Inactivation of the KlPMR1 gene of Kluyveromyces lactis results in defective cell-wall morphogenesis

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The P-type Ca\textsuperscript{2+}-ATPases are the transporters responsible for calcium homeostasis in the cell compartments of eukaryotes. The KlPMR1 gene of Kluyveromyces lactis encodes a P-type Ca\textsuperscript{2+}-ATPase, which is functionally and structurally homologous to Pmrlp of Saccharomyces cerevisiae, the calcium pump localized in the Golgi membranes. In this work, a novel involvement of KlPmrlp in cell-wall morphogenesis of K. lactis is reported. Klpmr1A cells exhibited the loss of outer-chain extension in the glycosylation of secreted proteins. The absence of KlPmrlp resulted in the accumulation of round, large cells with an abnormally thick cell wall, as revealed by transmission electron microscopy. The deletant strain also showed a delocalized deposition of chitin in the lateral cell wall accompanied by an unbalanced ratio of insoluble to soluble glucans. These morphological defects were accompanied by the presence of irregularly shaped nuclei and by a DNA content greater than 2n. Addition of 10 mM Ca\textsuperscript{2+} to the medium of the Klpmr1A strain reversed the chitin-deposition impairment, recovered the alteration to the glucan ratio and restored a normal thickness of the cell wall. The mutant cells resumed wild-type size, shape and nuclear morphology but the DNA content indicated the persistence of defects in the co-ordination between DNA replication and cell division. The glycosylation defects were completely unaffected by the calcium supplement. These results indicate that calcium homeostasis controlled by KlPmrlp plays an important role in the cell-wall morphogenesis of K. lactis.

Keywords: yeast, glycosylation, calcium homeostasis, Ca\textsuperscript{2+}-ATPase

INTRODUCTION

The asymmetric distribution of calcium ions in the subcellular compartments of eukaryotic organisms plays a central role in the control of many cellular processes. The structures and mechanisms responsible for the modification, sorting and delivery of proteins to specific locations are part of the cellular machinery that is deeply influenced by the ionic context. The Ca\textsuperscript{2+} concentration in the endoplasmic reticulum (ER) has been implicated in the retention of resident proteins (Booth & Koch, 1989) as well as in the release of secretory proteins (Sambrook, 1990). Protein folding (Gething & Sambrook, 1992), interaction of misfolded proteins with the BiP chaperone (Suzuki et al., 1991) and protein degradation are also affected by the Ca\textsuperscript{2+} content in the ER (Wileman et al., 1991). A high concentration of calcium has also been reported in the Golgi apparatus (Pezzati et al., 1997). In this compartment, calcium ions are involved in the glycosylation of secreted proteins (Rudolph et al., 1989), the control of protein trafficking and sorting (Carnell & Moore, 1994), the selective aggregation of cargo proteins (Chanat & Huttner, 1991), and precursor processing in yeast (Fuller et al., 1989) and higher eukaryotes (Oda, 1992). In Saccharomyces cerevisiae, changes in the intracellular calcium concentration control and interconnect several signalling pathways, one of the relevant targets being the regulation of the cell cycle and related morphogenetic events:
(Means, 1994; Cid et al., 1995; Clapham, 1995; Iida et al., 1990; Mizunuma et al., 1998). A recent report (Mondéret et al., 1997) established that the polarity of the secretory machinery, probably together with N-linked glycosylation processes, also plays a role in controlling polarized growth and cell-wall morphogenesis in yeast cells. A set of complex connections can thus be envisaged linking Ca\(^{2+}\) homeostasis, secretion processes and the biogenesis of the cell wall in yeast.

