Synthetic Lethal Interaction of the Mitochondrial Phosphatidylethanolamine and Cardiolipin Biosynthetic Pathways in *Saccharomyces cerevisiae*

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* Saccharomyces cerevisiae* mitochondria contain enzymes required for synthesis of the phospholipids cardiolipin (CL) and phosphatidylethanolamine (PE), which are enriched in mitochondrial membranes. Previous studies indicated that PE may compensate for the lack of CL, and *vice versa*. These data suggest that PE and CL have overlapping functions and that the absence of both lipids may be lethal. To address this hypothesis, we determined whether the *c rd1Δ* mutant, which lacks CL, was viable in genetic backgrounds in which PE synthesis was genetically blocked. Deletion of the mitochondrial PE pathway gene *psd1* was synthetically lethal with the *c rd1Δ* mutant, whereas deletion of the Golgi and endoplasmic reticulum pathway genes *psd2* and *dpl1* did not result in synthetic lethality. A 20-fold reduction in phosphatidylcholine did not affect the growth of *c rd1Δ* cells. Supplementation with ethanolamine, which led to increased PE synthesis, or with propanolamine, which led to synthesis of the novel phospholipid phosphatidylpropane, failed to rescue the synthetic lethality of the *c rd1Δ psd1Δ* cells. These results suggest that mitochondrial biosynthesis of PE is essential for the viability of yeast mutants lacking CL.

The phospholipid composition of the mitochondrial membrane is unique in that it is highly enriched in cardiolipin (CL) and phosphatidylethanolamine (PE). CL is a dimeric glycerocephospholipid that is synthesized exclusively in mitochondria and plays an important role in oxidative phosphorylation and mitochondrial membrane biogenesis (3). In contrast, PE biosynthesis occurs via multiple pathways (4). PE is commonly present in all subcellular membranes, although PE levels are highest in the mitochondrial membrane (2). PE and CL have similar physical properties in that they have a propensity toward the formation of nonbilayer, inverted hexagonal (HII) phase structures (5, 6). The local, transient formation of nonbilayer structures is thought to play an important role in vital cellular processes, such as vesicle formation, vesicle-mediated protein trafficking, and membrane fusion (7). In addition, nonbilayer lipids affect integration of proteins into the membrane, their lateral movement within the membrane, and the function and folding of certain integral membrane proteins (8).

In the yeast *Saccharomyces cerevisiae*, phospholipid biosynthesis is compartmentalized in various subcellular organelles, including the Golgi body, endoplasmic reticulum, and mitochondria (Fig. 1). CL biosynthesis occurs in three steps, all catalyzed by enzymes present in the mitochondria. The first step, catalyzed by phosphatidylglycerol phosphate (PGP) synthase, is the synthesis of PGP from CDP-diacylglycerol and glyceraldehyde 3-phosphate. PGP phosphatase dephosphorylates PGP to phosphatidylglycerol. In the final step, CL synthase catalyzes the formation of CL from phosphatidylglycerol and CDP-diacylglycerol (9, 10). PGP synthase and CL synthase have been characterized in yeast, and the genes encoding these enzymes, *PGS1* (11, 12) and *CRD1* (13–15), have been identified. The biosynthesis of aminoglycerophospholipids occurs in the endoplasmic reticulum, vacuole/Golgi body, and mitochondrial compartments (Fig. 1). PE biosynthesis is accomplished by two *de novo* pathways involving decarboxylation of phosphatidylethanolamine (PS) by PS decarboxylase, including Psl1p-catalyzed PE synthesis in mitochondria and Psd2p-catalyzed PE formation in the vacuole/Golgi body (16, 17). In addition, PE can be synthesized from ethanolamine (Etn) via the Kennedy pathway (Fig. 1). The Kennedy pathway is also linked to sphingolipid catabolism through a reaction catalyzed by dihydroxyphosphosinoglycolipid synthase lyase (Dpl1p). This enzyme cleaves the phosphorylated sphingoid base to a long chain aldehyde and ethanolamine phosphate (18), which is then incorporated into PE via the Kennedy pathway (19). The presence of CL and PE biosynthetic machinery in mitochondria suggests that these phospholipids are required for mitochondrial function.

