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Characterization of the chitin biosynthesis process as a compensatory mechanism in the *fks1* mutant of *Saccharomyces cerevisiae*

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Abstract Deletion of the 1,3-β-D-glucan synthase gene *FKS1* in *Saccharomyces cerevisiae* induces a compensatory mechanism that is reflected in a significant increase in chitin synthase III (CSIII) activity, leading to high rates of chitin synthesis. Deregulation of CSIII activity is mainly due to the intracellular delocalization of Chs3p and Chs4p, the two main components of the CSIII active complex.

Key words: Chitin; Compensatory mechanism; Chitin synthase III activity

1. Introduction

The cell wall is the outermost structure of yeast cells and it combines considerable mechanical strength with a dynamic plasticity, thus guaranteeing cell survival in a fluctuating environment. The yeast cell wall also constitutes a permeability barrier that has dramatic influence in the tolerance of yeast cells to damaging environmental agents. The combination of both characteristics requires a very complex regulatory mechanism that is only now beginning to be understood (see [1] for a recent review). Current knowledge of the yeast cell wall has been reviewed recently and can be summarized as follows: the yeast cell wall comprises a fibrillar network of chitin and 1,3-β-D-glucan to which mannoproteins anchor, mostly through 1,6-β-D-glucan molecules [1,2]. Compromise between rigidity and plasticity in this structure at each point of the cell life must be achieved by a delicate balance between synthesis and degradation.

To ensure the stability of its cell wall, *Saccharomyces cerevisiae* has a dedicated signalling pathway known as the protein kinase C (PKC) pathway or the cell integrity signal transduction pathway [3]. The most relevant characteristic of this pathway is its activation in response to different types of injuries to the yeast cell wall [4]. Activation of this pathway leads to the expression of several genes related to cell wall synthesis or assembly [1,5,6], among them the *FKS2* gene, which encodes a subunit of the 1,3-β-D-glucan synthase [7]. *FKS2* belongs to the family of glucan synthase genes also formed by the *FKS1* and *FKS3* genes. While *FKS1* is expressed during vegetative growth, the *FKS2* gene is not, although its expression is induced under different growth conditions and in response to cell wall defects [5,7,8]. Induction of *FKS2* seems to compensate for the lack of *FKS1* gene in terms of 1,3-β-D-glucan synthase activity and in vivo function [8]. So far, no function for the *FKS3* gene has been established.

Yeast cells with severe cell wall defects, including mutations in the *FKS1* or *GAS1* genes, also show a significant increase in chitin synthesis [9,10] accompanied by a significant increase in the synthesis of several cell wall proteins [1]. Taken together, these data suggest that yeast cells react against cell wall injuries by activating a compensatory mechanism that ensures cell wall stability [10]. Several lines of indirect evidence suggest that this compensatory mechanism is mediated by the PKC signal transduction pathway [1].

S. cerevisiae contains only 2–3% of chitin in its cell walls; however, the synthesis of this polymer seems to be essential for cell survival [11,12]. Most of this chitin is synthesized by chitin synthase III (CSIII) activity. The mechanism of regulation of this activity seems to be complex, and so far five different gene products have been directly related to it. In addition to Chs3p, the catalytic subunit of CSIII, Chs4p and Chs7p seem to play a direct role in the regulation of this activity, while Chs5p and Chs6p would have more general roles in the intracellular trafficking of proteins [12].

The present report focuses on the molecular mechanism that triggers the increase in chitin synthesis in response to *FKS1* deletion. This increase is shown to be due to an increase in CSIII activity and the molecular mechanisms involved in this response are discussed.

2. Materials and methods

2.1. Strains, plasmid and growth conditions

Initial work was performed in the CVX12 genetic background [13]. However, the 15Dau genetic background was used throughout this work [14]. Different mutants were constructed in this strain by the one step gene replacement technique. The deletion cassette for *FKS1* disruption is described in Section 3 (Fig. 1A). Double *fks1 chs3* mutants were constructed by crossing strains 15Dau *fks1Δ2* with TC1B (W303α *chs3::LEU2*) [15] and further tetrad dissection. Yeast cells were typically grown in YEPD medium, but SD medium with the corresponding supplements was used for growing cells transformed with plasmids. The yeast genetic techniques used are as described [16]. All the techniques involved in DNA manipulation have previously been described [17].

