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The Golgi Ca^{2+}-ATPase KlPmr1p Function Is Required for Oxidative Stress Response by Controlling the Expression of the Heat-Shock Element *HSP60* in *Kluyveromyces lactis*

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The Golgi P-type Ca^{2+}-ATPase, Pmr1p, is the major player for calcium homeostasis in yeast. The inactivation of *KlPMR1* in *Kluyveromyces lactis* leads to high pleiotropic phenotypes that include reduced glycosylation, cell wall defects, and alterations of mitochondrial metabolism. In this article we found that cells lacking KlPmr1p have a morphologically altered mitochondrial network and that mitochondria (m) from *Klpmr1*Δ cells accumulate Ca^{2+} more slowly and reach a lower $[Ca^{2+}]_m$ level, when exposed to $[Ca^{2+}] < 5$ μM, than wild-type cells. The *Klpmr1*Δ cells also exhibit traits of ongoing oxidative stress and present hyperphosphorylation of KlHog1p, the hallmark for the activation of stress response pathways. The mitochondrial chaperone KlHsp60 acts as a multicopy suppressor of phenotypes that occur in cells lacking the Ca^{2+}-ATPase, including relief from oxidative stress and recovery of cell wall thickness and functionality. Inhibition of *KlPMR1* function decreases *KlHSP60* expression at both mRNA and protein levels. Moreover, *KlPRM1* loss of function correlates with both decreases in HSF DNA binding activity and *KlHSP60* expression. We suggest a role for *KlPMR1* in HSF DNA binding activity, which is required for proper *KlHSP60* expression, a key step in oxidative stress response.

INTRODUCTION

The biological functions of calcium are notably versatile and the control of intracellular Ca^{2+} homeostasis in eukaryotic cells is the result of complexly networked processes. Tight regulation of Ca^{2+} influx from outside and storage in various cell compartments keeps the free Ca^{2+} concentration in the cytoplasm ($[Ca^{2+}]_{cyt}$) in a narrow range between 50 and 200 nM. Changes in $[Ca^{2+}]_{cyt}$, by a variety of external or internal stimuli, are sensed by calcium-binding proteins, for example, calmodulin. The calmodulin-dependent protein phosphatase calcineurin and various calmodulin-dependent protein kinases control the functioning of several downstream transduction pathways.

In the budding yeast *Saccharomyces cerevisiae* the vast majority of cellular Ca^{2+} is stored in the vacuole (Dunn *et al.*, 1994). The secretory apparatus (ER + Golgi) also plays an important role in the equilibrium of intracellular calcium homeostasis. Two P-type Ca^{2+}-ATPases control the Ca^{2+} level in the secretory pathway: Pmr1p, a Ca^{2+}/Mn^{2+} pump that is localized in early-medial Golgi (Rudolph *et al.*, 1989; Antebi and Fink, 1992) and Cod1p/Spf1p, which has been associated with ER (Cronin *et al.*, 2002). They collaborate to maintain the Ca^{2+}/Mn^{2+} homeostasis required for the processing and quality control of secretory glycoproteins; Pmr1p is however regarded as the major contributor to Ca^{2+} homeostasis in the secretory pathway (Strayle *et al.*, 1999).

The absence of Pmr1p produces a notable imbalance in Ca^{2+} distribution across the cell compartments. Depletion of the secretory pathway Ca^{2+} stores is accompanied by increased calcium uptake from environment resulting in a 16-fold increase of $[Ca^{2+}]_{cyt}$ despite a compensatory increase in the expression of Pmc1p, the vacuolar Ca^{2+}-ATPase (Cunningham and Fink, 1994; Strayle *et al.*, 1999). Pmc1p increase relies on the activation of the calcineurin-dependent transcription factor Tcn1p (Stathopoulos and Cyert, 1997). Calcium depletion of ER and Golgi observed in *pmr1*Δ cells leads to defective folding and glycosylation of secretory proteins (Durr *et al.*, 1998).

Among several cell compartments, mitochondria have been recently revaluated as relevant players in Ca^{2+} signaling. Mitochondria can accumulate Ca^{2+} from the cytosol and tight physical connections have been described between mitochondria and ER membranes with the occurrence of Ca^{2+}"hotspots" at the close contacts between the two organelles (Rizzuto *et al.*, 2004). Furthermore, the mitochondrial role in Ca^{2+} signaling is as critically important as regulatory mechanisms that control the oxidative stress response. Indeed, an important aspect of mitochondrial activity is that the electron transport chain consumes 90% of cellular oxygen, giving rise to free radicals and consequently to a stress condition referred to as oxidative stress. When

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sufficiently intense, oxidative stress can reduce cell viability and lead to mitochondrial damage that is probably the most important event after oxidative stress (Yan *et al.*, 1997; Kowaltowski and Vercesi, 1999). Ca^{2+} signals originated under oxidative stress conditions as a mechanism of intracellular signaling for switching on protective responses (Gibson and Huang, 2004; Starkov *et al.*, 2004).

Heat shock proteins (Hsps) have been directly related with resistance to oxidative stress, although the molecular mechanisms that control such responses have not been completely defined. The importance of Hsps lies both in their abundance and, more importantly, in their mitochondrial location. It has been suggested that the Hsps recognize partially misfolded proteins and facilitate refolding, thus preventing irreversible aggregation (Martin *et al.*, 1992; Fenton *et al.*, 1994). Temperature and other stress-triggered expression of Hsps are mediated by heat-shock transcription factors (HSFs). Although HSFs have been extensively studied with respect to their role in thermotolerance, it has recently been shown that calcium plays either a positive or negative role in HSF activation, depending on the cellular context (Li *et al.*, 2004).

HSF is a highly conserved protein, present in all eukaryotic organisms studied from yeast to human and the regulation of its activity is a central mechanism of transcriptional regulation of HSP gene expression. Under normal growth condition HSF is maintained in an inert monomer state through association with molecular chaperons such as Hsp70 (Shi *et al.*, 1998). During heat shock, the HSF is converted from a transcriptionally inactive monomer to active trimeric form that is capable of binding to conservative promoter elements (heat shock elements [HSEs]) and exhibits transcriptional activity. However, in yeast, HSF is constitutively trimeric and partially bound to DNA, and its activity is primarily regulated at the level of transactivation (Nieto-Sotelo *et al.*, 1990; Sorger, 1990; Bonner *et al.*, 1992; Chen *et al.*, 1993; Jakobsen and Pelham, 1998).

We have previously described a link between the Ca^{2+} homeostasis controlled by KlPmr1p, the *Kluyveromyces lactis* Golgi Ca^{2+}-ATPase, and mitochondrial metabolism as highlighted by increased respiration rate and by altered transcription levels of genes coding for respiratory enzymes after inactivation of *KlPMR1* (Farina *et al.*, 2004). In the present study we report that the inactivation of the Golgi Ca^{2+}-ATPase affects mitochondrial calcium homeostasis; the organelles also exhibit altered morphology and undergo oxidative stress. Evidence that the mitochondrial chaperone gene *KlHSP60* acts as a multicopy suppressor of calcium-related defects present in *Klpmr1*Δ cells, including the mitochondria and cell wall alterations is presented. Our findings indicate that *KlPMR1* has an important role in protecting cells from oxidative stress. A mechanism to explain this protection linking *KlPMR1*, *KlHSP60*, mitochondrial homeostasis, and oxidative damage is suggested.

MATERIALS AND METHODS

Yeast Strains, Growth Conditions. and Plasmids Construction

The strains used in this study were MW278-20C (MATa, *ade2*, *leu2*, *uraA*), CPK1 (MAT a, *ade2*, *leu2*, *uraA*, *KlPMR1::Kan R*), JA6 (MATα, *ade1-600*, *ade T-600*, *trp1-11*, *uraA1-1*), and CPK2 (MATα, *ade1-600*, *ade T-600*, *trp1-11*, *KlPMR1::URA1*). Yeast strains were grown in YPD medium (1% yeast extract, 1% peptone, 2% glucose) or SD minimal medium (2% glucose, 0.67% yeast nitrogen base without amino acids) with the appropriate auxotrophic requirements. The manganese growth test was performed on YPD at different concentrations of $MnCl_2$ as indicated; the analysis of the EGTA sensitivity of the *KlPmr1*Δ strain was performed on YPD supplemented with 10 mM EGTA and/or 5 μM deferoxamine [DFO], Sigma, St. Louis, MO). For calcium ionophore experiments the A23187 (calcium ionophore III, Sigma) was used at different concentrations as indicated, incubating exponentially growing cells for 2 h at 28°C.

Fivefold serial dilution from concentrated suspensions of exponentially growing cells (5×10^6 cell/ml) were spotted onto synthetic YPD agar plates supplemented or not with 4 mM H_2O_2 or 20 mM EGTA, and the plates were incubated at 30°C for 24–48 h.

Construction of the pCXJ3-mtGFP plasmid: the open reading frame (ORF) encoding for the green fluorescent protein (GFP) was isolated as a *Eco*RI-*Xho*I fragment from plasmid pYX232 (Westermann and Neupert, 2000) and was ligated into *Eco*RI-*Sal*I-restricted pCXJ3 multicopy plasmid (Chen, 1996).

