomycin. Typical preparations yield approximately 100 mg of mitochondrial protein from 2 g (dry weight) of cells. Protein was quantified by the Bradford method.

2.5. Respiratory activity

Cellular and mitochondrial oxygen consumption was measured in an Oroboros high-resolution oxygraph at 30 °C as in [31] using absolute ethanol (1 μL/mL) as respiratory substrate. The uncoupler FCCP was added at a final concentration of 10 μM for cells and 1 μM for isolated mitochondria, where indicated.

Activities of cytochrome *bc*1 (complex III) and cytochrome *c* oxidase (complex IV) were quantified by cytochrome *c* reduction and oxidation, respectively [32,33]. Briefly, for complex III activity, mitochondria were diluted to a final concentration of 0.5 mg/mL in a buffer composed of 0.6 M mannitol, 20 mM HEPES pH 7, 0.01% Triton X-100, 50 μM potassium cyanide and 20 μM yeast cytochrome *c*. The reaction was