The deletion of the Cardiolipin-specific Phospholipase Cld1 Rescues Growth and Life Span Defects in the Tafazzin Mutant

IMPLICATIONS FOR BARTH SYNDROME*

Received for publication, October 22, 2013, and in revised form, November 26, 2013 Published, JBC Papers in Press, December 8, 2013, DOI 10.1074/jbc.M113.529487

Cunqi Ye1,2, Wenjia Lou1,2, Yiran Li1, Liliana A. Chatzispyrou3, Maik Hüttemann4, Icksoo Lee2**, Riekelt H. Houtkooper5, Frédéric M. Vaz6, Shuliang Chen7‡, and Miriam L. Greenberg1,8

From the 1Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202, the 2Center for Molecular Medicine and Genetics and 3Cardiovascular Research Institute, Wayne State University School of Medicine, Detroit, Michigan 48201, the 4College of Medicine, Dankook University, Cheonan-si, Chungcheongnam-do 330-714, Republic of Korea, and the 5Laboratory of Genetic Metabolic Diseases, Academic Medical Center, 1105AZ Amsterdam, The Netherlands

Background: Cardiolipin (CL) is decylated by Cld1 to monolysocardiolipin, which is transacylated by tafazzin (Taz1) to form unsaturated CL.

Results: Deletion of CLD1 rescues growth and respiration defects in taz1Δ, whereas overexpression is deleterious to growth and respiration.

Conclusion: Decreased CL/MLCL, not decreased unsaturated CL, causes defects in tafazzin-deficient cells.

Significance: Attenuation of CL phospholipases may potentially treat Barth syndrome.

Cardiolipin (CL) that is synthesized de novo is decylated to monoscardiolipin (MLCL), which is reacylated by tafazzin. Remodeled CL contains mostly unsaturated fatty acids. In eukaryotes, loss of tafazzin leads to growth and respiration defects, and in humans, this results in the life-threatening disorder Barth syndrome. Tafazzin deficiency causes a decrease in the CL/MLCL ratio and decreased unsaturated CL species. Which of these biochemical outcomes contributes to the physiological defects is not known. Yeast cells have a single CL-specific phospholipase, Cld1, that can be exploited to distinguish between these outcomes. The cld1Δ mutant has decreased unsaturated CL, but the CL/MLCL ratio is similar to that of wild type cells. We show that cld1Δ rescues growth, life span, and respiratory defects of the taz1Δ mutant. This suggests that defective growth and respiration in tafazzin-deficient cells are caused by the decreased CL/MLCL ratio and not by a deficiency in unsaturated CL. CLD1 expression is increased during respiratory growth and regulated by the heme activator protein transcriptional activation complex. Overexpression of CLD1 leads to decreased mitochondrial respiration and growth and instability of mitochondrial DNA. However, ATP concentrations are maintained by increasing glycolysis. We conclude that transcriptional regulation of Cld1-mediated decylation of CL influences energy metabolism by modulating the relative contribution of glycolysis and respiration.

Cardiolipin (CL) is a unique phospholipid that is predominant in mitochondrial membranes (1, 2). Unlike other membrane phospholipids, it contains two phosphatidyl moieties, four acyl chains, and two negative charges (3, 4). As the signature lipid of mitochondria, it comprises about 15% of total mitochondrial phospholipids (5) and interacts with a wide range of mitochondrial proteins (6–9), including the ADP/ATP carrier (10, 11) and respiratory complexes (12–15). CL-protein interactions stabilize respiratory chain supercomplexes (16, 17) and promote supramolecular associations between the ADP/ATP carrier and respiratory supercomplexes (11). Therefore, it is not surprising that mitochondrial respiration and energy production are highly correlated with CL biosynthesis (11, 18, 19). Interestingly, CL deficiency also leads to deficiencies in diverse cellular functions other than mitochondrial bioenergetics, including mitochondrial dynamics (20, 21), mitochondrial protein import (18, 22), cell wall biogenesis (23, 24), vacuolar function and morphology (25), cell cycle (26), aging (27), and apoptosis (28–30). As CL is engaged in a plethora of cellular activities, the regulation of CL synthesis is crucially important.

The synthesis of CL is well characterized in Saccharomyces cerevisiae. As seen in Fig. 1, Pgs1 catalyzes the committed step of CL synthesis by converting CDP-DAG and glycerol 3-phosphate to phosphatidyglycerol (PGP) (31), which is dephosphorylated to phosphatidylglycerol by the PGP phosphatase Gep4 (32, 33). CL synthase (Crd1) catalyzes the final step of de novo CL synthesis by condensing phosphatidylglyc-

* This work was supported by grants from the Barth Syndrome Foundation, Barth Syndrome Foundation of Canada, and Association Barth France (to M. L. G.), Wayne State University Thomas C. Rumble University Fellowship and Summer Dissertation Fellowship (to C. Y.), Wayne State University graduate enhancement research funds (to C. Y., W. L., and Y. L.), Academisch Medisch Centrum Ph.D. fellowship (to I. A. C.), and ZonMw/Veni Grant 91613050 and AMC postdoctoral fellowship (to R. H. H.).

1 Both authors contributed equally to this work.

2 Present address: Dept. of Cellular and Molecular Medicine, George Palade Labs, University of California at San Diego, La Jolla, CA 92093-0668.

3 To whom correspondence should be addressed: 5047 Gullen Mall, Dept. of Biological Sciences, Wayne State University, Detroit, MI 48202. Tel.: 313-577-5202; Fax: 313-577-6891; E-mail: mlgreen@sun.science.wayne.edu.