Construction of the pCXJ3-mtAeq plasmid: the cDNA encoding for the apoaequorin was isolated as a *Hin*dIII-*Xho*I fragment from the plasmid mtAEQ/p (Rizzuto *et al.*, 1995) and was ligated into *Hin*dIII-*Xho*I-restricted pCXJ3 plasmid, subsequently the mitochondrial signal sequence was PCR amplified from the plasmid pYX232 and ligated into *Sma*I-*Hin*dIII-restricted pCXJ3-Aeq plasmid.

Construction of the p70 and p10 plasmids: the *KlHSP70* and *KlHSP10* genes were PCR amplified from *K. lactis* DNA genome using the primers 5′-CCGGATTCATCACGTGACACACTGCCATT-3′ and 5′-CCGGATTCACCACGTCAATAGTTTCGGTT-3′, 5′-CCGGATTCAGAAGGGGGGTAGTTCAAAT-3′ and 5′-CCGGATTCGCTTTTGGAGATGAAAAATC-3′ respectively (the *Eco*RI restriction site is underlined). The PCR products were sequenced (MWG Biotech, Ebersberg, Germany) and successively cloned in *Eco*RI-digested pCXJ3 plasmid.

Ca^{2+} Measurements

Yeast cells were transformed with the pCXJ3-mtAeq plasmid, containing the targeted apoaequorin fragment, using electroporation (Sambrook *et al.*, 1989). Selection was carried out on YPD medium containing 100 μg/ml G418. Cells were grown overnight to log phase, and 40 OD_{600} units were harvested and washed with synthetic complete medium (Sherman *et al.*, 1986) containing 1.2 M sorbitol buffered with 10 mM HEPES, pH 7 (SP). After resuspension in 1 ml SP, 100 U of zymolyase 100T (Seikagaku Kogyo, Tokyo, Japan) and 3 μl 2-mercaptoethanol were added, and the cell suspension was incubated at room temperature for 40–60 min, until conversion to spheroplasts was observed, assessed by osmotic swelling after suspension in deionized water. Spheroplasts were washed and resuspended in SP. After addition of coelenterazine to a final concentration of 10 μM, the cell suspension was incubated for 2 h at 30°C in the dark. Cells were then harvested, washed twice with SP and resuspended in 100 μl SP, followed by addition of 100 μl low melting point agarose (1.5%, 37°C). Fifty microliters of this cell suspension was then placed onto 13-mm round coverslips and stored at 4°C until the agarose solidified. The coverslips were transferred into a perfused, thermostated chamber (22–25°C) and placed in close proximity to a cooled, low-noise photomultiplier with a built-in amplifier-discriminator (EMI 9789, as described in Rizzuto *et al.*, 1994). The spheroplasts were perfused first with an intracellular buffer (IB) mimicking intracellular conditions (Rizzuto *et al.*, 1998) containing 1 mM EGTA for 20 s and then with the same solution plus 100 μM digitonin for 60 s in order to permeabilize the cells. Subsequently, the cells were perfused with IB (Ca^{2+} free conditions) and then with IB containing 20 μM Ca^{2+}. The consumption of reconstituted mtAeq accompanying this process was quantitatively monitored by photon counting according to Brini *et al.* (1995). Permeabilization with 100 μM digitonin in the presence of 10 mM $CaCl_2$ finished the experiment by discharge of residual aequorin. The aequorin luminescence data were captured by an EMI C660 photon counting board.

Determination of the Intracellular Ca^{2+} Content

Cells corresponding to 20 OD_{600} were harvested by centrifugation and suspended in 3 ml of spheroplast buffer (SB) containing 1 M sorbitol, 50 mM Tris buffer, pH 7.5, 10 mM Mg^{2+}, and 30 mM dithiothreitol (DTT). After 15-min incubation at 30°C, the suspension was centrifuged, and the resulting pellets were resuspended in 5 ml of SB containing 2 mg of zymolyase 20T. The suspensions were then incubated at 30°C for 40–60 min, until conversion to spheroplasts was observed and assessed as above. The spheroplast suspension was then centrifuged and washed twice with SB devoid of DTT. Freshly prepared spheroplasts were incubated for 60 min at 30°C in standard reaction medium (125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH 7.2 and 500 μM ethanol) in the presence of 0.1 mg/ml bovine serum albumin (Sigma) and 10 μM Fura-2AM (Molecular Probes, Eugene, OR). The suspension was then centrifuged and washed twice in standard reaction medium. The excitation fluorescence spectrum of the suspension was determined by a fluorescence spectrophotometer with emission wavelength fixed at 510 nm. Intracellular Ca^{2+} content was calculated relative to the fluorescence of free Fura and Fura saturated with Ca^{2+}, as described in Grynkiewicz *et al.* (1985).

Electron Microscopy

Exponential-phase cultures of yeast strains were fixed with 2%, vol/vol, glutaraldehyde in distilled water for 1 h at room temperature. Cells were then processed as described in Uccelletti *et al.* (1999), and ultrathin sections were

stained with lead citrate and examined with a CM 10 Philips electron microscope at 80 kV (Eindhoven, The Netherlands).

Fluorescence Microscopy

The cells were harvested during the exponential phase on YPD and fixed with 1% formaldehyde for 30 min. The vital dye 2-(4-dimetylaminostyryl)-*N*-methylpyridinium iodide (DASPMI) was used at the final concentration of 10^6 M. Cells were then observed by confocal microscopy. Cells containing the pCXJ3-mtGFP plasmid were grown in YPD plus G418 at 30°C and harvested in logarithmic phase. The GFP was detected by fluorescence microscopy, and cells were photographed directly from the culture.

Yeast Transformation and Selection of Suppressor Genes

The CPK1 strain was transformed to saturation with the yeast genomic library constructed in the pKep6 multicopy vector (kindly provided by Wesolowsky-Louvel) by electroporation (Sambrook *et al.*, 1989). All the Ura^+ transformants were replica plated on to YPD medium supplemented with 20 mM EGTA (Sigma). The plasmids isolated from the $Ura^+/EGTA^R$ transformants were used to transform the *Klpmr1*Δ strain. Plasmids capable of restoring the $EGTA^R$ phenotype to the *Klpmr1*Δ after retransformation were analyzed. Molecular analysis by restriction enzymes of the genomic fragments from the isolated plasmids showed that one of these plasmids carrying a fragment of about 3000 base pairs (bp) was able to restore the $EGTA^R$ phenotype. This fragment was subcloned into multicopy vector pCXJ3 to obtain the p60 plasmid and sequenced (MWG Biotech). The 2880-bp fragment contained the full ORF of *KlHSP60* plus 1000 bp upstream and 100 bp downstream.

Northern Blot Analysis

Total RNA of *K. lactis* strains was extracted by the hot phenol method (Schmitt *et al.*, 1990). The RNAs were quantified by absorption (OD_{260}) and separated by denaturing agarose electrophoresis. After electrophoresis the RNAs were transferred to nylon membranes and hybridized with ^{32}P-labeled random primed probes (Roche, Lewes, East Sussex, United Kingdom). All the probes were PCR amplified from the *K. lactis* DNA genome (the sequences were kindly provided by Prof. Bolotin-Fukuhara, Paris). The 900-bp PCR product of *KlIDP1* was obtained using primers 5′-CGATTGCCATTGCCCTAAGT-3′ and 5′-TGAAAGAGGCATGAGCGAAC-3′; the 800-bp PCR product of *KlSDH2* was obtained using primers 5′-TCTGCGATATCTACCTGGATTC-3′ and 5′-TATCCGTTTCCGTTAAGTTTTCAGA-3′; the 800-bp PCR product of *KlACO1* was obtained using primers 5′-ATGTTGTCTGCTCGTGTTGC-3′ and 5′-CGGATGTAGTGGCACCGATT-3′ and the 1700-bp PCR product of *KlHSP60* was obtained using primers 5′-GTACCGTAAAGCCAGGCAAT-3′ and 5′-GCATACCTGGCATACCACCTG-3′.

Respiration and Enzyme Activity

Measurements of respiratory activity were performed according to Ferrero *et al.* (1981). Preparation of mitochondria and determination of succinate dehydrogenase activity (SDH; EC 1.3.99.1) were carried out according to Lodi and Ferrero (1993). The SDH activity was expressed as nmol/min/mg protein.

Measurement of Intracellular Oxidation Levels

The oxidant-sensitive probe dihydrorhodamine 123 (Sigma) was used to measure intracellular oxidation levels in yeast according to Cabiscol *et al.* (2002).

Stress Condition and Viability

Yeast cells were grown aerobically at 28°C in YPD medium for 25 h and were challenged with hydrogen peroxide. This was directly added to the growth media to final concentration of 20 mM. Untreated cultures were incubated in parallel over the same periods. Viability was determined by colony counts on YPD plates after 2 and 5 h of incubation at 28°C and was expressed as the percentage of the corresponding control cultures. The values are the mean of three independent experiments with a SD $< 15\%$.

