Swm1p, a subunit of the APC/cyclosome, is required to maintain cell wall integrity during growth at high temperature in *Saccharomyces cerevisiae*

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Abstract

Swm1p, a subunit of the APC cyclosome, was originally identified for its role in the later stages of the sporulation process and is required for spore wall assembly. In addition, this protein is required to maintain cell wall integrity in vegetative cells during growth at high temperature. Electron microscopy analyses of mutant cells grown at the restrictive temperature in the absence of osmotic support show that the cell wall is clearly abnormal, with large number of discontinuities that may be responsible for the observed lysis. The mutant cells show a 7-fold reduction in glucan synthase activity during growth at 38 °C and a 3.5-fold increase in the chitin content of the cell wall. The chitin is deposited in a delocalized manner all over the cell wall, where it accumulates in patches in abnormal regions. The excess chitin is mainly synthesized by the action of chitin synthase III (Chs3p), since it disappears in the *swm1 chs3* double-mutant.

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1. Introduction

Yeast cells are surrounded by the cell wall, a complex structure that combines considerable mechanical strength with a dynamic plasticity. The cell wall is essential to guarantee cell survival in a constantly changing environment, in which the cells are subject to many stresses, and it also plays an essential role in determining cell shape [1,2]. The budding yeast cell wall is composed of a fibrillar network of 1,3-β-glucan and chitin, to which different mannoproteins are attached [2,3]. Glucan accounts for 50–55% of the dry weight of the cell wall; the mannoproteins represent around 40%, while chitin is the minor component, accounting only for 2% of the cell wall dry weight [2]. Two homologous genes, *FKS1* and *FKS2*, are thought to encode the putative catalytic subunits of 1,3-β-glucan synthase [4,5]. Chitin is synthesized by three isozymes encoded by *CHS1*, *CHS2* and *CHS3* (for a review, see [6]), which are functionally distinct since Chs1p has a repair function during cytokinesis, Chs2p synthesizes the chitin in the septum, and Chs3p synthesizes the chitin ring that marks the new bud site and also the chitin present in the lateral walls [6,7].

Yeast cells must remodel this rigid structure dynamically to ensure a certain degree of physical strength in the cell wall during the different morphogenetic processes that occur in the yeast life cycle, such as bud emergence, septum formation, or mating.
Furthermore, the cell wall composition must be adjusted in response to growth conditions or to environmental changes that represent stress for the cell in order to maintain cell integrity (reviewed in [1,2,8]). Many \textit{S. cerevisiae} mutants defective in the synthesis of particular cell wall components, such as 1,3-\beta-glucan (\textit{fks1} mutants), 1,6-\beta-glucan (\textit{kre6} mutants) or some of the cell wall construction proteins (\textit{gas1} mutants), show a characteristic set of alterations in the composition or architecture of their walls. Chitin degradants), show a characteristic set of alterations in the cell wall due to the activity of Chs3p, can be observed in mutant cells.

## 2. Materials and methods

**Yeast strains and growth conditions.** Yeast cells were grown vegetatively on YEPD (1\% yeast extract, 2\% peptone, 2\% glucose) supplemented with 1M sorbitol when needed. Initial work was done using isogenic strains YPA24 (wt) and YPA207 (\textit{swm1}) [21]. Strains LS40 (\textit{swm1::kanMX4}), TD28A (\textit{sh2::URA3}), LS46 (\textit{chs1::URA3}), LS47 (\textit{chs1::URA3 swm1::kanMX4}) and LS58 (\textit{chs3::URA3 swm1::kanMX4}) are derived from TD28 (\textit{MATa ura3-52 ino1}). Strain JAY37 (\textit{chs3::URA3}), constructed in W303-1B background (\textit{MATx ura3 leu2 his3 trp1 ade2 can1}), was provided by Dr. J.A. Trilla (University of Salamanca).