4 The abbreviations used are: CL, cardiolipin; MLCL, monolysocardiolipin; BTHS, Barth syndrome; qPCR, quantitative PCR; PGP, phosphatidylglycerolphosphate; FCCP, trifluorocarbonylcyanide phenylhydrazide; HAP, heme activator protein; CDP-DAG, cytidine diphosphate-diacylglycerol.
eral and CDP-DAG to form CL with primarily saturated acyl chains (34–37). Following the de novo synthesis of CL on the matrix side of the inner mitochondrial membrane, CL undergoes remodeling in which acyl chains are exchanged. In this process, CL is deacylated to monolysocardiolipin (MLCL) by the CL-specific lipase Cld1 on the matrix side of the inner mitochondrial membrane (38, 39). MLCL is reacylated by the transacylase Taz1 in the mitochondrial periphery (40–43). Remodeled CL has more unsaturated acyl chains than CL synthesized de novo (38, 41, 44, 45). Although the CL remodeling genes and enzymes have been identified in yeast, the function of CL remodeling and the mechanisms underlying its regulation are not understood.

The importance of CL remodeling is underscored by the X-linked mitochondrial disorder Barth syndrome (BTHS), a cardiосkeletal myopathy that results from mutations in the tafazzin gene (the homologue of yeast TAZ1) (46–48). Tafazzin deficiency leads to a decrease in the CL/MLCL ratio and a decrease in CL species containing unsaturated fatty acids (41, 49–54). Which of these biochemical outcomes leads to the pathology in BTHS is not understood. Genetic inactivation of the CL-specific phospholipase calcium-independent PLA2-serves to elucidate the role of deacylation in mammalian cells. CL-specific phospholipases have not been identified, and multiple phospholipases supposedly catalyze the deacylation of CL (56), complicating experiments to elucidate the role of deacylation in mammalian cells. In contrast, CLD1 is the only CL-specific phospholipase in S. cerevisiae (38). The yeast cld1Δ mutant has decreased unsaturated CL compared with wild-type cells, but the CL/MLCL ratio is not altered. In this study, we demonstrate for the first time that deletion of CLD1 rescued both respiratory and fermentative growth defects as well as decreased chronological life span in yeast taz1Δ cells. This suggests that deacylation of CL in the absence of tafazzin is deleterious because it leads to a decrease in the CL/MLCL ratio. These findings argue against the current thought that defects in tafazzin-deficient cells result from decreased unsaturated CL. We further show that expression of CLD1 is regulated in response to conditions affecting mitochondrial respiration and controlled by the HAP transcriptional activator. Overexpression of CLD1 leads to decreased ATP production from mitochondrial respiration that is compensated by increased glycolysis. Based on these findings, we proposed that transcriptional regulation of CLD1 controls deacylation of CL, and the regulation of this process modulates cellular energy production.

**Experimental Procedures**

**Yeast Strains, Plasmids, and Growth Media**—The yeast S. cerevisiae strains and plasmids used in this study are listed in Tables 1 and 2. Single deletion mutants were obtained from the yeast knock-out deletion collection (Invitrogen). Double mutants were obtained by tetrad dissection. Parental ρ0 cells were used to generate ρ0 derivatives by growing in yeast extract peptone dextrose (YPD) medium containing 20 μg/ml ethidium bromide to the early stationary phase. ρ0 strains were confirmed by the inability to grow on yeast extract peptone glycerol ethanol (YPGE) medium, the absence of mitochondrial DNA by DAPI staining, and the failure to complement ρ− tester strains for growth on YPGE medium.

To construct a CLD1 overexpression plasmid, a 1338-bp sequence containing the entire open reading frame of CLD1 was amplified from yeast genomic DNA using an EcoRI-tagged forward primer CLD1_EcoRI_F (5′-TATAGACATGAATT-CAAAAGTGACTGCTAATGAGC-3′) and an XbaI-tagged reverse primer CLD1_XbaI_R (5′-ATTGGAGTTAAGGAAAGAATAGCGGCGA-3′). The PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega). The purified DNA fragments were ligated into pYPGK18 cut with EcoRI and XbaI, downstream of the PGK1 promoter. All the plasmids were amplified and extracted using...
standard protocols. The plasmids were transformed into yeast strains using a one-step transformation protocol.

Synthetic complete (SC) medium contained adenine (20.25 mg/liter), arginine (20 mg/liter), histidine (20 mg/liter), leucine (60 mg/liter), lysine (200 mg/liter), methionine (20 mg/liter), threonine (300 mg/liter), tryptophan (20 mg/liter), uracil (20 mg/liter), yeast nitrogen base without amino acids (Difco), all the essential components of Difco vitamin (inositol-free), 0.2% ammonium sulfate, and glucose (2%). Inositol (75 μM) was supplemented in all media used in this study. Synthetic dropout media contained all ingredients mentioned above, except for the amino acid used as a selectable marker, and were used to culture strains containing a plasmid.

**Chronological Life Span**—Yeast chronological life span is determined by survival of nondividing cells in a prolonged stationary culture (57). A standard protocol previously described was followed (58) to assess chronological life span. In brief, individual colonies were inoculated in 10 ml of SC glucose medium and incubated overnight. The cultures were then diluted in 50 ml of SC medium, and cells were allowed to grow until saturation. Viable cells were measured every 2 or 3 days by counting colonies that were serially diluted and plated on YPD plates and represented as percentage of cells at day 2. The viability is considered to be 100% at or before day 2.

**Spotting Assay**—Cells were pre-cultured in SC medium to the early stationary growth phase at 30 °C and washed with sterile water. Three-μl aliquots of a series of 10-fold dilutions of 0.5 units of A₅₅₀ cells were spotted onto the indicated plates and incubated at 30 °C.