Protein Extracts and Immunoblot Analysis

Yeast strains were grown to OD_{600} of 1.0 at 28°C in SD medium. Cell extracts were obtained by using glass beads in ice-cold lysis buffer according to Alonso-Monge *et al.* (2003). Blots were probed with monoclonal antibody (mAb) to phospho-p38 MAP kinase (Cell Signaling Technology, Beverly, MA) and with polyclonal antibody to *S. cerevisiae* Hog1 (Santa Cruz Biotechnology, Santa Cruz, CA) according to Alonso-Monge *et al.* (2003). Blots probed with mAb anti-Hsp60 (Stress-Gen, Victoria, British Columbia, Canada) were treated as described in Schwock *et al.* (2004).

Assay of Zymolyase Sensitivity

Cells (5×10^8) grown in SD medium were collected by centrifugation and resuspended in 4 ml of buffered sorbitol (20 mM Tris containing 1.2 M sorbitol and 10 mM $MgCl_2$, pH 7.2). After 10 min of treatment with 3% 2-mercaptoethanol, 12.5 U of zymolyase 100T (Seikagaku Kogyo) were added, and the cells were incubated at 30°C under gentle agitation. Spheroplasts lysis after dilution in water was determined by measurements of OD_{660}.

Extract Preparation and EMSA

Yeast cell extracts were prepared as described (Schneider *et al.*, 1986). The sequence spanning the nucleotides −682 to −467 of *KlHSP60* promoter from ATG (5′-TGCCTCACGATTACAGAAGAGGATAGAACTCTTGTGTGTAT ATAGGAATT/GGG-3′) was synthesized as complementary oligonucleotides, annealed in vitro, and end-labeled with Klenow enzyme by standard methods. Binding reactions were carried out in 20 μl volumes containing 1 mM $MgCl_2$, 20 mM HEPES (pH 8.0), 5% glycerol, 0.1% Nonidet P-40, 1 mM DTT, 0.5 ng radiolabeled double-stranded DNA, 2 μg poly(dI-dC), and 10 μg of protein. Reaction mixes were incubated for 20 min at 28°C and loaded onto 6% polyacrylamide gels. Gels were preelectrophoresed for 1 h at 100 V and electrophoresed for 3–4 h at 200 V at room temperature. Gels were dried onto Whatman 3MM paper, visualized with a PhosphoImager, and autoradiographed.

RESULTS

Changes in Mitochondrial Morphology and Calcium Homeostasis Occur in KlPmr1Δ *Cells*

The inactivation of the *K. lactis* Golgi Ca^{2+}-ATPase, KlPmr1p, was recently demonstrated to result in altered mitochondrial metabolism (Farina *et al.*, 2004). Because mitochondrial morphology and dynamic motion are strongly associated with alterations in mitochondrial energy metabolism (Butow and Avadhani, 2004; Pham *et al.*, 2004), it was pertinent to investigate changes in mitochondrial structures in *Klpmr1*Δ cells. The mutant strain and its isogenic wild-type counterpart were analyzed by electron microscopy. In ultrathin sections of the wild-type cells (Figure 1A) the mitochondria appeared as tubular structures, with normal morphology and typical cell peripheral distribution. Ultrastructural analysis of the *Klpmr1*Δ strain indicated instead the presence of spherical mitochondria, located at the cell periphery (Figure 1B). Mitochondrial intramembranes did not appear to be affected, because *cristae* were detectable in both tubular and spherical mitochondria. Compared with the wild-type counterpart, increased cell wall thickness was also observed in *Klpmr1*Δ cells as previously reported (Uccelletti *et al.*, 1999).

In parallel, wild-type and deleted strains were transformed with a plasmid carrying the GFP fused to the mitochondrial signal sequence from the subunit 9 of the F_0-ATPase from *Neurospora crassa*; this construct has been demonstrated to correctly deliver functional GFP into mitochondria (Westermann and Neupert, 2000). The fluorescence microscope observation of the mitochondrial matrix showed a punctuated pattern for the mutant cells instead of the regular tubular network of these organelles in wild-type cells, in agreement with the structures observed by electron microscopy (Figure 1, D and C, respectively). Identical profiles were also observed when the vital staining DASPMI was used to observe mitochondria (unpublished data). These experiments confirmed the altered mitochondrial structures in the *Klpmr1*Δ cells.

The inactivation of the Golgi Ca^{2+}-ATPase in *S. cerevisiae* results in altered calcium homeostasis with increased calcium concentration in the cytosol of the mutated cells (Strayle *et al.*, 1999). These data prompted the analysis of Ca^{2+} homeostasis alterations at the mitochondrial level in *K. lactis* after *KlPMR1* deletion. A plasmid (mtAEQ) carrying the cDNA of the photoprotein aequorin fused to the mitochondrial signal sequence, previously utilized for GFP localization, was introduced into wild-type and mutant cells and used for the determination of mitochondrial Ca^{2+} content. An experimental set-up consisting of a perfusion chamber connected to a photon-counting device (described by Riz-

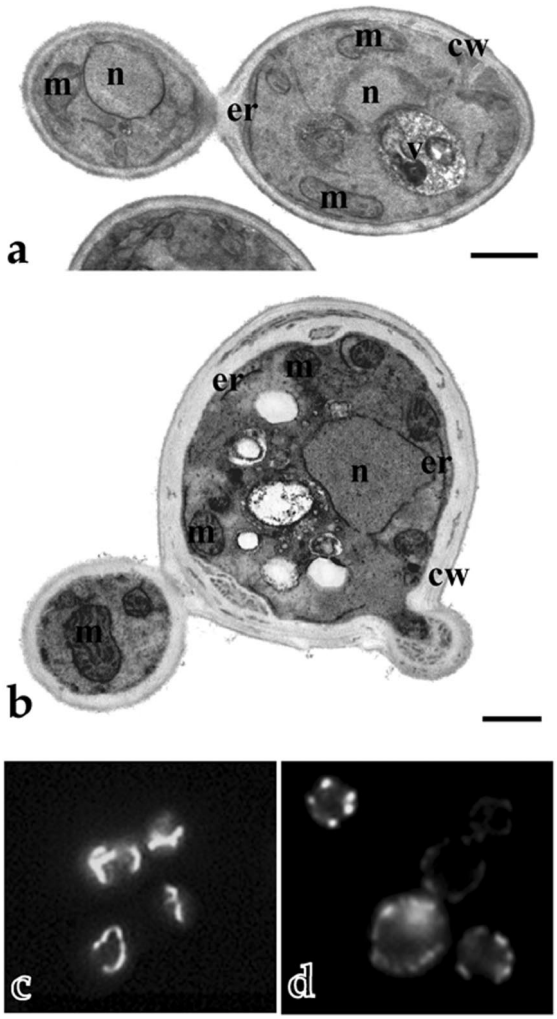

Figure 1. Klpmr1Δ cells have altered mitochondrial morphology. Micrographs of the wild-type and the *Klpmr1* Δ strains grown on minimal medium. Wild-type yeast cells show normal morphology and the typical peripheral distribution of mitochondria (a). In the *Klpmr1* Δ cells (b), the mitochondria appear as spherical structures, located at the cell periphery, without any visible inner membrane alterations. n, nucleus; m, mitochondrion; er, endoplasmic reticulum; v, vacuole; cw, cell wall. Bars, 2 μm. In panels c and d GFP fluorescence of the mitochondrial matrix in wild-type and mutant cells, respectively. Bars, 10 μm

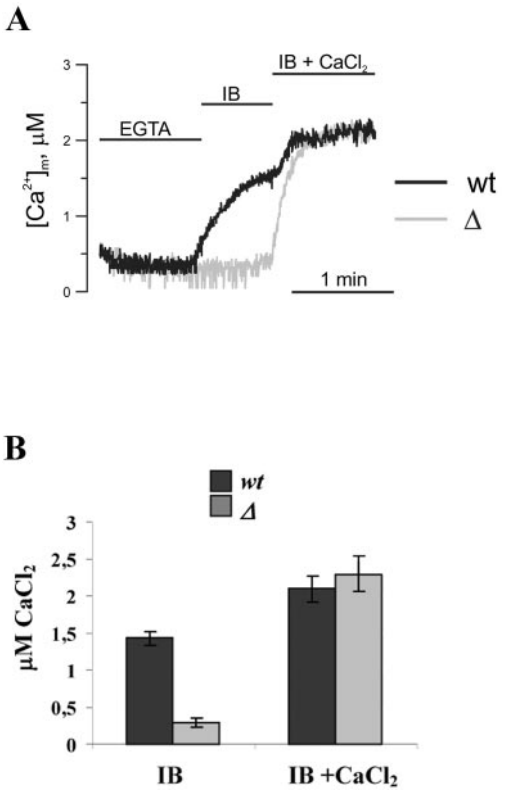

Figure 2. Measurement of calcium uptake in mitochondria of *KlPMR1* (wt) and *Klpmr1*Δ (Δ) cells.(A) A representative $[Ca^{2+}]$ trace in permeabilized cells expressing the mtAEQ, obtained during perfusion with either IB (Ca^{2+}-free condition) or with IB containing 20 μM Ca^{2+}. (B) Bar graph representation of data referring to the values of $[Ca^{2+}]_m$ measured in at least five independent experiments.

zuto *et al.*, 1994), which allowed efficient reconstitution of mitochondrial aequorin (~10^6 counts per coverslip) was utilized. Surprisingly, no differences in the mitochondrial calcium uptake per se were observed between wild-type and *Klpmr1*Δ cells. Indeed the two types of cells showed the same level of accumulation in presence of 20 μM Ca^{2+}. However mitochondria from *Klpmr1*Δ cells accumulate Ca^{2+} more slowly and reach a lower $[Ca^{2+}]_m$ level, when exposed to $[Ca^{2+}] < 5$ μM (Figure 2). This result could be part of a compensatory mechanism that protects mitochondria from the changes in the cytosolic calcium concentration that arises when the cells are depleted of the Ca^{2+}-ATPase. Intracellular calcium determinations, conducted using Fura-2AM as described by Kowaltowski *et al.*, 2000, indeed showed that the calcium content of the wild-type spheroplasts was almost half of the cation concentration present in the spheroplasts of *Klpmr1*Δ cells measured in the same conditions (137 ± 40 nM vs. 292 ± 30 nM, respectively).

