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Loss of Cardiolipin Leads to Longevity Defects That Are Alleviated by Alterations in Stress Response Signaling*[S]

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Perturbation of cardiolipin (CL) synthesis in yeast cells leads to defective respiratory chain function and mitochondrial DNA loss, both of which have been implicated in aging in mammals. The availability of yeast CL mutants enabled us to directly investigate the role of CL synthesis in aging. In this report, we show that the replicative life span of *pgs1*Δ, which lacks both CL and the precursor phosphatidylglycerol (PG), was significantly decreased at 30 °C. The life span of *crd1*Δ, which has PG but not CL, was unaffected at 30 °C but reduced at 37 °C. Life span extension induced by calorie restriction was not affected by the loss of CL. However, mild heat and osmotic stress, which extend life span in wild type cells, did not increase longevity in CL mutants, suggesting that the stress response is perturbed in these mutants. Consistent with this, longevity defects in *pgs1*Δ were alleviated by down-regulation of the high osmolarity glycerol stress response pathway, as well as by promoting cell integrity with the osmotic stabilizer sorbitol or via genetic suppression with the *kre5*^{W1166X} mutant. These findings show for the first time that perturbation of CL synthesis leads to decreased longevity in yeast, which is restored by altering signaling through stress response pathways.

The anionic mitochondrial phospholipid cardiolipin (CL)[5] is ubiquitous in eukaryotes. The importance of CL is underscored by the association of aberrant CL metabolism with several disorders, including Barth syndrome (1), thyroid dysfunction (2, 3), diabetes (4), myocardial ischemia (5), and aging (6, 7). The unique cellular localization of CL in the mitochondrial inner membrane suggests that it is closely associated with mitochondrial function, biogenesis, and genome stability (3, 8–10).

The identification of yeast genes encoding CL biosynthetic enzymes and subsequent construction of deletion mutants deficient in CL biosynthesis facilitated *in vivo* studies to elucidate the role of CL in mitochondrial function and cell viability. Disruption of *PGS1* encoding the first enzyme of the CL pathway, phosphatidylglycerolphosphate synthase, results in the complete loss of both phosphatidylglycerol (PG) and CL (11). Mutants lacking the *CRD1* gene encoding CL synthase contain no detectable CL but accumulate the upstream precursor PG (11–15). Perturbation of CL synthesis in *crd1*Δ mutant cells leads to mitochondrial dysfunction via uncoupling, instability of electron transport chain supercomplexes, and decreased function of individual complexes (14, 16–18). Mitochondria from the *crd1*Δ mutant exhibit decreased coupling during osmotic stress, heat stress, or increased respiration rate and are coupled only under optimal conditions (19, 20). In addition, *crd1*Δ mutant cells tend to lose mitochondrial DNA (mtDNA) and become petite (ρ^0) after prolonged growth at elevated temperatures (15). The *pgs1*Δ mutant, which lacks both PG and CL, loses mtDNA even when grown at 30 °C (21). Because respiratory function and mtDNA stability have been implicated in aging (22, 23), yeast CL mutants provide excellent models to elucidate the role of CL in these processes.

In addition to its central role in respiratory function, CL is also required for essential cellular processes that are not directly associated with respiration, several of which affect aging. The role of CL in essential functions was first suggested by the temperature sensitivity phenotype of CL mutants during growth on glucose, a carbon source that can be utilized by fermentation in the absence of respiratory function (15, 21, 24). Perturbation of the CL pathway in *crd1*Δ and *pgs1*Δ mutants leads to decreased growth at elevated temperatures (15). At 37 °C, the *crd1*Δ mutant can grow but cannot form colonies from single cells, whereas *pgs1*Δ cannot grow at all (15). One essential process requiring the CL pathway is cell wall biogenesis, which plays a role in aging in yeast (25). A link between the CL biosynthetic pathway and the cell wall was first indicated by a report that disruption of the promoter of *PGS1*, the gene that mediates the committed and rate-limiting step of CL biosynthesis (11), resulted in cell wall defects (26). Consistent with this, *pgs1*Δ temperature sensitivity was alleviated by supplementation with sorbitol, which provides osmotic support for the cell wall, and by genetic suppression with *kre5*^{W1166X}, a mutant that up-regulates cell wall-associated pathways (21). Further studies indicated that *pgs1*Δ exhibits cell wall defects due to defective activation of the protein kinase C (PKC)-activated Slt2 mitogen-activated protein kinase (MAPK) pathway (27). This pathway regulates cell wall biosynthesis and progression through the cell cycle in response to cell surface stress (28, 29).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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[5] The abbreviations used are: CL, cardiolipin; PG, phosphatidylglycerol; mtDNA, mitochondrial DNA; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; HOG, high osmolarity glycerol; CR, calorie restriction; SC, synthetic complete medium; WT, wild type; DAPI, 4′,6-diamidino-2-phenylindole.

TABLE 1
Yeast strains used in this study

Plasmid or strain	Characteristics or genotype	Source or reference
FGY3	*MATα, ura 3-52, lys2-801, ade2-101, trp1Δ1, his3Δ200, leu2Δ1*	Ref. 12
FGY2	Isogenic with FGY3 except *crd1Δ::URA3*	Ref. 12
ZQY1	Isogenic with FGY3 except *pgs1Δ::TRP1*	Ref. 71
FGY3_A (ρ^0)	Isogenic with FGY3 except *MAT***a**, *ADE2*, ρ^0	This study
QZY24B (ρ^0)	Isogenic with FGY3_A except *pgs1Δ::TRP1*	Ref. 71
QZY11A (ρ^0)	Isogenic with QZY24B except *kre5*^{W1166X}	Ref. 71
BY4742	*MATα his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	Euroscarf[a]
*sho1*Δρ^0	Isogenic with BY4742 except *sho1::KanMX*	Euroscarf
*can1*Δρ^0 (WT ρ^0)	Isogenic with BY4742 except *can1*Δ ρ^0	This study
9F (*pgs1*Δρ^0)	Isogenic with *can1*Δ ρ^0 except *pgs1::URA3*	This study
*pgs1*Δ*hsp12*Δ (ρ^0)	Isogenic with 9F except *hsp12::KanMX*	This study
*pgs1*Δ*sho1*Δ (ρ^0)	Isogenic with 9F except *sho1::KanMX*	This study
pPS1739	CEN, URA3, HOG1-GFP	Ref. 72

[a] Euroscarf, European *Saccharomyces cerevisiae* Archive for Functional analysis.

