Loss of Function of KRE5 Suppresses Temperature Sensitivity of Mutants Lacking Mitochondrial Anionic Lipids

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Disruption of PGS1, which encodes the enzyme that catalyzes the committed step of cardiolipin (CL) synthesis, results in loss of the mitochondrial anionic phospholipids phosphatidylglycerol (PG) and CL. The pgs1A mutant exhibits severe growth defects at 37°C. To understand the essential functions of mitochondrial anionic lipids at elevated temperatures, we isolated suppressors of pgs1A that grew at 37°C. One of the suppressors has a loss of function mutation in KRE5, which is involved in cell wall biogenesis. The cell wall of pgs1A contained markedly reduced β-1,3-glucan, which was restored in the suppressor. Stabilization of the cell wall with osmotic support alleviated the cell wall defects of pgs1A and suppressed the temperature sensitivity of all CL-deficient mutants. Evidence is presented suggesting that the previously reported inability of pgs1A to grow in the presence of ethidium bromide was due to defective cell wall integrity, not from “petite lethality.” These findings demonstrated that mitochondrial anionic lipids are required for cellular functions that are essential in cell wall biogenesis, the maintenance of cell integrity, and survival at elevated temperature.

INTRODUCTION

Cardiolipin (CL), a unique anionic phospholipid with dimeric structure, is ubiquitous in eukaryotes and primarily found in the mitochondrial inner membrane (Schlame et al., 2000). CL plays a key role in mitochondrial bioenergetics (Jiang et al., 2000; Koshkin and Greenberg, 2000, 2002; Schlame et al., 2000; Pfeiffer et al., 2003) and is also involved in mitochondrial biogenesis (Kawasaki et al., 1999; Jiang et al., 2000). Defective remodeling of CL is associated with Barth syndrome, a severe genetic disorder characterized by cardiomyopathy, neutropenia, skeletal myopathy, and respiratory chain defects (Vreken et al., 2000). The phenotype of Barth syndrome is dependent upon multiple factors that are not well understood (Barth et al., 1983, 1996). Elucidation of the functions of CL will help to clarify the abnormalities associated with this disorder.

The biosynthesis of CL is conserved in eukaryotic organisms. It occurs via three enzymatic reactions (Schlame et al., 2000), including formation of phosphatidylglycerolphosphate (PGP) from CDP-DAG and glycerol-3-P, dephosphorylation of PGP including formation of phosphatidylglycerolphosphate (PGP), and condensation of CDP-DAG and PG to form CL. Disruption of PGS1, the structural gene encoding PGP synthase, results in the complete loss of both PG and CL (Janitor et al., 1996; Chang et al., 1998a). The crd1Δ mutant, which lacks CL synthase, has no detectable CL but accumulates PG (Jiang et al., 1997; Chang et al., 1998b; Tuller et al., 1998; Jiang et al., 2000; Pfeiffer et al., 2003; Zhong et al., 2004). The human tafazzin gene (TAZ1), which is associated with Barth syndrome, encodes a transacylase that may be involved in the remodeling of CL (Xu et al., 2003). Deletion of the yeast homolog of this gene, TAZ1, leads to decreased CL, aberrant CL acyl species, and accumulation of monolysocardiolipin (Gu et al., 2004). Mutants deficient in CL biosynthesis exhibit growth defects at elevated temperatures. The laz1Δ mutant is temperature sensitive for growth on ethanol but grows well on other carbon sources at elevated temperature (Gu et al., 2004). The crd1Δ mutant loses viability on both fermentable and non-fermentable carbon sources at elevated temperature, and it does not form colonies from single cells seeded on YPD plates (Jiang et al., 1999, 2000; Zhong et al., 2004). The pgs1Δ mutant exhibits the most severe growth defects and cannot grow at all at 37°C, even on glucose (Chang et al., 1998a; Dzugosova et al., 1998). The temperature-sensitive growth defects observed in CL-deficient mutants suggest that CL plays an essential role in maintaining cell viability at elevated temperature. The greater degree of temperature sensitivity of the pgs1Δ mutant compared with the crd1Δ mutant indicates that PG can substitute for some essential functions of CL. Mitochondria from crd1Δ (Koshkin and Greenberg, 2000, 2002) and laz1Δ (Ma et al., 2004) exhibit defective energetic coupling at elevated temperatures. Although thermal sensitivity of the bioenergetic functions may explain temperature sensitivity of these mutants in non-fermentable medium, the reason for loss of viability on glucose is not known.

In addition to the temperature-sensitive growth defects, CL-deficient mutants exhibit decreased mitochondrial genome stability. Mutant cells of crd1Δ grown in the presence
of fermentable or nonfermentable carbon sources segregate large numbers of petites (respiratory incompetent cells) after prolonged culture at elevated temperature (Jiang et al., 2000; Zhong et al., 2004). The pgs1Δ mutant was initially determined to be “petite lethal” because the mutant cells did not survive ethidium bromide mutagenesis, which induces petite formation (Janitor and Subik, 1993; Dzugasova et al., 1998). However, 4,’6-diamidino-2-phenylindole (DAPI) staining of pgs1Δ revealed only the presence of nuclear DNA (Chang et al., 1998b). The absence of mitochondrial DNA (mtDNA) staining was attributed to a lack of elongated mitochondrial structure, but loss of mtDNA was not ruled out.