Several published studies are consistent with the possibility that CL and PE have redundant functions and that each can compensate for the loss of the other. The growth phenotype of *c rd1Δ* cells, which lack CL synthase and CL, is characterized by temperature sensitivity at 37 °C, reduced growth in nonfermentable media, and increased frequency of petite formation (20). Depletion of PE in *psd1Δ* cells produces a similar phenotype (16). Furthermore, the *Escherichia coli* AD93 strain, which lacks PE, has increased levels of CL (21), and the absence of CL in yeast *c rd1Δ* mutants results in increased PE (22). The null mutants *pgs1Δ, c rd1Δ, p s d1Δ, psd2Δ, pem1Δ, pem2Δ*, and *dpl1Δ* are viable in complex and synthetic media in the absence of external supplementation with Etn or choline, consistent with the redundancy of phospholipid functions in *S. cerevisiae*.

Based on the observed similarities in the phenotypes of *c rd1Δ* and *psd1Δ* cells, the physical properties of PE and CL, and the compensatory increases of PE in CL-deficient mutants and CL in PE-deficient mutants, we hypothesized that cells lacking both PE and CL are inviable. In support of this hypothesis, we determined that *c rd1Δ* is synthetically lethal with *psd1Δ* but not with mutants in the nonmitochondrial pathways for PE synthesis. Supplementation with ethanolamine or propanolamine failed to rescue the lethality of the *c rd1Δ psd1Δ* double mutant. These data indicate that the combined loss of synthesis of CL and PE from mitochondria is lethal to yeast cells.
The primers for PCR were forward primer (5′-AAAAGCTTCTGGA- TAGCATAGTTTGGTCC-3′) and reverse primer (5′-ACGGATC- CGTATGATTGCAATTACCG-3′) (13). Yeast deletion strains including BY4741 _psd1Δ::KanMX4_, BY4741 _psd2Δ::KanMX4_, BY4741 _dpl1Δ::KanMX4_, BY4741 _pem1Δ::KanMX4_, and BY4741 _pem2Δ::KanMX4_ were obtained from the yeast deletion collection (Invitrogen). The genotypes of these strains were confirmed by growth phenotypes and phospholipid profiles. Double mutant strains were created by sporulation of the diploids followed by tetrad dissection on either YPD medium or glucose-containing CSM medium with 2 mM or 20 mM Prn or Etn supplementation. The identities of all viable double mutant strains were also confirmed by their genotypes and characterization of their phospholipid profiles. The pYES6/CRD1 plasmid was constructed by inserting the CRD1 open reading frame sequence into pYES6/CT at the BamHI/XbaI site, upstream of the _V5_ tag and downstream from the _GAL1_ promoter. Primers were designed based on the SGD _CRD1_ gene sequence: forward primer (5′-AATTGATCCAT- GATTTCAATGTTGCCC-3′) and reverse primer (5′-CTTTTCTA- GAAGGATCGCATAATTACATT-3′). Restriction endonuclease sites BamHI and XbaI as indicated by the underlines, were added to the forward and reverse primers to facilitate subsequent DNA manipulations. The _CRD1_ stop codon was deleted in the reverse primer. The insertion was confirmed by sequencing, and functionality of _V5_-tagged Crd1p was checked by transforming the BY4741 _psd1Δ::KanMX4_, BY4741 _psd2Δ::KanMX4_, and BY4741 _pem1Δ::KanMX4_, and BY4741 _pem2Δ::KanMX4_ strains with plasmid containing a _CRD1_ tag and downstream of the _V5_ promoter. The expression of _V5_-tagged Crd1p was checked by transforming the BY4741 _psd1Δ::KanMX4_, BY4741 _psd2Δ::KanMX4_, and BY4741 _pem1Δ::KanMX4_, and BY4741 _pem2Δ::KanMX4_ strains with a linearized pUC19 plasmid containing a _CRD1_ tag and downstream of the _V5_ promoter. The expression of _V5_-tagged Crd1p was checked by transforming the BY4741 _psd1Δ::KanMX4_, BY4741 _psd2Δ::KanMX4_, and BY4741 _pem1Δ::KanMX4_, and BY4741 _pem2Δ::KanMX4_ strains with a linearized pUC19 plasmid containing a _CRD1_ tag and downstream of the _V5_ promoter. The expression of _V5_-tagged Crd1p was checked by transforming the BY4741 _psd1Δ::KanMX4_, BY4741 _psd2Δ::KanMX4_, and BY4741 _pem1Δ::KanMX4_, and BY4741 _pem2Δ::KanMX4_ strains with a linearized pUC19 plasmid containing a _CRD1_ tag and downstream of the _V5_ promoter.
Synthetic Lethal Interaction between CRD1 and PSD1