Isolation of KlHSP60, *a Multicopy Suppressor of Altered Calcium Homeostasis in* Klpmr1Δ *Cells*

To gain insights on the mechanism(s) of calcium homeostasis in which *KlPMR1* is involved, we performed a genetic screen to identify multicopy suppressors able to rescue the growth defects of cells lacking KlPmr1p on rich medium containing 15 mM EGTA, a calcium chelator. Three of the plasmids, isolated from the corresponding clones that survived the selection procedure, proved to be identical and were further analyzed since the 4500 base pairs *K. lactis* genome insert had a restriction pattern different from the *KlPMR1* gene itself. A smaller fragment of ~3000 bp, subcloned in the multicopy plasmid pCXJ3 to construct the p60 plasmid, was still able, once introduced in the *Klpmr1*Δ cells, to promote growth in presence of the chelator (Figure 3A). Sequencing analysis revealed that the *K. lactis* DNA fragment present in the p60 plasmid contained an ORF of 1780 bp with 1000 bp upstream of the putative ATG start codon and 100 bp downstream of the putative stop codon. The protein encoded by this ORF resulted to be 87% identical (94% similar) to the *S. cerevisiae* mitochondrial chaperone Hsp60 and with similar degrees of identity with Hsp60s from other yeast species and higher eukaryotes (Figure 3B). Because of these similarities the *K. lactis* ORF will be subsequently referred to as *KlHSP60*.

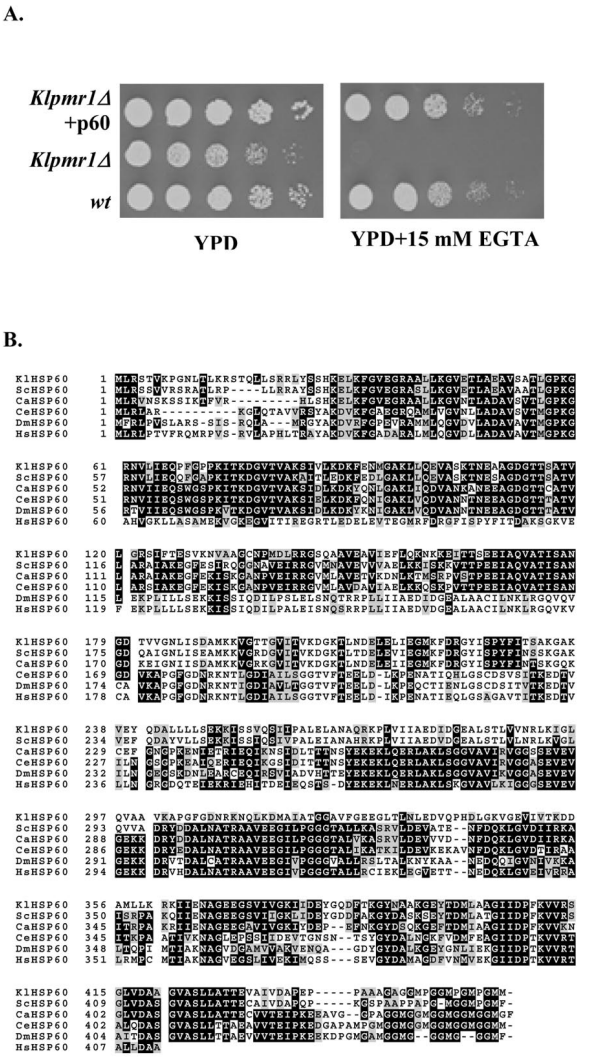

Figure 3. Isolation of *KlHSP60* as multicopy suppressor of KlPmr1p function. (A) Growth of yeast strains in the presence of 15 mM EGTA. YPD plates and YPD plates containing EGTA were spotted with 5 μl of 10-fold serial dilutions of cells grown in SD medium from *KlPMR1* (wt), *Klpmr1*Δ (Δ), and *Klpmr1*Δ transformed with the plasmid p60 (Δ+p60) strains. The growth at 28°C was monitored after 3 d; three independent transformants have been checked, obtaining identical results. (B) Comparison of the deduced amino acid sequence of KlHsp60 with related proteins. The BOXSHADE program was used; identical amino acids are shown on a black background, and a gray background indicates conservative substitutions. Accession numbers for the sequences used in the alignment are: *K. lactis* (Kl) XM455510, *S. cerevisiae* (Sc) M33301, *C. albicans* (Ca) AF085694, *Drosophila melanogaster* (Dm) NM078560, *Caenorhabditis elegans* (Ce) AAK84594.1, *Homo sapiens* (Hs) NP955472.1.

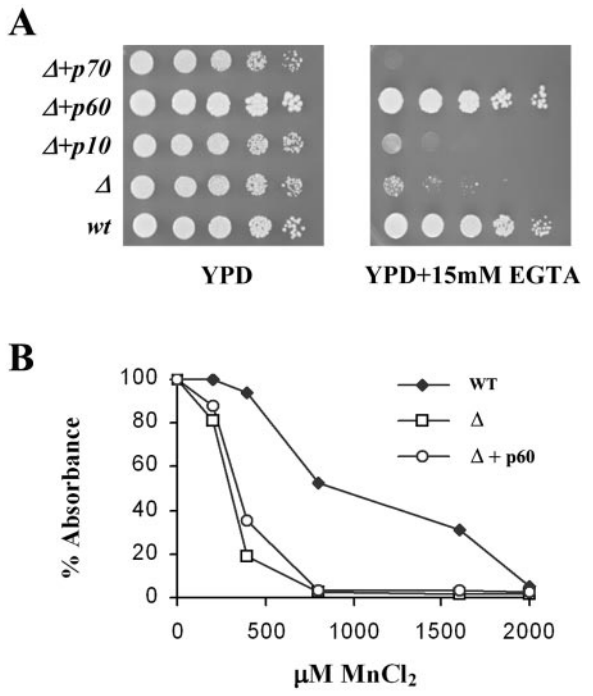

Figure 4. Specific genetic interaction between *KlPMR1* and *KlHSP60*. (A) Growth of the following strains: *KlPMR1* (wt), *Klpmr1*Δ (Δ), and *Klpmr1*Δ transformed with the three plasmids carrying the genes coding for the chaperones: KlHsp10, KlHsp60, and KlHsp70 (Δ+p10, Δ+p60 and Δ+p70, respectively). Serial dilutions of cells grown in minimal medium were spotted on YPD plates and YPD plates containing 15 mM EGTA. Growth at 28°C was scored after 3 d. Three different transformants of mutant cells with each plasmid gave the same results. (B) Growth of the following strains: *KlPMR1* (wt), *Klpmr1*Δ (Δ), and *Klpmr1*Δ transformed with the p60 plasmid (Δ+p60) in presence of different concentrations of $MnCl_2$ reported as percentages. The values are the mean of three independent experiments.

Hsp60 belongs to one of the two major chaperones classes, Hsp70 and Hsp60 in the mitochondrial matrix, that cooperate in the folding reaction of imported proteins in a sequential order. Preproteins first encounter mtHsp70 and only after being released they interact with the Hsp60 complex for proper folding. Hsp60 cooperates with the cochaperone Hsp10 that it is supposed to coordinate the behavior of the single Hsp60 monomers and regulate the ATP cycle (Dubaquie *et al.*, 1997).

The unexpected isolation of *KlHSP60* gene as multicopy suppressor of the calcium-related growth defect because of the lack of the Golgi Ca^{2+}-ATPase prompted the investigation of whether this phenotype correlated specifically with the chaperone Hsp60 or if it could be a general feature of mitochondrial chaperones. The *K. lactis* DNA sequences coding for proteins related to the *S. cerevisiae* Hsp70 and Hsp10 (respectively, 86 and 92% of similarity, between the two yeast species) were subcloned in the multicopy plasmid pCXJ3 to obtain the plasmids p70 and p10, respectively. The *Klpmr1*Δ cells were successively transformed with these plasmids for phenotype analysis. Neither overexpression of KlHsp70 or KlHsp10 was able to suppress the EGTA sensitivity of the mutant strain (Figure 4), indicating a novel and specific genetic interaction between *KlHSP60* and *KlPMR1*.