In a large-scale screen to identify genes involved in cell wall biogenesis, Lussier et al. (1997) reported that disruption of the PGS1 promoter results in several cell wall defects, including decreased glucosamine levels and hypersensitivity to cell wall-perturbing agents such as zymolyase, calcofluor white (CFW), papulacandin, and caffeine. The yeast strain pgs1Δ-1,3-glucan synthesis, strengthening the cell wall structure and enabling it to survive at elevated temperature.

To understand the essential functions of CL at elevated temperature, we took the genetic approach of isolating spontaneous suppressor mutants of pgs1Δ that grow at elevated temperatures, one of which was identified to have a loss of function allele of KRE5, which is involved in cell wall biogenesis. In this report, we demonstrated that the absence of mitochondrial anionic phospholipids PG and CL results in defective cell wall assembly. Disruption of KRE5 induces β-1,3-glucan synthesis, strengthening the cell wall structure in pgs1Δ and enabling it to survive at elevated temperature. These data suggest that mitochondrial anionic phospholipids are required for processes that are essential in cell wall biogenesis and the maintenance of cell integrity.

### MATERIALS AND METHODS

#### Materials

All chemicals used were reagent grade or better. The polymerase chain reaction (PCR) was performed using the native pfu enzyme kit from Invitrogen (Carlsbad, CA). The Yzmprep yeast plasmid mini prep kit was from ZymoResearch (Orange, CA). The Wizard Plus Miniprep DNA purification system was from Promega (Madison, WI). All other buffers and enzymes were purchased from Sigma-Aldrich (St. Louis, MO). Glucose, yeast extract, and peptone were purchased from Difco (Detroit, MI).

#### Yeast Strains and Growth Media

The Saccharomyces cerevisiae strains used in this work are listed in Table 1. Synthetic complete medium (SCD) contained amino acids adenine (20.25 mg/l), arginine (20 mg/l), histidine (20 mg/l), leucine (60 mg/l), lysine (200 mg/l), methionine (20 mg/l), threonine (300 mg/l), tryptophan (20 mg/l), and uracil (20 mg/l), vitamins, salts (essentially components of Difco Vitamin Free Yeast Base without amino acids), inositol (75 μM), and glucose (2%).

<table>
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<th>Plasmid or strain</th>
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<th>Source or reference</th>
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<td>Invitrogen</td>
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<td>pRS415-PGS1</td>
<td>derivative of pYES2/CT, expresses PGS1 from Gal1 promoter</td>
<td>He and Greenberg (2004)</td>
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<td>Centromere, URA3</td>
<td>Rose et al. (1987)</td>
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<td>Ycp50-KRE5</td>
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<td>This study</td>
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<td>C. Dieckmann</td>
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<td>102</td>
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<td>103</td>
<td>MAT a, ade1, cob (–)</td>
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<td>106</td>
<td>MAT a, met6, ox2 (–)</td>
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<td>107</td>
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<td>C. Dieckmann</td>
</tr>
<tr>
<td>T158c/S14a</td>
<td>Diploid prototroph S. cerevisiae</td>
<td>ATCC (46427)</td>
</tr>
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</table>
selection. Sporulation medium contained potassium acetate (1%), glucose (0.05%), and the essential amino acids. Complex media contained yeast extract (1%), peptone (2%), and glucose (2%) (YPD) or glycerol (3%) and ethanol (1%) (YPGE). Complex YDPS medium was YPD supplemented with 1 M sorbitol. Solid medium contained agar (2%) in addition to the above-mentioned ingredients.

**DAPI Stain**

Yeast cells were grown to early stationary phase, fixed in 70% ethanol at room temperature for 30 min, and stained with 1 μg/ml DAPI for 5 min. Cells were viewed with an Olympus BX41 epifluorescence microscope, WU filter, and a 100× oil immersion objective. Images were captured with a Q-color3 camera and represent at least 200 observed cells.

**Isolation of Extragenic Suppressors of pgsΔ**

Disruption of the PGS1 gene was performed as described previously (Zhang and Greenberg, 2003). Haploid pgsΔ mutants of opposite mating types were obtained. YPD medium was inoculated from single colonies of pgsΔ cells and grown for 24 h. About 10^6 cells from each independent culture were plated on a fresh YPD plate and incubated at 39°C. Single colonies from each plate were reexamined for growth at 39°C. Cells that grew at 39°C were analyzed further. To test the dominant and recessive character of the suppressor mutation, suppressor mutants were crossed to the parent strain, and growth of the diploid cells at 39°C was examined. Genetic complementation analysis was carried out with recessive mutants.