Table One

| Yeast strains and plasmids used in this study | Genotype/Characteristics | Source/Reference |
|---------------------------------------------|--------------------------|-----------------|
| BY4741 (WT)                                 | MAT a, his 301, leu 200, met 1500, ura 300 | Invitrogen      |
| BY4742 (WT)                                 | MAT a, his 301, leu 200, lys 200, ura 300 | Invitrogen      |
| VGY1 (BY4742 crd1Δ)                         | MAT a, his 301, leu 200, lys 200, ura 300, crd1Δ:URA3 | This study      |
| BY4741 psd1Δ                                | MAT a, his 301, leu 200, met 1500, ura 300, psd1Δ:KanMX4 | Invitrogen      |
| BY4741 psd1Δ                                | MAT a, his 301, leu 200, met 1500, ura 300, psd1Δ:KanMX4 | Invitrogen      |
| BY4741 dpl1Δ                                | MAT a, his 301, leu 200, met 1500, ura 300, dpl1Δ:KanMX4 | Invitrogen      |
| BY4741 pem1Δ                                | MAT a, his 301, leu 200, met 1500, ura 300, pem1Δ:KanMX4 | Invitrogen      |
| BY4741 pem1Δ                                | MAT a, his 301, leu 200, met 1500, ura 300, pem2Δ:KanMX4 | Invitrogen      |
| VGY2 (crd1Δ psd2Δ)                          | MAT a, his 301, leu 200, lys 200, ura 300, crd1Δ:URA3, psd2Δ:KanMX4 | This study      |
| VGY3 (crd1Δ dpl1Δ)                          | MAT a, his 301, leu 200, met 1500, ura 300, crd1Δ:URA3, dpl1Δ:KanMX4 | This study      |
| VGY4 (crd1Δ pem1Δ)                          | MAT a, his 301, leu 200, met 1500, ura 300, crd1Δ:URA3, pem1Δ:KanMX4 | This study      |
| VGY5 (crd1Δ pem2Δ)                          | MAT a, his 301, leu 200, met 1500, ura 300, crd1Δ:URA3, pem2Δ:KanMX4 | This study      |
| pYES6/CT                                   | S. cerevisiae expression vector derived from the parental pYES2 vector | Invitrogen      |
| pYES6/CRD1                                  | pYES6 derivative vector containing CRD1 gene | Q. He          |

Table Two

Synthetic lethality of crd1Δ and psd1Δ mutants

Heterozygous diploids were sporulated and tetrads were dissected on YPD medium for all the crosses except for BY4741/2 (CRD1/crd1Δ:URA3 PS1/PD1Δ:KanMX4) + pYES6/CRD1, which were dissected on YPI medium.

| Diploid strain | Number of tetrads | Recovery of viable Ura+ Kan− spores | Parental ditotype | Tetratype | Nonparental ditotype |
|----------------|-------------------|-------------------------------------|------------------|-----------|---------------------|
| BY4741/2 (wild type) | 20 | NA* | NA | NA | NA |
| BY4741/2 (CRD1/crd1Δ:URA3 PS1/PD1Δ:KanMX4) | 63 | No | 9 | 40 | 14 |
| BY4741/2 (CRD1/crd1Δ:URA3 PS1/PD1Δ:KanMX4) + pYES6/CRD1 | 23 | Yes | 3 | 12 | 8 |
| BY4741/2 (CRD1/crd1Δ:URA3 PS2/PD2Δ:KanMX4) | 19 | Yes | 3 | 11 | 5 |
| BY4741/2 (CRD1/crd1Δ:URA3 DPL1/dpl1Δ:KanMX4) | 17 | Yes | 0 | 14 | 3 |
| BY4741/2 (CRD1/crd1Δ:URA3 pem1Δ/pem1Δ:KanMX4) | 16 | Yes | 7 | 9 | 0 |
| BY4741/2 (CRD1/crd1Δ:URA3 pem2Δ/pem2Δ:KanMX4) | 16 | Yes | 3 | 9 | 4 |

* NA, not available. Both the wild-type strains BY4741 and BY4742 are Ura+; therefore, URA+ spores were not expected.

