In *Schizosaccharomyces pombe* chs2p has no chitin synthase activity but is related to septum formation

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Abstract Chitin synthesis occurs in most fungi through the action of different chitin synthase (CS) isoenzymes. In *Schizosaccharomyces pombe* the *chs2+* gene codes for a protein with significant similarity to CS enzymes, but lacking most of the residues considered to be essential for activity, including the QRRRW domain. Here we show that chs2p is a functional protein that localises to the growing edge of the septum but is not a CS enzyme. Strong over-expression is lethal, while moderate expression leads to a severe defect in septum formation. These results suggest that chs2p has remained through evolution to play an alternative role in septation.
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1. Introduction

The cell wall is the exoskeleton of fungal cells and the integrity of this structure is essential for cell survival [1,2]. Chitin, a crystalline polymer composed of (1,4)-β-*N*-acetyl-glucosamine, contributes to the rigidity of the cell wall. In *Saccharomyces cerevisiae* synthesis of this polymer, that has been shown to be essential for cell viability [3], is carried out by the action of three chitin synthase (CS) enzymes: CSI repairs the excessive action of chitinase after cytokinesis [4], CSII is responsible for the synthesis of the primary septum, and CSIII is required for synthesis of the chitin ring at the neck between mother and daughter cells, at the lateral wall of vegetative cells and spores and at the projection of the shmoos during mating [3–6].

In 1990 the *Candida albicans CHS1* gene was isolated by expression in a *S. cerevisiae chs1* mutant [7]. The comparison of the *ScCHS1* and *ScCHS2* genes with the *CaCHS1* allowed the design of polymerase chain reaction (PCR) primers that were used to screen genomic DNA for the presence of CS enzymes in 14 fungal species [8]. It was found that all the tested fungi had at least one CS. Since then, many CS genes

have been isolated from different organisms. All these genes can be grouped into different families and classes according to different signature domains [8,9]. Detailed study of the CS genes has shown that a QRRRW motif, together with several isolated residues in the central portion of the protein, is present in all of them [10]. A QXXRW domain is present in other glycosyl transferases [11] and has been proposed as the catalytic domain of these enzymes. Mutations in the QRRRW domain result in a loss of function in vivo and of enzymatic activity in vitro of the *ScCHS1*, *ScCHS2* and *ScCHS3* genes [12,13].

It has not been possible to establish the presence of chitin in the *Schizosaccharomyces pombe* cell wall [14–17]. However, a CS activity has been found in this fission yeast [14,15]. In the genome of this organism there are two genes with similarity to CS genes from other fungi. *chs1+* shares 47%, 45% and 28% identity with the *ScCHS2*, *ScCHS1* and *ScCHS3* genes, respectively, and codes for a protein responsible for the CS activity [15]. This enzyme is essential for ascospore maturation and for the synthesis of a wheat-germ agglutinin (WGA)-reactive material in the spore cell wall [15]. *chs2+* is 32% and 29% identical to *ScCHS2* and *ScCHS1*, respectively, while it has no significant identity with the *ScCHS3* gene. It is noteworthy that chs2p lacks the QRRRW domain and several amino acids essential for CSII activity. Ding et al. found that a chs2::GFP fusion localised to the septum when they analysed protein localisation by the use of a green fluorescent protein (GFP)-fusion genomic DNA library expressed in a plasmid [18].

In this work we confirm that chs2p is expressed and localises to the inner edge of the growing septa, showing that it is not a pseudogene. We also show that this protein is not a CS enzyme and present evidence that indicates a role in septum formation.

2. Materials and methods

2.1. Strains, growth conditions and plasmids

All *S. pombe* strains are derivatives of the WT 972 *h−* and 975 *h+* and were grown on YES, EMM or ME media and sporulated on SPA plates [19]. For Calcofluor sensitivity analysis YES medium was buffered with 50 mM potassium phtalate pH 6.0 to prevent precipitation of the dye. Quantitative mating experiments were carried out as described in [15]. *S. cerevisiae* strains are in the 15Daub genetic background [20] and were grown on SD medium (6.7 g/l yeast nitrogen base without aminoacids, 20 g/l glucose or galactose, 20 g/l agar for solid medium) supplemented with uracil when required. All general techniques have already been described [19,21]. *chs2Δ* null mutant was constructed in a diploid strain by the one-step gene replacement technique [22] and further tetrad dissection. The *chs2::ura4+* deletion