**Real Time Quantitative PCR (RT-qPCR) Analysis**—Cells were grown to the indicated growth phase and harvested at 4 °C. Total RNA was extracted using hot phenol (59) and purified using the RNeasy Mini Plus kit (Qiagen, Valencia, CA). Complementary DNA (cDNA) was synthesized using the first strand cDNA synthesis kit (Roche Applied Science) according to the manufacturer’s manuals. RT-qPCRs were performed in a 20-μl volume using Brilliant III Ultra-Faster SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA). Triplicates were included for each reaction. The primers for RT-qPCR are listed in Table 2. RNA levels were normalized to ACT1. Relative values of mRNA transcripts are shown as fold change relative to indicated controls. Primer sets were validated according to Methods and Applications Guide from Agilent Technologies. Optimal primer concentrations were determined, and primer specificity of a single product was monitored by a melt curve following the amplification reaction. All the primers were validated by measurement of PCR efficiency and have calculated reaction efficiencies between 95 and 105%.

**Measurement of Respiration**—Cell respiration was analyzed in a closed 500-μl chamber equipped with a micro Clark-type oxygen electrode (Oxygraph Plus System, Hansatech Instruments) at 30 °C. Cells grown to the logarithmic phase were mixed in fresh growing media using a protein concentration of 2 mg/ml following measurements of basal respiration. State 4 and state 3 respiration was determined in the presence of 4 μM oligomycin and 5 μM FCCP, respectively. KCN (0.2 mM) was added at the end of the experiment to inhibit cytochrome c oxidase to normalize for (subtract) cytochrome c oxidase-independent oxygen consumption. Oxygen consumption was recorded on a computer and analyzed with the Oxygraph plus software. Respiration rates are defined as consumed O₂ (nmol)/min-total protein (mg).

**Determination of ATP Concentrations**—Yeast cells were cultured to the logarithmic phase and flash-frozen with liquid nitrogen. ATP levels were determined by the bioluminescence method described previously (60).

**Determination of Ethanol Concentrations**—Yeast cells were cultured in 10 ml of growth medium for the indicated times to the logarithmic phase after inoculation at A₅₅₀ of 0.05, and cells were pelleted by a 5-min centrifugation at 3000 rpm. Supernatants were used to determine ethanol concentrations in the media. An ethanol colorimetric assay kit from BioVision was used to assay ethanol concentrations according to the manufacturer’s manual.

**Mitochondrial Aconitase Activity**—Cultures (2 liters) of yeast cells in the mid-logarithmic phase were harvested for isolation of mitochondria. Mitochondria were isolated as described previously (61). Briefly, spheroplasts generated by zymolyase treatment were ruptured by Dounce homogenization, and mitochondria were obtained by differential centrifugation. Total mitochondrial protein concentration was determined using the BCA protein assay (Pierce Protein). Mitochondrial aconitase activity was determined in mitochondrial extracts (50 μg of protein) using an aconitate-isocitrate dehydrogenase-coupled assay, in which NADPH formation was monitored at A₃₄₀ for 1 h (62).

**Determination of CL by Mass Spectrometry**—Total lipid extracts from 10 mg of cells (dry weight) were analyzed by HPLC-MS as described previously (63).

**RESULTS**

**Deletion of CLD1 Rescues Growth and Life Span Defects of the Taz1Δ Mutant**—Decaylation of CL in the absence of tafazzin leads to a decreased ratio of CL/MLCL and decreased unsaturated CL (41, 50, 52, 53), either of which may be responsible for cellular defects in tafazzin-deficient cells. We wished to distinguish between decreased CL/MLCL ratio versus decreased unsaturated CL as the mechanism underlying the defects in the taz1Δ mutant. Blocking CL deacylation by deletion of CLD1
CLD1 Expression Regulates Mitochondrial Functions

FIGURE 2. Deletion of CLD1 rescues growth and chronological life span defects in taz1Δ. A, serial 10-fold dilutions of WT, crd1Δ, cld1Δ, taz1Δ, and cld1Δtaz1Δ cells were spotted on synthetic complete medium with 2% glucose or 2% ethanol as carbon sources. Plates were incubated at 30 °C for 3 days. B, serial 10-fold dilutions of respiration-incompetent (ρ0) cells of the above mutants were spotted on synthetic complete medium with 2% glucose. C, chronological life span of WT, crd1Δ, cld1Δ, taz1Δ, and cld1Δtaz1Δ cells was determined as described under “Experimental Procedures.” The data depicted in the figure are representative of three experiments.

prevents the decrease in the CL/MLCL ratio (38). However, the CL that is synthesized de novo but not remodeled is mostly saturated, in contrast to remodeled CL in wild type (WT) cells, which is mostly unsaturated (38). To determine whether the decreased CL/MLCL ratio is responsible for taz1Δ defects, we determined the effects of CLD1 deletion in taz1Δ mutants. Interestingly, deletion of CLD1 rescued the respiratory growth defect of the taz1Δ mutant (Fig. 2A). Because mitochondrial respiration varies in strains with different genetic backgrounds (64) and the presence of polymorphic mitochondrial DNA can contribute to differences in mitochondrial respiration (65), we assayed the effects of CLD1 deletion independent of mitochondrial respiration. To do so, we constructed ρ0 strains (which lack mitochondrial DNA) of the WT and CL mutants. Although CL-deficient cells grow normally on glucose (Fig. 2A), which can be fermented, growth on glucose is compromised in the mutants if they lack mitochondrial DNA (Fig. 2B). Deletion of CLD1 rescued this growth defect (Fig. 2B). We predicted that CL-deficient cells would exhibit a decreased chronological life span similar to the decreased replicative life span observed in these cells (27). As shown in Fig. 2C, both crd1Δ and taz1Δ mutants exhibited a dramatic decrease in chronological life span. Deletion of CLD1 partially rescued the decrease in taz1Δ life span, as the life span of taz1Δcld1Δ was almost similar to that of WT (Fig. 2C). The observation that deletion of CLD1 suppresses the defects in taz1Δ indicates that deacylation of CL is deleterious in the absence of tafazzin and that the decreased CL/MLCL ratio but not decreased CL unsaturation is likely the primary cause of taz1Δ defects.

