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Chitin synthase I and chitin synthase II are not required for chitin synthesis *in vivo* in *Saccharomyces cerevisiae*

(yeast cell wall/septum/chitin synthase III/morphology/multinucleate)

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ABSTRACT **In *Saccharomyces cerevisiae*, the polysaccharide chitin forms the primary division septum between mother cell and bud. Two related enzymes, chitin synthase I and chitin synthase II (UDP-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxyglucosyltransferase, EC 2.4.1.16), have been identified and their structural genes, *CHS1* and *CHS2*, respectively, have been cloned and sequenced. Gene disruption experiments led to the conclusion that *CHS2* is essential for cell division [Silverman, S. J., Sburlati, A., Slater, M. L. & Cabib, E. (1988) *Proc. Natl. Acad. Sci. USA* 85, 4735–4739], whereas *CHS1* is not. We repeated the disruption of *CHS2* and determined that it is not essential for vegetative growth. The viability of *chs1*::*HIS3* *chs2*::*TRP1* spores is influenced by strain background and germination conditions. The double disruption mutant has no detectable chitin deficiency *in vivo*, as judged by quantitative assay and by staining cells with Calcofluor. Assay of membrane preparations from the double disruption mutant indicates the presence of chitin synthetic activity. Unlike the *CHS* gene products, this third activity is not stimulated by trypsin. Characterization of the double disruption mutant revealed abnormalities in morphology and nuclear migration.**

Chitin, a linear chain of β-1,4-*N*-acetylglucosamine residues, plays a structural role in the cell walls of fungi and the exoskeletons of arthropods. In the yeast *Saccharomyces cerevisiae*, chitin is localized primarily in the division septum between mother and daughter cells, implying that its synthesis must be regulated temporally and spatially (1). The molecular basis of this regulation is unknown.

Two chitin synthases (UDP-acetamido-2-deoxy-D-glucose: chitin 4-β-acetamidodeoxyglucosyltransferase, EC 2.4.1.16) have previously been detected in *S. cerevisiae* membrane preparations (2). Both are zymogens that require partial proteolysis for maximal activity *in vitro*. The structural genes for chitin synthase I and chitin synthase II, designated *CHS1* (3) and *CHS2* (4, 5), respectively, have been cloned and sequenced. Comparison of the deduced amino acid sequences shows that the *CHS1* and *CHS2* proteins are related; over a region of ≈660 amino acids, they are 42% identical (5).

In the presence of trypsin, chitin synthase I is the major activity *in vitro* (2), and Cabib *et al.* (6) have proposed a role for this enzyme in cell wall repair. However, *chs1*::*URA3* mutants have as much chitin *in vivo* as wild-type cells (3), and direct evidence that this enzyme makes chitin *in vivo* is still lacking.

Based on gene disruption experiments, Silverman *et al.* (4) concluded that *CHS2* is essential for growth. Cabib *et al.* (2, 6) have cited this result as evidence that *CHS2* is required for chitin synthesis, which in turn is required for septation and cell division. However, results from other laboratories raise questions about the role of chitin in septation. Roncero *et al.* (7) and Bulawa and Robbins (8) have isolated mutants that contain <10% of the wild-type level of chitin *in vivo*, and these cells divide normally. Thus, growth and septation can proceed in the absence of most of the chitin. This discrepancy prompted us to reinvestigate the function of *CHS2*. In the present study, we show that under certain conditions, *chs2* null alleles are viable. Surprisingly, these mutants have no detectable deficiency in chitin *in vivo*, but they do display abnormalities in morphology and division.

MATERIALS AND METHODS

Strains and Plasmids. The *S. cerevisiae* strain YPH274 (*MATa*/*MATα ura3-52*/*ura3-52 lys2-801*^{amber}/*lys2-801*^{amber}*ade2-101*^{ochre}/*ade2-101*^{ochre} *trp1-Δ1*/*trp1-Δ1 his3-Δ200*/*his3-Δ200 leu2-Δ1*/*leu2-Δ1*) is the parent of all strains constructed in this study; it is derived from two isogenic haploids (9). The following plasmids were sources of the indicated genes: pSS2 (4), *CHS2*; pMS-1(3), *CHS1*; pJH-H1, *HIS3*; and pJH-W1, *TRP1*. The plasmids pJH-H1 and pJH-W1 were generously provided by J. Hill (Carnegie–Mellon University). Molecular cloning techniques were standard (10). pSS352 is the 6-kilobase (kb) *Hin*dIII–*Sph* I fragment of pSS2 ligated into the same sites of the vector YEp352 (11).