**Microscopy techniques.** For light microscopy, cells were fixed in formaldehyde (4\%) and stained with Calcofluor White as previously described [22]. Samples were viewed and photographed using a Leica DMXRA microscope equipped with Nomarski optics and epifluorescence. Pictures were taken with a Photometrics Sensys CCD camera. Samples for electron microscopy were prepared exactly as previously described [20] from cells that had been growing for 8 h at 38 °C in YEPD.

**RNA isolation and Northern analysis.** Total RNA was prepared as previously described [20] at different time intervals after transfer to the restrictive temperature (38 °C). For Northern blot analysis, 5 µg of RNA was used. The DNA probes used to detect the different transcripts were obtained by PCR amplification of internal fragments of the coding region of each gene, which were radioactively labelled using the random priming method.

**Viability assays.** Cells grown at the restrictive temperature (38 °C) were centrifuged, resuspended in methylene blue solution (0.02% methylene blue in 50 mM KH₂PO₄) and incubated for 5 min before viable and dead cells were counted by direct microscopic observation.

**Biochemical determinations.** Chitin contents were determined enzymatically as described [23]. Glucan synthase (GS) and chitin synthase (CS) activities were determined in total cellular extracts as described [23,24]. GS activity was assayed in the presence and absence of GTP, and the three CS activities were routinely measured with and without trypsin.

**Detection of dually phosphorylated \textit{Slt2p}.** Cell extract preparation and Western analysis were performed exactly as described [25]. Anti-phospho-p44/42 MAP kinase antibody (New England Biolabs) was used to detect the active \textit{Slt2p} protein. Anti-Pgk1 (Molecular Probes) was used as a loading control.
3. Results

3.1. swm1 mutants have an altered chitin distribution during growth at restrictive temperature

We have recently reported that SWM1 is required for growth at elevated temperatures, since mutants lacking this gene are unable to grow in the absence of osmotic support [20]. In that report, it was shown that swm1 mutant strains grown at 38 °C formed chains of connected cells that were unable to complete cell separation due to a defect in nuclear accumulation of the Ace2p transcription factor at the end of mitosis. In addition, longer incubation times at the restrictive temperature resulted in cell lysis of some of the components of the chains, indicating that depletion of Swm1p produces defects in the maintenance of cell wall integrity.

To further characterize the nature of the lysis phenotype, the distribution of chitin in wild-type and mutant cells was assessed using Calcofluor White. In wild-type cells, chitin is primarily deposited at the neck between the mother and the daughter cells as a circular bud scar (Fig. 1). In contrast, swm1 cells have an aberrant chitin deposition, since this polymer is spread continuously over the surface of the cells and some patches are abnormally disposed at random locations of the cell wall. It has been described that mutants with cell wall defects (such as fks1 or gas1 mutants) or mutants with defects in polarized growth (such as the thermosensitive mutants act1, cdc24 or myo2) show a significant increase in chitin synthesis and in some cell wall proteins as part of a cellular response to ensure cell viability [3,9,26]. Thus, these results suggest that swm1 mutant cells may also have a defect in cell wall construction during growth at 38 °C.

3.2. The ultrastructure of the cell wall in swm1 cells is abnormal

To analyse the nature of the cell wall defects in further detail, wild-type and mutant cells were grown in the absence of osmotic support for 8 h at 38 °C and then prepared for electron microscopy (EM). In wild-type cells, the normal structure of the cell wall could be observed, in which an inner layer, which often appeared electron-transparent, was surrounded by an osmiophilic layer mainly corresponding to mannoproteins (Fig. 2A). Examination of swm1 cells revealed that the cell wall contained both layers, although the thickness of this structure was greater than in wild-type cells, especially in the cells at the ends of the chains (Fig. 2C). In addition...
to thick septa [20], some cells had a laminar structure in the cell wall, where several juxtaposed layers were observed (Fig. 2D, arrowhead). A lack of osmotic support also resulted in the lysis of individual cells in the chains (Fig. 2B). Detailed inspection of the cell walls of the lysed cells disclosed the presence of a large number of defects and discontinuities in the cell wall (arrowheads), especially in the regions close the bud neck, which may be the ultimate cause of cell death. Abnormal bud scars were also frequent in the mutant cells incubated at the restrictive temperature (Fig. 2E). These results show that Swm1p is required for proper cell wall assembly and the maintenance of cell integrity during growth at high temperature.