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cassette was constructed as follows: PCR amplifications allowed us to obtain 1 kb DNA fragments corresponding to the upstream or downstream non-coding regions in which appropriate sites for restriction enzymes had been introduced. These fragments and the *ura4*$^+$ gene (as a *Hin*dIII/*Xba*I DNA fragment) were sequentially cloned into the KS vector. Double *chs1::his3*$^+$ *chs2::ura4*$^+$ mutants were obtained by crossing the single mutants and selecting his+ ura+ clones by random spore analysis. The selected clones were verified by PCR analysis. In order to over-express the *chs2*$^+$ gene, removal of a small intron was carried out by site-directed mutagenesis and verified by sequencing; then, sites for the *Sal*I enzyme were generated before the ATG and after the STOP codons so the open reading frame (ORF) was cloned as a *Sal*I/*Sal*I DNA fragment into the *Sal*I site of the pREP3X plasmid (bearing the strongest thiamine-inducible *nmt1* promoter [23]) or the *Xho*I site of the pREP41X and pREP81X plasmids (medium- and weak-strength promoters [23]). Construction of a chimera carrying the *chs2*$^+$ ORF flanked by the upstream and downstream sequences of the *ScCHS2* gene was achieved by PCR amplification of these non-coding sequences so *Sal*I sites were created before the ATG and after the STOP codons. The *chs2*$^+$ ORF was then cloned in-between as a *Sal*I/*Sal*I fragment.

2.2. Biochemical determinations

CS activity determination was performed as described in [15].

2.3. Microscopy

Calcofluor, WGA, and 4′-6′-diamidin-2-phenylindole (DAPI) staining were performed as described in [15]. In order to stain cells with methylene blue, 2 μl of the corresponding culture was mixed with 2 μl of a 0.6% solution of the dye and observed under a bright-field microscope. A chs2::GFP fusion was constructed as follows: a restriction site for the *Not*I enzyme was created by site-directed mutagenesis just before the STOP codon. The GFP S65T protein was cloned as a *Not*I/*Not*I DNA fragment. The fusion was cloned into the pJK148 plasmid and integrated in the genome. Studying its phenotype when expressed from the pREP41X vector assessed functionality of this fusion. Images were captured with a Leica DM RXA and processed with Adobe Photoshop software.

3. Results

It had been described that a chs2::GFP fusion protein identified in a large screening of protein localisation was located at
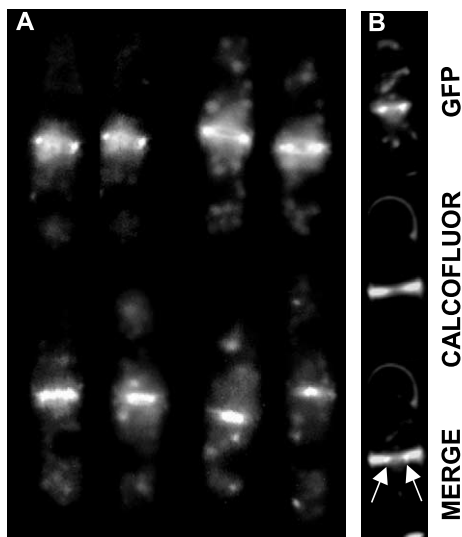