Disruption of *CHS1* and *CHS2*. The *chs1*::*HIS3* mutation was constructed by replacing the 2.4 kb of *CHS1* coding sequence between the *Pst* I sites with the *HIS3* gene. The *chs2*::*TRP1* mutation was constructed by replacing the 1.9 kb of *CHS2* coding sequence between the *Bgl* II and *Sal* I sites with *TRP1*. A detailed description of these constructions will be reported elsewhere. A linear fragment containing the *chs1*::*HIS3* allele was used for transformation (12) of YPH274. His$^+$ transformants were sporulated and subjected to tetrad analysis. Two *chs1*::*HIS3* haploids, CBY15 and CBY18, were mated to give CBY19. This diploid was transformed with a linear fragment containing the *chs2*::*TRP1* allele. Three independent Trp$^+$ transformants, CBY20, CBY21, and CBY22, were used for the experiment described in Fig. 1. Sister strains 42.1A, 42.1B, 42.1C, and 42.1D were obtained from the germination of CBY21 on SD plates.

Media and Growth Conditions. Cells were grown at 30°C. Three different media were used: YPD, SD, and YPG. SD medium was supplemented with nutrients as required by the strain. For solid media, agar was added to 2%. Diploids were sporulated in 0.3% potassium acetate at 25°C (12).

Chitin Synthase Assay. Membranes were isolated from exponentially growing cells as described by Orlean (13). Chitin synthase activity was measured by incubating 10–20 μl of membranes with 1 mM UDP[U-^{14}C]GlcNAc (625 cpm/nmol)/40 mM GlcNAc/50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$ at 25°C. The reaction was stopped with 10% trichloroacetic acid and the amount of product was quantitated (3).

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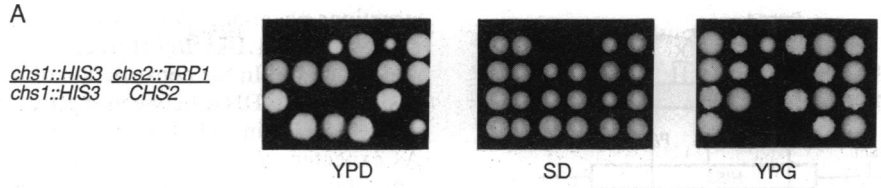
Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DIC, differential interference contrast.

A



B

DIPLOID	MEDIUM	VIABILITY (LIVE:DEAD) 4:0	3:1	2:2	1:3	0:4	VIABLE/TOTAL	*TRP⁺/trp⁻*
chs1::HIS3 chs2::TRP1 / *chs1::HIS3 CHS2*	YPD	1	14	18	2	1	84/144	17/66
	SD	28	5	1	0	1	129/140	67/62
	YPG	15	11	6	2	4	107/152	55/52
chs1::HIS3 CHS2 / *chs1::HIS3 CHS2*	YPD	12	1	0	0	0	51/52	-
	SD	12	1	0	0	0	51/52	-
	YPG	10	1	1	1	0	46/52	-

FIG. 1. Germination and growth of tetrads from *chs2::TRP1/CHS2* diploid on YPD, SD, and YPG media. (*A*) Strains CBY19, CBY20, CBY21, and CBY22 were sporulated and ≈12 tetrads from each were dissected onto an agar slab. The slabs were transferred to each of the plates indicated above and incubated at 30°C. After 4 days on YPD, 5 days on SD, and 7 days on YPG, the plates were photographed. Representative sections from CBY22 are pictured above. On YPD, the difference in colony size was more pronounced with a shorter incubation period. (*B*) Summary of tetrad analysis from *A* above. The results from CBY20, CBY21, and CBY22 were combined.