Calcium gradients are maintained through the action of the Ca\(^{2+}\)-ATPases, members of a conserved family of cation transporters, the so-called P-type pumps. Genes encoding P-type Ca\(^{2+}\)-ATPases of the early secretory pathway have been isolated from a variety of organisms; the Ca\(^{2+}\) pump encoded by the PMR1 gene of \textit{S. cerevisiae} is perhaps the best characterized (Rudolph et al., 1989; Harmsen et al., 1993; Sorin et al., 1997; Dürr et al., 1998). This protein localizes in the membranes of the Golgi apparatus (Antebi & Fink, 1992) and inactivation of PMR1 gave rise to a complex set of phenotypes. Mutant cells exhibited calcium-related growth defects (Rudolph et al., 1989), reduced glycosylation of secreted invertase (Rudolph et al., 1989; Dürr et al., 1998), enhanced release of heterologous proteins (Moir et al., 1987; Harmsen et al., 1993, 1997) and defective sorting of carboxypeptidase Y (Antebi & Fink, 1992). Recent studies have demonstrated that Mn\(^{2+}\) can partially substitute for the Ca\(^{2+}\) requirement and that Pmr1p can operate as a common transporter for Ca\(^{2+}\) and Mn\(^{2+}\) (Dürr et al., 1998). Most noticeably, these authors demonstrated that the ion balance depending on Pmr1p, the Ca\(^{2+}\) pump localized in the Golgi membranes, could affect the export of incorrectly folded proteins from the ER, a topologically distinct compartment. Taken together, the above results emphasize the importance of Pmr1p functions in the early secretory pathway of \textit{S. cerevisiae}.

We have recently cloned KIPMR1, the gene of \textit{K. lactis} encoding the Pmr1p functional homologue (EMBL accession number AJ001018) and we have demonstrated that the disruption of KIPMR1 resulted in calcium-related growth defects and hypersecretivity to EGT (Uccelletti et al., 1999). \textit{K. lactis} has been reported to have superior secretory capabilities to \textit{S. cerevisiae} (Van den Berg et al., 1990; Rocha et al., 1996; Tokunaga et al., 1997; Walsh & Bergquist, 1997), but its secretory system is almost unexplored. In the context of studies aimed at investigating secretory processes in \textit{K. lactis}, we provide here evidence of a novel connection between the calcium homeostasis mediated by KIPmr1p and the morphogenesis of the cell wall in \textit{K. lactis}.

### METHODS

#### Yeast strains, media and standard methods.

Strains of \textit{S. cerevisiae} and \textit{K. lactis} used in this study are listed in Table 1. The strains were grown on YPD medium (1\%, w/v, yeast extract; 1\%, w/v, peptone; 2\%, w/v, glucose). CaCl\(_2\) was added to YPD medium to a final concentration of 10 mM. The \textit{S. cerevisiae} pmr1A strain was derived from strain AH201 by using the PCR-based gene disruption procedure developed by Wach et al. (1994). The details of the disruption of KIPMR1 in \textit{K. lactis} strain MW 278-20C have been reported elsewhere (Uccelletti et al., 1999).

#### Analysis of external invertase.

Preparation of invertase extracts, native PAGE of invertase and activity staining were as described by Ballou (1990) with the following minor modifications: the concentration of polyacrylamide gels was raised to 3.5\% (w/v) and 15 cm long slab gels were run for 18 h at 15 mA constant current to better resolve the highly glycosylated forms of invertase.

#### Fluorescence microscopy.

Chitin staining was performed as follows. Aliquots of 10\% cells of overnight-grown cultures in YPD or YPD + 10 mM CaCl\(_2\) were centrifuged and washed twice with PBS (10 mM Na\(_2\)HPO\(_4\), 18 mM KH\(_2\)PO\(_4\), 138 mM NaCl, 1 mM MgCl\(_2\), 1 mM KCl). Cell pellets were then resuspended in 100 \(\mu\)l calcofluor white (1 mg ml\(^{-1}\); Sigma) and incubated for 10 min at room temperature. The cells were then washed three times with 1 ml PBS and resuspended in 300 \(\mu\)l distilled water (Pringle, 1991). Aliquots were applied to slides and observed with an UV filter in a fluorescence microscope. To visualize filamentous F-actin structures, cells were stained with 3-3\(\mu\)M rhodamine-phalloidin (Molecular Probes) as described by Finger et al. (1997). DNA analysis was performed by DAPI (4,6-diamidino-2-phenylindole; Sigma) staining. Cells were harvested in exponential phase and fixed with 70\% (v/v) ethanol for 30 min. DAPI was added to a final concentration of 1 \(\mu\)g ml\(^{-1}\) and cells were observed with a fluorescence microscope.