**Plasmid Complementation**

A yeast genomic DNA library in plasmid YCp50 (Rose et al., 1987) was used to clone the suppressor genes by complementation. Suppressor mutant cells were transformed with library DNA, plated on Ura− plates, and incubated at 25°C until colonies formed. Transformants were replicated onto YPD plates, and growth at 39°C was tested. Plasmid DNA was extracted from transformants that lost the capability to grow at 39°C, amplified in Escherichia coli DH5α, and retransformed into the suppressor mutant to confirm complementation of the suppressor phenotype. The DNA inserts of the positive clones were sequenced using primer YCp50 forward (5′-TTGGAAGCATTACATGAC-TACG-3′) and YCp50 reverse (5′-ATGGTCTCCGGCCTAGAGATCAT-C′). The cells were pelleted while the gelatin was still warm, and the pellets were left to cool on ice. The gelatin-embedded pellets were cut out of their tubes and sliced into thin strips, which were fixed in 2.5% buffered glutaraldehyde for 1 h. Postfixation, dehydration, and resin infiltration were carried out in a microwave processor using a protocol modified from Giberson and Demaree (1999). A Polco BioWave Microwave processor (Ted Pella, Redding, CA) was used for all irradiation steps. A flat chamber through which cold water was circulated was placed on the floor of the processor, and specimens were placed on top. The gelatin-embedded yeast cells were placed into glass vials containing ice-cold aqueous 2% osmium tetroxide and irradiated at full power for 40 s at a maximum temperature of 30°C, left at room temperature for 5 min, cooled on ice, and irradiated for an additional 40 s at full power. The osmium tetroxide was removed and replaced with cold water. Acetone dehydration was performed using the following steps: 1× 50%, 1× 70%, 1× 90%, 2× 100% acetone. Each step was performed in the microwave processor with 100% power for 40 s at a temperature maximum of 37°C. Infiltration with uncatalyzed epoxy resin consists of 50% full-power irradiation for 15 min at 45°C and full power in 1.1 acetone; resin followed by a similar irradiation in 100% resin. The specimens were then removed from the microwave processor and placed on a rotating table where they were infiltrated for 3 d in epoxy resin, changing the resin each day. Finally, the specimens were embedded in epoxy resin containing a catalyst and left to polymerize overnight at 60°C. Sections were prepared using an Ultrcut S ultra-microtome (Leica Microsystems, Deerfield, IL) equipped with a diamond knife (Diatome US, Hatfield, PA). Sections, on metal grids were contrasted with uranyl acetate and lead citrate, and imaged in a CM120 BioTwin transmission electron microscope (FEI, Hillsboro, OR) operating at 80 kV.

**Alkali-Insoluble β-Glucan Quantification**

Yeast cells were grown in 50–100 ml of YPD or synthetic Ura− medium to early stationary phase. Cells were harvested and washed once with distilled water. Half the cells were used to determine the dry weight, and the other half were prepared for alkali extraction following the protocol described previously (Boone et al., 1990). The insoluble pellet that remained after zymolase digestion was removed with centrifugation and dialyzed against distilled water using Slide-a-Lyse 7000-Da molecular weight cut-off cassettes (Pierce Chemical, Rockford, IL). Total alkaline insoluble β-1,3- and β-1,6-glucan was determined by analysis of the carbohydrate content of the supernatant before dialysis by using the phenol-sulfuric acid method (Dubois et al., 1956). Analysis of the carbohydrate content of the retained fraction after dialysis determined the proportion of β-1,6-glucan.

**Alkali-Soluble β-1,3-Glucan Quantification**

Alkali-soluble β-1,3-glucan immunodetection was performed as described previously (Lusser et al., 1998). Briefly, cells were grown to early stationary phase at 30°C in YPD, harvested, and washed once with 5 ml of water. Cell pellets were resuspended in 100 μl of water with 100 μl of glass beads and subjected to five cycles of vortexing for 30 s, interspersed with 30 s incubations on ice. Total cellular β-glucan was measured by the Bradford assay before alkali extraction (1.5 N NaOH, 1 h, 75°C). A set of 1:2 serial dilutions of the alkali-soluble fractions was spotted on nitrocellulose membrane. The immunoblotting was performed in Tris-buffere saline/Tween 20 containing 5% nonfat dried milk powder by using a 1000-fold dilution of anti-β-1,3-glucan primary antibody (Biosupplies Australia, Victoria, Australia), and a 5000-fold dilution of alkaline phosphatase conjugated goat anti-mouse secondary antibody (Promega). The membranes were developed with an AP detection kit (Promega). Dot blots were scanned with a ScanMaker 6800 scanner, and signals were quantitated with Adobe Photoshop software, by using the histogram function.

**Chitin Quantification**

Yeast cells were grown in 50–100 ml cultures to early stationary phase. Chitin levels were determined as described previously (Reissig et al., 1955). Briefly, 600–800 mg of cells was harvested. Half the cells were used to determine the cell dry weight, and the other half were transferred to 13 × 100 borosilicate tubes, resuspended in 4 ml of 6% KOH, and incubated at 80°C for 90 min to remove the mannan layer of the cell wall. After alkali treatment, 0.4 ml of glacial acetic acid was added. Cells were centrifuged at 4000 × for 4 min and washed twice with cold water. Chitinase from Serratia marcescens (0.4 U) was resuspended in 2 ml of 50 mM sodium phosphate buffer (pH 6.3) and added to samples. Digestion was carried out at 30°C overnight, and 400 μl of supernatant was incubated for 1 h at 37°C with cytohelicase (Sigma-Aldrich). A 100-μl portion of each sample, blank or standard, was mixed to 100 μl of 0.27 M potassium-tetraborate pH 9.0, boiled for 3 min, and then cooled on ice. Color was developed by addition of 3 ml of freshly diluted DMAB reagent (Ehrlich’s reagent, consisting of 10 g of p-dimethylamino-ylaminobenzaldehyde in 12.5 ml of concentrated HCl and 87.5 ml of glacial acetic acid, diluted 1:10 with glacial acetic acid). Absorbance at 490 and 585 was measured, and sensitivity was determined the proportion of