2.2. Biochemical determinations

Chitin was determined enzymatically as described [14]. 1,3-β-D-Glucan synthase and CS activities were determined in total cellular extracts as described [13,14]. All three CSs were routinely measured with and without trypsin; however, only the data on optimum activity after trypsin treatment are presented. mRNA expression levels were determined by Northern blot experiments [17] using a *FKS2* fragment as the probe exactly as described [8]. Protein expression levels were de-

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termined in total cellular extracts by Western blot analysis as described [14]. To do so, the different strains were transformed with centromeric plasmids carrying the HA-tagged versions of Chs3p [18], Chs4p [19] or Chs7p [14].

2.3. Direct and indirect immunofluorescence

Chitin was localized after calcofluor staining as described [18]. Chs3p-3HA and Cdc11p were localized intracellularly by indirect immunofluorescence [14]. Chs3p-3HA was detected using the mouse HA.11 anti-HA antibody (Berkeley Antibody Co., 1:100 dilution). Rabbit anti-Cdc11p antibodies (Santa Cruz, 1:50 dilution) were used for the detection of Cdc11 protein. Chs4p was detected by laser confocal microscopy in cells transformed with a *CHS4-GFP* chimera [19]. All procedures were carried out in cells of the 15Dau background with the exception of the immunolocalization of Chs3p-3HA which was carried out in the Y1306 strain [20].

3. Results

Previous work in our laboratory led to the characterization of a deletion of the *FKS1* gene in *S. cerevisiae* [13]. This deletant (*fks1Δ1*) shows a strong reduction in 1,3-β-D-glucan synthase activity, accompanied by a slow-growth phenotype (Fig. 1B) that can be suppressed by supplementing the medium with 5 mM $CaCl_2$ (Fig. 1B). Additionally, the lack of *FKS1* induces expression of the *FKS2* gene (Fig. 1C). These phenotypes are similar to those observed in several other *fks1* partial deletions described by other groups [8,21–23]. Further work with this partial deletion strain (*fks1Δ1*) revealed some unexpected phenotypes for a null mutant: *FKS1* truncated mRNA could be detected, and diploid strain *fks1Δ1/pbr1-1* [13] was papulacandin B sensitive (data not shown), indicating that a truncated *fks1Δ1* protein may compete with the mutated *pbr1-1* protein, which is known to confer papulacandin B resistance [13]. We therefore decided to construct a new and complete deletion of this gene. To do so, the 5′ and 3′ regions of the *FKS1* gene were amplified by polymerase chain reaction (PCR) and ligated using an appropriate version of the *URA3* marker as linker. In this construction, most of the *FKS1* open reading frame (from nucleotide 158 to 4937, approximately 85%) was replaced by the auxotrophic marker (Fig. 1A). The deletion cassette was used to replace the *FKS1* wild-type copy of the CVX12 strain. Correct replacement was confirmed by Southern blot and/or PCR with primers external to the deletion cassette. The null *fks1* mutant (*fks1Δ2*) showed some interesting phenotypes: its growth was indistinguishable from that of the wild-type strain (Fig. 1B), and it had a minor defect in 1,3-β-D-glucan synthase (Fig. 1D). Accordingly, the Ca^{2+} concentration had no effect on its growth. This strain also showed an induction of the *FKS2* gene comparable to that observed in the partial deletion (Fig. 1C). Apparently, absence of the function of *FKS1* has a weaker effect than that previously observed with the partial deletions, although induction of the compensatory mechanism seems to be very similar.