#### Flow cytometric analyses.

Cells were grown until late-exponential phase, collected and washed with PBS, sonicated and fixed in 70\% (v/v) ethanol. After treatment with 1 mg RNAase ml\(^{-1}\) for 1 h at 37 °C, the cells were washed twice with PBS and stained with propidium iodide. The DNA content distributions were then determined by fluorescence signal intensity from a flow cytometer (Becton Dickinson) equipped with an argon-ion laser (excitation wavelength 488 nm, laser power 200 mW). The sample flow rate during analyses did not exceed 500-600 cells s\(^{-1}\). Typically, 40000 events were analysed per sample.

#### Electron microscopy.

Exponential-phase cultures of KIPMR1 and KIPmr1A cells were fixed with 2\% v/v, glutaraldehyde in distilled water for 1 h at room temperature. Cells were washed five times with water and post-fixed with freshly prepared 4\% (w/v) KMnO\(_4\) in double-distilled H\(_2\)O for 2 h at 4 °C. After five washes with water, cells were incubated with 2\% uranyl acetate for 2 h at room temperature, then were washed five times with water and dehydrated in increasing (30--100\%) v/v concentrations of ethanol. The samples were infiltrated overnight at 4 °C in a 1:1 mixture of ethanol and Epon 812 embedding medium. The mixture was replaced with pure Epon 812 and the samples allowed to polymerize at 60 °C for 48 h. Ultrathin sections were cut with a Reichert automatic ultramicrotome, stained with lead citrate and examined with a CM 10 Philips electron microscope at 80 kV.

#### Cell-wall analyses.

The total alkali-insoluble (1,3 and 1,6)-\(\beta\)-D-glucans were isolated as described by Boone et al. (1990) and quantified as the hexose content before dialysis (Roemer & Bussey, 1991). The alkali-soluble glucans were measured by precipitating carbohydrates from alkali-extraction supernatants with 2 vols ethanol at -20 °C. The total carbohydrate content of each fraction was determined as hexoses by the borosulphuric acid method (Badin et al., 1953). The chitin level was determined as glucosamine content following the procedure described by Popolo et al. (1997). The data reported are the means of three independent experiments.
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### Table 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tr>
<td><em>K. lactis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW 278-20C</td>
<td>MATa uraA leu2 ade2 lac4-8</td>
<td>M. Wesolowski-Louvel*</td>
</tr>
<tr>
<td>CPK1</td>
<td>MATa uraA leu2 ade2 lac4-8</td>
<td>Uccelletti et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>KlPMR1::KanR</td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AH201</td>
<td>MATa his3 trp1 ure3</td>
<td>A. Hinnen†</td>
</tr>
<tr>
<td>CPS1</td>
<td>MATa his3 trp1 ure3 PMR1::KanR</td>
<td>This work</td>
</tr>
</tbody>
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† Hans-Knoll Inst., Jena, Germany.

### RESULTS

**Influence of the Klpmr1Δ allele on the glycosylation process**

A Ca²⁺-ATPase, localized in the Golgi apparatus, can be expected to influence the glycosylation of secretory proteins during transport through the organelle (Rudolph et al., 1989). We therefore looked for this phenotype in the *K. lactis* disruptant. We used invertase as a reporter protein that is glycosylated along the secretory pathway. The invertase extracted from the *K. lactis* KlPMR1 strain appeared, on native gel electrophoresis, as a diffuse band in the upper part of the gel due to the heterogeneous N-linked glycosylation of the protein (Fig. 1 lanes 1 and 2). The Klpmr1Δ strain produced instead only the core-glycosylated form of invertase, as indicated by the higher mobility of the band in Fig. 1 (lane 3). Very similar results, shown in Fig. 1, lanes 6 (pmr1Δ), 7 and 8 (WT), were obtained in *S. cerevisiae*, where the glycosylation defect of pmr1Δ has been assigned to a Golgi compartment (Rudolph et al., 1989; Antebi & Fink, 1992; Harmsen et al., 1993). In *S. cerevisiae*, this defect can be partially reversed by external addition of millimolar amounts of Ca²⁺ (Antebi & Fink, 1992); in fact the presence of 10 mM Ca²⁺ in the medium results in the partial glycosylation of invertase (compare lane 5 with lane 6 in Fig. 1). In contrast, the glycosylation defect of Klpmr1Δ is not corrected by the external addition of Ca²⁺ (compare lanes 3 and 4 in Fig. 1). The use of 20 mM Ca²⁺ gave identical results (not shown).