RESULTS

The crd1Δ and psd1Δ Mutants Are Synthetically Lethal—To test the hypothesis that crd1Δ and psd1Δ are synthetically lethal, we constructed a crd1Δ:URA3 strain in MATα background and mated it with a MATα psd1Δ:KanMX4 strain. The resulting heterozygous diploids were sporulated, and meiotic tetrad analysis was carried out. In 63 tetrads dissected, 54 tetrads contained less than four viable spores, and no viable crd1Δ psd1Δ spores (Ura− Kan+) were recovered (TABLE TWO), indicating that the crd1Δ psd1Δ double mutant is synthetically lethal. The lethality was not due to defective spor germination, since microscopic analysis indicated that tiny colonies of no more than a few hundred cells formed from the spores that were deduced to be crd1Δ psd1Δ (not shown). In order to demonstrate a conditional synthetic lethal relationship in the crd1Δ psd1Δ double mutant, the heterozygous diploid BY4741/2 (CRD1/crd1Δ:URA3 PS1/PD1Δ:KanMX4) was transformed with the pYES6/CRD1 plasmid, from which the expression of VS-tagged CRD1 is controlled by the GAL1 promoter, and meiotic tetrad analysis of the sporulated diploid was carried out. We chose the pYES6/CT expression system, because it allowed controlled expression of epitope-tagged Crd1p, and thus growth phenotype could be correlated with expression of the Crd1p protein. Viable crd1Δ psd1Δ spores were recovered on YPI medium in which Crd1p is induced (TABLE TWO). These cells exhibited normal growth on YPI (Fig. 2A) and reduced, but discernible, growth on YPD (Fig. 2B). Growth on YPD medium could be attributed to low level expression of Crd1p from the leaky GAL1 promoter (Fig. 2C), which was sufficient to synthesize expressed as a percentage of radiolabel incorporated into total phospholipids.

Mitochondrial Phospholipid Determination—Cells were grown in CSM in the absence or presence of 2 mM Prn. Mitochondria were isolated from 5–6 g of cell pellet as described by Daum et al. (26). Total mitochondrial phospholipids were extracted and purified as described (27). Isolated total phospholipids were applied to silica gel 60 plates and separated by two-dimensional TLC (25). The developed TLC plates were dried and exposed to iodine vapor to visualize phospholipid spots.

Western Blot Analyses—Protein extraction was carried out as per the Invitrogen product manual. The extraction buffer contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, and protein inhibitor mixture at the concentration of 1 tablet/10 ml of buffer. Protein quantification was carried out using the Bio-Rad protein assay reagent. Protein extracts (50 µg) were separated by SDS-PAGE using precast (4–20%) gradient polyacrylamide gels. Protein from the separating gel was electroblotted onto a polyvinylidene difluoride membrane. The membrane was incubated with the monoclonal anti-V5 antibodies (1:3000 dilution; Invitrogen) and then washed and incubated with alkaline phosphatase-linked secondary antibodies. The protein bands were detected using a 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium color development kit.

Other Procedures—Standard molecular techniques were used in this study. Plasmid purification from E. coli was performed using the Wizard Miniprep DNA Purification system. Transformations of E. coli cells were performed using a BTX electroporation system. Yeast transformations were carried out by the lithium acetate method (28).
almost 70% of wild-type levels of CL (Fig. 2D). However, the growth phenotype in liquid YPD medium of the crd1Δpsd1Δ cells containing the pYES6/CRD1 plasmid was similar to crd1Δpsd1Δ cells containing the plasmid that were cultured in liquid YPI medium (Fig. 2F). Phenotypic differences between culturing in solid or liquid medium have been previously observed in the psd1Δ and crd1Δ mutants. The temperature-sensitive phenotype of psd1Δ cells is apparent at 37 °C in liquid medium but is not observed in solid medium at 37 °C (25), whereas the reverse is true for the crd1Δ mutant (29).