For proteolytic activation of the *CHS2* gene product, membranes were treated with trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone). After 10 min at 30°C, a 2-fold excess of soybean trypsin inhibitor was added. These samples were assayed as described above.

Measurement of Chitin *in Vivo*. Extraction of cells with KOH, digestion of ghosts with chitinase, cleavage of chitobiose with hexosaminidase, and measurement of GlcNAc were performed as described (3), except that chitinase was from *Streptomyces plicatus* and hexosaminidase was from *Helix pomatia* (β-glucuronidase).

Microscopy. Cells were fixed, stained, and mounted as described (14). A Zeiss Axioplan microscope was used.

RESULTS

Strains with *chs2* Null Mutations Are Viable. The structure of the *chs2::TRP1* null mutation is shown in Fig. 2. The 1.9 kb of coding sequence between the *Bgl* II site and the *Sal* I site has been replaced by *TRP1*; the extent of this deletion is identical to disruption allele no. 1 previously characterized by Silverman *et al.* (4). Because *CHS1* and *CHS2* encode related proteins (5), they may be able to substitute for one another *in vivo*. To eliminate this possibility, we constructed a *chs1::HIS3/chs1::HIS3* diploid (see *Materials and Methods*), designated CBY19. This strain was transformed with a DNA fragment containing the *chs2::TRP1* gene, and three independent Trp^+ colonies, designated CBY20, CBY21, and CBY22, were sporulated and then dissected on YPD plates. After 2 days of incubation at 30°C, each tetrad had produced only two colonies of easily visible size. However, after 2 additional days, 39% of the tetrads had produced three colonies (Fig. 1). In each of these cases, one of the three colonies was small and Trp^+, whereas the other two were large and Trp^-.

To test the effect of a functional *CHS1* gene on viability, we repeated the disruption of *CHS2* with a *CHS1/CHS1* diploid (YPH274). Results similar to those reported above were obtained (data not shown).

The Viability of *chs1::HIS3 chs2::TRP1* Strains Is Influenced by Growth Medium. Microscopic examination of *chs1::HIS3 chs2::TRP1* mutants grown in YPD (rich medium with glucose as the carbon source) revealed several morphological abnormalities (see below), but these could be significantly corrected by growth in SD (synthetic minimal medium with glucose as carbon source and ammonium sulfate as nitrogen source) or YPG (rich medium with glycerol as carbon source).

We tested the effect of these media on spore viability. Tetrads from the *chs1::HIS3/chs1::HIS3 chs2::TRP1/CHS2* strains (CBY20, CBY21, CBY22) and from the *chs1::HIS3/chs1::HIS3 CHS2/CHS2* control (CBY19) were dissected and germinated on YPD, SD, and YPG. Photographs of the plates from the dissection of CBY22 are shown in Fig. 1*A*. (Identical results were obtained with CBY20 and CBY21; not shown.) As described above, germination on YPD gave low spore viability (58%; Fig. 1*B*), and the colonies differed in size. On SD and YPG, spore viability was higher (92% and 70%, respectively; Fig. 1*B*), and the colonies were of more uniform size. On these media, unlike YPD, Trp^+ and Trp^- colonies were recovered with equal frequency, indicating no bias against the survival of the *chs1::HIS3 chs2::TRP1* double disruption mutant (Fig. 1*B*). The viability of spores from the *chs1::HIS3/chs1::HIS3 CHS2/CHS2* control was high on all three media (Fig. 1*B*).

On YPG, dissection of *chs1::HIS3/chs1::HIS3 chs2::TRP1/CHS2* asci gave two types of colonies: either round and smooth or ruffled and granular (Fig. 1*A*). Tetrad analysis showed that the colony morphologies segregated 2:2; the round, smooth morphology cosegregated with Trp^- and the ruffled, granular morphology cosegregated with Trp^+.

When we disrupted *CHS2* in a different strain, one that was not derived from YPH274, we obtained results similar to those originally reported by Silverman *et al.* (4); we recovered only two colonies per tetrad on YPD. However, we were able to obtain viable *chs2* haploids in two ways. First, when spores were germinated on YPG, *chs2* colonies were obtained at a low frequency. Second, when a high copy plasmid carrying *CHS2* and *URA3* (pSS352) was introduced into the *chs2/CHS2* diploid, sporulation and dissection of the transformant gave two *CHS2*/pSS352 colonies and two *chs2*/pSS352 colonies; all four colonies lost the plasmid at similar frequencies, as judged by growth on medium containing 5-fluoroorotic acid (data not shown).