In order to characterize the phenotypes associated with the lack of *FKS1* function, we constructed the null *fks1* mutant in our standard genetic background: strain 15Dau. The 15Dau *fks1Δ2* strain showed similar phenotypes to those described for the CVX12 background (Fig. 1B–D). However, the null mutant was characterized by a slower growth than the wild-type, a phenotype that could only be weakly corrected by the

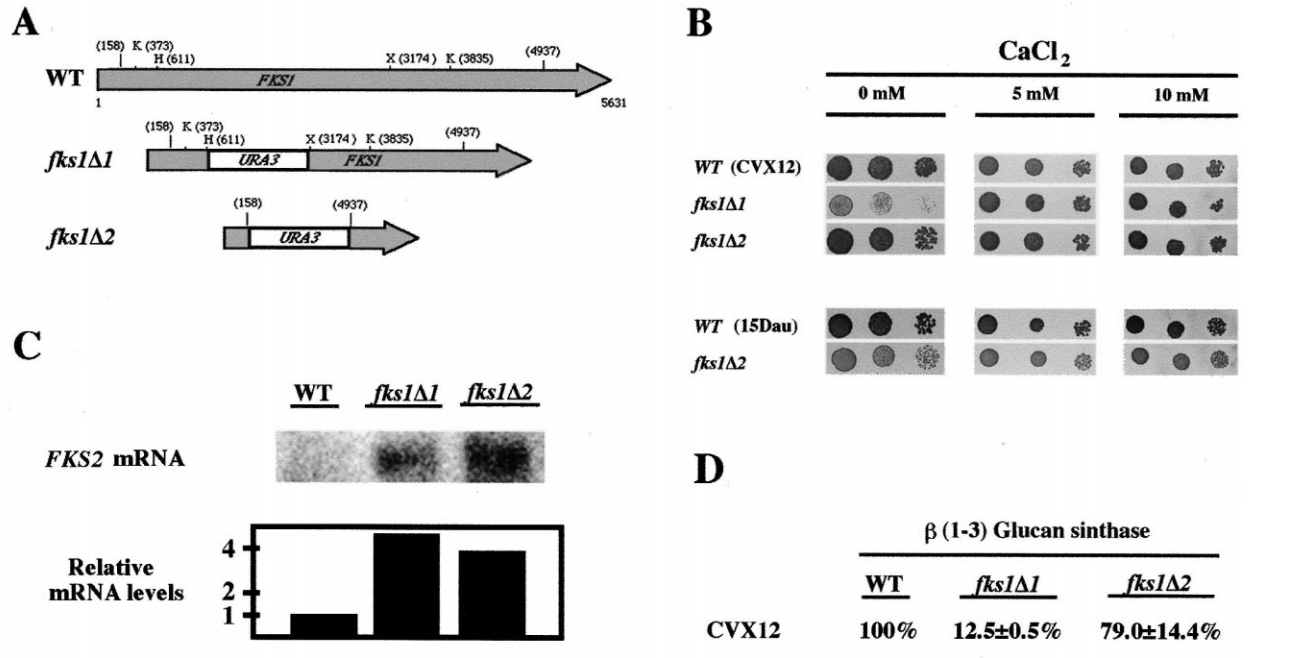

Fig. 1. Characterization of *fks1* mutants. A: Schematic representation of different *FKS1* constructs. WT, wild-type; *fks1Δ1*, partial deletion; and *fks1Δ2*, complete deletion. Relevant restriction sites and positions are indicated. K, *Kpn*I; H, *Hin*dIII; and X, *Xba*I. B: Cell growth of different strains on YEPD medium with or without $CaCl_2$. Cultures were grown overnight and plated onto the indicated medium at 1/10, 1/100 and 1/1000 dilutions. The relevant genotype of each strain is indicated on the left. Strains are derivatives of CVX12 (upper part) or 15Dau (lower part). C: Expression levels of *FKS2* gene measured in Northern blot experiments (see Section 2 for details). Autoradiographic images and quantitative determinations by Phosphorimager are shown. Relative amounts of *FKS2* mRNA are referred to actin levels. All strains are in the CVX12 genetic background. D: Relative values of 1,3-β-D-glucan synthase activity of the indicated strains in the CVX12 genetic background.