Disruption of Nonmitochondrial Pathways for PE Biosynthesis Does Not Affect the Growth of crd1Δ Cells—Although mitochondrial PSD1 is the major source of PE (4), it can be synthesized in significant amounts via multiple nonmitochondrial pathways. This was confirmed by analysis of the total cellular phospholipid composition of psd2Δ and dpl1Δ mutants in YPD and CSM. Phospholipid extraction was carried out from cells grown to the late logarithmic phase in glucose-containing medium in order to simulate the growth conditions of spores obtained from tetrads dissected on glucose-containing medium. As shown in TABLES THREE and FOUR, the decrease in total PE levels in psd2Δ cells was similar to the decrease in PE observed in psd1Δ cells, suggesting that the decrease in cellular PE per se does not result in inviability of the crd1Δpsd1Δ cells. This was confirmed by the recovery of viable crd1Δpsd2Δ colonies (TABLE TWO). The dpl1Δ mutation resulted in only a minor decrease in PE (TABLE FOUR), and crd1Δdpl1Δ double mutants were also recovered (TABLE TWO). Mitochondrial membranes are highly enriched in PC, comprising almost 40% of total mitochondrial phospholipids (2). However, disruption of PC synthesis did not lead to loss of viability of crd1Δ cells, since viable crd1Δpsd1Δ and crd1Δpem2Δ double mutants were also recovered (TABLE TWO). Growth of these double mutants was similar to that of single mutant or wild-type cells in YPD or CSM (Fig. 3), even with up to a 20-fold reduction in PC in the crd1Δpem2Δ cells (TABLE THREE).

Synthetic Lethality of crd1Δpsd1Δ Is Not Rescued by Ethanolamine or Propanolamine—Storey et al. (25) reported an increase in PE levels upon Etn supplementation and synthesis of a novel phospholipid, phosphatidylpropanolamine, upon Prn supplementation in CSM. We wished to determine whether synthetic lethality of crd1Δpsd1Δ could be rescued by increasing the concentration of Etn or Prn, which rescue the temperature sensitivity of psd1Δ cells. Steady-state phospholipid analysis of psd1Δ cells in YPD medium showed that PE is not significantly increased with Etn supplementation, even at a concentration of

### Table Three

**Phospholipid composition of yeast strains grown in CSM to the early stationary growth phase**

Data are expressed as a percentage of radiolabel 32P incorporated into total phospholipids. Mean values of two independent measurements are shown. PDME, phosphatidylphosphatidylglycerol; PA, phosphatidic acid; PG, phosphatidylinositol; PI, phosphatidylcholine; ND, not detectable.

| Strain       | PDME (%) | CL (%) | PA (%) | PE (%) | PG (%) | PS (%) | PI (%) | PC (%) |
|-------------|----------|--------|--------|--------|--------|--------|--------|--------|
| BY4741 (WT) | 0.31     | 3.26   | 3.37   | 14.55  | 0.35   | 8.39   | 23.85  | 44.66  |
| BY4742 (WT) | 0.28     | 3.22   | 3.08   | 14.54  | 0.32   | 8.31   | 23.62  | 44.48  |
| psd1Δ       | 0.24     | 2.48   | 4.11   | 10.42  | 0.26   | 10.90  | 20.71  | 50.43  |
| crd1Δ       | ND       | ND     | 2.19   | 16.16  | 2.97   | 8.72   | 24.89  | 42.83  |
| dpl1Δ       | 0.32     | 3.83   | 3.54   | 14.28  | 0.31   | 6.15   | 26.74  | 42.86  |
| psd2Δ       | ND       | ND     | 3.83   | 3.75   | 9.73   | 0.23   | 7.81   | 24.80  | 48.03  |
| pem1Δ       | ND       | 4.01   | 6.59   | 37.03  | 0.39   | 7.83   | 27.05  | 14.59  |
| pem2Δ       | 3.19     | 2.97   | 2.77   | 51.91* | 0.50   | 7.07   | 28.51  | 2.23   |
| crd1Δpsd1Δ  | ND       | ND     | 2.13   | 8.56   | 2.97   | 10.39  | 26.46  | 47.86  |
| crd1Δdpl1Δ  | ND       | ND     | 3.75   | 17.15  | 3.68   | 6.11   | 24.72  | 41.83  |
| crd1Δpem1Δ  | ND       | ND     | 5.93   | 40.90  | 3.52   | 6.78   | 27.26  | 13.79  |
| crd1Δpem2Δ  | 2.70     | ND     | 2.99   | 53.71* | 3.31   | 7.24   | 26.54  | 2.29   |