It has been previously demonstrated that KlPmr1p is the functional homologue of the *S. cerevisiae* counterpart Ca^{2+}/Mn^{2+} pump (Uccelletti *et al.*, 1999); it is possible that, in *K. lactis*, inactivation of the corresponding gene could result in altered manganese homeostasis and such alteration could be suppressed by overexpressing *KlHSP60*. The ability of the mutant cells to growth in presence of various concentrations of the cation was compared with that of wild-type cells. As reported in Figure 4B the *Klpmr1*Δ strain showed hypersensitivity toward manganese; the mutant cells showed a drastic reduction of growth when the medium was supple-

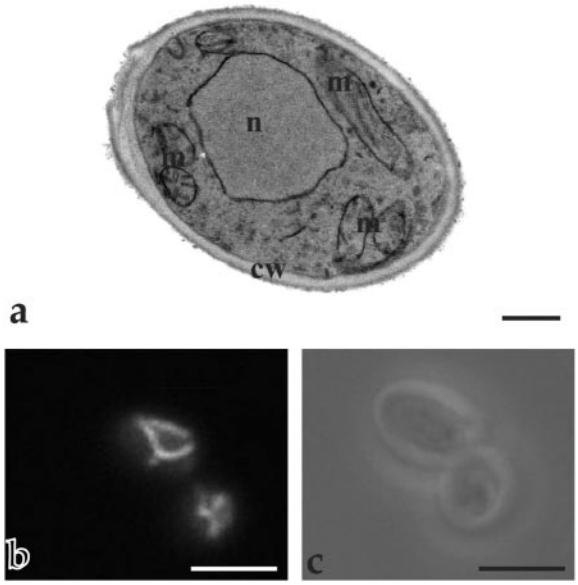

Figure 5. Mitochondrial morphology in *Klpmr1*Δ cells overexpressing *KlHSP60.* (a) Ultrathin section of *Klpmr1*Δ cells overexpressing *KlHSP60*; n, nucleus; m, mitochondrion; cw, cell wall. Bar, 2 μm. (b) DASPMI staining and (c) phase-contrast display of the same strain. Bar, 5 μm.

mented with 400 μM of manganese: 20% of mutant cells survived versus 94% of wild-type counterparts. A nearly identical behavior was observed for the *Klpmr1*Δ strain transformed with the p60 plasmid; this indicates that *KlHSP60* is not involved in manganese detoxification.

Altered Mitochondrial Phenotypes in the Klpmr1Δ *Strain Are Recovered by Increased Levels of* KlHSP60

To understand the relationship of calcium homeostasis with mitochondrial morphology in the mutant cells, the mitochondrial phenotypes in the cells carrying the p60 plasmid were analyzed.

The DASPMI staining (Figure 5B), supported also by the electron microscopy experiments (Figure 5A), showed a recovery of the network morphology (typical of wild-type mitochondria) for the mutant cells transformed with the p60 plasmid. Extension of part of the mitochondrial reticulum into a newly forming bud was also visible. The mitochondrial reticulum was positioned at the cell periphery and extended through the entire cell (Figure 5).

The suppression of morphological alterations by the *KlHSP60* gene was also accompanied by a reduction in the oxygen consumption as well as in SDH activity: values previously observed as increased in the mutant cells compared with wild-type cells (Farina *et al.,* 2004). In fact, the presence of the p60 plasmid in the mutant strain resulted in a reduction in oxygen consumption also with respect to wild-type cells (55 ± 4 vs. 67 ± 7 μl O_2/h/mg dw, respectively). The same pattern of behavior was observed when the SDH activity was measured, in this case the *Klpmr1*Δ strain carrying the p60 plasmid showed a reduction of 50% with respect to the wild-type strain (Table 1).

We previously demonstrated that the transcription of several genes involved in mitochondrial functions was altered in the *Klpmr1*Δ strain. Among them, *KlIDP1* (isocitrate dehydrogenase) as well as *KlSDH2* (a subunit of the SDH) and

Table 1. Respiratory activity and succinate dehydrogenase activity

Strain	EAU (nmol succ/min mg protein)	QO_2 (ml O_2 h/mg dry weight)
Wild type	130	67
KlPmr1Δ	180	95
KlPmr1Δ + p60	75	55

Cells were grown on YNB medium to stationary phase. The values reported represent the mean of three independent experiments; the single values agree within ±10%.

KlACO1 (aconitase) were down-regulated in mutant cells. Because the overexpression of the mitochondrial chaperone was able to recover the mitochondrial structures and oxygen consumption to wild-type levels, it was thought that the *Klpmr1*Δ cells transformed with the plasmid p60 would have normal mRNA levels of the above genes. Northern analyses were performed on total RNA extracts from wild type, *Klpmr1*Δ, and the mutant carrying the p60 plasmid grown in minimal medium. *KlIDP1*, *KlACO1*, and *KlSDH2* were utilized as probes (Figure 6). Densitometric analysis revealed that the same amounts of these transcripts were present in wild type and in the mutant strain transformed with p60 plasmid, whereas it was possible to observe their reduction in the *Klpmr1*Δ cells.

The suppression of these mitochondrial phenotypes strongly suggests that KlHsp60 is involved in *K. lactis* mitochondrial signaling between mitochondrion and nucleus when calcium homeostasis is altered by inactivation of *KlPMR1*.

Oxidative Stress Is Present in Klpmr1Δ *Cells and Can Be Suppressed by Increasing the Expression of KlHsp60*

The sensitivity of the *Klpmr1*Δ strain as compared with wild-type counterpart to hydrogen peroxide was analyzed, keeping in mind that phenotypes related to oxidative stress could occur because increases in oxygen consumption and in cytosolic calcium levels were present in such a mutant. The survival of the cells as percentage of CFUs (colony forming units) after challenging the cells with 20 mM hydrogen peroxide for 2 or 5 h with respect to untreated cells was monitored (Figure 7A). A drastic reduction in the survival rate was indeed observed in cells deleted in *KlPMR1* already after 2 h of treatment: 17 versus 80% of the wild-type counterpart. After 5 h the percentage of CFUs from mutant cells decreased to 10%, whereas the CFUs from wild-type strain was 47%.

Cabiscol *et al.* (2002) showed that, in *S. cerevisiae,* the level of Hsp60 is critical in the protection against oxidative stress, so the level of the *HSP60* mRNA in wild-type and mutant cells was analyzed. Densitometric analysis of the Northern blot showed a reduction of 50% of the signal for the mRNA from the mutant cells (Figure 7B). This reduction was also revealed at the protein level, where Hsp60 antibodies were used against protein extracts from wild-type and mutant cells (Figure 7C). As a control, the mutant cells transformed with the plasmid p60 showed an increased expression of the chaperone (Figure 7C).

Deletion of *KlPMR1* function decreased expression of KlHsp60, indicating that *KlPMR1* function is required for the expression of *KlHSP60* mRNA. The 1-kb region of the putative *KlHSP60* promoter contains several sequences that match the consensus-binding site of HSF. As HSF may pro-

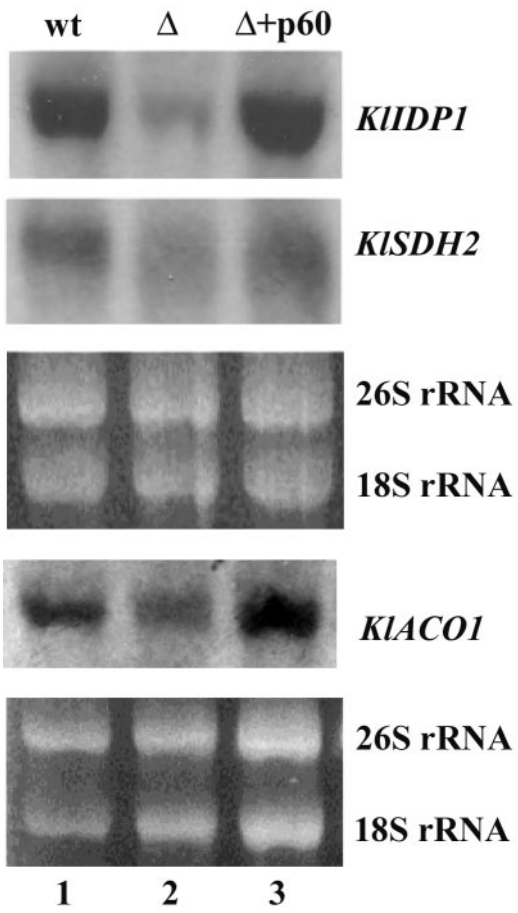

Figure 6. Expression pattern of mitochondrial enzyme genes. Northern blot analyses of *KlIDP1*, *KlSDH2*, and *KlACO1* in *KlPMR1* (wt), *Klpmr1*Δ (Δ), and *Klpmr1*Δ transformed with the plasmid p60 (Δ+p60). Total RNA was extracted from these strains (lanes 1, 2, and 3, respectively) grown on SD medium. The same amount of RNA (10 μg) was loaded on each lane. The ethidium bromide-stained gels of the autoradiogram are shown in the lower part of the panel. Densitometric quantification of mRNA was performed by the computer program Phoretix 1D and mRNA loading was normalized using the rRNAs bands.