**Disruption of PGS1 Leads to Loss of Mitochondrial DNA**

The pgsΔ mutant in the FGY3 strain background was generated as described previously (Zhang and Greenberg, 2003). 100% of the culture was transferred to 13 × 100 borosilicate tubes, resuspended in 4 ml of 6% KOH, and incubated at 80°C for 90 min to remove the mannans layer of the cell wall. After alkali treatment, 0.4 ml of glacial acetic acid was added. Cells were centrifuged at 4000 × for 4 min and washed twice with cold water. Chitinase from Serratia marcescens (0.4 U) was resuspended in 2 ml of 50 mM sodium phosphate buffer (pH 6.3) and added to samples. Digestion was carried out at 30°C overnight, and 400 μl of supernatant was incubated for 1 h at 37°C with cytohelicase (Sigma-Aldrich). A 100-μl portion of each sample, blank or standard, was mixed to 100 μl of 0.27 M potassium-tetraborate pH 9.0, boiled for 3 min, and then cooled on ice. Color was developed by addition of 3 ml of freshly diluted DMAB reagent (Ehrlich’s reagent, consisting of 10 g of p-dimethylamino-ylaminobenzaldehyde in 12.5 ml of concentrated HCl and 87.5 ml of glacial acetic acid, diluted 1:10 with glacial acetic acid). Absorbance at 490 and 585 was measured, and sensitivity was determined the proportion of

**Chitin Distribution**

Yeast cells grown to early stationary phase were harvested by centrifugation at 2000 × g. Chitin stain was performed using Oregon Green 488 from Molecular Probes following the procedures from the manufacturer and observed using Olympus BX41 NIB filter. Images captured represent at least 200 observed cells.

**RESULTS**

Disruption of PGS1 Leads to Loss of Mitochondrial DNA

The pgsΔ mutant in the FGY3 strain background was generated as described previously (Zhang and Greenberg, 2003).
with heterozygous diploids were tested by complementation that whereas mtDNA was not evident. To exclude the possibility pg51A dent haploid wild-type cells, only nuclear DNA was visible in pg51A mutant cells derived from sporulation of heterozygous diploids were tested by complementation with pg51A tester strains for growth on nonfermentable medium (YPGE). Representative crosses are shown in Figure 1B. None of the 28 pg51A strains was complemented by any of the pg51A tester strains. As a control, diploid cells that carried complementary pg51A mutations grew on YPGE. This demonstrates that pg51A cells grown on glucose medium exhibited loss of mtDNA, even at the optimal growth temperature.

Disruption of KRE5 Suppresses Temperature Sensitivity of pg51A

To gain insight into the role of anionic phospholipids at elevated temperature, we used the genetic approach of isolating spontaneous suppressors of pg51A temperature sensitivity. Eighteen recessive suppressor mutants that grew at nonpermissive temperatures were isolated, and these identified three complementation groups. One of the suppressors, QZY11A, was characterized further. In addition to complementation of growth at 37°C (Figure 2A), the suppressor was transformed with empty vector YCp50 (+vec), or with YCp50 containing KRE5 (+KRE5) were serially diluted, spotted on YPD plates, and incubated at the indicated temperatures. (B) Wild-type (FGY3), pg51A (QZY24B), and suppressor (QZY11A) cells were grown to mid-log phase in YPD and examined microscopically.

Biochemical activity of Kre5p has not been determined. However, analysis of truncated versions of Kre5p indicated that all major regions of the protein are required for function (Levinson et al., 2002). Disruption of KRE5 results in decreased β-1,6-glucan and resistance to K1 killer toxin, the

Figure 1. Disruption of PGS1 results in loss of mtDNA. (A) Isogenic wild-type (FGY3), p51 p5, and pg51A (QZY24B) cells were grown in YPD to early stationary phase. DNA was visualized by staining with DAPI as described in Materials and Methods. (B) Haploid pg51A and p5 tester strains (100–107) were crossed on a YPD plate. Diploid cells were selected on synthetic minimal medium. Mitochondrial function was determined by assessing growth on nonfermentable medium (YPGE).

Figure 2. KRE5 complements the suppressor phenotype. (A) Cells from wild-type (FGY3), p5, pg51A (QZY24B), the suppressor (QZY11A), and the suppressor transformed with empty vector YCp50 (+vec), or with YCp50 containing KRE5 (+KRE5) were serially diluted, spotted on YPD plates, and incubated at the indicated temperatures. (B) Wild-type (FGY3), p5, pg51A (QZY24B), and suppressor (QZY11A) cells were grown to mid-log phase in YPD and examined microscopically.

Figure 3. The suppressor mutant carries a loss of function allele of KRE5. (A) DNA of the KRE5 locus from pg51A (QZY24B) and the suppressor (QZY11A) was sequenced as described in Materials and Methods. The single nonsense mutation in the coding sequence of KRE5 causes deletion of 201 amino acids from the C terminus of the protein. (B) K1 killer toxin producing cells (T158c/S14a) were spotted on plates preseeded with wild-type (FGY3), p5, pg51A (QZY24B), and suppressor (QZY11A) cells and incubated at 30°C for 2 d. Sensitivity to K1 killer toxin is indicated by the presence of a killing zone surrounding cells. (C) The suppressor mutant (QZY11A) was crossed to the wild-type strain (FGY3). Diploid cells were sporulated, and meiotic tetrad analysis was performed. Genotypes of the haploid spores from six tetrads are shown.