* Value indicates the sum of PE and phosphatidylmonomethylethanolamine.
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**TABLE FOUR**

Phospholipid composition of yeast strains grown in YPD medium to the late logarithmic/early stationary growth phase

Data are expressed as a percentage of radiolabel 32P incorporated into total phospholipids. Mean values of two independent measurements are shown. PDME, phosphatidylidimethylethanolamine; PA, phosphatic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine; ND, not detectable.

|          | PDME  | CL   | PA   | PE   | PG   | PS   | PI   | PC   |
|----------|-------|------|------|------|------|------|------|------|
| BY4741 (WT) | 3.92  | 2.84 | 3.33 | 17.71| 0.24 | 7.83 | 20.95| 42.15|
| BY4742 (WT) | 3.76  | 2.88 | 3.28 | 17.74| 0.22 | 7.96 | 21.71| 41.38|
| psd1Δ    | 3.00  | 1.30 | 2.97 | 11.83| 0.21 | 8.62 | 25.22| 45.60|
| crd1Δ    | 3.89  | ND   | 3.37 | 19.58| 2.11 | 9.08 | 21.00| 39.53|
| dpl1Δ    | 2.68  | 3.28 | 3.54 | 15.17| 0.25 | 7.88 | 25.75| 40.71|
| psd2Δ    | 1.15  | 2.74 | 2.72 | 10.88| 0.19 | 9.06 | 23.59| 48.84|
| pem1Δ    | ND    | 2.76 | 3.24 | 21.88| 0.26 | 8.72 | 21.93| 40.28|
| pem2Δ    | 0.35  | 2.76 | 2.79 | 26.28*| 0.28 | 8.79 | 21.08| 36.63|
| crd1Δpsd1Δ| 1.04  | ND   | 2.71 | 13.40| 2.08 | 9.98 | 22.02| 47.47|
| crd1Δdpl1Δ| 3.21  | ND   | 3.92 | 18.13| 2.14 | 8.83 | 21.69| 41.03|
| crd1Δpem1Δ| 0.05  | ND   | 4.47 | 22.15| 1.79 | 8.85 | 22.80| 38.83|
| crd1Δpem2Δ| 0.23  | ND   | 3.03 | 26.66*| 1.83 | 8.58 | 21.85| 36.39|

* Value indicates the sum of PE and phosphatidylmonomethylethanolamine.

20 mM (Fig. 4A). Similarly, there was minimal synthesis of phosphatidylpropanolamine upon Prn supplementation in YPD medium (Fig. 4A). However, consistent with the findings of Storey et al. (25), supplementation with Etn in CSM medium resulted in a 2-fold increase in PE levels (Fig. 4B). Prn supplementation resulted in the synthesis of phosphatidylpropanolamine, comprising up to 30% of total phospholipid levels (Fig. 4B). The synthesis of PE and phosphatidylpropanolamine upon Etn and Prn supplementation occurs in the endoplasmic reticulum. To determine whether these phospholipids could be imported into mitochondria of mutant cells, we analyzed the phospholipid composition of mitochondria from cells grown in the presence of Prn. Consistent with the findings of Storey et al. (25), we observed the presence of phosphatidylpropanolamine in crd1Δ and psd1Δ mitochondria (data not shown). However, crd1Δpsd1Δ colonies could not be recovered on medium supplemented with either Etn or Prn (TABLE FIVE). The tiny colonies from spores deduced to be crd1Δpsd1Δ did not have a greater number of cells than those germinated in the absence of Etn and Prn (data not shown). These results clearly demonstrate that the absence of both mitochondrial PE and CL biosynthesis is lethal.