**KlPMR1 is involved in the morphogenesis of the cell wall**

Microscopic observations revealed that the strain carrying the disrupted KlPMR1 allele had a clumpy appearance and a substantial fraction of the cells were abnormally round and larger than wild-type. In addition, many cells carried more than one bud (data not shown). These observations suggest that the cells could be impaired in establishing normal polarized growth. To observe this phenotype better, cells of Klpmr1Δ strains were grown in YPD, harvested and stained with calcofluor white or rhodamine-phalloidin. When the Klpmr1Δ cells were stained with the calcofluor white, a remarkable alteration of the localization of chitin was observed. In wild-type cells, the localized deposition of chitin marked the bud-emergence sites (Fig. 2a); in contrast, the *K. lactis* deletant strain exhibited fluorescence distributed across the entire cell wall (Fig. 2b). The calcofluor-stained material was largely diffuse and the intensity decreased with the distance from the bud-emergence sites. A similar defect, although much less pronounced, was detectable in *S. cerevisiae* pmr1Δ (our unpublished results). The morphological defects were

![Fig. 1. Effect of PMR1 deletion on native invertase glycosylation. Cells were grown in YPD (lanes 1, 3, 6 and 7) or YPD + 10 mM Ca²⁺ (lanes 2, 4, 5 and 8). Samples were subjected to native gel electrophoresis (3.5% polyacrylamide) and stained for invertase activity. Fully glycosylated invertase is present in lanes 1 and 2 (*K. lactis* KlPMR1), and 7 and 8 (*S. cerevisiae* PMR1). Underglycosylated invertase is produced by *K. lactis* Klpmr1Δ (lane 3) and by *S. cerevisiae* pmr1Δ (lane 6). The addition of 10 mM Ca²⁺ partially restores the glycosylation of invertase in *S. cerevisiae* pmr1Δ (lane 5) while the defect of *K. lactis* Klpmr1Δ is unaffected by the Ca²⁺ addition (lane 4).](image-url)
Fig. 2. Delocalization of chitin deposition in KlpmrlA cells. Cells were grown to late-exponential phase in YPD (a, b) or in YPD + 10 mM Ca\(^{2+}\) (c, d) and stained with calcofluor. All photographs were taken at the same magnification and exposure time. (a) MW278-20C; KlPMR1 (b) CPK1; KlpmrlA (c) MW278-20C; KlPMR1 (d) CPK1; KlpmrlA.

Fig. 3. Polarization of cortical F-actin patches is not perturbed in a KlpmrlA strain. Cells were grown in YPD to late-exponential phase, fixed in 3.7% formaldehyde and stained with rhodamine-phalloidin. All photographs were taken at the same magnification and exposure time. (a) MW278-20C; KlPMR1 (b) CPK1; KlpmrlA.

almost completely reversed when the KlpmrlA cells were grown in YPD + 10 mM Ca\(^{2+}\) (Fig. 2d). Under these conditions, chitin deposition was localized at the point of bud emergence, and the size and shape of the cells were almost indistinguishable from the wild-type cells grown under the same conditions (Fig. 2c). These results indicate that in the KlpmrlA strain, the apparatus responsible for the synthesis and localized deposition of chitin is impaired. This is supported by the observation that the KlpmrlA strain was hypersensitive to Nikkomycin Z, a competitive inhibitor of the major chitin synthase, Chs3p (Gaughran et al., 1994). In fact, while the growth of the K. lactis deletant strain was inhibited by 100 pM Nikkomycin Z, the wild-type strain was still able to grow in presence of 1 mM inhibitor (data not shown).