Southern Analysis of the *chs1::HIS3 chs2::TRP1* Mutant. One explanation for the recovery of viable Trp^+ His^+ colonies was that the DNA fragments carrying the disrupted *chs* alleles did not integrate into the expected genes. To eliminate this possibility, Southern analyses were performed on six strains: YPH274 (wild-type), CBY21 (*chs1::HIS3/chs1::HIS3 chs2::TRP1/CHS2*), and four haploid segregants from CBY21, 42.1A, 42.1B, 42.1C, and 42.1D. For the analysis of

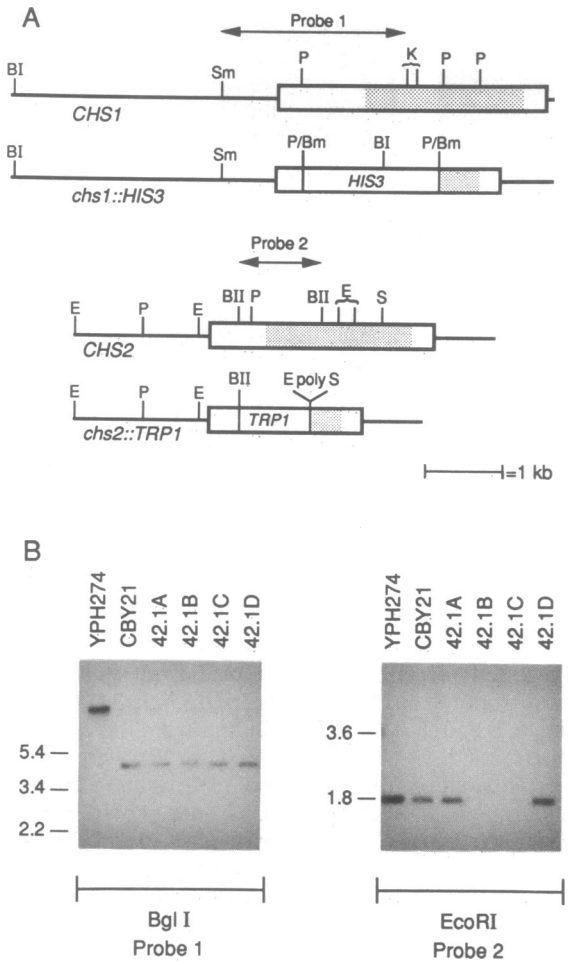


FIG. 2. Structure of the *CHS1*, *chs1*::*HIS3*, *CHS2*, and *chs2*::*TRP1* genes and Southern analysis of genomic DNA from YPH274, CBY19, 42.1A, 42.1B, 42.1C, and 42.1D. (*A*) The open boxes show the extent of the open reading frames as determined from the DNA sequences. The shaded areas encode a conserved region of 660 amino acids. The black line represents chromosomal DNA. Restriction site abbreviations: BI, *Bgl* I; BII, *Bgl* II; Bm, *Bam*HI; E, *Eco*RI; K, *Kpn* I; P, *Pst* I; S, *Sal* I; and Sm, *Sma* I. P/Bm was generated by cutting with *Pst* I, making blunt ends with T4 DNA polymerase, and adding *Bam*HI linkers. Poly is a polylinker (*Sac* I, *Kpn* I, *Sma* I, *Bam* HI, *Xba* I) that originated from pJH-W1. (*B*) Cells were grown overnight in YPD. Chromosomal DNA was isolated (12) and digested with the indicated restriction enzymes. Southern blotting was performed as described (3, 10). Probe 1 is the 2.4-kb *Sma* I to *Kpn* I fragment from pMS-1. Probe 2 is the 1.1-kb *Bgl* II fragment from pSS352. Purified fragments were radiolabeled by nick-translation. The blots were washed with 0.3 M NaCl/0.03 M sodium citrate (2× SSC) at 65°C. The numbers to the left of each autoradiogram give the size (kb) and position of molecular weight standards. The theoretical sizes of the radioactive bands for each allele are as follows: >7.6 kb for *CHS1* (YPH274); 4.8 kb for *chs1*::*HIS3* (CBY21, 42.1A, 42.1B, 42.1C, and 42.1D); 1.8 kb for *CHS2* (YPH274, 42.1A, and 42.1D); 1.8 kb for *chs2*::*TRP1*/*CHS2* (CBY21); and no band for *chs2*::*TRP1* (42.1B and 42.1C). The blot on the right was stripped and reprobed with a 1.4 kb *Pst* I fragment containing the beginning of *CHS2*; the expected results were obtained (data not shown).