mote expression of *KlHSP60,* the impact of *KlPMR1* deletion on the DNA-binding activity of HSF was investigated. HSF activity was assayed in nuclear extract derived from both wild-type and *Klpmr1*Δ cells, using a 50-bp fragment derived from the *KlHSP60* promoter as a probe in electrophoretic mobility shift assay (EMSA). Compared with wild-type control cells (Figure 7D, lane 2), HSF DNA binding activity was decreased up to 80–90% in extracts prepared from *KlPMR1* deleted cells (Figure 7D, lane 3). In lane 4, complex formation with the HSE-labeled oligonucleotides was efficiently competed when wild-type extracts were preincubated with a 100-fold molar excess of cold HSP-promoter oligonucleotides, and in lane 5, treatment with a 100-fold molar excess of cold nonspecific oligonucleotides before the addition of the probe did not have competitive effects, indicating that the binding to HSE is specific. The changes taking place in cellular calcium in the *Klpmr1*Δ strain were analyzed to see if they were responsible for the reduced expression of KlHsp60. Wild-type cells were incubated with different concentrations of the calcium ionophore A23187 to increase cytosolic calcium levels (Ohsumi and Anraku, 1983). Western blot analysis on the corresponding protein extracts found a decrease in the KlHsp60 level together with a drastic reduction for the HSF-binding activity of wild-type extracts (Figure 7E).

At this point we asked if the presence of the p60 plasmid could be able to relieve the H_2O_2 sensitivity that occurs in the mutant strain. Growth in the presence of hydrogen peroxide of *Klpmr1*Δ cells in comparison to wild type and to the mutant strain transformed with the p60 plasmid was analyzed (Figure 8A). The increased gene dosage of *KlHSP60* clearly restored the growth capabilities of the mutant cells on H_2O_2 to wild-type level. In addition, the accumulation of reactive oxygen species (ROS) in the mutant and wild-type strains was analyzed by incubating the cells with the fluorescent dye dihydrorhodamine 123. This compound accumulates inside the cells and is oxidized by ROS to the corresponding fluorescent cromophore. Cells lacking *KlPMR1* showed an intense intracellular staining for at least 40% of the population compared with the marginal fluorescence presented in <5% of the wild-type cells (Figure 8B, b and a, respectively). The staining was almost reduced to wild-type level, <10%, by overexpression of the mitochondrial chaperone in the mutant strain (Figure 8Bc), in agreement with the reduced oxygen consumption observed in these cells.

It has been shown that in *S. cerevisiae* the iron chelator, DFO, protects low Hsp60-expressing cells from oxidative stress (Cabiscol *et al.,* 2002). Because in *K. lactis,* EGTA sensitivity of *Klpmr1*Δ cells was suppressed by increasing dosage of the mitochondrial chaperone, it is possible that the growth of mutant cells would be improved by the addition of DFO. In fact addition of 5 μM DFO, together with EGTA to the growth medium, doubled the growth of the *Klpmr1*Δ strain with respect to the growth in presence of EGTA alone. The presence of the iron chelator alone in the growth medium of the mutant cells improved the growth capability up to a wild-type level (Figure 8C). Similar results (unpublished data) were also obtained when DFO was replaced by 10 μg/ml PBN, a quencher of intracellular ROS (Madeo *et al.,* 1999).

Hog1 MAPK Is Activated in Golgi Ca^{2+}-ATPase-deleted Cells

In comparison with wild-type cells, the *Klpmr1*Δ mutant shows increased cell wall thickness and some dark-stained rims appeared within the amorphous layer (Figure 9, A and B). Notably, in cells lacking *KlPMR1* (Figure 9C), but transformed with the p60 plasmid, the thickness of the cell wall was reverted and resembled that of the wild-type parent. In addition using an assay based on the rate of formation of spheroplasts from exponentially growing yeast cells (Ovalle *et al.,* 1998), we found that cell wall functionality was affected in *Klpmr1*Δ cells. Indeed, the cell wall of *Klpmr1*Δ cells was more resistant to lysis than that of wild-type cells (Figure 9D) when treated with zymolyase, a preparation essentially containing 1,3-β-glucanase activity. The presence of the p60 plasmid in the mutant strain allowed these cells to respond to the zymolyase treatment exactly as the wild type, indicating a recovery in the cell wall architecture. Interestingly, this phenotype could be ascribed to the activation of a signaling pathway that generates a compensatory mechanism required for counterbalancing the decay in mechanical strength of the altered cell wall. Indeed, it has been shown that both *C. albicans* and *S. cerevisiae* cells under oxidative stress produce an adaptive response that protects them from lethal effects and activation of components of the cell wall biosynthesis represents at least one of the signals that adapt

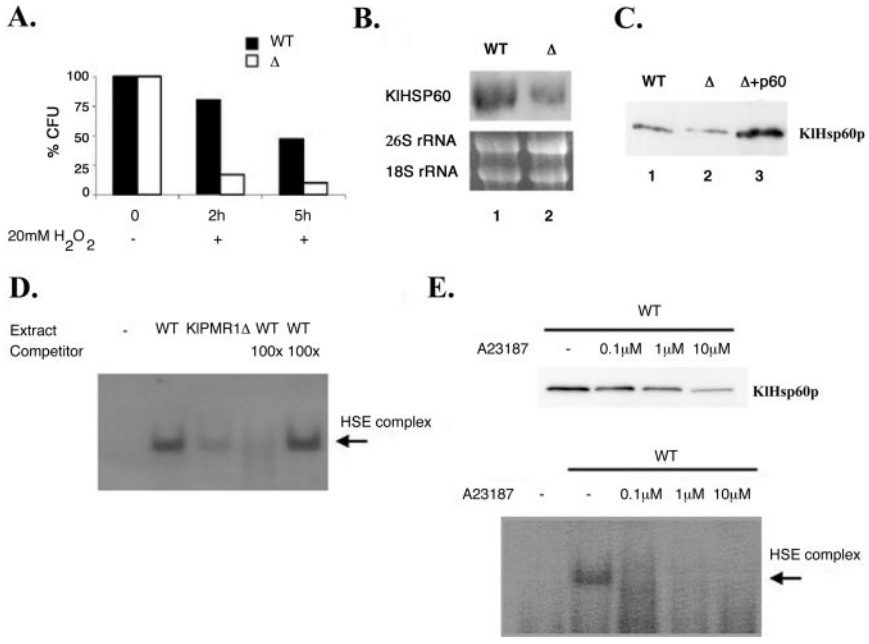

Figure 7. Oxidative stress in *KlPMR*-deleted cells. (A) *KlPMR1* and *Klpmr1*Δ cells grown in YPD were challenged with 20 mM H_2O_2 for 2 and 5 h. Cell viability (CFU) is expressed as the percentage of the corresponding control cultures with an SD < 10%. The values are the mean of three independent experiments. (B) Northern blot analysis of *KlHSP60* in *KlPMR1* and *Klpmr1*Δ cells. The same amount of total RNA (20 μg) from the strains was loaded on each lane; the ethidium bromide-stained gel of the autoradiogram is shown in the bottom part of the panel. Densitometric quantification of mRNA was performed by the computer program Phoretix 1D and mRNA loading was normalized using the rRNAs bands. (C) Western blot analysis of KlHsp60 in total protein extracts from *KlPMR1* (wt), *Klpmr1*Δ (Δ), and *Klpmr1*Δ transformed with the plasmid p60 (Δ+p60; lanes 1, 2, and 3, respectively) was performed as described in *Material and Methods*. Densitometric quantification of bands was analyzed by the computer program Phoretix 1D. (D) Suppression of HSF binding activity by *KlPMR1* deletion. HSF activity analyzed by EMSA in nuclear extracts from both wild-type and *Klmr1*Δ cells in lanes 2 and 3, respectively. In lane 4 wild-type extracts were preincubated with a 100-fold molar excess of cold HSP-promoter oligonucleotides, and in lane 5 with a 100-fold molar excess of cold nonspecific oligonucleotides before the addition of the probe; in lane 1 free-probe. (E) Analysis of KlHsp60 expression and HSF-binding activity in wild-type cells pretreated with different concentrations of calcium ionophore. In the top part of the panel Western blot analysis of KlHsp60 in total protein extracts from *KlPMR1* (wt); in the bottom part of the panel HSF activity was analyzed by EMSA in nuclear extracts from *KlPMR1* (wt).