**DISCUSSION**

In this study, we demonstrated a synthetic lethal interaction between the crd1Δ mutant and the psd1Δ mutant, which cannot synthesize PE in the mitochondria. Disruption of nonmitochondrial pathways of PE biosynthesis in crd1Δ cells did not result in synthetic lethality. Increasing cellular PE and synthesis of phosphatidylpropanolamine also did not rescue the synthetic lethality of the crd1Δpsd1Δ double mutant. These data indicate that cells cannot survive if synthesis of both PE and CL in the mitochondria is eliminated. This suggests that CL and PE have overlapping functions in the mitochondria and that loss of both is lethal.

The reduction in total cellular PE per se was not responsible for synthetic lethality in the crd1Δ background, since the decrease in total cellular PE in psd2Δ cells was similar to the decrease observed in psd1Δ cells (TABLES THREE and FOUR). Up to a 20-fold decrease in PC, the most abundant mitochondrial phospholipid, due to a mutation in PEM2 (TABLE THREE), did not result in synthetic lethality, as evidenced by the recovery of viable crd1Δpem2Δ colonies (TABLE TWO). Consistent with the specific requirement of PE in the crd1Δ background, we observed severely diminished growth of the crd1Δpsd2Δ mutant in CSM compared with double mutants of the PC pathway (Fig. 3). These results point to a specific requirement for either mitochondrial PE biosynthesis or the mitochondrial PE biosynthetic enzyme Psd1p for the viability of CL-deficient cells. The presence of Psd1p itself in mitochondria may not be sufficient to allow survival of crd1Δ cells, since Janitor et al. (30) have shown that disruption of the CHO1 gene in the CL-deficient pgs1Δ background results in synthetic lethality. Cho1p catalyzes the synthesis of PS, which is the substrate for Pdh1p. Therefore, the absence of PS results in an inability to synthesize mitochondrial PE via Psd1p. Thus, pgs1Δ and cho1Δ are synthetically lethal despite the presence of Psd1p.

It is well documented that in the yeast *Saccharomyces cerevisiae*, mitochondrial biogenesis and respiration is triggered in nonfermentable medium or in...
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FIGURE 4. Etn and Prn supplementation in psd1Δ results in increased cellular PE and phosphatidylpropanolamine (PP) in CSM but not in YPD. psd1Δ cells were grown in the presence of 2 or 20 mM Etn or Prn in YPD (A) or CSM (B) to the early stationary growth phase. Cells were harvested by centrifugation, and lipids were extracted as described under “Experimental Procedures.” Phospholipids were separated by one-dimensional TLC and visualized by phosphorimaging. PE and phosphatidylpropanolamine bands were quantified using ImageQuant software. Data are expressed as the percentage of radiolabel 32P incorporated into total phospholipids and represent the average of three independent experiments.

TABLE FIVE

| Supplementation | Number of tetrads | Recovery of viable crd1Δpsd1Δ cells | Parental ditype (tetrads with four viable spores) | Tetraparental ditype (tetrads with three viable spores) | Nonparental ditype (tetrads with two viable spores) |
|-----------------|------------------|----------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| YPD             | 63               | No                               | 9                                             | 40                                            | 14                                            |
| YPD + 2 or 20 mM Etn | 33               | No                               | 6                                             | 17                                            | 10                                            |
| YPD + 2 or 20 mM Prn | 53               | No                               | 6                                             | 35                                            | 12                                            |
| CSM             | 28               | No                               | 6                                             | 18                                            | 4                                             |
| CSM + 2 or 20 mM Etn | 46               | No                               | 7                                             | 30                                            | 9                                             |
| CSM + 2 or 20 mM Prn | 44               | No                               | 8                                             | 19                                            | 17                                            |

Supplementation of Etn and Prn does not rescue synthetic lethality of crd1Δpsd1Δ cells

The BY4741/2 (CRD1/crd1Δ:URA3 PSD1/psd1Δ:KanMX4) heterozygous diploid was pregrown with the indicated supplementation. Diploids were sporulated, and tetrads were dissected on plates containing the same concentration of supplement.