3.3. swm1 mutants show an increase in chitin and altered glucan and chitin synthase activities

To confirm the changes observed by microscopic observation, biochemical analysis of the chitin content of isolated cell walls from wild-type and mutant cells was carried out (Fig. 3A). While no apparent differences between wild-type and mutant cells were observed during growth at 28 °C, in agreement with the absence of a phenotype at this temperature, growth at 38 °C resulted in a 3- to 4-fold increase in the chitin content of the cell wall of swm1 mutants as compared to isogenic wild-type cells (7.1 vs. 2.0 nmol GlcNAc, respectively).

Since the previous observation suggested the existence of a defect in cell wall composition, glucan synthase (GS) and chitin synthase (CS) activities were measured in wild-type and mutant cells cultured under permissive and restrictive conditions. For GS activity, no apparent differences were observed between wild-type and mutant cells at 28 °C (Fig. 3B). However, a significant reduction (7-fold) in GS activity was detected in swm1 mutant cells at 38 °C. The three CS activities were also measured in wild-type and swm1 cells. The greatest differences were found for CSI activity, which was 2.4-fold higher in swm1 cells than in the wild-type at 38 °C (Fig. 3C). By contrast, CSII and CSIII activities remained basically unaltered under all conditions examined (data not shown). To test whether these differences were due to a transcriptional defect, Northern blot analysis was performed in wild-type and swm1 mutants during growth at 28 °C and 10 h after transfer to the restrictive temperature (Fig. 3D). No significant difference was observed between wild-type and mutant cells when the expression patterns of the FKS1 and FKS2 genes (encoding the putative catalytic subunits of the glucan synthase) were compared. As previously described [5], FKS2 expression was strongly induced

Fig. 3. Analysis of some cell wall-related parameters of wild-type and swm1 mutants. Wild-type (strain YPA24, white rectangles) and mutant cells (strain YPA207, grey rectangles) were grown for 10 h at 28 °C or at 38 °C in YEPD medium before several analyses were performed. Data represent the means of three independent measurements, with error bars showing the standard deviation. (A) Chitin content of the cell wall of wild-type and mutant strains (indicated as nmols of NAcGlc released per mg of cells). (B) Glucan synthase (GS) activity measured in the presence of GTP (expressed as nmols of UDP-Glc incorporated per minute per mg of protein). (C) Chitin synthase (CSI) activity measured after trypsin activation (expressed as nmols of NAcGlc incorporated per hour per mg of protein). For panels B and C, only data for optimum GS and CSI activities are presented. (D) Expression of the genes encoding catalytic subunits of glucan and chitin synthases. RNA was purified from wild-type (strain TD28) and swm1 cells (strain LS40) at 0 and 10 h after transfer to 38 °C. RNA blots were hybridized with radioactively labelled probes for FKS1, FKS2 and CHS1. The ACT1 gene was used to test for equal loading of RNA in all lanes.
during growth at high temperature both in the wild-type and the mutant strain. For CHS1, a modest increase in expression in the mutant strain was detected at 10 h after transfer to 37 °C, in agreement with previously described results [27]. Taken together, these results suggest that SWM1 is required for proper cell wall assembly at elevated temperature, and that its deletion affects proteins related to the assembly of this structure mainly at post-transcriptional level.