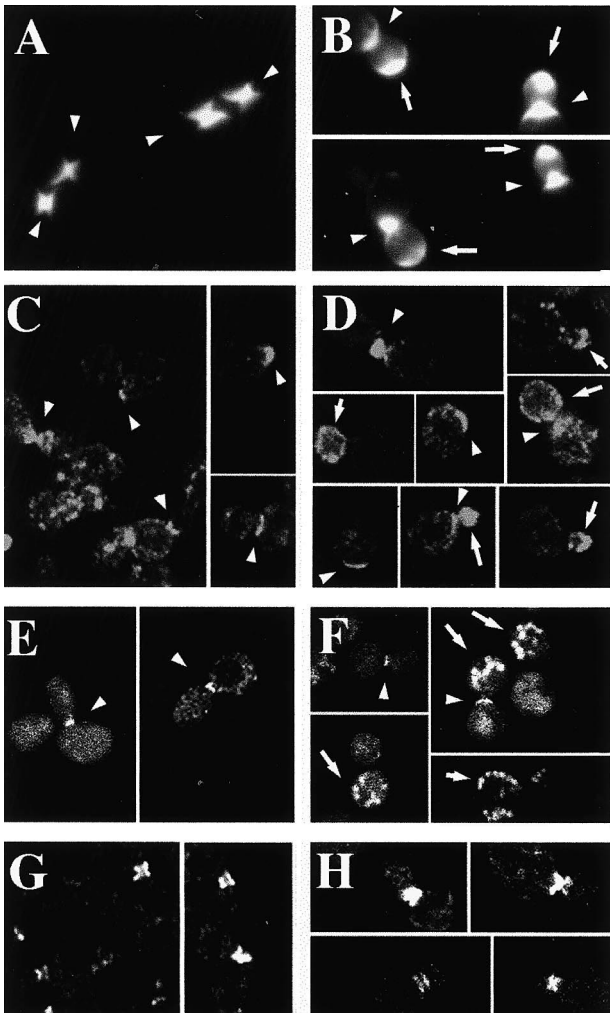

Fig. 2. Localization of several proteins in wild-type and *fks1* null mutant strains. A,B: Chitin localization after calcofluor staining. C,D: Immunolocalization of Chs3p-3HA. E,F: Cellular localization of Chs4-GFP protein. G,H: Immunolocalization of Cdc11 protein. Cells shown in A, C, E and G are from the wild-type strains while B, D, F and H are from *fks1Δ2* strains. Arrowheads indicate wild-type localization, while arrows indicate altered distribution. For specific details in the protocols and strains, see Section 2.

addition of $CaCl_2$ to the medium (Fig. 1B). The 15Dau *fks1Δ2* strain showed an altered distribution of chitin after the addition of calcofluor to the medium (Fig. 2A,B): chitin was not only localized at the septum site but also at the tip of the daughter cell. This altered distribution was also accompanied by a significant increase in the rate of chitin synthesis, since mutant strains had approximately 6-fold more chitin than controls (data not shown). Double *fks1Δ2 chs3* mutants were viable, and after the addition of calcofluor they showed the characteristic staining pattern of a chitin-deficient mutant (data not shown). This result suggests that the increase in chitin levels observed in the *fks1* null mutant is directly related to CSIII activity. In order to confirm this apparent relationship, we measured the different CS activities in the wild-type and *fks1Δ2* mutant strains. CSIII increased almost 3-fold in the mutant strain (Table 1). The increase was basically independent of addition of trypsin as an activator of CSIII activity. Under the same conditions, CSII activity remained basically unaltered; however, a strong increase in CSI activity was also detected in the *fks1Δ2* mutant.

Table 1
CS activities in different strains

CS activity[a]	Strains[b]		Activation factor[c]
	wild-type	*fks1Δ2*	
CSI	37.0 ± 11.1	301.6 ± 92.5	8.2
CSII	19.4 ± 3.8	25.6 ± 8.5	1.3
CSIII basal	5.2 ± 1.4	13.3 ± 2.4	2.6
CSIII total	6.9 ± 1.3	19.9 ± 2.1	2.9

[a]CS activities were measured as described in Section 2 and expressed as nmol incorporated per mg of protein per hour. For CSI and CSII activities, only values after activation with trypsin (total activity) are shown.
[b]Strains are 15Dau (wild-type) and LGY1 (*fks1Δ2*).
[c]Activation factor represents the increase in CSs from the *fks1* null mutant as compared to the wild-type.