Fig. 1. chs2p localises to the inner side of the growing septum. A: Cells bearing the chs2::GFP chimera were photographed under the fluorescence microscope; representative cells undergoing cytokinesis are shown. B: Cells were stained with Calcofluor and photographed using different filters to visualise chs2p (GFP), cell wall (Calcofluor) or both (merge). Arrows point to the growing edge of the septum.
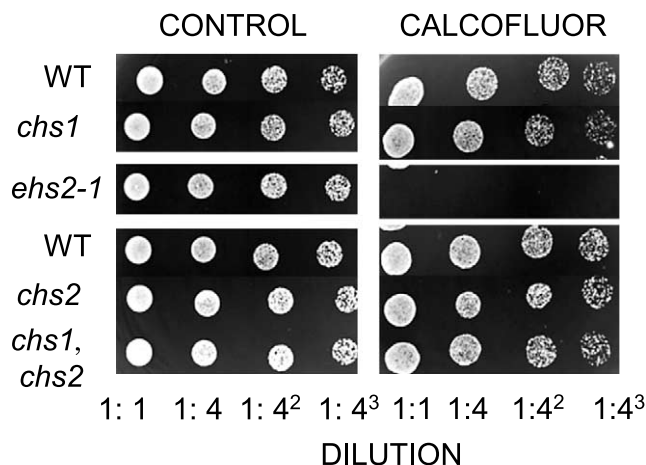

Fig. 2. Growth in the presence of Calcofluor. Serial 1/4 dilutions from the indicated strains were grown in buffered YES medium supplemented with Calcofluor (2 mg/ml). Plates were incubated at 28°C for 60 h.

the septum. chs2p in this fusion was expressed from a plasmid, lacked one amino acid, and it was not shown to be functional [18]. In order to confirm this result we constructed a full-length chs2::GFP fusion and integrated it at the chromosome. Observation under fluorescence microscope revealed that chs2p only localises to the septum. However, the width of the fluorescent mark varied from cell to cell (Fig. 1A). Additionally, Calcofluor staining showed that, in all the cells undergoing cytokinesis, chs2p clearly precedes the primary septum in its inward growth (an example is shown in Fig. 1B). These results indicate that chs2p co-localises with the contractile ring.

In order to determine if *chs2*$^+$ is essential for cell wall synthesis or morphogenesis we constructed a *chs2Δ* null mutant by replacing this gene with the *ura4*$^+$ marker in a diploid strain. Tetrad analysis showed that *chs2::ura4*$^+$ cells are viable. Since the *chs1*$^+$ gene shows a low-level expression during vegetative growth it was possible that it could compensate for the absence of *chs2*$^+$. In order to study this possibility we constructed a *chs1Δ chs2Δ* mutant. The double mutant turned out to be viable. We then carried out a detailed study of the single and double mutants. First, we studied the growth curve and the morphology of the cells in different media (YEPD, YES, EMM or ME) at different temperatures (20, 25, 28, 32 or 37°C). We could not find any difference between the wild-type (WT) and the mutant strains. Then, we analysed septum formation by staining cells with Calcofluor. The staining pattern was similar in all the strains analysed, showing that neither chs1p nor chs2p are essential for septum formation. In order to investigate if the *chs2*$^+$ gene plays a role in cell wall synthesis, we investigated if *chs2Δ* or *chs1Δ chs2Δ* mutants showed an enhanced sensitivity to this dye, as *S. pombe* mutants affected in glucan synthesis do [24,25], or if they were resistant to high concentrations of the drug, as is the case for *S. cerevisiae* mutants affected in chitin synthesis [26]. Since it has been described that *S. pombe* WT cells are resistant to 1 mg/ml Calcofluor [24], a concentration that inhibits cell wall synthesis in other fungi [27], we supplemented the medium with 0.4, 0.8, 1.0 and 2.0 mg/ml of Calcofluor. The hypersensitive *ehs2-1* mutant [25] was used as a control. We found that *S. pombe* is able to grow at 2 mg/ml Calcofluor and that

there was no difference between the mutants and the WT controls at any of the tested concentrations (Fig. 2). Additionally, when grown in liquid medium supplemented with the drug, all the strains presented multiseptated cells, but there was no difference in the number or structure of the septa between the WT and the *chs2Δ* cells. Finally, we wanted to check if Calcofluor enhances chitin synthesis in the *S. pombe* cell wall, as happens in *S. cerevisiae* [27], and if there was a difference in this enhancement between the *chs2Δ* mutants with respect to the WT. To do so, *S. cerevisiae* and *S. pombe* WT strains, and the *chs1Δ*, *chs2Δ*, and *chs1Δ chs2Δ* mutants from *S. pombe*, were treated with 100 μg/ml of the drug, extracted with 6% NaOH for 1 h at 37°C and stained with WGA–FITC (fluorescein isothiocyanate). Only the *S. cerevisiae* cells were stained with the lectin. Finally, we investigated if *chs2⁺* is necessary for mating or sporulation. Quantitative mating experiments showed that the *chs2Δ* cells conjugated and sporulated properly while the *chs1Δ chs2Δ* mutant behaved as the single *chs1Δ*. Taken together, all these results show that the *chs2⁺* gene does not play any prominent role during vegetative growth, mating or sporulation.