2003). Consistent with a previous report (Chang et al., 1998a), the haploid pg51A mutant was viable but did not grow on a nonfermentable carbon source. To determine whether pg51A cells contained a mitochondrial genome, cells were stained with DAPI (Figure 1A). In contrast to isogenic wild-type cells, only nuclear DNA was visible in pg51A cells, whereas mtDNA was not evident. To exclude the possibility that pg51A cells contained incomplete mtDNA, 28 independent haploid pg51A mutant cells derived from sporulation of heterozygous diploids were tested by complementation with p5 tester strains for growth on nonfermentable medium (YPGE). Representative crosses are shown in Figure 1B. None of the 28 pg51A strains was complemented by any of the p5 tester strains. As a control, diploid cells that carried complementary p5 mtDNA mutations grew on YPGE. This demonstrates that pg51A cells grown on glucose medium exhibited loss of mtDNA, even at the optimal growth temperature.

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KRE5 encodes an N-glycoprotein of ~200 kDa that localizes to the endoplasmic reticulum (Levinson et al., 2002).
binding of which requires β,1,6-glucan (Meaden et al., 1990). The kre5W1166X mutant was assayed for K1 killer toxin sensitivity. The absence of a killer zone in the suppressor mutant (Figure 3B) suggests that kre5W1166X is a loss of function allele. To confirm that the suppressor phenotype resulted from the kre5W1166X allele, diploid cells heterozygous for pgs1Δ and kre5W1166X were sporulated, and suppression of temperature sensitivity of the pgs1Δ mutant was analyzed in 18 tetrads. Suppression of temperature sensitivity cosegregated with K1 killer toxin resistance (Figure 3C). Loss of kre5p often leads to extremely slow growth (Meaden et al., 1990) or inviability in some strain backgrounds (Shahinian et al., 1998). Interestingly, the single mutant kre5W1166X in the FGY3 strain background exhibited only slightly compromised growth phenotypes. The above-mentioned genetic analysis demonstrated that the loss of function allele kre5W1166X is an extragenic suppressor of pgs1Δ, which enables it to grow at elevated temperature.

Disruption of PGS1 Leads to a Defective Cell Wall

Identification of a mutant in cell wall synthesis as a suppressor of pgs1Δ temperature sensitivity suggests that pgs1Δ temperature sensitivity results from perturbation of cell wall biosynthesis and/or structure. We therefore examined the cell wall properties of pgs1Δ and the suppressor strain. As seen in Figure 4B, the wild-type cell wall has a finely delineated dark-staining mannoprotein layer. In contrast, the cell wall of the suppressor was twice as thick and exhibited a rough appearance, similar to that reported for the kre5 mutant (Simons et al., 1998; Levinson et al., 2002). However, aberrations in cell wall morphology were not observed in pgs1Δ cells.

To gain further insight into how kre5W1166X suppressed pgs1Δ temperature sensitivity, we characterized the cell wall composition of pgs1Δ and the suppressor strain. Levels of the major cell wall components were measured, including β,1,3 and β,1,6-glucan and chitin (Table 2). Because pgs1Δ cells lose mtDNA and are all ρ0 cells (Figure 1), we compared cell wall composition in pgs1Δ and isogenic wild-type ρ0 cells. Interestingly, the loss of mtDNA from wild-type cells resulted in decreased alkaline-soluble and -insoluble β,1,3-glucan (50 and 12% decrease, respectively) and a decrease (37%) in β,1,6-glucan. Disruption of PGS1 led to an even more pronounced decrease in β,1,3-glucan. Alkaline-soluble and -insoluble β,1,3-glucan was reduced to 76 and 68% of the isogenic ρ0 wild-type levels. In contrast, β,1,3 glucan was dramatically increased in the suppressor mutant to levels greater than those observed in wild-type cells. Alkaline-insoluble β,1,6-glucan was reduced approximately twofold in the pgs1Δ mutant compared with the isogenic ρ0 wild-type and to an even greater extent in the suppressor mutant.

In contrast to β-glucans, chitin levels were not affected by loss of mtDNA (Table 2). In both pgs1Δ and the suppressor mutant, chitin levels were ~3 times higher than in wild-type cells (Table 2). Examination of the distribution of chitin by using specific fluorescent probes revealed the presence of chitin predominantly in the bud scars of wild-type cells (Figure 5A). In pgs1Δ and suppressor mutants, however, chitin staining was uniformly distributed to the lateral cell wall, and the bud scar was hardly distinguishable from the rest of the cell wall. Hyperaccumulation of chitin in cell wall mutants is mediated by chitin synthase III (CSIII) (Osmond et al., 1999; Valdivieso et al., 2000). When grown in the presence of 0.1 mM Nikkomycin Z, a known inhibitor of CSIII (Gaughran et al., 1994), viability of the pgs1Δ mutant was reduced by 40% (our unpublished data). In contrast, the
Table 2. Cell wall composition in pgs1Δ and suppressor mutants

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<td>YPD</td>
<td>7.7 ± 2.5</td>
<td>156.8 ± 1.4</td>
</tr>
<tr>
<td>pgs1Δ ρ^0</td>
<td>YPD</td>
<td>21.0 ± 1.6</td>
<td>98.8 ± 18.8</td>
</tr>
<tr>
<td>pgs1Δ ρ^0 + PGS1</td>
<td>Ura^-0</td>
<td>25.8 ± 5.3</td>
<td>131.6 ± 33.5</td>
</tr>
<tr>
<td>pgs1Δ ρ + vec</td>
<td>Ura^-0</td>
<td>21.4 ± 2.1</td>
<td>69.6 ± 22.7</td>
</tr>
<tr>
<td>pgs1Δ kref5^(W166X) ρ^0 + vec</td>
<td>Ura^-0</td>
<td>7.4 ± 1.8</td>
<td>118.4 ± 17.3</td>
</tr>
<tr>
<td>pgs1Δ kref5^(W166X) ρ^0 + KRE5</td>
<td>Ura^-0</td>
<td>27.1 ± 0.9</td>
<td>99.3 ± 9.6</td>
</tr>
</tbody>
</table>