cells to peroxide stress. Activation of the Hog pathway has been shown to play an important role in such adaptative response. (Toone *et al.,* 1998; Alonso-Monge *et al.,* 2003; Chauman *et al.,* 2003; Bilsland *et al.,* 2004). Hallmark of such responses is the phosphorylation of the Hog1 MAPK. Investigation of the steady state levels of the Hog1 phosphorylation in *Klpmr1*Δ cells with respect to the wild-type counterparts was undertaken. Western blot analysis with the mAb against phospho-p38 in wild type, mutant, and mutant carrying p60 plasmid detected a band of ~50 kDa, a size in agreement with that obtained in the case of *S. cerevisiae* (Brewster *et al.,* 1993; Figure 10). In fact, from the *K. lactis* genome sequence deposited in EMBL (Dujon *et al.,* 2004) the Hog1p-related protein resulted highly similar (81% identity) to the *S. cerevisiae* counterpart. On the basis of this, after stripping of the anti-phospho-p38 antibody, we immunoblotted the same membrane with a commercial ScHog1 polyclonal antibody, which recognized a band of the same size and that was then used as a loading control. Image densitometry revealed that, when the anti-phospho-p38 mAb was used, the intensity of the signal from *Klpmr1*Δ protein extracts was fivefold of that from wild type (Figure 10). Interestingly, the presence of the p60 plasmid in *Klpmr1*Δ strain was able to reduce to wild-type levels the activation of KlHog1, as seen by its phosphorylation status. In summary, our data indicate that there is a complete suppression of cell wall defects back to wild-type levels in cells lacking the Golgi Ca^{2+}-ATPase by increased dosage of the mitochondrial chaperone. Furthermore, high levels of pHog1 correlate with low levels of *KlHSP60* expression, suggesting that, also in *K. lactis,* oxidative stress results in the activation of the Hog pathway and that it could act as a compensatory response that protects cells from the lethal effects of oxidative stress.

DISCUSSION

In yeast whole cell Ca^{2+} concentration appear to be regulated in the submicromolar range primarily by transporters found in the plasma membrane, the vacuole, and the Golgi complex. The system is not as well understood as its counterpart in mammalian cells, in particular with regard to interactions with mitochondria.

A study that used yeast mitochondria loaded with a Ca^{2+} indicator (fluo-3) showed that the matrix space Ca^{2+} concentration is established by a simple equilibration with the extramitochondrial concentration (Uribe *et al.,* 1992). One difference from higher eukaryotes is that yeast mitochondria do not contain a calcium uniporter but a general diffusion pore in the inner membrane and are subject to a permeability transition (Jung *et al.,* 1997, 2004). In the yeast *K. lactis* a similar system is probably present; in fact the uncoupler FCCP does not affect Ca^{2+} accumulation in mitochondria (our unpublished observations).

The inactivation of KlPmr1p, the Golgi Ca^{2+}-ATPase of *K. lactis*, affects the functioning of mitochondria as illustrated by the increase in respiratory activity and by the changes in the transcription level and activity of some mitochondrial enzymes (Farina *et al.,* 2004). We report here that when *KlPMR1* is deleted, mitochondrial structures are also altered, whereas $[Ca^{2+}]_{cyt}$ is increased and that mitochondrial calcium homeostasis is subtly impaired. Mitochondrial integrity is a crucial factor in maintaining protection against oxidative stress and we, consistently, show here that *KlPMR1* inactivation results in an increased oxidative stress sensitivity. However, we found that the mitochondrial chaperone KlHsp60 can act as a multicopy suppressor of the calcium-related phenotypes originated by the inactivation of *KlPMR1,* but does not suppress the manganese hypersensitivity of the mutant cells. In *S. cerevisiae* Hsp60 is an essential

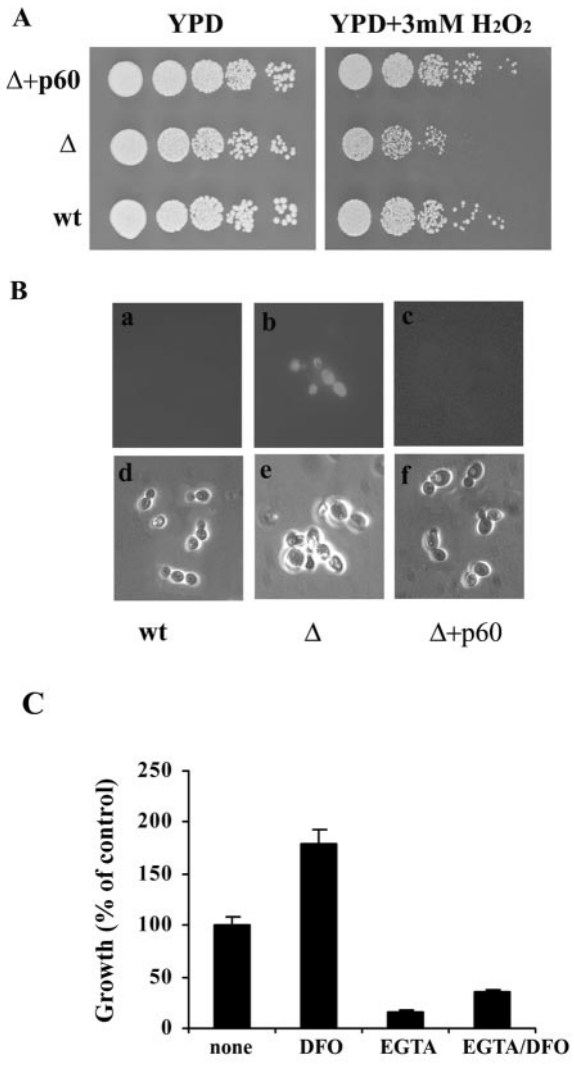

Figure 8. Rescue of oxidative stress in the *Klpmr1*Δ strain by overexpression of *KlHSP60*. (A) Growth of yeast strains in the presence of 3 mM H_2O_2. YPD plates and YPD plates containing 3 mM H_2O_2 were spotted with 5 μl of 10-fold serial dilutions of cells grown in SD medium from *KlPMR1* (wt), *Klpmr1*Δ (Δ), and *Klpmr1*Δ transformed with the plasmid p60 (Δ+p60) cell. Growth at 28°C was monitored after 3 d and three independent transformants of *Klpmr1*Δ with p60 gave identical results. (B) Cell fluorescence (a–c) and phase contrast display of cells (d–f) after 2-h incubation with dihydrorhodamine 123. *KlPMR1* (wt; a, d); *Klpmr1*Δ (Δ; b, e); *Klpmr1*Δ transformed with p60 (Δ+p60; c, f). (C) Growth of *Klpmr1*Δ strain in presence of 10 μM EGTA and/or 10 mM DFO reported as OD_{600} percentage with respect to cells grown in YPD.

chaperone involved in the proper folding of many proteins imported in the mitochondrial matrix or in the intermembrane space. Such function requires the participation of the cochaperone Hsp10 and is ATP dependent (Dubaquie *et al.*, 1997). In *K. lactis* the role of KlHsp60 in suppressing the alterations occurring in *Klpmr1*Δ cells appears to be specific because it cannot be substituted by the overexpression of the cochaperone KlHsp10 or by the overexpression of the other mitochondrial chaperone KlHsp70. *HSP60* is reported as a constitutively expressed gene under normal growth conditions because of its relevance for proper mitochondria functionality and is essential even at physiological temperature (Cheng *et al.*, 1989). The amount of such protein is increased as a result of several kinds of stress, including heat stress (Madura and Prakash, 1990). Hsp60 has been described as a component of the oxidative stress defense because of its protective role in supporting Fe/S proteins such as Aco1p (aconitase) and Sdh2p (SDH; Cabiscol *et al.*, 2002). The *Klpmr1*Δ cells have a reduced amount of KlHsp60 and the transcription of the corresponding gene is also reduced. As a consequence the mutant strain is under oxidative stress, as indicated by increased respiration rate and the amount of ROS accumulated in the cells. Increased levels of KlHsp60 cause a reduction in O_2 uptake and SDH activity even below wild-type values. The ability of KlHsp60 to function as a multicopy suppressor of *Klpmr1*Δ may thus be due, at least in part, to an increased capacity to deal with ongoing oxidative stress. This suggests a direct and localized role of KlHsp60 in maintaining mitochondrial integrity and in control of ROS-generating activities, both of which are crucial factors in determining cell viability. This role was further supported by the highly improved growth of the mutant cells in presence of the iron chelator DFO; such a molecule was also able to ameliorate the growth of the mutant in presence of EGTA.

Expression of Hsp(s) is mediated by HSFs. In *K. lactis*, a single gene encodes HSF (Jakobsen and Pelham, 1991). We report here that the 1-kb region of the putative *KlHSP60* promoter contains several sequences that match the consensus-binding site of HSF. We have explored the impact of *KlPMR1* deletion on the DNA-binding activity of HSF and found that HSF DNA-binding activity was decreased by 80–90% in extracts prepared from *KlPMR1*-deleted cells. The calcium ionophore experiments strongly support the view that the cytosolic calcium changes occurring in *Klpmr1*Δ strain are responsible for the reduced KlHsp60 expression mediated by HSF. The suppression in *Klpmr1*Δ cells of the oxidative stress related phenotypes by increasing the levels of *KlHSP60* indicates that a *PMR1/HSP60* module is the principal route for detoxification of endogenous oxidative stress and HSF-mediated expression of *KlHSP60* is required in order to maintain operative this module.