In response to stress, cells are under the coordinate control of both the PKC-Slt2 and high osmolarity glycerol (HOG) signaling pathways. The PKC-Slt2 pathway functions to withstand increased turgor pressure resulting from heat or hypotonic stress (*i.e.* shifting cells from high to low osmolarity) by increasing the expression of genes for cell wall remodeling (30–32). In contrast, the HOG pathway is activated when cells are shifted from low to high osmolarity (hypertonic conditions). This pathway is mediated by Hog1p and two independent membrane sensors, Sho1p and Sln1p (33). Hog1p is activated by phosphorylation, whereupon it moves into the nucleus and phosphorylates the transcriptional regulators Msn2p/Msn4p, Sko1p, etc. (30). Glycerol and trehalose synthesis is subsequently induced, causing increased intracellular turgor pressure, which counterbalances the high external osmolarity (30). In this light, cell wall defects resulting from a defective PKC-Slt2 pathway, as observed in *pgs1*Δ, could be lethal if exacerbated by HOG signaling.

The MAPK and stress pathways discussed above are highly conserved from yeast to mammals and have been shown to affect life span in yeast in several ways (34–37). First, regulation of MAPK pathways in response to heat and osmotic stress exerts complex effects on chronological and replicative life span (38). Activation of the transcriptional activators Msn2p/Msn4p by Hog1p induces the general stress response and leads to the induction of many downstream targets, including superoxide dismutase (33). Although this pathway is beneficial to cells under stress, increased expression of the transcription factor Msn2p/Msn4p and/or superoxide dismutase *SOD2* decreases the replicative life span of yeast (38, 39). Second, increased cell integrity extends yeast replicative life span, possibly by preventing cell lysis in old cells (40). In addition to these factors, the replicative life span of yeast cells is regulated by calorie restriction (CR) and stress (34, 41), both of which involve activation of Sir2p. Sir2p mediates chromatin silencing and promotes longevity by suppressing the formation of extrachromosomal ribosomal DNA circles (37, 42–44).

In this report, we show for the first time that loss of the mitochondrial anionic phospholipids CL and PG leads to decreased replicative life span. The longevity defects in CL mutants can be alleviated by genetic or osmotic remediation of the cell wall and, strikingly, by down-regulation of the HOG pathway. Our findings suggest that mitochondrial anionic phospholipids play a novel role in the cellular stress response.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were reagent grade or better. PCR was performed using the Taq polymerase enzyme kit from Promega (Madison, WI). The Wizard Plus Miniprep DNA purification system was from Promega (Madison, WI). All other buffers and enzymes were purchased from Sigma. Glucose, yeast extract, and peptone were purchased from Difco.

Yeast Strains and Growth Media—The *Saccharomyces cerevisiae* strains used in this work are listed in Table 1. Synthetic complete medium (SC) contained the amino acids and the nucleotides 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), and uracil (20 mg/liter), vitamins, salts (essentially components of Difco Vitamin Free Yeast Base without amino acids), and glucose (2%). Synthetic drop-out medium contained all ingredients except uracil (Ura^-), histidine (His^-), or lysine and methionine (Lys^-Met^-). Sporulation medium contained potassium acetate (1%), glucose (0.05%), and essential amino acids. Complex medium (YPD) contained yeast extract (1%), peptone (2%), and glucose (2%). Complex YPDS medium was YPD supplemented with 1 M sorbitol. Solid medium contained agar (2%). Limited glucose media for CR contained 0.5% rather than 2% glucose.

Life Span Analysis—Single cells from each strain were obtained and aligned on a YPD or YPDS plate, ~60 cells/plate. At 2–3-h intervals, cells were visualized under the dissection microscope to identify mature buds. Buds were removed and counted to determine the number of daughter cells produced by each mother cell, indicative of the replicative life span. Life spans were quantified at optimal or elevated temperatures by microscopic determination of the number of times a mother cell produced a daughter cell, and the average replicative life spans (in generations) were determined as described (36, 37). The statistical significance of differences in life spans was determined by analysis of variance ($p < 0.05$) and shown for all experiments in Table 2.

*Targeted Screen for pgs1*Δ *Suppressors*—A *pgs1*Δ strain was generated in the BY4742 (*MATα*) background by disrupting *PGS1* and linking the *pgs1*Δ mutation to the dominant selectable marker *URA3* and the reporter construct *MFA1pr-HIS3,* which is only expressed in the *MAT***a** background. Seventy-seven mutants, each with a deletion in a stress response gene,

TABLE 2

Life spans of WT and CL mutant strains

The mean and maximum (Max) replicative life spans, S.E., and total number of the cells analyzed (*n*) are shown for each life span measurement in the indicated strain, medium, and temperature. *p* value was calculated for each measurement versus its corresponding standard(s) (standards A–N).

Genetic background	Strain, medium, temperature	Mean	Max	S.E.	*n*	$p < 0.05$	Standard
FGY3	WT, YPD, 30 °C	16.7	40	0.78	117		A
	WT, YPD, 37 °C	17	39	0.83	145		B
	WT, YPD, 37 °C[a]	15.4	31	0.75	97		C
	WT, YPDS, 30 °C	25.4	52	0.79	163	A	D
	WT, YPD, heat shock	25.2	47	0.81	119	A	E
	WT, osmotic shock	25.9	66	1.06	115	A	F
	WT, CR, 30 °C	26.5	49	0.63	190	A	
	*crd1*Δ, YPD, 30 °C	15.9	32	0.71	113		G
	*crd1*Δ, YPD, 37 °C	8.7	28	0.51	127	B, G	H
	*crd1*Δ, YPD, 37 °C[a]	6.4	20	0.38	175	C, G, H	
	*crd1*Δ, YPDS, 30 °C	24.1	50	0.81	112	G	
	*crd1*Δ, YPD, heat shock	16.7	30	0.58	120	E, G[b]	
	*crd1*Δ, osmotic shock	12.6	52	0.75	146	F, G[b]	
	*crd1*Δ, CR, 30 °C	23.2	52	0.5	208	G	
	*pgs1*Δ, YPD, 30 °C	5.7	17	0.31	106	A	I
	*pgs1*Δ, YPDS, 30 °C	15.1	58	0.31	106	D, I	
	*pgs1*Δ, CR, 30 °C	3.1	9	0.13	242	I	
FGY3_A ρ^0	WT, YPD, 30 °C	19	34	0.79	101	A	J
	*pgs1*Δ, YPD, 30 °C	7.1	13	0.26	109	I, J	K
	*pgs1*Δ*kre5*^{W1166X}, YPD, 30 °C	15.3	36	0.77	107	J, K	
BY4742 *can1*Δ ρ^0	WT, YPD, 30 °C	25.3	67	1.39	108		L
	*pgs1*Δ, YPD, 30 °C	17.1	31	0.54	113	L	M
	*pgs1*Δ*hsp12*Δ, YPD, 30 °C	19	44	0.98	114	M[b]	
	*pgs1*Δ*sho1*Δ, YPD, 30 °C	24.1	51	0.88	118	M	

[a] Experiments in which virgin cells were obtained at 37 °C.
[b] *p* value larger than 0.05 *versus* its corresponding standard(s).

were obtained from the deletion collection generated in the BY4741 (*MAT***a**) background (Research Genetics). The gene mutated in each strain was replaced by a kanamycin (Geneticin) resistance marker (*KanMX*). After *pgs1*Δ was mated with each of the 77 deletion strains, diploids were selected on Met$^-$Lys$^-$ double drop-out medium, and sporulation was induced. *MAT***a** spores were selected on His$^-$ drop-out medium, and double mutant meiotic progeny were selected on Ura$^-$ drop-out medium with 200 mg/liter Geneticin, as described (45). Growth of double mutants was examined at 39 °C to identify suppressors of *pgs1*Δ temperature sensitivity.