CLD1 Expression Is Highly Regulated in Response to Growth Phase, Glucose Availability, and Respiratory Activity—The finding that cld1Δ rescued respiratory defects in taz1Δ suggested that CLD1 expression plays a role in respiration. We first compared expression of CL biosynthetic genes, including PGS1, GEP4, CRD1, CLD1, and TAZ1, in logarithmically growing cells (in which energy is generated primarily from glycolysis) and in cells in the stationary phase (during which energy is generated from respiration). Expression of all the CL biosynthetic genes was increased in the stationary phase (Fig. 3A). However, although PGS1, GEP4, CRD1, and TAZ1 were increased about 3–5-fold, CLD1 was increased by about 10-fold in the early stationary phase and more than 30-fold in the later stationary phase (Fig. 3A). The large increase in CLD1 expression suggests that levels of unsaturated CL may be increased during the stationary phase. This was in fact observed (Fig. 3B). Specifically, in the C68 cluster, the most unsaturated CL (C68:4, m/z 699.5) was abundant, whereas a more saturated species (C68:2, m/z 701.5) was less abundant in stationary phase cells. Conversely, the C68:4 CL was much less abundant than C68:2 in logarithmically growing cells. This is also evident in the C60 cluster as the most saturated CL (C60:0, m/z 647.4) was absent from stationary cells but was clearly present in logarithmically
CLD1 Expression Regulates Mitochondrial Functions

FIGURE 3. Increased CLD1 expression in the stationary phase is concomitant with increased CL unsaturation. A, WT cells were grown in SC medium to the early logarithmic (EL), mid-logarithmic (ML), early stationary (ES), and stationary (S) growth phases, and PGS1, GEP4, CRD1, CLD1, and TAZ1 expression was quantified by RT-PCR as described under “Experimental Procedures.” Values of each gene were normalized to the internal control ACT1 and are represented as fold change relative to those in early logarithmic phase. Data shown are mean ± S.E. (n = 3). B, cells grown in YPD in the logarithmic and stationary phases were extracted for CL acyl composition analysis by HPLC-mass spectrometry, as described under “Experimental Procedures.” C, WT and cld1Δ cells grown in SC media in the logarithmic and stationary phases were extracted for CL acyl composition analysis by HPLC-mass spectrometry, as described under “Experimental Procedures.”

Growing cells. Deletion of CLD1 prevents CL remodeling and leads to decreased unsaturated CL (38). As expected, cld1Δ exhibited a decreased degree of unsaturated CL compared with WT regardless of growth phase (Fig. 3C). Interestingly, unsaturated CL levels were greater in stationary phase than in log phase cld1Δ cells. This finding suggests that an as yet unidentified mechanism regulates CL saturation in the absence of Cld1.

Increased CLD1 expression in the stationary phase, during which glucose is exhausted and cells shift from fermentation to oxidative phosphorylation, suggested that CLD1 may be transcriptionally regulated in response to glucose availability and the need to respir. To test this prediction, we examined the expression of CLD1 in response to acute removal of glucose and in respiration-deficient cells (ρ0 cells). As expected, expression of CLD1 but not the other CL biosynthetic genes was greatly increased in response to glucose starvation, by 6- to 10-fold, during the 30- and 60-min starvation, respectively (Fig. 4A). Furthermore, CLD1 transcription was increased in the stationary phase in ρ0 cells but not in respiration-incompetent ρ0 cells (Fig. 4B). These findings indicate that CLD1 expression is up-regulated during respiratory conditions and in response to glucose deprivation.

Using the promoter database of S. cerevisiae to search for putative regulatory elements in the upstream region of the CLD1 gene, we identified consensus sequences for Hap2 and Mig1 (Fig. 4C), transcription factors that mediate activation of respiratory gene expression and glucose repression, respectively (66–68). Consistent with this observation, the HAP complex regulates CLD1. As seen in Fig. 4D, CLD1 expression in the stationary phase was greatly reduced in hap2Δ, hap3Δ, hap4Δ, and hap5Δ mutants, indicating that the HAP complex up-regulates CLD1 transcription. Mig1 has been shown to repress gene expression in the presence of glucose (68, 69). If Mig1 repressed CLD1 transcription in the presence of glucose, CLD1 transcription would be increased in mig1Δ cells. However, CLD1 transcription in mig1Δ cells was decreased in these con-
ditions (Fig. 4E). Thus, Mig1 appears to be a positive regulator of CLD1 expression. This is consistent with reported activator activity of Mig1 (70, 71). Taken together, these findings indicate that expression of CLD1 is increased in response to respiration conditions, and this increase is mediated by the HAP and Mig1 transcriptional factors.

Constitutive Overexpression of CLD1 Leads to Decreases in Respiration and Mitochondrial Aconitase Activity and Instability of Mitochondrial DNA—As expression of CLD1 is deleterious to tafazzin-deficient cells, we predicted that increased CLD1 expression alters metabolism and perturbs cell growth. Consistent with this, cell growth was decreased when CLD1 was overexpressed (Fig. 5A). One possible explanation for this is that increased CLD1 expression perturbs respiration. In support of this, basal respiration in mitochondria from cells that overexpressed CLD1 was about half that of control cells (Fig. 5B). This difference was even more pronounced comparing the maximum respiratory capacity that was achieved by uncoupling the respiratory chain with FCCP. Therefore, constitutive overexpression of CLD1 decreases mitochondrial respiration.