Glanuc and chitin levels were measured as described in Materials and Methods in wild-type (FGY3), ρ^0, pgs1Δ (QZY24B), and suppressor mutant pgs1Δ kref5^(W166X) (QZY11A) cells grown in YPD or YPDS; pgs1Δ (QZY24B) cells transformed with empty vector (pYES2/CT (+vec)) or pYES2/CT-PGS1 (+PGS1); and pgs1Δ kref5^(W166X) suppressor mutant (QZY11A) cells transformed with empty vector YCP50 (+vec) or the genomic clone of KRE5 (+KRE5) grown in synthetic ura^- medium. Alkaline insoluble glucan and chitin are expressed as micrograms per milligram of cell dry weight. Alkaline soluble β-1,3-glucan in cells grown in complex medium (top) was expressed as a percentage of that of wild-type (FGY3) cells. Alkaline soluble β-1,3-glucan in cells grown in synthetic ura^- medium (bottom) was expressed relative to pgs1Δ (QZY24B) cells transformed with pYES2/CT-PGS1. Data represent three independent experiments.

wild-type and suppressor mutant tolerated 1 mM Nikkomycin Z with no obvious loss of viability. Hypersensitivity to Nikkomycin Z suggested that increased chitin is essential for the survival of pgs1Δ. We wished to determine whether a further increase in chitin synthesis led to increased viability of pgs1Δ cells at 37°C. Glucosamine was recently shown to induce chitin synthesis via CSIII in wild-type cells as well as in cell wall mutants (Bulik et al., 2003). As shown in Figure 5B, in the presence of 10 mM glucosamine, growth of pgs1Δ was slightly improved at 30°C. Chitin levels in pgs1Δ cells were doubled at 37°C compared with 30°C in the presence of 10 mM glucosamine (our unpublished data). However, at 37°C, pgs1Δ cells exhibited only limited growth and lost viability after 20 h (Figure 5B). Thus, 10 mM glucosamine did not restore growth to levels observed in the presence of the suppressor mutation. Supplementation with 5–20 mM glucosamine led to a shortened lag in growth and increased saturation optical density of the pgs1Δ mutant at 30°C, but was not sufficient to support growth at elevated temperature (our unpublished data).

When transformed with a plasmid-borne copy of the PGS1 gene under the control of the P_GAL1 promoter, β-1,3-glucan levels in the pgs1Δ mutant significantly increased and chitin decreased (Table 2). This was observed even during growth in glucose, in which low levels of expression of PGS1 from this plasmid are sufficient to restore growth of pgs1Δ at elevated temperature and synthesis of PG and CL (He and Greenberg, 2004). Expression of the plasmid-borne copy of KRE5 in the suppressor mutant restored β-1,6-glucan and decreased β-1,3-glucan levels (Table 2). Together, these experiments indicate that disruption of PGS1 leads to multiple cell wall defects resulting in defective growth at elevated temperature. Increasing cell wall synthesis, particularly β-1,3-glucan and chitin, restored growth at 37°C.

Osmotic Stabilization of the Cell Wall Restores Growth of pgs1Δ at Elevated Temperature

Suppression of pgs1Δ temperature sensitivity by increased β-1,3-glucan synthesis suggested that osmotic stabilization of the cell wall might alleviate cell wall stress and support growth of pgs1Δ at elevated temperature. To address this possibility, we examined the effect of sorbitol on cell wall composition and growth of pgs1Δ at 37°C. Mutant cells of pgs1Δ grown in the presence of 1 M sorbitol contained 1.8 and 2.4-fold increased alkaline soluble β-1,3-glucan and alkaline insoluble β-1,6-glucan levels, respectively, compared with levels observed in pgs1Δ cells grown in YPD lacking sorbitol, whereas chitin levels were significantly reduced (Table 2). Consistent with this finding, the majority of pgs1Δ cells displayed the wild-type pattern of chitin staining of bud scars and exhibited normal cell size in the presence of sorbitol (data not shown). Supplementation with sorbitol restored growth of pgs1Δ in two strain backgrounds, FGY3 and GA74D (Figure 6A). Sorbitol also supported colony formation of cnd1Δ on YPD (Figure 6B), as well as growth of taz1Δ on ethanol (data not shown) at 37°C.

As this report has shown, pgs1Δ cells in the FGY3 background grown on YPD are all ρ^0 cells. However, the pgs1Δ mutant in the GA74D strain background, GA74D3C, was previously thought to be “petite lethal”, because the mutant cells did not survive ethidium bromide mutagenesis, which induces loss of mtDNA (Janitor and Subik, 1993; Dzugasova et al., 1998). A likely explanation for the inability of pgs1Δ cells to grow in the presence of ethidium bromide is that pgs1Δ ρ^0 cells in the GA74D strain background fail to survive due to loss of cell wall integrity. To resolve this discrepancy, we examined the effects of ethidium bromide on pgs1Δ cells in the presence or absence of osmotic support. Consistent with the previous report (Janitor and Subik, 1993; Dzugasova et al., 1998), pgs1Δ (GA74D3C) cells failed to grow on plates containing 25 μg/ml ethidium bromide. However, when supplemented with 1 M sorbitol, the cells grew in the presence of ethidium bromide (Figure 7). Failure to complement ρ^- tester strains for growth on YPGE (our unpublished data) confirmed the loss of mtDNA. The pgs1Δ ρ^0 mutant in this genetic background was viable on YPD but exhibited slower growth than the isogenic ρ^- strain. Consistent with these observations, a greater decrease in alkaline-soluble and -insoluble β-1,3-glucan was observed in pgs1Δ ρ^0 cells than in pgs1Δ ρ^- cells (our unpublished data). These data suggest that pgs1Δ cells can survive ethidium bromide treatment in the presence of increased osmotic support.
In summary, disruption of PGS1 leads to a defective cell wall and inability to grow at elevated temperature. Stabilization of the cell wall, either by increased synthesis of β-1,3-glucan or increased osmotic support, restores growth at 37°C. These findings show that mitochondrial anionic phospholipids are essential in cellular functions required for cell wall biogenesis and maintenance of cell integrity.