Loss of polarized deposition of chitin has often been associated with defects in the organization of the F-actin cortical cytoskeleton (Liu & Bretscher, 1992; Mulholland et al., 1997). We examined the aspect and distribution of F-actin patches as revealed by rhodamine-phalloidin staining. This experiment did not reveal any obvious differences in F-actin patch distribution between KlPMR1 (Fig. 3a) and KlpmrlA (Fig. 3b) cells. In both strains, F-actin patches were concentrated at the sites of active growth, namely in small buds and the neck between large buds and mother cells (Fig. 3b). This suggests that the impairment of chitin deposition caused by the deletion of KlPMR1 is not accompanied by alterations in the F-actin cortical cytoskeleton as revealed by this procedure. The morphological aspects of the KlpmrlA phenotype were also investigated by electron microscopy. We employed potassium permanganate as a fixative and stain of the cells, because it selectively reveals cellular membranes and does not require removal of the cell wall. In the wild-type strain, the cell wall was well defined and had the same thickness all around the cell (Fig. 4a). A higher magnification (see inset) the characteristic structure of the cell wall was evident, with the darker layer of mannoproteins on the outer part of the cell wall. The cell walls of KlPMR1 cells grown in YPD (Fig. 4a) or YPD + 10 mM Ca\(^{2+}\) (Fig. 4c) were morphologically indistinguishable. Analysis of the KlpmrlA strain revealed a marked difference in the thickness of the cell wall. The cell wall of the mutant cells (Fig. 4b) appeared structurally similar to that of wild-type, but with a clear increase in the thickness (see inset) evenly distributed all around the cell. Moreover, the cells were larger than the wild-type, as already described in Fig. 2. When mutant cells were grown in presence of 10 mM Ca\(^{2+}\) (Fig. 4d) the thickness of the cell wall (see inset) resembled that of wild-type cells.

The morphological modifications we observed in KlpmrlA cells prompted us to perform a quantitative analysis of the main components of the cell wall. The results are reported in Table 2 and showed that the deletant strain had altered proportions of glucans as compared with the isogenic wild-type. The alkali-insoluble glucans showed a 33% increase in the KlpmrlA strain, while the alkali-soluble fraction was reduced by half. Due to these changes, the ratio of alkali-soluble to alkali-insoluble was reduced to less than one-third of the corresponding wild-type value. The level of chitin was almost unaffected by the deletion of KlPMR1. Similar analyses were performed using cells grown in the presence of 10 mM CaCl\(_2\). Under these conditions, the alkali-insoluble glucans of the deletant strain were reduced below the level of the wild-type and the alkali-soluble glucans were also lower than the corresponding
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Fig. 4. Increase of the cell-wall thickness in the Klpmr1Δ deletant and reversion to the wild-type phenotype by the addition of 10 mM Ca\(^{2+}\). Cells were grown to late-exponential phase in YPD (a, b) or in YPD+10 mM Ca\(^{2+}\) (c, d). (a) MW278-20C; KlPMR1 (b) CPK1; Klpmr1Δ (c) MW278-20C; KlPMR1 (d) CPK1; Klpmr1Δ. Bars, 2 μm.

Table 2. β-Glucan and chitin levels in KlPMR1 and Klpmr1Δ cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>10 mM CaCl(_2)</th>
<th>Alkali-insoluble glucans*</th>
<th>Alkali-soluble glucans*</th>
<th>Soluble/insoluble ratio</th>
<th>Chitin†</th>
</tr>
</thead>
<tbody>
<tr>
<td>KlPMR1</td>
<td>−</td>
<td>294 ± 33</td>
<td>235 ± 9</td>
<td>0.79</td>
<td>2.27 ± 0.27</td>
</tr>
<tr>
<td>Klpmr1Δ</td>
<td>−</td>
<td>393 ± 21</td>
<td>104 ± 5</td>
<td>0.26</td>
<td>2.90 ± 0.39</td>
</tr>
<tr>
<td>KlPMR1</td>
<td>+</td>
<td>302 ± 27</td>
<td>241 ± 35</td>
<td>0.79</td>
<td>2.15 ± 0.22</td>
</tr>
<tr>
<td>Klpmr1Δ</td>
<td>+</td>
<td>251 ± 42</td>
<td>143 ± 14</td>
<td>0.57</td>
<td>2.04 ± 0.09</td>
</tr>
</tbody>
</table>

* Carbohydrate content (measured by the borosulfuric acid method) expressed as mean ± SD [μg (mg dry cell wt)\(^{-1}\)].