A possible mechanism to account for decreased respiration in CLD1-overexpressing cells is suggested by the obser-
vation that over 60% of cells became cytoplasmic petites. The respiratory growth deficiency of the petites was not complemented by crossing to ρ−/H9267 tester strains, and mitochondrial DNA was not observed in the petite cells stained with DAPI. As aconitase is required for mitochondrial genome maintenance (72), we tested the possibility that aconitase activity might be decreased in cells overexpressing CLD1. In fact, the kinetics of aconitase enzymatic activity in mitochondria from CLD1-overexpressing cells exhibited a 60% decrease compared with cells overexpressing empty vector (Fig. 5C). Taken together, these studies indicate that increasing CL deacylation by constitutive overexpression of CLD1 impairs
cell growth and respiration and decreases mitochondrial DNA stability, suggesting that deacylation of CL is an important control point for mitochondrial function.

Increased Fermentation Compensates for Decreased Respiration in Cells Overexpressing CLD1—As respiration was decreased in cells overexpressing CLD1, we expected to see a concomitant decrease in ATP synthesis. The contribution of mitochondria to cellular ATP production can be estimated by the decrease in oxygen consumption resulting from the addition of oligomycin, an inhibitor of ATP synthesis. Under basal conditions, the decrease in respiration caused by oligomycin was significantly less in mitochondria from CLD1-overexpressing cells than in controls (Fig. 5B), suggesting that mitochondrial ATP synthesis was decreased. Interestingly, however, total ATP levels were actually higher in CLD1-overexpressing cells (Fig. 6A). This suggested that cells may compensate for the respiratory loss by increasing ATP generation from fermentation. Consistent with this, ethanol production was significantly higher in CLD1-overexpressing cells than in controls (Fig. 6B).

To determine whether up-regulation of genes in glycolysis/fermentation could account for increased ethanol production, we analyzed expression of GAPDH and PGK1, which encode enzymes that catalyze key steps in glycolysis (glyceraldehyde-3-P dehydrogenase and phosphoglycerate kinase, respectively), as well as ADH1 and ADH2, which encode the fermentation enzyme alcohol dehydrogenase. As seen in Fig. 6C, expression of ADH1 and ADH2 was increased 2-fold, which most likely accounts for the increase in ethanol production. Expression of GAPDH and PGK1 was not altered. These findings indicate that overexpression of CLD1 leads to decreased mitochondrial respiration and ATP synthesis, which is compensated by increasing glycolysis.

DISCUSSION

A deficiency in CL reacylation catalyzed by tafazzin is deleterious in eukaryotes (41, 49–53), most notably in humans where it leads to the life-threatening disorder BTHS (46–48). The loss of tafazzin results in perturbation of CL metabolism. Specifically, the CL/MLCL ratio is decreased, as are the levels of unsaturated CL species. Although many studies suggest that the deleterious effects of tafazzin deficiency result from the absence of unsaturated CL (45, 49, 73–75), no reports to date have distinguished between decreased unsaturated CL and decreased CL/MLCL as the cause of the cellular defects. In this study, we addressed this question by characterizing the effects of CLD1 deletion on tafazzin-deficient yeast cells. The cld1Δ mutant has decreased unsaturated CL (similar to the taz1Δ mutant), but the CL/MLCL ratio is not decreased. We report that cld1Δ rescues growth and respiration defects of the taz1Δ mutant, indicating that the decreased CL/MLCL ratio, and not decreased unsaturated CL, leads to the defects in tafazzin-deficient cells.

Interestingly, the double mutant cld1Δtaz1Δ exhibited defective growth in glycerol/ethanol medium at 37 °C as reported in Beranek et al. (38). We tested growth of WT, cld1Δ, taz1Δ, and cld1Δtaz1Δ cells in media containing glucose, glycerol, ethanol, or glycerol/ethanol as carbon sources. Indeed, we found that the double mutant grew poorly compared with WT when glycerol/ethanol was used as carbon source, similar to the observation of Beranek et al. (38). However, in these carbon sources, we did not observe respiratory growth defects in taz1Δ at 30 °C. Although taz1Δ exhibited decreased growth in glycerol/ethanol at 37 °C, high temperature stress complicates respiration defects. In marked contrast,
we observed that taz1Δ cells exhibit a significant respiratory growth defect in ethanol medium at 30 °C. Under these conditions, we observed that the double mutant rescued the respiratory defects of taz1Δ. As cld1Δ restores CL levels but not unsaturated CL species in taz1Δ, this finding indicates that rescue of respiratory growth of taz1Δ by cld1Δ results from restoration of CL levels.