A common experimental strategy used to uncover the function of a protein is to over-express the corresponding gene. As shown in Fig. 3A, *chs2⁺* over-expression from the pREP3X plasmid (carrying a strong promoter) is lethal, leading to cell lysis that is not remedied by the presence of sorbitol, an osmotic stabiliser, indicating that it is not due to a cell wall defect (Fig. 3B). Growth is impaired even under repressing conditions, which are known not to be very tight [23]. Upon over-expression from the pREP41X or pREP81X plasmids (medium- and weak-strength promoters, respectively) cells are viable, although in the former case there is a slow-growth

phenotype. In both cases aberrant cells with several septa can be observed (Fig. 3C), and their number is proportional to the strength of the promoter (50% for the pREP41X-*chs2⁺* plasmid and 10% for the pREP81X-*chs2⁺*). Of these aberrant cells, $44 \pm 10.5\%$ present several septa with one nucleus per compartment, while $25 \pm 3.5\%$ of them have several nuclei in one of the compartments and $18 \pm 7.5\%$ have one enucleated compartment. Additionally, $5.2 \pm 1.5\%$ of the cells present a 'cut' phenotype, with the nucleus cut by the septum and $6.6 \pm 1.8\%$ of them present thickened septa. These results clearly show that an enhanced expression of the *chs2⁺* gene interferes with septation.

We then tried to find out whether chs2p is a CS enzyme. First, we measured activity in the *chs2::ura4⁺* null mutant, using the WT and *chs1Δ* mutant as controls. Although the activity in the *chs2Δ* mutant was lower than in the WT strain, it was not so low as in the *chs1Δ* cells (Table 1). In the case of the cells that bear the pREP41X-*chs2⁺* plasmid, the CS activity was higher than in the WT control, but much lower than in cells that over-express the *chs1⁺* gene. In both cases differences of activity with respect to the WT control were small but reproducible, and were consistent with a low CS activity of chs2p. However, in *chs1Δ* cells carrying the pREP41X-*chs2⁺* plasmid, CS activity was as low as in the *chs1Δ* mutant (Table 1). Thus, the activity detected in WT cells over-expressing the *chs2⁺* gene is dependent on *chs1⁺*. Another possibility was that in *S. pombe* there was a *chs2⁺*-dependent CS activity but that its biochemical properties were different from those of the activity dependent on *chs1⁺*. To check on this, we obtained membrane extracts from the *chs1Δ* cells that over-express the *chs2⁺* gene and measured activity in the absence or presence of different amounts of trypsin, at different pH val-
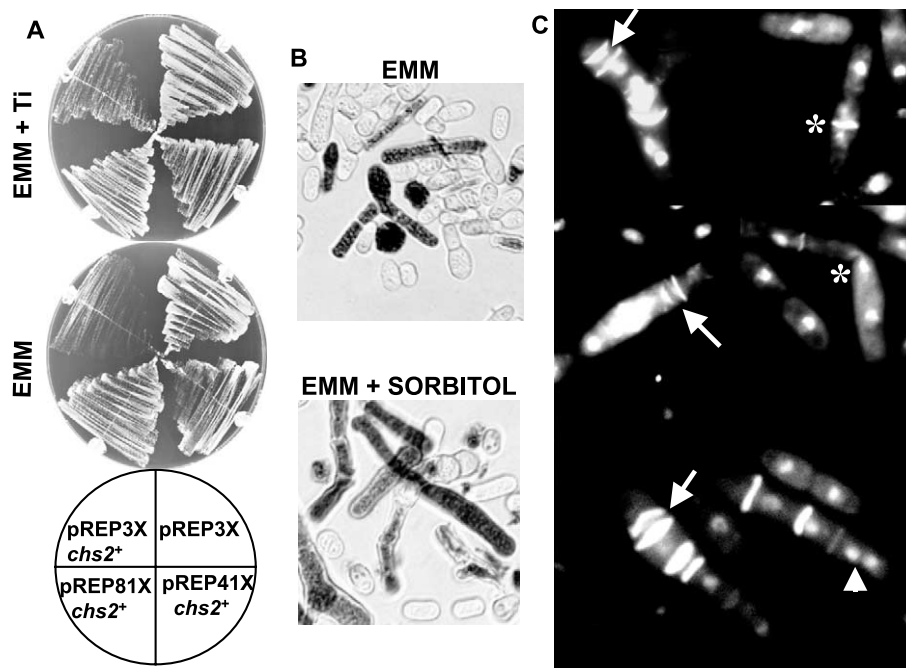