CSIII activity depends on several proteins for a proper functioning. While Chs3p seems to be the catalytic subunit of this enzyme, Chs4p participates in the activation and/or proper localization of the active complex [15,24]. Chs5p and Chs6p play different roles in the sorting of Chs3p but their exact roles are not fully understood [12]. Chs7p is required for the proper export of Chs3p from the endoplasmic reticulum and its levels seem to act as a major regulator of chitin synthesis levels [19]. In order to investigate the participation of the different components in the increase in CSIII activity, we determined the protein levels of Chs3p, Chs4p and Chs7p in the *fks1Δ2* mutant. As shown in Fig. 3, neither Chs3p nor Chs4p levels seemed to be affected. However, we did detect a significant increase (approximately 25 times) in Chs7p expression.

Cellular localization can also be a major way of regulating intracellular activity and we therefore determined the cellular localization of several Chs proteins in the *fks1Δ2* mutants. In the *fks1* null mutant, Chs3p was only partially localized to the septum site, also showing a clear localization to the membrane of the daughter cell (Fig. 2D). This distribution was in fair agreement with that of chitin (Fig. 2B), and clearly different from that observed in the wild-type strain, in which virtually

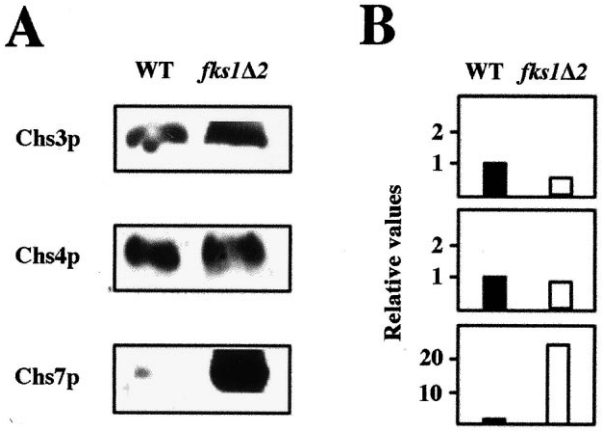

Fig. 3. Expression levels of Chs proteins in the null *fks1Δ2* mutant. A: Western blot showing protein amounts in wild-type and *fks1Δ2* strains. B: Relative levels of Chs proteins as determined by quantitative densitometry of the Western blots presented in A. Values are referred to wild-type levels.

all the Chs3p detected was located at the septum (Fig. 2C), the site of chitin synthesis (Fig. 2A). Chs4p was also mislocalized, since only a fraction of the protein was localized at the septum (Fig. 2F) while a significant part of it formed aggregates inside the cytoplasm. These aggregates did not seem to define a specific distribution but rather a mislocalization. Curiously, the cellular machinery that supports septum formation, the septin ring [25], was in its proper place as determined by the distribution of Cdc11p (Fig. 2G,H).

4. Discussion

This study was conducted to gain insight into the molecular mechanisms involved in the increase in chitin levels observed after *FKS1* deletion. To do so, we first constructed a complete deletion of the *FKS1* gene since our experience with the previously described *fks1* deletion [13] had indicated that this partial deletion could afford some deleterious phenotypes (see Section 3). The results obtained seem to support this conclusion since the partial deletion displayed a slow-growth phenotype that was not observed in the complete deletion. In addition, the reduction in 1,3-β-D-glucan synthase activity was much stronger in the partial than in the complete deletion. The results with the partial deletion are consistent with those obtained by other authors [8,21,22]. The deleterious effect of the previously described *fks1* deletion seems to be associated with a low expression of the *FKS2* gene since an increase in the expression of this gene, mediated by the addition of Ca^{2+} to the medium, restored wild-type levels of growth and 1,3-β-D-glucan synthase [8]. In our case, the slow-growth phenotype of the partial deletion was also overcome by growth in 5 mM Ca^{2+}; however, the addition of Ca^{2+} had no effect on the complete deletion (Fig. 1B). Our results indicate that the expression of *FKS2* in these strains is very similar and hence the different phenotypes observed cannot be attributed to differences in *FKS2* expression. The most plausible explanation is that partial *fks1Δ1* deletion would produce a truncated protein, as expected from the observation of a truncated mRNA (data not shown), that would compete with the highly homologous product of the *FKS2* gene. Only the high levels of expression of *FKS2* achieved after the addition of Ca^{2+} can overcome this competitive effect. In the complete deletion, this overexpression would not be necessary due to the absence of the truncated protein. This hypothesis also seems to be supported by the fact that diploid *fks1Δ1/pbr1-1* is more sensitive than diploid *fks1Δ2/pbr1-1* to papulacandin B, a new indication that the truncated *fks1Δ1* protein could compete with the mutated *pbr1-1* protein which confers papulacandin B resistance [13]. The slow-growth phenotype observed after *FKS1* complete deletion in the 15Dau strain seems to be related to the genetic background employed since it was neither observed in other strains nor significantly alleviated by the addition of Ca^{2+}.