Although deletion of CLD1 does not appear to affect growth, expression of the gene is deleterious in the absence of reacylation, as taz1Δ cells that have the wild type CLD1 gene are defective, whereas those carrying the cld1Δ mutation grow normally. To gain insight into the mechanism underlying the deleterious effects of increased CLD1, we characterized growth and mitochondrial function of cells overexpressing this gene. Interestingly, overexpression of CLD1 resulted in increased ATP levels (Fig. 6A) despite a significant reduction in mitochondrial respiration (Fig. 5B). Two possibilities may explain this seemingly surprising finding. First, overexpression of CLD1 leads to a growth slowdown, and therefore less ATP is required and utilized to maintain cellular functions. Second, CLD1 overexpression shifts metabolism from respiration toward glycolysis and fermentation (Fig. 7), compensating for defective oxidative phosphorylation. This indicates that regulation of Cld1-mediated deacylation of CL influences energy metabolism by modulating the relative contribution of glycolysis and respiration. CL is an essential component of oxidative phosphorylation complexes. For example, it was identified in the crystal structure of cytochrome c oxidase (15), the proposed rate-limiting enzyme of the electron transport chain (reviewed in Ref. 76), and is required for optimal enzyme function and activity (77). Therefore, reduced mitochondrial respiration in CLD1-overexpressing cells would be expected if the CL pool is modified. (We hypothesize that during stationary growth, when oxidative phosphorylation is used, CL may be tuned toward increased membrane fluidity or association with the supercomplexes.) Furthermore, such alterations lead to mitochondrial DNA instability.

Apparently, there is yet another level of regulation of the CL metabolic pathway intersecting with cytochrome c oxidase regulation. We show here that CLD1 gene regulation is mediated
**CLD1 Expression Regulates Mitochondrial Functions**

CLD1 expression is regulated by carbon sources, and the activity of Clld1 is increased by dissipating the mitochondrial membrane potential (39). They suggested that CL remodeling functions to increase oxidative phosphorylation efficiency and/or replace oxidized CL.

Our findings have implications for understanding the mechanism underlying BTHS. Many studies of BTHS have concluded that the disorder is due to the complete lack of the "normal" unsaturated (tetratinoleoyl or L4) CL in the heart (45, 49, 73–75). However, this study indicates that in yeast, a total lack of the normal unsaturated CL species is not deleterious to cells. The large number of mammalian phospholipases complicates the ability to distinguish between decreased CL/MLCL versus decreased unsaturated CL in human cells. Gross and co-workers (85) reported that ablation of phospholipase calcium-independent PLA_{2\gamma} in the mouse reduced MLCL levels by only ~50% indicating that other phospholipases deacylate CL. Mass spectrometry analysis of phospholipase activity identified at least four phospholipases that deacylate CL in vitro (56). The identification of mammalian CL-specific phospholipases may ultimately enable this question to be addressed.

*Note Added in Proof*—During the review of this paper, a study was published reporting that remodeled and unremodeled cardiolipin are functionally indistinguishable in yeast (Baile *et al.*, Nov. 27, 2013 (87)). Consistent with our findings that deletion of CLD1 rescued *taz1Δ* growth and life span defects, Baile *et al.* showed that defective growth of *taz1Δ* was rescued by *cld1Δ* in different genetic backgrounds, which further supports the conclusion that CLD1 expression is deleterious in the absence of TAZ1. Interestingly, they demonstrated that CL remodeling is not required for mitochondrial morphology or optimal oxidative phosphorylation activity. Despite the importance of CL in mitochondrial morphology and functions, the remodeling of CL seems to be dispensable. In contrast, our findings suggested that excessive CL remodeling is deleterious as CLD1 overexpression leads to decreased respiration and instability of mitochondrial DNA. Taken together, the findings in both studies have important implications for BTHS, because if decreased CL/MLCL and not altered CL acyl composition is the cause of the pathology, attenuation of CL-specific phospholipase activity may be a potential strategy to treat BTHS patients.

**REFERENCES**

1. Hostetter, K. Y., van den Bosch, H., and van Deenen, L. L. (1972) The mechanism of cardiolipin biosynthesis in liver mitochondria. *Biochim. Biophys. Acta* **260**, 507–513
2. Joshi, A. S., Zhou, J., Gohil, V. M., Chen, S., and Greenberg, M. L. (2009) Cellular functions of cardiolipin in yeast. *Biochim. Biophys. Acta* **1793**, 212–218
3. Pangborn, M. C. (1947) The composition of cardiolipin. *J. Biol. Chem.* **168**, 351–361
4. Lecocq, J., and Ballou, C. E. (1964) On the structure of cardiolipin. *Biochemistry* **3**, 976–980
5. Jakovcic, S., Getz, G. S., Rabinowitz, M., Jakob, H., and Swift, H. (1971) Cardiolipin content of wild type and mutant yeasts in relation to mitochondrial function and development. *J. Cell Biol.* **48**, 490–502
6. Schlame, M., Rua, D., and Greenberg, M. L. (2000) The biosynthesis and functional role of cardiolipin. *Prog. Lipid Res.* **39**, 257–288
7. Claypool, S. M. (2009) Cardiolipin, a critical determinant of mitochondrial carrier protein assembly and function. *Biochim. Biophys. Acta* **1788**, 2059–2068
8. Schlame, M., and Ren, M. (2009) The role of cardiolipin in the structural

---

**FIGURE 7. Proposed model.** Regulation of Clld1-mediated deacylation of CL influences energy metabolism. CLD1 expression is up-regulated in response to increased respiration. Increased CLD1 expression modulates the relative contributions of oxidative phosphorylation and glycolysis to cellular energy production. We speculate that the function of CL deacylation, which is increased during respiratory conditions that are known to increase oxidative stress, is to remove peroxidized acyl chains from damaged CL.

by the Hap2/3/4/5p transcription factor complex (Fig. 4E), which is also a crucial regulator of cytochrome c oxidase subunit V isoforms Va and Vb (78). These isoforms result in an enzyme with higher affinity for oxygen when the substrate is scarce. Furthermore, overexpression of components of the Hap2/3/4/5p complex rescues cytochrome c oxidase deficiencies (79). In another example of coordinate control, regulation of COX4 translation requires Pgls, the enzyme that catalyzes the committed step of CL synthesis (80). Taken together these findings suggest an integrated and concerted response to environmental stress that affects the CL pathway and oxidative phosphorylation, both of which are interconnected.