Fig. 3. chs2⁺ over-expression causes cell lysis and aberrant septation. A: Cells carrying the indicated plasmids were streaked on minimal medium supplemented with thiamine (EMM+Ti; repressing conditions) or EMM (derepression) and incubated at 32°C for 48 h. B: Cells expressing the *chs2⁺* gene from the pREP3X plasmid cultivated in EMM medium with or without 1.2 M sorbitol were stained with methylene blue. Dark cells are those that have been stained because they have lost selective permeability. C: Cells with the pREP81X-*chs2⁺* plasmid were fixed in cold 70% ethanol and stained with DAPI and Calcofluor so nuclei and cell walls were observed. Arrows indicate cell compartments without a nucleus; the arrowhead points to a cell with two nuclei; the asterisks mark cells with a 'cut' phenotype.

Table 1
CS activities in different *S. pombe* strains

Strain	Plasmid	Activity[a]	
		Basal[b]	Total[c]
WT	None	0.30 ± 0.06	0.56 ± 0.06
chs1Δ	None	0.01 ± 0.01	0.02 ± 0.02
chs2Δ	None	0.10 ± 0.04	0.33 ± 0.08
chs1Δ chs2Δ	None	0.00 ± 0.01	0.00 ± 0.01
WT	pREP41X-*chs2*+	1.01 ± 0.2	2.15 ± 0.52
chs1Δ	pREP3X-*chs1*+	86 ± 12	109 ± 0.05
chs1Δ	pREP41X-*chs2*+	0.01 ± 0.01	0.03 ± 0.03

[a]CS activity is expressed as nmol of substrate incorporated per mg of protein per hour.
[b]Activity measured directly on the membrane extracts.
[c]Activity measured after optimal activation with trypsin.

ues (from 6.0 to 8.5) and using different divalent cations as putative inducers. We could not detect significant values of activity in any case, which confirms the previous result.

We had previously shown that the *ScCHS2* gene, when expressed in *S. pombe*, produces a CS activity that is not able to complement the sporulation defect of *chs1Δ* mutants [15]. We wanted to ascertain if expression of the *chs1*+ or *chs2*+ genes had an effect on the lethality of a *S. cerevisiae chs1Δ chs2Δ chs3Δ* strain. For this purpose, the HVY337 strain, that has a deletion on each of the *CHS* genes and bears an integrated plasmid with the *CHS2* ORF under the control of the *GAL1* promoter, was transformed with the following plasmids: pRS426, pRS426 carrying the *ScCHS2* gene, pRS426 with the *chs2*+ ORF cloned between the *ScCHS2* promoter and terminator sequences, and pVT101U carrying the *chs1*+ gene under the control of the *ADH1* promoter. These strains were maintained in SD medium with galactose where the *CHS2* gene provides an active CSII activity. To test viability the strains were transferred to an SD plate to repress expression of the integrated *CHS2* gene. Only the cells that express the *ScCHS2* gene survived (results not shown), demonstrating that neither *chs1*+ nor *chs2*+ substitute for *ScCHS2* or *ScCHS3* to promote cell growth in *S. cerevisiae*.