4′,6-Diamidino-2-phenylindole (DAPI) Stain—Yeast cells were grown in liquid medium at 30 °C, harvested in early stationary phase, fixed in 70% ethanol at room temperature for 30 min, and stained with 1 μg/ml DAPI for 5 min, as described (21). Cells were viewed with an Olympus BX41 epifluorescence microscope using a WU filter and a ×100 oil immersion objective, and images were captured with a Q-color3 camera and represent at least 200 observed cells.

Hog1p Phosphorylation—Dually phosphorylated Hog1p was determined as described previously with minor modifications (46, 47). Yeast cells were grown in YPD medium to A_{550} of ~1.0 and then treated with NaCl (0.8 M). Cell extracts were prepared, and proteins were separated by SDS-PAGE and visualized as described previously (46, 47). Dually phosphorylated Hog1p was detected by anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) (9211S; 1:1000; New England Biolabs). Anti-Hog1p was used to detect total Hog1p (Hog1 yC-20; 1:5000; Santa Cruz Biotechnology).

Hog1p Localization—Plasmid pPS1739 expressing *HOG1*-GFP (kindly provided by Dr. Pamela Silver) was transformed into wild type and *pgs1*Δ cells in the FGY3 background. Transformants were grown to A_{550} of ~1.0 in SC Ura$^-$ supplemented with 1 M sorbitol liquid medium (sorbitol is required for growth of *pgs1*Δ). Cells were treated with NaCl (0.8 M) for 2 min, stained with DAPI, and visualized as described above.

Slt2p Phosphorylation—Dually phosphorylated Slt2p was determined as discussed previously with minor modifications (27). Midlog phase cells were diluted to A_{550} of ~0.3 and grown at 30 or 37 °C for 2 h. Cell extracts were prepared, and proteins were separated by SDS-PAGE and visualized as described previously (27). Dually phosphorylated Slt2p (Thr^{202}/Tyr^{204}) was detected by anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) (9101S; 1:1000; Cell Signaling Technology), and anti-Mpk1p was used to detect total Slt2p (Mpk1 yN-19; 1:100; Santa Cruz Biotechnology).

RESULTS

Perturbation of the CL Pathway Leads to a Shortened Life Span—To address the hypothesis that a functional CL pathway is required for normal life span, virgin cells of isogenic wild type, *crd1*Δ, and *pgs1*Δ strains in the FGY3 genetic background were obtained at 30 °C, and life span was determined as described under "Experimental Procedures." Because *pgs1*Δ mutant cells lose mtDNA when grown at 30 °C, life spans of isogenic wild type ρ^0 cells were analyzed for comparison. In some strain backgrounds, ρ^0 cells were reported to have increased replicative life spans, possibly due to up-regulation of retrograde signaling (48). Consistent with this, loss of mtDNA from wild type cells in the FGY3 genetic background causes a small extension in replicative life span (Fig. 1*A*). At 30 °C, *pgs1*Δ was found to have a shortened life span of 5.7 generations, whereas *crd1*Δ cells displayed a normal life span of 15.9 generations (similar to the wild type life span of 16.7 generations) (Fig. 1*A*). When virgin cells were obtained at 30 °C and life span was determined at 37 °C, the life span of *crd1*Δ cells was significantly reduced to 8.7 (Fig. 1*B*). Life span was further reduced slightly when virgin cells were obtained at 37 °C (Fig. 1*C*). The *pgs1*Δ mutant failed to survive under these conditions. Thus, dis-

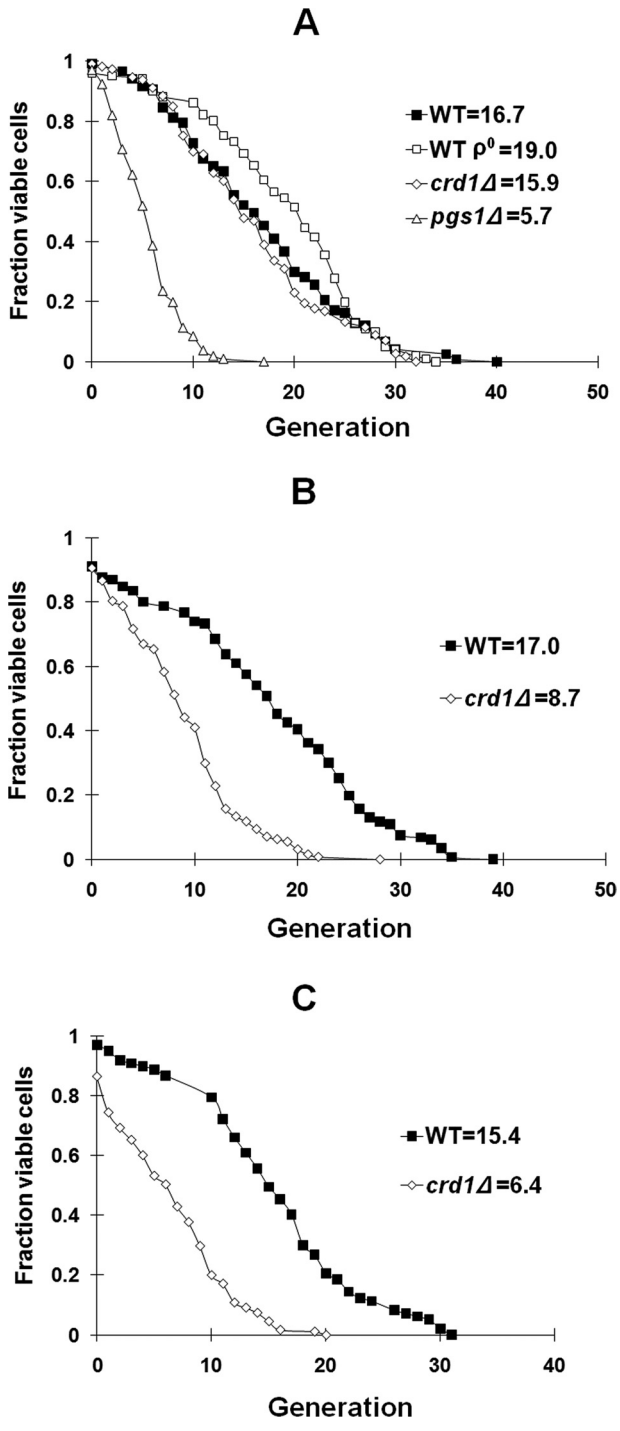

FIGURE 1. **Decreased life span in CL mutants.** Isogenic cells in the FGY3 genetic background were seeded on YPD plates. Virgin cells from each strain were obtained at 30 °C, and life spans were quantified at 30 °C (*A*) and 37 °C (*B*) by microscopic determination of the number of times a mother cell produced a daughter cell. The number after the strain indicates the average replicative life span (in generations). *pgs1*Δ has a shortened life span at 30 °C, and reduced life span in *crd1*Δ was observed at 37 °C. *C*, virgin cells from each strain were obtained at 37 °C, and life spans were quantified at 37 °C as described.