Our findings suggest that increased CLD1 is deleterious to cells because it decreases respiration. However, CLD1 expression was increased during respiratory growth and regulated by the Hap complex (Fig. 4), the transcriptional activator that responds to respiratory growth signals. This raises the following question. What is the function of the CL remodeling pathway? It also raises the following corollary question. Why is CLD1 expression increased in response to respiratory conditions? We speculate that the function of CL remodeling is to remediate the deleterious effects of respiration (Fig. 7). In support of this possibility, superoxides generated by respiratory complex III cause peroxidation of CL and decreased cytochrome c oxidase activity (81–83). Exogenous supplementation of CL, but not peroxidized CL or other phospholipids, rescued both reduced activity of cytochrome c oxidase and increased generation of reactive oxygen species in the reperfused heart (83, 84). In this light, CL remodeling may be a mechanism whereby damaged fatty acyl chains are replaced. Although different approaches have been used, this proposed model is similar to the model described in Baile *et al.* (39), in which they suggested a feedback loop between oxidative phosphorylation and CL remodeling. Specifically, they found that
CLD1 Expression Regulates Mitochondrial Functions

leads to longevity defects that are alleviated by alterations in stress response signaling. J. Biol. Chem. 284, 18106–18114
28. Gonzalez-V, and Gottlieb, E. (2007) Cardiolipin: setting the beat of apoptosis. Apoptosis 12, 877–885
29. Houtkooper, R. H., and Vaz, F. M. (2008) Cardiolipin, the heart of mitochondrial metabolism. Cell. Mol. Life Sci. 65, 2493–2506
30. Schug, Z. T., and Gottlieb, E. (2009) Cardiolipin acts as a mitochondrial signalling platform to launch apoptosis. Biochim. Biophys. Acta 1788, 2022–2031
31. Chang, S. C., Heacock, P. N., Clancy, C. J., and Dowhan, W. (1998) The PEL1 gene (renamed PSG1) encodes the phosphatidylglycerol-phosphate synthase of Saccharomyces cerevisiae. J. Biol. Chem. 273, 9829–9836
32. Osman, C., Haag, M., Wieland, F. T., Brügger, B., and Langer, T. (2010) A mitochondrial phosphatase required for cardiolipin biosynthesis: the PGP phosphatase Gep4. EMBO J. 29, 1976–1987
33. Kelly, B. L., and Greenberg, M. L. (1990) Characterization and regulation of phosphatidylglycerol phosphate phosphatase in Saccharomyces cerevisiae. Biochim. Biophys. Acta 1046, 144–150
34. Chang, S. C., Heacock, P. N., Mileykovskaya, E., Voelker, D. R., and Dowhan, W. (1998) Isolation and characterization of the gene (CLS1) encoding cardiolipin synthase in Saccharomyces cerevisiae. J. Biol. Chem. 273, 14933–14941
35. Tuller, G., Gravina, A., Schleifner, G., Schild, C., Bock, J. F., and Dam, G. (1998) YDL142c encodes cardiolipin synthase (Clk1p) and is non-essential for aerobic growth of Saccharomyces cerevisiae. FEBS Lett. 421, 15–18
36. Tamai, K. T., and Greenberg, M. L. (1990) Biochemical characterization and regulation of cardiolipin synthase in Saccharomyces cerevisiae. Biochim. Biophys. Acta 1046, 214–222
37. Jiang, F., Rizavi, H. S., and Greenberg, M. L. (1997) Cardiolipin is not essential for the growth of Saccharomyces cerevisiae on non-permeable carbon sources. Mol. Microbiol. 26, 481–491
38. Beranek, A., Rechberger, G., Knauer, H., Wolinski, H., Kohnle, S. D., and Leber, R. (2009) Identification of a cardiolipin-specific phospholipase encoded by the gene CLD1 (YGR110W) in yeast. J. Biol. Chem. 284, 11572–11578
39. Baile, M. G., Whited, K., and Claypool, S. M. (2013) Deacetylation on the matrix side of the mitochondrial inner membrane regulates cardiolipin remodeling. Mol. Biol. Cell 24, 2008–2020
40. Testet, E., Laroche-Traine, J., Noubhani, A., Coulon, D., Runoust, O., Camougard, N., Manon, S., Lessire, R., and Besoulle, J. J. (2005) Ypr140wp, ‘the yeast tafazzin’, displays a mitochondrial lysophosphatidylcholine (lyso-PC) acyltransferase activity related to triacylglycerol and mitochondrial lipid synthesis. Biochem. J. 387, 617–626
41. Gu, Z., Valianpour, F., Chen, S., Vaz, F. M., Hakkaart, G. A., Wanders, R. J., and Greenberg, M. L. (2004) Aberrant cardiolipin metabolism in the yeast taz1 mutant: a model for Barth syndrome. J. Biol. Chem. 279, 43089–43094
42. Brandner, K., Mick, D. U., Frazier, A. E., Taylor, R. D., Meisenger, C., and Rehling, P. (2005) Taz1, an outer mitochondrial membrane protein, affects stability and assembly of inner membrane protein complexes: implications for Barth syndrome. Mol. Biol. Cell 16, 5022–5021