† Glucosamine content in zymolyase-undigestible pellet fractions expressed as mean ± SD [μg (mg dry cell wt)\(^{-1}\)].

fraction of the wild-type cells. This resulted in a ratio of the alkali-soluble to alkali-insoluble glucans having an intermediate value between the wild-type and deletant values. It is also noteworthy that deletion of *PMR1* in *S. cerevisiae* did not result in detectable alterations of the cell wall thickness and composition (data not shown).
Fig. 5. Abnormal DNA content of Klpmr1Δ cells and partial recovery by the addition of 10 mM Ca2+. The wild-type and disruptant cells were grown in YPD (panels a–d) or YPD + 10 mM Ca2+ (panels e–h); samples were subjected to DAPI staining and fluorescence-activated cytometry. All photographs were taken at the same magnification and exposure time. (a, b) MW278-20C; KlPMR1 (c, d) CPKI; Klpmr1Δ (e, f) MW278-20C; KlPMR1 (g, h) CPKI; Klpmr1Δ.

Influence of KIPMR1 on cell-cycle progression

Morphogenetic events, intracellular calcium levels and the cell cycle are extensively interconnected in yeast cells (Iida et al., 1990; Cid et al., 1995). Defects in polarization of cell growth have been correlated with a delay in the G2 phase of the cell cycle, as reported by Lew & Reed (1995). For this reason, we looked at the DNA content in KlPmrlΔ cells and in their wild-type counterparts by flow cytometry analysis and DAPI staining. The flow cytometric analysis from DNA of KIPMR1 cells growing on YPD (Fig. 5a) showed two peaks, one corresponding to cells with a pre-replication DNA content (n) and the other corresponding to cells with a post-replication DNA content (2n). In addition, this strain had a single well-defined nucleus shown by DAPI staining (Fig. 5b). When the KIPMR1 strain was grown on YPD + 10 mM Ca2+, a similar pattern of DNA distribution was obtained (Fig. 5c), although a decrease in 2n cells was observed. This difference could be barely seen comparing the DAPI staining of KIPMR1 cells grown in YPD + 10 mM Ca2+ (Fig. 5f) with that of the same strain grown in YPD (Fig. 5b); in both cases a single, well-defined nucleus was evident in each cell. In Klpmr1Δ cells growing on YPD (Fig. 5c), the flow cytometry data indicated that most of the cells had a DNA content greater than 2n. The abnormal DNA content of Klpmr1Δ cells was also revealed by the pattern of DAPI staining (Fig. 5d). Many cells had irregularly shaped nuclei and sometimes more than one nucleus was visible. The cells often presented a `Mickey Mouse’ shape with two large buds, both containing nuclei (Fig. 5d). This must be taken into account in the interpretation of the flow cytometry data since these ‘Mickey Mouse’ groups are measured as a single cell in the flow cytometry analysis. DAPI staining of the Klpmr1Δ cells grown in the presence of millimolar amounts of calcium (Fig. 5h) did not reveal the persistence of multiple nuclei in single cells. A single and well-defined nucleus was indeed observed in each cell; these nuclei had a larger size and stronger intensity of DAPI staining, compared to their wild-type counterparts (compare Fig. 5h and 5f). Under these conditions, flow cytometry analysis (Fig. 5g) indicated a normal distribution of DNA, although the majority of the cells had a 2n DNA content while a minor fraction had a 4n DNA content, thus appearing as a typical flow cytometry profile of a diploid strain. All these results indicate that the variations of the DNA content we observed in the null mutant are probably linked, albeit not exclusively, to alterations in calcium homeostasis.

DISCUSSION

Calcium homeostasis in the Golgi apparatus is associated with the glycosylation and maturation of proteins since the resident enzymes involved are calcium dependent; this has been reported for Gdalp (Abejion et al., 1993) and Kex2p (Wagner et al., 1987). The Klpmr1Δ mutant is not able to extend the mannose outer-chains of invertase; this result strongly suggests that the impaired step is probably in the Golgi apparatus. This glycosylation defect was not relieved by the addition of external calcium; this is an important difference with S. cerevisiae pmr1Δ, where the defect was partially reversed by 10 mM Ca2+ (Antebi & Fink, 1992). This result suggests a different sensitivity to calcium concentration of the enzymes involved in the glycosylation processing in the two yeasts, or that significant differences in calcium homeostasis exist between K. lactis and S. cerevisiae.