ruption of the first step of the CL pathway (*pgs1*Δ), resulting in loss of both PG and CL, leads to a shortened life span at 30 °C and the inability to replicate at elevated temperatures, whereas disruption of the last step of the pathway (*crd1*Δ), which results in the ability to synthesize PG but not CL, affects life span at elevated temperatures but not at 30 °C.

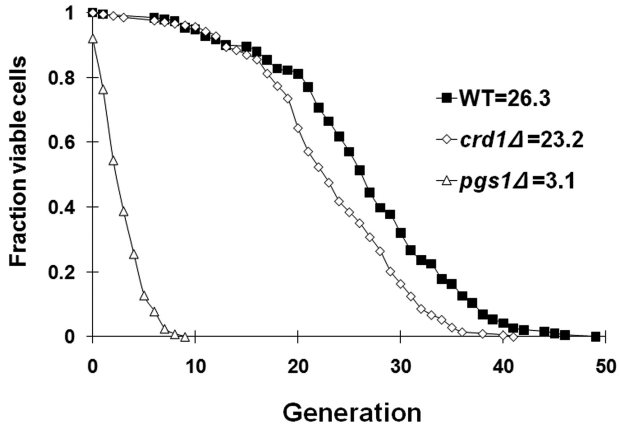

FIGURE 2. **CL is not required for CR induced life span extension.** Virgin wild type, *crd1*Δ and *pgs1*Δ cells in the FGY3 background were obtained and maintained on YPD with 0.5% glucose. Life span was determined at 30 °C.

CL Is Not Required for CR-induced Life Span Extension—As discussed above, CR resulting from low glucose in the media can induce life span extension in yeast (44). To determine if perturbation of the CL pathway affects life span extension in response to CR, we examined the life span of *crd1*Δ on low glucose (CR) medium at 30 °C. CR increased life span from 16.7 to 26.5 generations in wild type cells and from 15.9 to 23.2 in the *crd1*Δ mutant (compare Figs. 1*A* and 2), indicating that CL is not required for life span extension induced by CR. However, the life span of *pgs1*Δ was reduced rather than extended by CR (Fig. 2), suggesting that more severe mitochondrial defects in *pgs1*Δ prevent life span extension triggered by CR.

CL Is Required for Stress-induced Life Span Extension—Mild stress conditions, including heat and osmotic shock, are known to extend yeast life span (34, 36, 37). To determine if mild stress extended the life span of CL mutants, virgin cells were obtained at 30 °C, heat-shocked by immediate exposure to elevated temperature (37 °C) for 2 generations (4 h), and then shifted back to 30 °C. Alternatively, mild hypotonic shock was achieved by shifting virgin cells obtained on YPDS to YPD. The results indicated that heat or hypotonic shock extended wild type life span from 16.7 to ~25 generations (compare Figs. 1*A* and 3). Strikingly, no increase in the life span of *crd1*Δ was observed in response to either heat stress (compare Figs. 1*A* and 3*A*) or osmotic stress (compare Figs. 1*A* and 3*B*). Therefore, CL is required for life span extension in response to heat or osmotic stress. Virgin cells of the *pgs1*Δ mutant failed to produce daughter cells under these stress conditions.

Osmotic Stabilization and Genetic Suppression of Cell Wall Defects Extend Life Span in CL Mutants—We have shown previously that temperature-sensitive growth of the *pgs1*Δ mutant is at least partially due to cell wall defects, which can be alleviated by sorbitol (an osmotic stabilizer) or genetically suppressed by a mutation in the *KRE5* gene, leading to increased synthesis of cell wall components (21, 27). As discussed above, defects in yeast cell wall biogenesis are associated with a shortened life span (40). Thus, if the decreased life span of *pgs1*Δ results from perturbation of cell wall synthesis, we would expect that the life span of this mutant would increase upon alleviation of cell wall defects. To address this possibility, we examined the life span of *pgs1*Δ in the presence of the osmotic

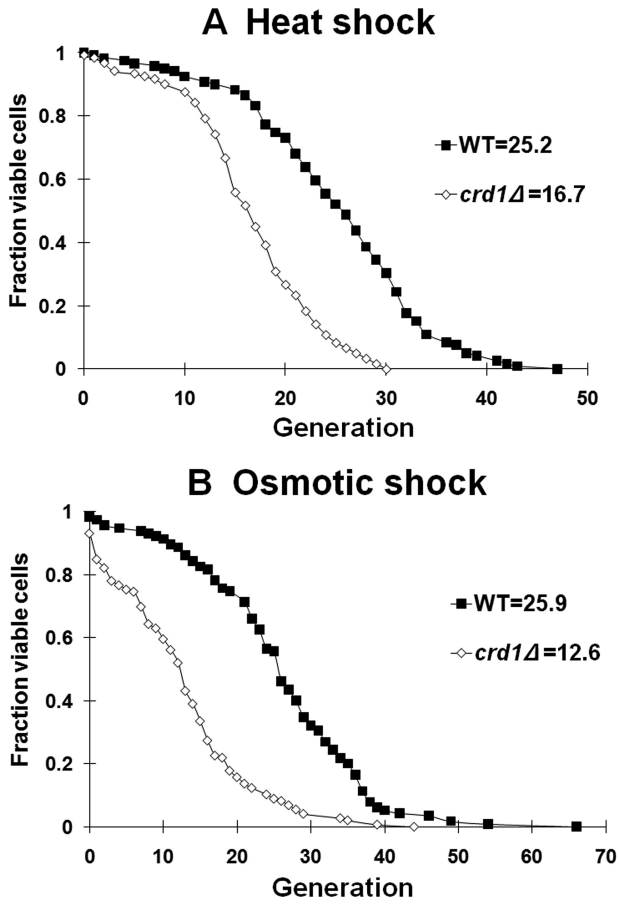

FIGURE 3. **CL is required for stress-induced life span extension.** *A*, heat shock. Single cells of wild type and *crd1*Δ in the FGY3 background were seeded on YPD plates. Virgin cells were obtained at 30 °C and grown for 2 generations at 37 °C (heat shock), and life spans were determined at 30 °C. *B*, osmotic shock. Single cells of wild type and *crd1*Δ were seeded on YPDS plates (osmotic shock). Virgin cells were obtained and transferred onto YPD plates. Life span was determined at 30 °C.