**DISCUSSION**

The pgs1Δ mutant, which lacks mitochondrial anionic phospholipids PG and CL, exhibits severe temperature sensitivity for growth even on fermentable carbon sources (Chang et al., 1998a; Dzugasova et al., 1998), suggesting that anionic phospholipids are required for essential cellular functions. This report shows that mutants lacking these lipids are defective in cell wall biogenesis. Reorganization of the cell wall in the pgs1Δ kre5W1166X suppressor or osmotic stabilization with sorbitol restores growth at elevated temperature. The pgs1Δ mutant exhibited a marked decrease in β-1,3-glucan (Table 2), the lack of which greatly impairs the mechanical strength of the cell wall and severely threatens viability of cells at elevated temperature (Klis et al., 2002). As a result, pgs1Δ mutant cells become enlarged and rounded (Figure 2), resembling another cell wall mutant, gas1Δ, which is also defective in β-glucan synthesis (Popolo et al., 2004).

Figure 5. Increased chitin deposition in pgs1Δ and the suppressor mutant. (A) Cells from wild-type (FGY3), p0, pgs1Δ (QZY24B), and the suppressor (QZY11A) were grown in YPD to early stationary phase. Chitin was visualized by staining with Oregon Green 488 as described in Materials and Methods. Chitin distribution was visualized by focusing on two planes. (B) Cells from pgs1Δ (QZY24B) and the suppressor mutant (QZY11A) were grown in YPD in the presence or absence of 10 mM glucosamine at the indicated temperatures. Viable cells were determined by serial dilution and plating.
Cell wall biogenesis is a highly regulated process. β-1,3-glucan is synthesized by β-1,3-glucan synthase (GS) localized on the plasma membrane (Qadota et al., 1996). GS is composed of a catalytic subunit encoded by the two homologous genes FKS1 and FKS2 (Inoue et al., 1995; Mazur et al., 1995), and a regulatory subunit, the small GTPase, Rho1p (Drgonova et al., 1996; Qadota et al., 1996). The Rho-type GTPase is generally regulated by switching between a GDP-bound inactive state and a GTP-bound active state (Wei et al., 1997; Ihara et al., 1998). Various factors are involved in the regulation of β-1,3-glucan synthesis by Rho1p in yeast cells. The putative cell surface sensor protein Wsc1p plays a critical role in stimulating nucleotide exchange of Rho1p through the GDP/GTP exchange factor, Rom2p (Philip and Levin, 2001). Exchange of GDP for GTP stimulates Rho1p, leading to activation of GS activity. Lrsg1p, a GTPase-activating protein, promotes formation of GDP-bound Rho1p, thus negatively regulating β-1,3-glucan synthesis (Watanabe et al., 2001). Thus, overexpression of ROM2 or WSC1 (Sekiya-Kawasaki et al., 2002), or loss of function of LRG1 (Watanabe et al., 2001) restores the impaired β-1,3-glucan synthesis observed in GS mutants. In addition, posttranslational modification of Rho1p by the geranylgeranylation group is required for binding of Rho1p to GS and activation of GS activity (Inoue et al., 1999). Other factors affect β-1,3-glucan synthesis by regulation of the catalytic subunit of GS. Movement of Fks1p driven by actin is required for the construction of a uniform and solid cell wall (Utsugi et al., 2002). Transcription of FKS2 is up-regulated in response to cell wall stress induced by heat, cell wall mutations, and cell wall-perturbing agents (Zhao et al., 1998; de Nobel et al., 2000). Lagorce et al., 2003; Garcia et al., 2004). Deletion of KRE5 leads to a 114-fold up-regulation of FKS2 in response to an impaired cell wall (Kapteyn et al., 1999). Restored β-1,3-glucan levels in pgs1Δ mutant cells in the presence of the kre5Δ suppressor mutation (Table 2) could be mediated by up-regulation of FKS2 expression. In fact, increased β-1,3-glucan is a general characteristic shared by several kre mutants, along with defective β-1,6-glucan synthesis (Roemer et al., 1994; Dijkgraaf et al., 1996; Shahnian et al., 1998; Shahnian and Bussey, 2000).