3.4. Chs3p is responsible for the chitin increase detected in the cell wall of swm1 mutants

It has been reported that the Chs3p protein (CSIII activity) is responsible in vivo for the increase in chitin contents observed in fks1 or gas1 mutants, although in vitro a strong increase in CSI activity was also detected [11,12]. To test whether the same was true in swm1 mutants, the CHS1 or CHS3 genes were deleted in our strain background and the resulting strains were stained with Calcofluor White. Inactivation of CHS3 dramatically reduced the staining in the double mutant as compared with the single swm1 mutant (Fig. 4A). In contrast, the staining pattern in the double swm1 chs1 mutant was identical to that of the single swm1 deletants (data not shown). These results indicate that, as has been reported for other cell wall mutants, Chs3p is indeed the enzyme responsible for the increase in chitin accumulation that is observed in swm1 cells during growth at high temperature.

To test whether the increase in chitin was a result of the activation of the cell wall salvage pathway or whether it was merely the result of the cell cycle defect, the viability of the swm1 and swm1 chs3 was measured during incubation at the restrictive temperature. Samples taken at different times after transfer of the cells to 38 °C were stained with methylene blue (a dye that accumulates in dead cells and stains them blue). The results revealed that viability was similar in both strains (Fig. 4B), suggesting that the increase in chitin in the cell wall does not aim to protect cells from lysis, but could reflect a loss of coordination of cell cycle events.

3.5. Slt2p MAP kinase is normally activated during growth at high temperature

Mutants with cell wall defects, such as fks1 or gas1, display a high level of active Slt2p/Mpk1p in comparison with a wild-type strain, as measured by using an specific antibody that recognizes the active dually phosphorylated form of this kinase [25]. To test whether a similar increase in activation also occurs in swm1 cells, the Slt2p phosphorylation state was analysed. Wild-type, swm1 or slt2 mutants (as a control) were grown at 28 or 38 °C and then cell extracts were prepared and probed with anti-phospho-p44 antibodies (Fig. 5). No
activation of the MAP kinase was detected during growth at the permissive temperature, in accordance with the absence of a phenotype at this temperature. Growth at the restrictive temperature did not result in a detectable increase in Slt2p phosphorylation as compared to that of the wild-type strain.

4. Discussion

In this study, we characterize the phenotypes associated with the loss of the Swm1p protein during vegetative growth. This protein has been identified as one of the core subunits of the APC, a complex required for cell cycle progression [14–16]. We have recently reported that swm1 null mutants show a delay in exit from mitosis during growth at high temperature, resulting in the accumulation of high levels of cyclin Clb2p and the associated CDK kinase activity [20]. However, this delay in CDK inactivation does not result in a cell cycle block, since the cells are able to divide and form chains of connected cells. swm1 mutants are unable to complete the mother-daughter separation process, forming chains of cells that remain associated, and this defect is produced by the inability of the transcription factor Ace2p, which controls the expression of a group of genes involved in cell separation [28,29], to accumulate in the nucleus of the daughter cell at the end of mitosis.

In addition to the cell separation defect, swm1 mutants show a thermosensitive phenotype that can be remedied by osmotic support, suggesting the existence of a cell wall defect. Here, we focused on the analysis of this defect, which results in cell lysis during growth at the restrictive temperature. One of the main defects observed was a noticeable decrease in GS activity in swm1 cells after 8 h of incubation at 38 °C, although this was not due to a decrease in the transcription of the genes encoding the catalytic or regulatory subunits of this enzyme, as assessed by Northern analysis. The reduction in GS activity was accompanied by an increase in the chitin content of the cell wall dependent on Chs3p, a cellular response that has been described in other mutants affected in cell wall construction to ensure cell integrity [9,11,12]. However, the fact that the viability of the swm1 chs3 double mutant during growth at high temperature is almost identical to that of the swm1 mutant suggests that the chitin excess is not intended to protect the cells from lysis, but instead would be a consequence of the loss of coordination of cell cycle events. From EM observations, it was clear that the cell wall displayed large number of discontinuities, which resulted in the release of the cytoplasmic content and, consequently, in cell death. The defects were often located close to the septal region, suggesting that the cells were lysing either at the neck, or at the bud or birth scars. Interestingly, these defects are different from those described for pkc1 mutants, which arrest at the G2-M transition and lyse at the tips of small buds, where there is noticeable thinning of the cell wall [30]. Thus, the lysis of swm1 cells during growth at the restrictive temperature cannot simply be due to a failure to activate the Pkc1p/Slt2p pathway. In fact, Slt2p kinase is normally activated in mutant cells at high temperature and the growth of the double slt2 swm1 mutants is similar to that of swm1 cells during growth at 38 °C (unpublished observations). All these observations suggest that swm1 cells are deficient in maintaining cell integrity during growth at high temperature as a consequence of defects in the coordination between cell wall synthesis and cell cycle events.