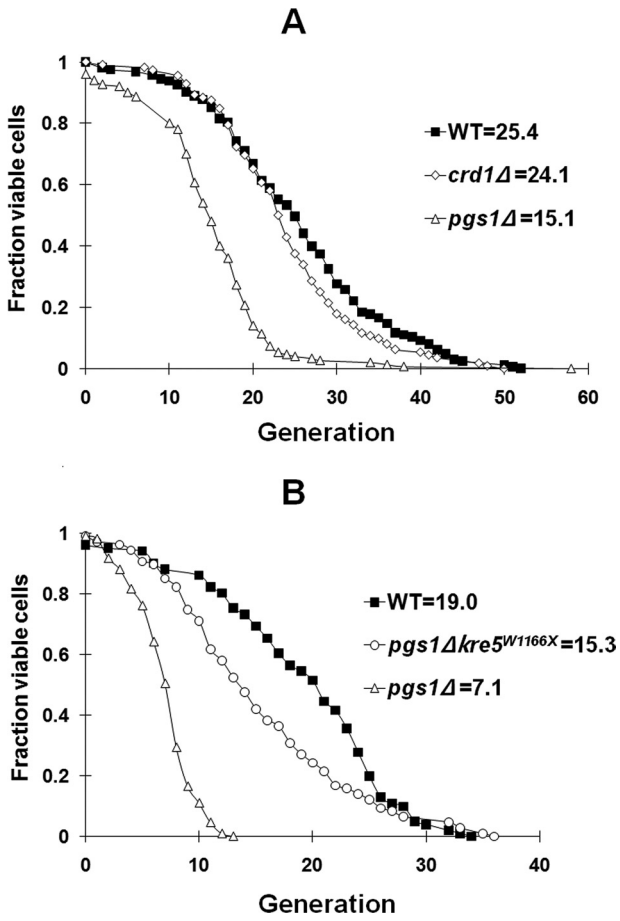

FIGURE 4. **Alleviating cell wall defects extends life span in CL mutants.** *A*, effect of sorbitol. Single cells of isogenic wild type, *crd1*Δ, and *pgs1*Δ strains in the FGY3 background were seeded on YPDS plates. Virgin cells from each strain were obtained at 30 °C, and life spans were quantified. *B*, effect of *kre5*^{W1166X} mutation. Isogenic single cells in the FGY3-A background were seeded on YPD plates. Virgin cells from each strain were obtained, and life spans were quantified at 30 °C.

stabilizer sorbitol or the *kre5*^{W1166X} mutation. In the presence of sorbitol, the life span of *pgs1*Δ at 30 °C increased from 5.7 to 15.1 (compare Figs. 1*A* and 4*A*). To determine if the *kre5*^{W1166X} suppressor extended the life span of *pgs1*Δ, we compared the life spans of *pgs1*Δ and *pgs1*Δ*kre5*^{W1166X}. The *kre5*^{W1166X} mutation extended the life span of *pgs1*Δ from 7.1 to 15.3 generations (Fig. 4*B*), most likely due to reorganization of the cell wall and enhancement of cell wall stability.

*Perturbation of the HOG Pathway Restores Growth and Longevity Defects of pgs1*Δ—The inability of CL mutants to increase life span in response to stress (Fig. 3) suggested that the mechanism linking CL to decreased life span was via perturbation of the stress response in the mutants. To address this possibility, a targeted screen for suppressors of *pgs1*Δ temperature sensitivity among stress pathway mutants was carried out. A search of the *Saccharomyces* Genome Database (available on the World Wide Web) identified 77 nonessential genes that play a role in the HOG, PKC-Slt2, and related stress response pathways (supplemental Table 1). A modified synthetic genetic array procedure (45) was utilized to screen for suppressors among mutants of these genes. Briefly, stress pathway deletion mutants were mated with *pgs1*Δ, diploids were sporulated, and meiotic progeny containing mutations in both *PGS1* and each of the stress pathway genes were selected by Geneticin resistance (stress pathway mutant) and uracil prototrophy (*pgs1*Δ mutant). Suppressors of the *pgs1*Δ temperature sensitivity defect were identified by growth of the double mutant at 39 °C. Of the 77 double mutants, 20 grew at 39 °C and thus identified potential suppressors of *pgs1*Δ temperature sensitivity (Table 3). Interestingly, eight of these identified genes are required for the HOG pathway, including *SHO1*, *STE50*, *SSK2*, *SSK22*, *PBS2*, *MSN2*, *HSP12*, and *YAP* (30), suggesting that down-regulation of the HOG pathway leads to alleviation of *pgs1*Δ defects. To further test this possibility, double mutants combining *pgs1*Δ with a HOG pathway mutant were constructed. Attempts to obtain the *pgs1*Δ*hog1*Δ mutant were not successful. However, the double mutants *pgs1*Δ*sho1*Δ and *pgs1*Δ*hsp12*Δ were obtained by meiotic tetrad analysis and tested for suppression of *pgs1*Δ temperature sensitivity at 39 °C. We observed that the *pgs1*Δ*sho1*Δ

TABLE 3
Potential suppressors of *pgs1*Δ temperature sensitivity

ATH1	*MRK1*	*ROM2*	*SSK22*
GAC1	*MSN2*	*SHO1*	*STE50*
HOR2	*PBS2*	*SKN7*	*WSC3*
HSP12	*PIL1*	*SSA2*	*WSC4*
IML3	*PSR1*	*SSK2*	*YAP1*

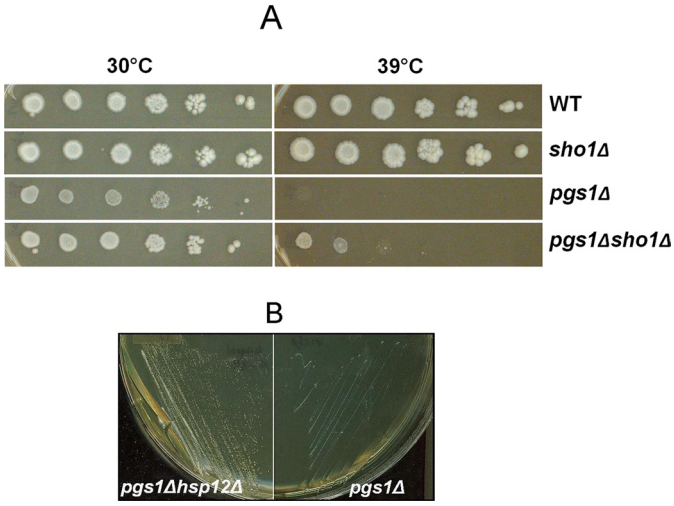

FIGURE 5. **Down-regulation of the HOG pathway alleviates growth and longevity defects of *pgs1*Δ.** *A*, isogenic cells in the BY4742 ρ^0 background were serially diluted, spotted on a YPD plate, and incubated at 39 °C. *B*, single cells of isogenic *pgs1*Δ and *pgs1*Δ*hsp12*Δ strains in the BY4742 ρ^0 background were streaked on a YPD plate and incubated at 39 °C. *C*, single cells of isogenic wild type, *pgs1*Δ, *pgs1*Δ*hsp12*Δ, and *pgs1*Δ*sho1*Δ strains in the BY4742 ρ^0 background were seeded on YPD plates. Virgin cells from each strain were obtained, and life spans were quantified at 30 °C.