Decreased β-glucan levels in pΔ cells and in mutants lacking mitochondrial anionic lipids suggest the existence of a regulatory link between mitochondrial biogenesis and cell wall synthesis. It has been suggested that cytoplasmic petite mutants isolated after ethidium bromide mutagenesis have altered cell wall assembly (Wauters et al., 2001). In this study, we have shown for the first time that loss of mtDNA alone led to a significant decrease in β-glucan. These defects were exacerbated in the pgs1Δ mutant (Table 2). Our findings that greater cell wall defects were observed in pgs1Δ than in the wild-type pΔ cells suggests that, along with oxidative phosphorylation, other mitochondrial functions requiring PG and/or CL may be required for cell wall biogenesis. A link between mitochondrial functions and cell wall biogenesis has been implicated in several previous studies as well. In addition to pgs1, Lussier et al. (1997) reported that mutations in four other genes with mitochondrial associated functions, IMP2’, IFM1, SMP2, and COX11 have cell wall defects. Three of these genes (IFM1, SMP2, and COX11) are required for mtDNA stability (Vambutas et al., 1991; Irie et al., 1993; Tzagoloff et al., 1993). A genomewide screen for deletion mutants that exhibit increased resistance to K1 killer toxin, which indicates alterations in the cell surface, identified 17 deletion mutants affecting genes for respiration and ATP metabolism (Page et al., 2003). All of the mutants are respiratory deficient, and four are involved in mitochondrial genome maintenance.

The identification of cell wall defects in mutants with mitochondrial dysfunction suggests that mitochondria may play a general role in the regulation of cell wall biogenesis. Several enzymes involved in β-1,3-glucan synthesis were found to have dual localization in the plasma membrane and mitochondrial membranes. The role of these enzymes in regulating mitochondrial membrane biogenesis and cell wall biogenesis remains to be determined.
PGS1 Is Essential for Cell Integrity

mitochondria. Both the catalytic and the regulatory subunit of GS are localized on the plasma membrane at the site of cell wall synthesis (Qadota et al., 1996). Interestingly, both Fks1p and Rho1p are also present in mitochondria (Sickmann et al., 2003). Gas1p, a putative β-1,3-glucan—remodeling enzyme (Popolo and Vai, 1999; Mouyna et al., 2000), the loss of which also results in reduced β-1,3-glucan in the cell wall (Popolo et al., 1993; Ram et al., 1998), is attached to the plasma membrane via a glycosyl-phosphatidylinositol anchor (Conzelmann et al., 1988; Nuoffer et al., 1991). Gas1p also is found in mitochondria (Grandier-Vazeille et al., 2001; Sickmann et al., 2003). It is not known whether dual plasma membrane/mitochondria localization of these enzymes has any physiological relevance. It is tempting, however, to assume that mitochondria are required for the maturation or modification of those enzymes, in which case mitochondrial dysfunction would result in decreased enzyme activity and cell wall defects. Alternatively, mitochondrial dysfunction may trigger signals that prevent proper mobilization of those enzymes to the site of cell wall biosynthesis.

Our finding that pgs1Δ in the FGY3 strain background exhibited loss of mtDNA even at optimal growth temperature suggests that PG and CL are required for maintaining mtDNA. Mutants lacking only CL exhibit a strain-dependent decrease in mtDNA stability at elevated temperature (Jiang et al., 2000; Zhong et al., 2004). We have noticed that crd1Δ mutants from different strain backgrounds differ greatly with respect to the temperature at which growth is defective and mtDNA becomes unstable (Zhong et al., 2004). In addition, crd1Δ was less thermotolerant on synthetic medium than on rich medium (Zhong et al., 2004). Although pgs1Δ in the GA74D strain background does not lose mtDNA on YPD at 30°C, it cannot grow on synthetic medium with glycerol and ethanol as carbon source (Dzugasova et al., 1998), suggesting that it may lose mtDNA under this condition. Furthermore, the results presented here show that pgs1Δ is not “petite lethal,” and the previously reported inability of pgs1Δ to survive ethidium bromide (Janitor and Subik, 1993; Dzugasova et al., 1998) was due to defective cell wall integrity. Pgs1Δ ρ0 cells in the GA74D strain background were obtained after ethidium bromide treatment in the presence of osmotic support and were viable on YPD (Figure 7B). Those petite cells exhibited slower growth than the isogenic ρ+ strain, which presumably resulted from the exacerbated cell wall defects caused by loss of mtDNA. The further compromised growth observed in pgs1Δ ρ0 cells suggests a “synthetic sick” interaction between pgs1Δ and the ρ0 mutation. This interaction predicts that the number of the ρ0 cells surviving the loss of mtDNA would be low. This seems paradoxical in light of our finding that lack of PG and CL in the pgs1Δ mutant strain resulted in 100% petite formation on YPD. Interestingly, mutations in ATP15 and ATP16, two structural genes encoding ε and δ subunit of F1-ATPase, lead to similar phenotypes. On one hand, atp15 and atp16 exhibited an extremely high frequency of petite formation. However, the petite mutants have severe growth defects. Like PGS1, ATP15 and ATP16 also were thought to be essential in a petite background (Giraud and Velours, 1997; Lai-Zhang et al., 1999; Contamine and Picard, 2000).

In summary, we have isolated and identified an extra- 

genic suppressor of pgs1Δ, the loss of function allele of KRE5, kre5Δ/H9267. Characterization of pgs1Δ and the suppressor strain strongly suggests that temperature sensitivity of CL-deficient mutants and the previously reported “petite lethal” phenotype of pgs1Δ mutant cells were primarily due to defective cell wall integrity. This work is the first demonstration of defective cell wall biosynthesis in mutants lacking mitochondrial anionic phospholipids PG and CL. Our findings thus provide new insights into the essential functions of these lipids and point to a regulatory role of mitochondria in cell wall biogenesis.

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