4. Discussion

In the fission yeast *S. pombe*, the major cell wall structural components are glucose homopolymers: β-glucans, α-glucan, and galactomannan [28]. Chitin is an important structural component in many fungal cell walls. Its presence in fission yeast remains controversial, but sequencing of its genome has revealed that this organism has several genes with similarity to CS enzymes and with some of their regulators. This finding was surprising and prompted us to study if these genes had a role in cell wall synthesis and/or in morphogenesis or if they were pseudogenes that had been left behind during evolution. Cloning and characterisation of the *chs1*+ gene showed that it does code for a CS activity that is essential for the spores to mature properly [15]. The *chs2*+ gene is more intriguing because, although it shares significant identity with CS from different fungi, it lacks many of the residues that have been shown to be essential for activity [10]. Study of this gene could give information about the structure–function relationship in CS enzymes and about its potential role in fission yeast.

CS activity determination indicated that chs2p is not a CS enzyme. Since the *chs2*+ gene is more similar to *ScCHS2*, a gene that leads to enhanced CS activity when over-expressed, than it is to *ScCHS3*, which requires over-expression of some

regulators to produce a high activity [29], it seems improbable that we are getting a negative result because some regulator is limited. The residual activity detected in cells over-expressing the *chs2*+ gene requires the presence of *chs1*+ and therefore could be attributed to an indirect activation of the *chs1*+-dependent activity by interference with some regulator through conserved sequences. The lack of enzymatic activity of chs2p is in agreement with the data obtained in *S. cerevisiae*, where single-site mutations that eliminate the third arginine or the tryptophan residues from the QRRRW domain in the *CHS1*, *CHS2* or *CHS3* genes eliminate the corresponding activity [12,13], and therefore underscores the relevance of this domain in the activity of CS enzymes.

It has been shown that the *ScCHS1* gene cannot complement the *S. cerevisiae chs2Δ chs3Δ* double mutant [3] and that the *ScCHS2* gene codes for a CS activity in *S. pombe* that is not able to complement the *chs1Δ* mutant [15], indicating that each activity must have specific requirements in every organism. Still, we decided to study the behaviour of the *S. pombe chs*+ genes when expressed in *S. cerevisiae*. Not surprisingly, the *chs2*+ gene is not able to complement the *S. cerevisiae chs1Δ chs2Δ chs3 Δ* mutant. Additionally, the *chs1*+-dependent activity does not complement the triple *chs* mutant from *S. cerevisiae* either. Northern analysis showed that the *chs1*+ mRNA is expressed in the budding yeast, but that it is processed since several small-size molecules were detected (results not shown).

Characterisation of null *chs2Δ* and *chs1Δ chs2Δ* mutants showed that *chs2*+ has no essential function in *S. pombe*, and that both *chs*+ genes are not redundant. Chitin cannot be detected in Calcofluor-treated *S. pombe* cells, suggesting that in this organism the drug is not able to increase chitin synthesis, probably because there is not a CS at the membrane of vegetative cells. On the other hand, high-level expression of *chs2*+ leads to a cell lysis that is not remedied by an osmotic stabiliser, indicating that chs2p does not participate in cell wall synthesis with essential functions. Low-level and medium-level expression causes a severe alteration of the septation process. Additionally, some cells have several nuclei or are enucleated; the same phenomenon was observed in *S. cerevisiae chs2Δ chs3Δ* mutants [3], suggesting that, in both organisms, aberrant septation interferes with nuclear segregation. Finally, a functional chs2::GFP fusion protein is only detected at the growing edge of the septum, confirming that this protein is able to interact with the septum machinery. It is possible that at some time chs2p was a functional CS enzyme required for *S. pombe* to synthesise chitin at the septum, but that in the process of evolution the fission yeast has substi-

tuted chitin by (1,3)-β-glucan [30] with the emergence of different specialised glucan synthases. Meanwhile, the *chs2*+ gene has accumulated mutations that have eliminated its CS activity because of a lack of selective pressures. It is possible that chs2p from both micro-organisms share some sequences that promote their interaction with some neck or septum components. In budding yeast this interaction would result in chitin synthesis at the primary septum, while in *S. pombe* it could contribute to septation by strengthening the septal region. An excess of chs2p would destabilise the septum while other structural proteins could compensate lack of this protein in the null mutant. Experiments directed to study the relationship between *chs2*+ and other genes involved in septum synthesis are in progress.

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