and *pgs1*Δ*hsp12*Δ double mutant progeny were ρ^0. Therefore, the corresponding controls used for experiments with this mutant were also ρ^0. Consistent with suppression, *pgs1*Δ*sho1*Δ cells grew at 39 °C (Fig. 5*A*). To determine if deletion of *SHO1* also alleviated the life span defects of *pgs1*Δ, we examined the life span of *pgs1*Δ*sho1*Δ cells on YPD plates at 30 °C. As seen in Fig. 5*C*, deletion of *SHO1* extended the life span of *pgs1*Δ by 7 generations. Similarly, *pgs1*Δ*hsp12*Δ showed slightly improved growth at 39 °C and a modest increase in life span (Figs. 5, *B* and *C*). In these double mutants, the degree of suppression of temperature-sensitive growth correlated with the effect on life span. These findings suggest that the life span defects of *pgs1*Δ result at least partially from activation of the HOG pathway.

*Hog1p Is Not Overactivated in pgs1*Δ—Suppression of *pgs1*Δ mutant phenotypes by mutants that down-regulate the HOG pathway suggests the possibility that HOG signaling is increased in *pgs1*Δ cells. To determine if phosphorylation of Hog1p is increased in *pgs1*Δ, a specific antibody against dually phosphorylated p38 was used, as described previously (46, 47). There was no increase in phosphorylated Hog1p in *pgs1*Δ cells compared with wild type cells in the BY4742 background (Fig. 6*A*) or in the FGY3 background (data not shown). Furthermore, *pgs1*Δ did not exhibit differences in cellular localization of Hog1p before or after NaCl treatment (which induces Hog1p activation) compared with wild type (Fig. 6*B*). These findings argue that the HOG pathway is not overactivated in *pgs1*Δ.

*Down-regulation of the HOG Pathway Alleviates the Cell Wall Defects in pgs1*Δ—We observed previously that *pgs1*Δ cells in the FGY3 background exhibited sensitivity to caffeine and CFW as well as defective activation of the PKC/Slt2 pathway (21, 27). Increased sensitivity to caffeine and CFW (Fig. 7*A*) and a slight decrease in Slt2p phosphorylation (Fig. 7*B*) are also observed in *pgs1*Δ cells in the more robust BY4742 genetic background. Because activation of the PKC-Slt2 pathway reduces intracellular pressure (30), the loss of Slt2p activation may lead to increased intracellular turgor pressure in *pgs1*Δ cells. It is possible that activation of the HOG pathway, which leads to increased intracellular turgor pressure (30), may further exacerbate this defect. Thus, we predicted that down-regulation of the HOG pathway in *pgs1*Δ may alleviate the cell integrity defects caused by loss of Slt2p activation. Consistent with this possibility, deletion of *SHO1* alleviates the sensitivity to caffeine and CFW in *pgs1*Δ (Fig. 7*A*), although phosphorylation of Slt2p is not restored to wild type levels (Fig. 7*B*).

DISCUSSION

In this study, we show for the first time that 1) the replicative life span of yeast cells is dependent upon the presence of the mitochondrial anionic phospholipids CL and PG, and 2) life span in the *pgs1*Δ mutant can be restored by up-regulating the cell integrity pathway and down-regulating the HOG stress response pathway.

As expected, longevity defects were more severe in *pgs1*Δ than in *crd1*Δ. PG has been shown to compensate for the loss of CL for some functions (8). The shortened life span of *crd1*Δ at elevated temperature (Figs. 1, *B* and *C*) and the inability of *crd1*Δ cells to extend life span under stress conditions (Fig. 3) argue that PG cannot completely compensate for the lack of CL in these functions. Although an intact CL pathway is required for normal longevity under stress conditions, our data suggest that CL synthesis is not required for life span extension in response to CR. On the other hand, CR failed to extend life span in *pgs1*Δ cells (Fig. 2), which lack both PG and CL and exhibit more severe mitochondrial defects than *crd1*Δ cells, including mitochondrial DNA loss at 30 °C (21). Because mitochondrial functions are required for CR-induced life span extension (49), our findings suggest that loss of both ionic phospholipids PG and CL, but not CL alone, compromises mitochondrial function too severely to extend life span in response to CR.

The *pgs1*Δ mutant has significantly reduced β-1,3-glucan levels in the cell wall, resulting in a more spherical appearance and larger cell size than wild type cells (21). This defect is due at least in part to defective activation of the PKC-Slt2 MAPK pathway (27). Genetic suppression by $kre5^{W1166X}$ restores Slt2p activation and β-1,3-glucan synthesis in *pgs1*Δ (27). As discussed earlier, a defective cell wall is linked to decreased life span (40, 50). Consistent with this, suppression of cell wall defects by $kre5^{W1166X}$ led to a marked extension of *pgs1*Δ life span (Fig. 4*B*). Sorbitol, which enables growth of cell wall mutants by osmotic stabilization (21, 51–53), supports the growth of CL mutants at elevated temperature (21) and also restores *pgs1*Δ life span (Fig. 4*A*). Both sorbitol and genetic suppression by $kre5^{W1166X}$ may extend life span of CL mutants by promoting cell integrity. Although previous studies have demonstrated that *hypertonic* stress extends life span (34, 37), we show for the first time that *hypotonic* shock also extends life span in wild type cells (compare Figs. 1*A* and 3*B*). Up-regulation of the PKC-Slt2 pathway is required for adaptation to hypotonic shock (31, 54). As we have shown previously, Slt2p is constitutively activated in $kre5^{W1166X}$ (27). Therefore, we speculate that *pgs1*Δ did not survive heat shock or hypotonic shock due to lack of Slt2 activation and that $kre5^{W1166X}$ suppresses the