Lately logarithmic growth phase of cells grown in glucose-containing (fermentable) medium. The CRD1 gene expression, CL biosynthesis, and CL levels are in nonfermentable medium and upon entering the stationary growth phase in glucose-containing medium (29, 31, 32). Mitochondrial PE biosynthesis is essential for growth in nonfermentable medium (4). These results point to the requirement of CL and PE for respiratory functions. Thus, our results showing crd1Δpsd1Δ synthetic lethality in fermentable medium are surprising. This suggests that continuous synthesis of mitochondrial PE or CL is required for essential processes other than mitochondrial respiration. A recent report has shown that Psd1p is the major source of mitochondrial PE (33). The same authors also observed a small but significant level of import of microsomal PE into mitochondria. Others (25) have shown that Etn supplementation could rescue the temperature-sensitive phenotype of psd1Δ cells, consistent with import of PE into the mitochondria. Thus, we tried to rescue the crd1Δpsd1Δ synthetic lethality by increasing PE levels via Etn supplementation. However, a 2-fold increase in PE due to Etn supplementation (Fig. 4B) failed to rescue the crd1Δpsd1Δ synthetic lethal phenotype. Phosphatidylpropanolamine, a novel phospholipid not normally present in yeast, rescues the growth defects of PE-deficient psd1Δ and psd1Δpsd2Δ cells and has been shown to efficiently incorporate into mitochondrial membranes (25, 34). However, we did not recover viable crd1Δpsd1Δ cells even under conditions in which phosphatidylpropanolamine formed 30% of total phospholipids (Fig. 4B). The possibility that import of phosphatidylpropanolamine may be defective in CL-deficient mitochondria was ruled out, since we observed a significant amount of phosphatidylpropanolamine in crd1Δ and psd1Δ mitochondria (data not shown), suggesting that CL is not essential for the mitochondrial import of nonbilayer phospholipids. Taken together, these data indicate that mitochondrial synthesis of PE is critical for survival of crd1Δ cells.

What function of Psd1p-synthesized PE is required in the absence of CL? One of the most likely roles of PE is to meet a requirement for nonbilayer formation in the inner mitochondrial membrane in the absence of CL. Whereas externally synthesized PE or phosphatidylpropanolamine (data not shown) can be transported to mitochondria, these nonbilayer phospholipids may not be transported efficiently to the inner mitochondrial membrane to compensate for the lack of both PE and CL, and thus supplementation does not rescue the synthetic lethality of crd1Δpsd1Δ cells. In support of this hypothesis, Burgemeister et al. (33) reported a 3-fold reduction in inner mitochondrial membrane PE levels in the psd1Δ mutant as compared with wild type cells, indicating that PE synthesized in the microsomes or via Psd2p catalysis was not efficiently incorporated into the inner mitochondrial membrane. Previous studies in E. coli have shown that bacteria need a critical amount of nonbilayer-forming phospholipid for survival (21). This observation is supported by the recent report showing that incorporation of a foreign nonbilayer-forming glycolipid, α-monomucosidicylglycerol, restores cellular function in PE-deficient E. coli cells (35). PE is also required for the formation of glycosylphosphatidylinositol-linked proteins (4, 36), which play an essential role in cell viability by maintaining structural integrity of the cell wall. A recent report from our laboratory indicated an important role of CL and its precursor, phosphatidylglycerol, in maintaining cell wall integrity (37). Based on these results, we speculated that loss of viability of crd1Δpsd1Δ cells could be the result of compromised cell wall stability. However, 1 M sorbitol, which rescues the growth defects of the pgs1Δ mutant by stabilizing the cell wall, did not rescue the lethality of crd1Δpsd1Δ (data not shown), indicating that synthetic lethality is
not due to loss of cell wall integrity. Genetic screens designed to identify suppressors of crd1Δpsd1Δ synthetic lethality, currently in progress, will elucidate the potential function(s) of mitochondrial nonbilayer phospholipids.

The large scale synthetic lethal screen of Tong et al. (38) did not report crd1Δpsd1Δ synthetic lethality, because it focused on genes involved in cell polarity, cell wall biogenesis, chromosome segregation, and DNA synthesis and repair. However, their work provided the framework to identify synthetic lethal interactions between genes of interest. Their study indicated a high likelihood of synthetic lethal interactions between genes with the same mutant phenotypes and subcellular localization. Identification of a synthetic lethal interaction between crd1Δ and psd1Δ in this study supports their prediction.

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Synthetic Lethal Interaction of the Mitochondrial Phosphatidylethanolamine and Cardiolipin Biosynthetic Pathways in *Saccharomyces cerevisiae*

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