The involvement of KlPmrlp in the morphogenesis of the cell wall is supported by fluorescence-staining experiments, by electron microscopy and by the analysis of cell-wall components, which demonstrated that in the
Klpmr1Δ strain there is a delocalization of the chitin, a noticeable increase in the cell wall thickness and an alteration of glucan alkali soluble/insoluble ratio.

Alterations in the cell-wall composition have been reported following the loss of Ggp1p/Gas1p, a protein directly involved in the biogenesis of cell walls of S. cerevisiae (Popolo et al., 1993). In the ggp1Δ mutant, an unbalanced increase of the alkali-soluble glucans took place and a more than tenfold increase in chitin deposition in the cell wall was described as a part of a compensatory mechanism required for counter-balancing the decay in mechanical strength of the altered cell wall (Popolo et al., 1997; Kapteyn et al., 1997; Ram et al., 1998). The absence of KlPmr1p resulted instead in a delocalization of chitin without a significant change in the total amount of this polymer. The altered distribution of chitin was accompanied by a large displacement of glucans from the alkali-soluble to the alkali-insoluble fraction. It is worthwhile remembering that alkali insolubility has been explained by a linkage between chitin and glucan chains (Kollar et al., 1995) and that the alkali-insoluble glucans are, in the main, responsible for cell-wall rigidity (Manners et al., 1973).

The present results indicate that the Klpmr1Δ cells have a reorganized cell-wall architecture with a different distribution of chitin and glucans as compared to their wild-type counterparts. The increased sensitivity of the Klpmr1Δ cells to Nikkomycin Z and their round appearance argue in favour of this hypothesis. The cell-wall alterations we observed could then be regarded as a variation of compensatory mechanisms having a common theme: changes in the amount and/or distribution of cell-wall components to achieve a structure of sufficient mechanical effectiveness. In this case, however, the mechanism is not triggered by the absence of a component of the cell wall, but is linked to alterations in calcium balance caused by the absence of a Ca²⁺-ATPase presumably located in the early secretory pathway.

The Klpmr1Δ strain exhibited a phenotype strikingly similar to that of the mcd mutants of S. cerevisiae (Mondésert & Reed, 1996; Mondésert et al., 1997); these mutants show defects in N-linked glycosylation, DNA content and chitin localization but have unaffected F-actin polarization. These authors proposed that changes in N-linked glycosylation may be involved in a signalling pathway between secretion polarity and the cell-cycle progression, thus playing an important role in controlling polarized growth and cell-wall morphogenesis in yeast cells. Here, chitin deposition and mannosyl outer-chain extension of reporter proteins were defective in the Klpmr1Δ mutant, whereas the polarization of cortical F-actin was unaffected. This indicates that, as in mcd mutants, impaired deposition of chitin in this deletant strain is not associated with defective organization of the F-actin. The relevant point is that the morphological defects observed in the Klpmr1Δ background appear to originate mainly from an altered Ca²⁺ balance. In fact, chitin deposition, cell-wall thickness and the overall cell morphology of the K. lactis deletant strain, reverted to wild-type in the presence of high concentrations of calcium, whereas the glycosylation defects were not relieved.

In Klpmr1Δ cells grown in the presence of high calcium, DAPI staining showed wild-type nuclei and the DNA content resumed a more regular distribution; this latter aspect could be also due to the suppression of cell-separation defects, often present in mutants with altered cell walls. The flow cytometry analysis, however, showed that haploid Klpmr1Δ cells grown in the presence of high calcium had a diploid-like DNA content. This could suggest that the co-ordination mechanism(s) linking DNA replication and the control of cell division was affected in cells devoid of KlPmr1p and that this defect was not connected to morphogenetic events. According to Mondésert et al. (1996, 1997), this defect could be possibly related to persistent glycosylation impairment, not alleviated by external calcium.

The results presented here indicate that the alterations of calcium balance originated by the absence of KlPmr1p play a relevant role in controlling cell-wall morphogenesis in K. lactis; this interpretation may also involve a compensatory mechanism similar to that already described (Popolo et al., 1997; Ram et al., 1998). Moreover, our experiments suggest that in K. lactis, we can separate the effect of alterations in Ca²⁺ homeostasis on cell-wall biogenesis from the effect on glycosylation processes and cell-cycle progression.

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