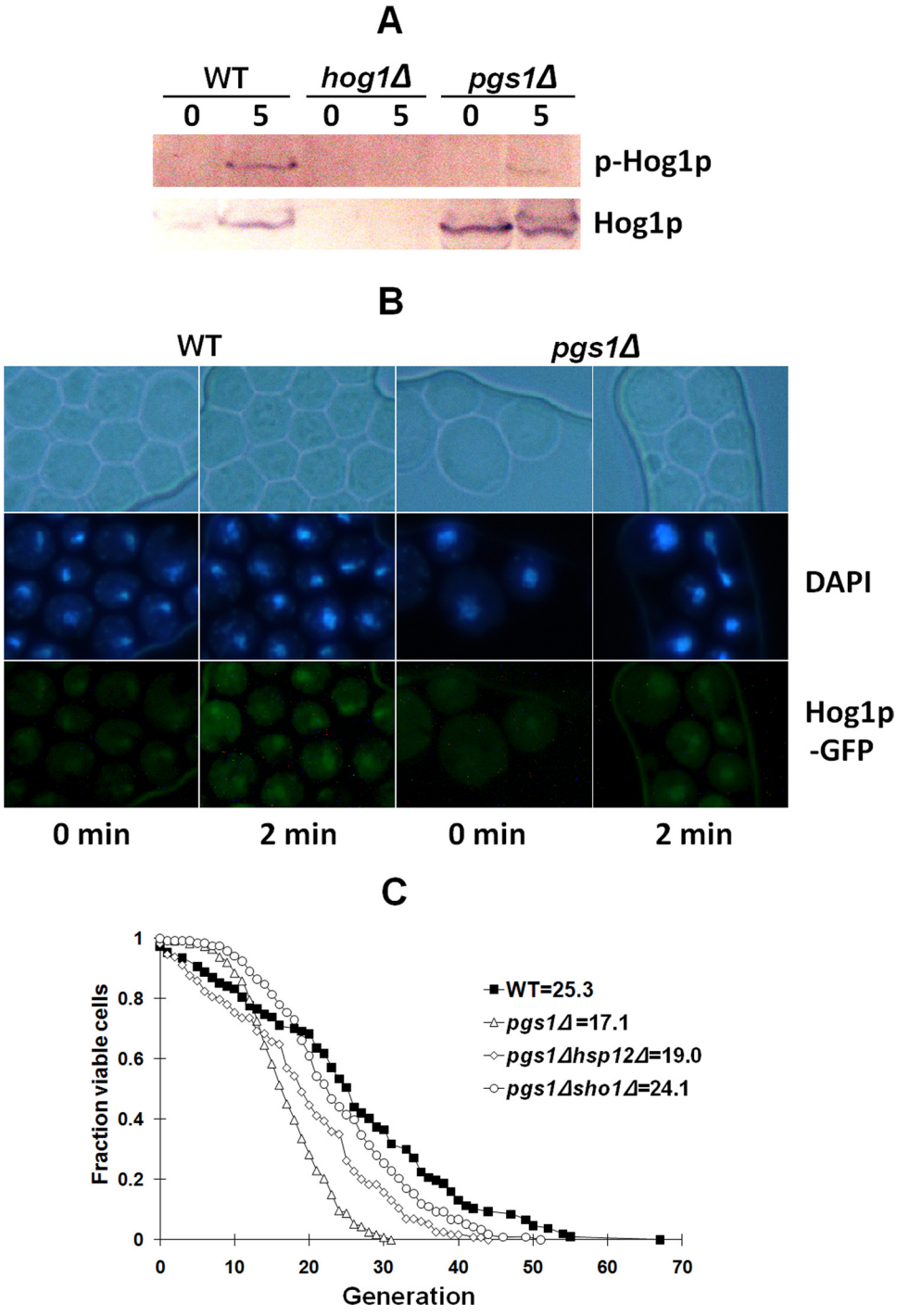

FIGURE 6. **Hog1p is not overactivated in *pgs1*Δ cells.** *A*, isogenic wild type and *pgs1*Δ cells in the BY4742 ρ^0 background were grown in YPD liquid medium to midlog phase and treated with 0.8 M NaCl for 5 min. The dually phosphorylated Hog1p (*p-Hog1p*) and total Hog1p proteins were detected by Western analysis, as described previously (46, 47). A representative membrane is shown for three independent experiments. *B*, isogenic wild type and *pgs1*Δ cells in the FGY3 background were transformed with the plasmid pPS1739, on which *HOG1* is fused to GFP, grown in SC Ura$^-$ supplemented with 1 M sorbitol liquid medium (sorbitol is required for *pgs1*Δ to grow) to A_{550} of ~1, treated with 0.8 M NaCl, stained with DAPI, and visualized as described above.

growth and life span defects of *pgs1*Δ by up-regulation of the PKC-Slt2 pathway.

In addition to restoration of life span by improved cell integrity, down-regulation of the HOG pathway by deletion of *SHO1* or seven other HOG pathway genes also restored the growth of *pgs1*Δ at elevated temperatures. Deletion of *SHO1* robustly extended *pgs1*Δ life span, whereas deletion of *HSP12* had only a modest effect on *pgs1*Δ life span (Fig. 5*C*). Interestingly, overexpression of *YPD1*, a negative regulator of the HOG pathway, also enabled growth of *pgs1*Δ at elevated temperatures.[6] These findings suggested that the HOG pathway is overactivated in *pgs1*Δ or that HOG signaling is deleterious to the mutant in the absence of high osmolarity. Previous studies have shown that *hypertonic* stress extends life span by activation of the HOG pathway (34, 37), and our finding that sorbitol, which provides high osmolarity, also extends life span (Fig. 4*A*) is consistent with these reports. However, under conditions of normal or low osmolarity when HOG signaling is not required, activation of this pathway can cause consequences that are more deleterious than beneficial to *pgs1*Δ. Suppression of *pgs1*Δ defects by deletion of *SHO1* supports this possibility. The central step of HOG pathway activation is the phosphorylation of Hog1p, which results in translocation of Hog1p from the cytoplasm into the nucleus, where it phosphorylates transcriptional regulators (30). Neither the phosphorylation pattern nor the cellular localization of Hog1p was altered in *pgs1*Δ (Fig. 6), indicating that the HOG pathway is not overactivated in the mutant. More likely, the normal activation of the HOG pathway and/or its downstream target(s) are deleterious to CL pathway mutants under conditions of normal osmolarity.

Why is HOG pathway activation deleterious to CL mutants? One possibility relates to the observation that the HOG and PKC-Slt2 pathways appear to exert opposite effects on cellular turgor pressure (30, 55–58) (Fig. 8). The HOG pathway brings about an increase in intracellular turgor pressure, whereas the PKC-Slt2 pathway causes a decrease in intracellular turgor pressure (30, 57). Thus, it is possible that the inability of *pgs1*Δ cells to reduce the intracellular turgor pressure due to

[6] V. Gohil, unpublished data.