43. Claypool, S. M., Boonheung, P., McCaffery, J. M., Loo, J. A., and Koehler, C. M. (2008) The cardiolipin transacylase, tafazzin, associates with two distinct respiratory components providing insight into Barth syndrome. Mol. Biol. Cell 19, 5143–5155
44. Vaz, F. M., Houtkooper, R. H., Valianpour, F., Barth, P. G., and Wanders, R. J. (2003) Only one splice variant of the human TAZ gene encodes a functional protein with a role in cardiolipin metabolism. J. Biol. Chem. 278, 43089–43094
45. Xu, Y., Kelley, R. I., Blanck, T. J., and Schlame, M. (2003) Remodeling of cardiolipin by phospholipid transacylation. J. Biol. Chem. 278, 51380–51385
46. Barth, P. G., Scholte, H. R., Berden, J. A., Van der Klein-Van Moorsel, J. M., Luyp Houwen, I. E., Van ‘t Veer-Korthof, E. T., Van der Harten, J. J., and Sobota-Pløjhar, M. A. (1983) An X-linked mitochondrial disease affecting cardiac muscle, skeletal muscle and neutrophil leukocytes. J. Neurol. Sci. 62, 327–355
47. Barth, P. G., Wanders, R. J., Vreken, P., Janssen, E. A., Lam, J., and Baas, F.
(1999) X-linked cardioskeletal myopathy and neutropenia (Barth syndrome) (MIM 302060). J. Inherit. Metab. Dis. 22, 555–567
48. Barth, P. G., Valianpour, F., Bowen, V. M., Lam, J., Duran, M., Vaz, F. M., and Wanders, R. J. (2004) X-linked cardioskeletal myopathy and neutropenia (Barth syndrome): an update. Am. J. Med. Genet. A 126, 349–354
49. Schlamle, M., Kelley, R. F., Feigenbaum, A., Towbin, J. A., Heerdt, P. M., Schieble, T., Wanders, R. J., DiMauro, S., and Blanck, T. J. (2003) Phospholipid abnormalities in children with Barth syndrome. J. Am. Coll. Cardiol. 42, 1994–1999
50. Valianpour, F., Wanders, R. J., Barth, P. G., Overmars, H., and van Gennip, A. H. (2002) Quantitative and compositional study of cardiolipin in platelets by electrospray ionization mass spectrometry: application for the identification of Barth syndrome patients. Clin. Chem. 48, 1390–1397
51. Vreken, P., Valianpour, F., Niˇjmants, L. G., Grivel, L. A., Plecko, B., Wanders, R. J., and Barth, P. G. (2000) Defective remodeling of cardiolipin and phosphatidylglycerol in Barth syndrome. Biochem. Biophys. Res. Commun. 279, 378–382
52. Xu, Y., Condell, M., Plesken, H., Edelman-Novemsky, I., Ma, J., Ren, M., and Schlame, M. (2006) A Dro sophila model of Barth syndrome. Proc. Natl. Acad. Sci. U.S.A. 103, 11584–11588
53. Azehan, D., Vaz, F., Houtkooper, R. H., James, J., Moore, V., Tokunaga, C., Kulik, W., Wansapura, J., Toth, M. J., Strauss, A., and Khuchua, Z. (2011) Cardiac and skeletal muscle defects in a mouse model of human Barth syndrome. J. Biol. Chem. 286, 899–908
54. Houtkooper, R. H., Rodenburg, R. J., Thiels, C., van Lenthe, H., Stet, F., Poll-The, B. T., Stone, J. E., Stewart, C. G., Wanders, R. J., Smeitink, J., Kulik, W., and Vaz, F. M. (2009) Cardiolipin and monolysocardiolipin analysis in fibroblasts, lymphocytes, and tissues using high-performance liquid chromatography–mass spectrometry as a diagnostic test for Barth syndrome. Anal. Biochem. 387, 230–237
55. Malhotra, A., Edelman-Novemsky, I., Xu, Y., Pleken, H., Ma, J., Schlamle, M., and Ren, M. (2009) Role of calcium-independent phospholipase A2 in the pathogenesis of Barth syndrome. Proc. Natl. Acad. Sci. U.S.A. 106, 2337–2341
56. Hsu, Y. H., Dumlao, D. S., Cao, J., and Dennis, E. A. (2013) Assessing phospholipase A2 activity toward cardiolipin by mass spectrometry. PLoS One 8, e59267
57. Fabrizio, P., and Longo, V. D. (2003) The chronological life span of Saccharomyces cerevisiae. Aging Cell 2, 73–81
58. Hu, J., Wei, M., Mirisola, M. G., and Longo, V. D. (2013) Assessing chronological aging in Saccharomyces cerevisiae. Methods Mol. Biol. 965, 463–472
59. Köh rer, K., and Domdey, H. (1991) Preparation of high molecular weight RNA. Methods Enzymol. 194, 398–405
60. Lee, I., Pecinova, A., Pecina, P., Neel, B. G., Araki, T., Kucherlapati, R., Roberts, A. E., and Hüttemann, M. (2010) A suggested role for mitochondrial functional activators HAP/NF-Y rescue a cytochrome c oxidase defect in yeast and human cells. Hum. Mol. Genet. 19, 775–788
61. Su, X., and Dowhan, W. (2006) Translational regulation of nuclear gene COX4 expression by mitochondrial content of phosphatidylglycerol and cardiolipin in Saccharomyces cerevisiae. Mol. Cell. Biol. 26, 743–753
62. Paradies, G., Ruggiero, F. M., Petrosillo, G., and Quagliariello, E. (1998) Peroxidative damage to cardiac mitochondria: cytochrome c oxidase and cardiolipin alterations. FEBS Lett. 424, 155–158
63. Baile, M. G., Sathappa, M., Lu, Y. W., Pryce, E., Whit ed, K., McCaffery, J. M., Han, X., Alder, N. N., and Cl ay pond, S. M. (2014) Unremodeled and remodeled cardiolipin are functionally indistinguishable in yeast. J. Biol. Chem. 289, 1768–1778