Deletion of *FKS1*, as well as of *GAS1*, induces a compensatory mechanism in the cell that has been indirectly related to the PKC signal transduction pathway [1]. This compensation includes an increase in chitin levels and in those of certain cell wall proteins, as well as alterations in the cross-links between chitin and other molecules [1,6]. The *fks1Δ2* mutant also showed a significant increase in chitin levels that was accompanied by delocalization of this polymer (Fig. 2, see below). In this strain the increase in chitin synthesis was accompanied by a significant increase in CSI and CSIII levels, but not in CSII activity (Table 1). Although the increase in CSI was the highest, there are several reasons to suggest that CSIII activity is directly responsible for the higher amount of chitin: (a) double *fks1 chs3* mutants lacked chitin as determined by calcofluor staining; (b) CSI has never been shown to synthesize detectable amounts of chitin in vivo, while the CSIII makes more than 90% of cellular chitin [12]; and (c) *chs1 gas1* double mutants contain very high levels of chitin [26].

In our hands, the *fks1 chs3* double mutant was viable, in contrast to what has been reported recently [27]. We were working with a null *fks1* mutant while the other authors selected for point *fks1* mutations. It is therefore possible that these point mutations might have additional phenotypes (see above). Furthermore, differences due to different genetic backgrounds cannot be ruled out. This result indicates that chitin induction in the *fks1* mutant does not reflect the necessity of the cells to increase chitin synthesis, but rather that this increase is a consequence of the induction of a more general compensatory mechanism.

The observed increase in CSIII activity does not seem to be related to differences in the expression of the *CHS* genes. We have been unable to find any significant differences in the levels of Chs3p, Chs4p (Fig. 3) or even Chs6p (not shown), and we only detected a significant increase in Chs7p levels. Recently, Chs7p has been shown to be a limiting step in chitin synthesis [19]. However, since no increase in Chs3p levels was detected, in our hands the increase in the expression of Chs7p remains unclear. By contrast, we did detect significant changes in the intracellular localization of Chs3p and Chs4p. The distribution of Chs3p in *fks1Δ2* mutants was not restricted to the septa but extended to the surface of daughter cells (Fig. 2D) in a distribution approximately resembling that of chitin (Fig. 2B). In addition, most of Chs4p was observed as aggregates in the cytoplasm, a clear indication that this protein was delocalized. The delocalization process seems to be specifically related to chitin synthesis since cell polarity was apparently normal, as shown by the fact that budding and growth occurred normally and the septin ring was located in its normal place (Fig. 2H). The most plausible explanation is that the targeting of Chs3p to the septum [24] would be altered in the *fks1* null mutant, leading to a delocalization of the CSIII complex. It is likely that this delocalization increases the amount of Chs3p that reaches the membrane (compare fluorescence intensities in Fig. 2C,D), producing the observed increases in CSIII and in chitin synthesis.

In sum, we conclude that deletion of the *fks1* gene produces an increase in chitin synthesis that is due to an activation of the CSIII activity mediated by an unusual localization of the active CSIII complex.

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