The major findings of this report are schematized in Figure 11. The alteration of calcium homeostasis, as a result of the absence of Klpmr1p, disrupts the mitochondria-nucleus signaling of the ongoing oxidative stress mechanisms and the activation of the protective system involving *HSF/KlHSP60* is not achieved. In *Klpmr1*Δ cells the expression of *KlHSP60* is reduced and such cells are, in fact, more sensitive toward external ROS-generating molecules. When the amount of KlHsp60 is restored, by increasing the gene copy number in the mutant cells, the normal mitochondrial tubular network is reconstituted, the oxidative stress phenotypes disappear, the transcription levels of mitochondrial enzymes recover the wild-type values and resistance to H_2O_2 is indistinguishable from that of wild-type cells.

Mutants of *S. cerevisiae* lacking superoxide dismutase suffer from oxidative damage that can be suppressed by mutations in *PMR1*; in such cases changes in manganese homeostasis have been demonstrated to be part of the mechanism involved (Lapinskas *et al.*, 1995). Polychlorinated biphenyls inhibit cell growth of *PMR1*-deficient *S. cerevisiae* cells through accumulation of intracellular hydrogen peroxide (Ryu *et al.*, 2003). We found that inactivation of *PMR1* in *S. cerevisiae* caused hypersensitivity toward H_2O_2; however, no increase in O_2 uptake or reduction in *HSP60* transcription was present (our unpublished results). *S. cerevisae* is a facultative aerobic yeast whereas *K. lactis* is obli-

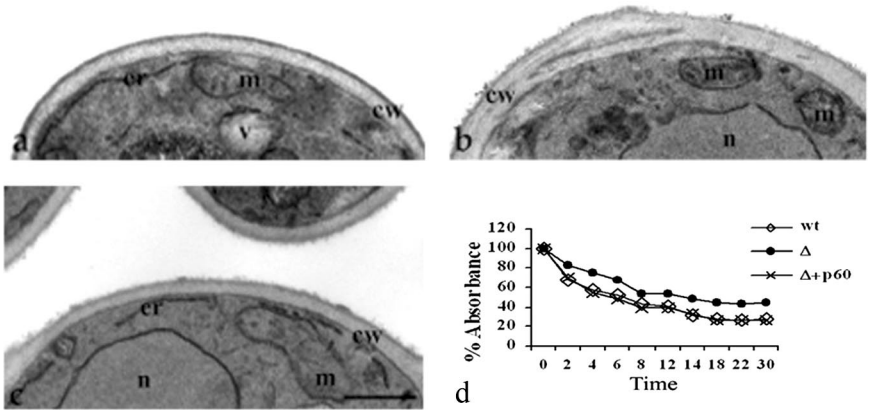

Figure 9. Increased gene level of *KlHSP60* rescues the normal thickness of the cell wall in *Klpmr1*Δ cells. Cells were grown in SD medium and prepared for electron microscopy as described in *Materials and Methods.* (a, b, and c) Sections from *KlPMR1* (wt), *Klpmr1*Δ (Δ), and *Klpmr1*Δ transformed with p60 (D+p60) cells, respectively. n, nucleus; m, mitochondrion; er, endoplasmic reticulum; cw, cell wall. Bars, 2 μm. (d) Cell lysis sensitivity after treatment with zymolyase reported as decrease in OD_{600} percentage after osmotic shock is shown.

gate respiratory yeast; this could be reflected in a different organization of the responses to oxidative stress.

Dealing with stresses requires coordination and regulation of the components of the various defense systems. For oxidative stress, several signaling pathways have been implicated in mammals and yeasts. The yeast relative of p38 family, Hog1p, the MAPK of the high-osmolarity glycerol (HOG) pathway, was initially known to activate upon osmostress, resulting in glycerol accumulation as a defense mechanism for the cells (Brewster *et al.,* 1993). Now this pathway is also known to be activated upon heat or citric acid stress (Winkler *et al.,* 2002; Lawrence *et al.,* 2004). Recently it has been demonstrated that, in *S. cerevisiae* as in *Schizosaccharomyces pombe* and *Candida albicans,* the Hog1 MAPK cascade is activated upon oxidative stress (Toone *et al.,* 1998; Alonso-Monge *et al.,* 2003; Bilsland *et al.,* 2004) and that the MAPK-activated protein kinase (MAPKAPK) Rck2, a member of the Calcium/calmodulin-dependent protein Kinase family (Teige *et al.,* 2001), is involved in the response pathway (Bilsland *et al.,* 2004). In *S. cerevisiae* and *C. albicans* the HOG pathway contributes also to cell wall modeling, e.g., by influencing the expression of genes encoding cell wall-modifying enzymes (Jiang *et al.,* 1995; Alonso-Monge *et al.,* 2001).

The strategy used by yeast cells to counteract oxidative stress appears to be complex. Thus, resistance to oxidative stress is associated not only with increased activity of enzyme(s), which control ROS-generating activities, but is also partially superimposed on the so-called compensatory

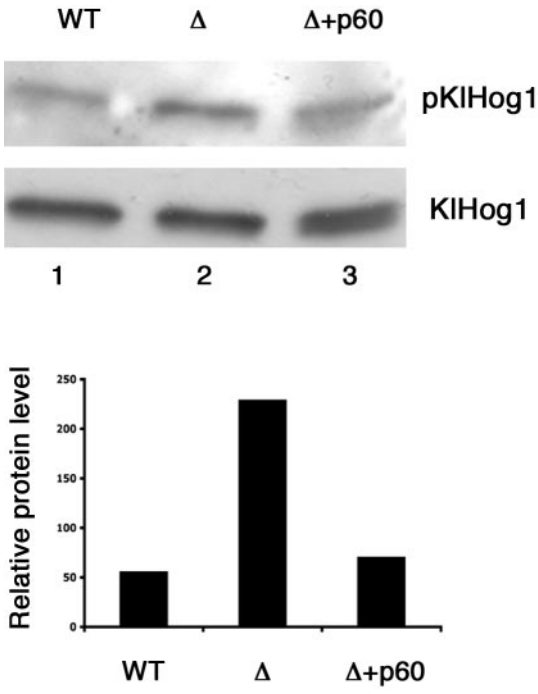

Figure 10. Hog1 activation in Golgi Ca^{2+}-ATPase *KlPMR1* deleted cells. Western blot analysis to detect Hog1 phosphorylation in total protein extracts from *KlPMR1* (wt), *Klpmr1*Δ (Δ), and *Klpmr1*Δ transformed with the plasmid p60 (Δ+p60) (lanes 1, 2, and 3, respectively). The same blot was assayed with a mAb raised against phospho-p38 and a polyclonal antibody raised against *S. cerevisiae* Hog1 as described in *Materials and Methods.* In the lower part of the panel densitometric quantification of bands analyzed by the computer program Phoretix 1D.

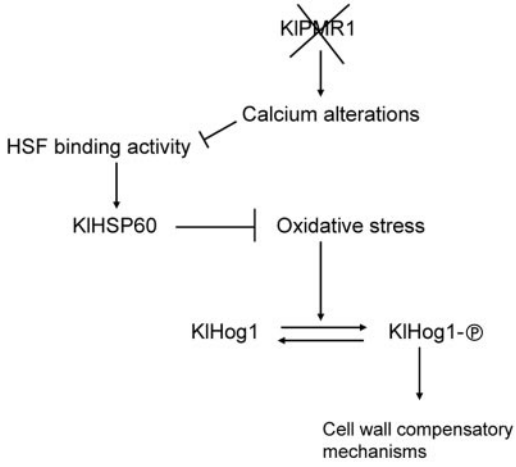

Figure 11. A model for *KlPMR1* mediated oxidative stress protection. KlPmr1p controls calcium homeostasis and deletion of the corresponding gene results in reduction of HSF DNA-binding activity, which is required for sustained *KlHSP60* expression. The loss of the KlHsp60 deprives cells of a key mediator of oxidative stress defense, this results in increase of ROS and oxidative stress thereby leading to activation of Hog1-dependent defense pathways, which include cell wall compensatory responses.

mechanism(s) aimed at preserving cell integrity through cell wall remodeling.

Cell wall thickness and functionality were affected in *Klpmr1*Δ cells. At the same time *Klpmr1*Δ cells have reduced amounts of KlHsp60 and are under oxidative stress. The activation of KlHog1p could originate from any one of these conditions as well as from the increased cytosolic calcium due to the lack of the Golgi Ca^{2+}-ATPase. In *S. cerevisiae* hyperosmotic shock induces the HOG pathway and this activation is accompanied by an increase in the cytosolic calcium concentration due, in this case, to an influx of extracellular calcium (Matsumoto *et al.*, 2002). The overexpression of KlHsp60 in *Klpmr1*Δ cells is able to suppress all the calcium-related phenotypes and also results in restoration of wild-type phosphorylation levels of KlHog1p as well as relief from oxidative stress. This supports KlHsp60 as a key player in the cross-talk between the calcium and HOG signaling taking place in *K. lactis*.

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