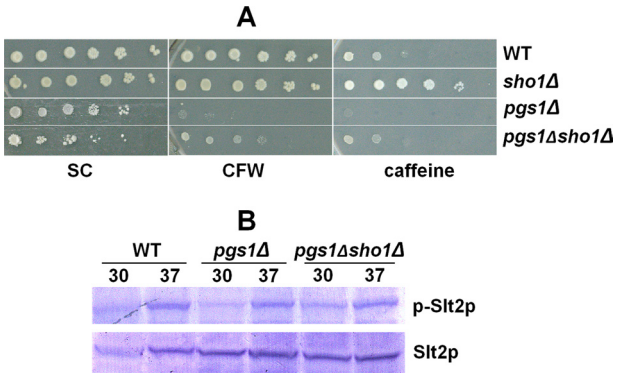

FIGURE 7. **Down-regulation of the HOG pathway partially alleviates cell integrity defects of *pgs1*Δ.** *A*, cells of isogenic wild type, *sho1*Δ, *pgs1*Δ, and *pgs1*Δ*sho1*Δ strains in the BY4742 ρ^0 background were serially diluted, spotted on SC plates supplemented with 12 mM caffeine or 10 μg/ml CFW, and incubated at 30 °C. *B*, cells of isogenic wild type, *pgs1*Δ, and *pgs1*Δ*sho1*Δ strains in the BY4742 ρ^0 background were grown to midlog phase and kept at 30 or 37 °C for 2 h. Dual phosphorylation of Slt2p was detected as described previously. A representative membrane is shown for three independent experiments.

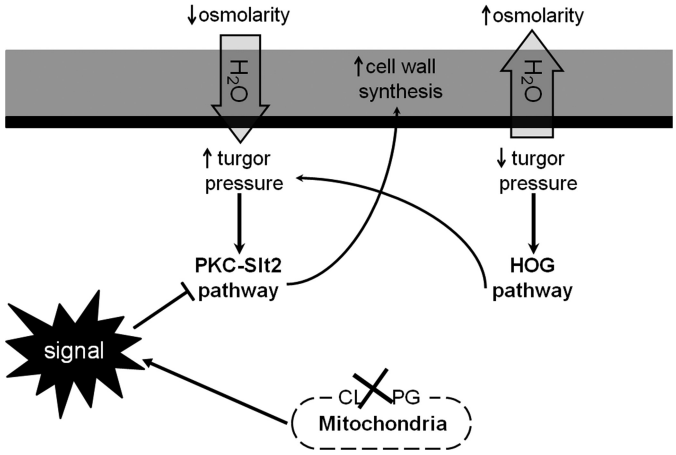

FIGURE 8. **Perturbation of PKC and HOG signaling in *pgs1*Δ.** Under hypertonic or cold stress conditions, extracellular osmolarity is increased, which causes an efflux of water from the cell, reducing the turgor pressure on the cell wall. Activation of the HOG pathway by increased osmolarity leads to *increased* intracellular turgor pressure to counteract the extracellular osmolarity. In contrast, low extracellular osmolarity resulting from heat or hypotonic stress causes an influx of water into the cell, which increases the turgor pressure. To withstand the increased turgor pressure, the PKC-Slt2 pathway is activated to induce cell wall synthesis (30, 55–58). Loss of PG/CL may generate a signal (as yet unidentified) that is detected by regulators or components of the PKC-Slt2 pathway, resulting in down-regulation of the pathway. In this case, activation of the HOG pathway will exacerbate the cell wall defects in *pgs1*Δ cells by further increasing the turgor pressure.

loss of Slt2p activation is exacerbated by a further increase in turgor pressure resulting from activation of the HOG pathway. Down-regulation of the HOG pathway may thus suppress the temperature sensitivity and longevity defects in *pgs1*Δ (Fig. 5) by relieving the increased turgor pressure. Consistent with this hypothesis, deletion of *SHO1* from *pgs1*Δ cells conferred resistance to caffeine and CFW, although phosphorylation of Slt2p was not fully restored to wild type levels (Fig. 7). HOG pathway activation may be deleterious for other reasons. For example, increased activation of HOG1 causes severe growth inhibition, resulting in G_1 and G_2 arrest (59, 60). However, it is unlikely that cell cycle arrest accounts for the temperature sensitivity and life span defects of *pgs1*Δ, since this mutant did not exhibit increased activation of the HOG pathway (Fig. 6), increased expression of *SWE1* (data not shown), or defective cell cycle progression (data not shown).

In addition to the possibilities discussed above, an increase in the total Hog1p level may contribute to the deleterious effect of the HOG pathway. As seen in Fig. 6*A*, *pgs1*Δ cells seem to have more total Hog1p than wild type cells. Although the amount of phosphorylated Hog1p was not increased in *pgs1*Δ, the increase in total Hog1p may affect targets in the cytosol, causing deleterious effects. The identification of cytosolic targets of Hog1p will help to test this possibility.

The specific process fulfilled by PG/CL with respect to life span is not clear. We hypothesize that mitochondrial dysfunction due to loss of these lipids generates a signal (as yet unidentified) that is detected by regulation of components of the PKC-Slt2 pathway, leading to down-regulation of the pathway and decreased life span (Fig. 8). Consistent with this, increased activation of this pathway restores life span to near wild type levels (Fig. 4*B*). An alternative possibility is that PG/CL directly interacts with signaling pathway components. This could occur if PG/CL were present in the nucleus, consistent with some reports in mammalian cells (61, 62). Alternatively, PG/CL in the mitochondrial outer membrane in contact sites (63, 64) may interact with cytoplasmic components of the signaling pathways.

The HOG pathway is highly conserved. The mammalian homolog of Hog1p, p38, is activated during the onset of senescence (65), and activation of p38 promotes apoptosis (66, 67). p38 is also a signal relay protein that responds to osmotic stress. A role for p38 in cardiomyopathy is suggested by findings that inhibition of p38α reduces cardiomyopathy induced by overexpression of the β-adrenergic receptor (68). p38 also plays an important role in the cardiac expression of proinflammatory cytokines and in the development of cardiac dysfunction relative to the inflammatory response (69). The effects of CL deficiency on mammalian p38 have not been studied. However, because p38 is involved in processes that affect and exacerbate cardiomyopathy, it is tempting to speculate that perturbation of p38 may contribute to the variation in phenotypic severity seen in Barth syndrome, a genetic disorder characterized by cardiomyopathy due to mutations in the CL remodeling enzyme tafazzin (70).

In summary, we show for the first time that mitochondrial anionic phospholipids are required for normal replicative life span. Decreased life span in CL mutants can be restored by promoting cell integrity or by down-regulation of the HOG pathway. Our findings suggest that activity of the HOG pathway and/or downstream targets of this pathway are deleterious in the absence of a functional CL pathway. Down-regulation of the HOG pathway partially alleviates the cell wall defects caused by loss of Slt2p activation in *pgs1*Δ. These findings provide new insights into the functions of CL and point to a role for CL in the response to cellular stress.

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