To explain the absence of phenotypes at 28 °C, it has been proposed that deletion of SWM1 might affect the stability of the APC complex only during growth at the restrictive temperature [20]. Lysis of the swm1 mutant was suppressed by osmotic stabilizers, such as 1M sorbitol. However, this did not overcome the primary defect of swm1 cells, since at elevated temperatures chains of cells with thick septa still accumulated, although they no longer underwent the dramatic loss of viability seen in the absence of osmotic support. High osmolarity causes the intracellular accumulation of glycerol [31], which can result in the stabilization of the mutant APC complex, allowing a partial function of this complex at high temperature and the suppression of the lysis defect. Although we cannot rule out this possibility, it is more likely that lysis suppression occurs by stabilization of the cell wall. As shown previously, at high temperatures swm1 cells show a delay in exit from mitosis [20] that might result in a defect in maintaining cell integrity. An attractive hypothesis is that there could exist a tight cell-cycle regulation of the GS activity to activate cell wall synthesis at specific moments of the cell cycle, such as during bud growth or septum synthesis. Such a mechanism might be important for coordinating events in the nucleus with the cell cortex during the cell division cycle. In swm1 mutants, the delay in exit from mitosis might hinder the timely activation of GS, thereby compromising cell integrity, and might also result in abnormal localization of Chs3p, thus increasing the chitin content of the cell wall. However, direct evidence of such a mechanism is lacking. At the permissive temperatures, swm1 cells are not particularly fragile or sensitive to cell wall disruption by low concentrations of SDS or Calcofluor White (unpublished observations). An alternative explanation is that the cell integrity defect would be secondary to the cell cycle defect. For example, mitotic arrest is accompanied by a large increase in cell size, which may render the cells more fragile and likely to lyse. This might be overcome by osmotic stabilization. Preserving the large arrested cells longer may simply allow them more time to complete the inactivation of the CDK activity and to exit from mitosis.
Although the nature of the defect that results in cell lysis in swm1 mutants is currently unknown, our results suggest that a correct coordination between cell cycle events and cell wall synthesis is important for cell survival. It is interesting to note that SPB duplication and cell integrity/morphogenesis are also linked. Yeast Cdc31p, the homologue of centrin, is essential for SPB duplication, since mutations in this gene block the formation of the satellite precursor [32]. In addition to SPB duplication, Cdc31p also plays a role in morphogenesis and cell integrity via interaction with the PAK kinase Kic1p [33]. Mutations in the kinase domain of Kic1p and certain cdc31 alleles result in abnormal bud morphology and cell lysis, and extensive genetic interactions between the PKCl pathway and cdc31 alleles that affect SPB duplication have been described [34]. Furthermore, mutations in the SPC110 gene, which encodes a calmodulin-binding protein of the SPB, also produce defects in cell integrity in addition to the spindle formation defect [35]. Interestingly, the morphology of kic1 mutants is very similar to the defects observed in swm1 [33], suggesting the existence of different moments when coordination of cell wall synthesis and other cell cycle events could occur. Alternatively, it is possible that the delay in exit from mitosis seen in swm1 mutants would indirectly affect the events that occur in the next G1/S transition, SPB duplication and bud emergence, via Cdc31p and Kic1p. Further experiments will address this questions.

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