the *CHS1* constructions, genomic DNA was digested with *Bgl* I and probed with a 2.4-kb *Sma* I to *Kpn* I fragment containing the beginning of the *CHS1* gene and upstream flanking sequence. In the construction of the *chs1*::*HIS3* disruption, insertion of the *HIS3* gene introduced a new *Bgl* I site (Fig. 2*A*). The fragment from CBY21 was smaller than the one from YPH274, as expected (Fig. 2*B*). Since CBY21 is homozygous, all of the strains in tetrad 42.1 also have the smaller, 4.8-kb, fragment. For analysis of the *CHS2* constructions, genomic DNA was digested with *Eco*RI and probed with a 1.1-kb *Bgl* II fragment within the *CHS2* open reading frame. In the construction of the *chs2*::*TRP1* disruption, 1.9 kb of DNA between the *Bgl* II and *Sal* I sites was deleted, removing all of the DNA homologous to the probe. As expected, there was no detectable hybridization to the DNA from the Trp^+ haploids, 42.1B and 42.1C (Fig. 2*B*). These data verify the construction and integration of both *chs* disruption alleles.

***CHS2* Is Not Required for Chitin Synthesis *in Vivo*.** To determine how much chitin is made *in vivo* by chitin synthase II, cells from tetrad 42.1 were grown in YPD, SD, and YPG, and chitin levels were quantitated (Fig. 3). No reduction in cellular chitin was observed in the *chs1*::*HIS3 chs2*::*TRP1* mutants (42.1B and 42.1C) under any of the growth conditions tested. On the contrary, in YPD, the mutant contains twice as much chitin as the *chs1*::*HIS3 CHS2* control. These results are consistent with the analysis of cells by staining with Calcofluor (see below). We detect no loss of chitin in the *chs1*::*HIS3 chs2*::*TRP1* mutant *in vivo*, implying that most of the chitin in cells is made by a third chitin synthase.

The *chs1*::*HIS3 chs2*::*TRP1* Mutants Contain a Trypsin-Independent Chitin Synthase. Because previous work established that the *CHS1* and *CHS2* genes encode chitin synthase zymogens (3, 4, 15), we performed assays with and without trypsin preincubation. If *CHS1* and *CHS2* encode the only chitin synthases in vegetative cells, then the *chs1*::*HIS3 chs2*::*TRP1* mutant should lack enzymatic activity under both conditions.

The four strains from tetrad 42.1 were grown in YPD and membranes were prepared. When assayed directly, without trypsin pretreatment, the *chs1*::*HIS3 chs2*::*TRP1* membranes incorporated [^{14}C]GlcNAc into an acid-insoluble product (Fig. 4). We believe that this activity is a chitin synthase because it was 60% inhibited by 1 μM polyoxin D, a specific inhibitor of fungal chitin synthases (16), and because the radiolabeled reaction product was sensitive to partially purified *St. plicatus* chitinase (data not shown). If chitin synthase II were active under these conditions, then *chs1*::*HIS3 CHS2* membranes (42.1A and 42.1D) would have higher specific activities than *chs1*::*HIS2 chs2*::*TRP1* membranes (42.1B and 42.1C). This was not observed; all of the strains had similar specific activities (Fig. 4 and Fig. 5 *Inset*, control), suggesting that most of the *CHS2* gene product is in the zymogen form. Taken together, these data indicate that a third chitin synthase is present that is the major activity under the present assay conditions. We will refer to this enzyme as chitin synthase III.

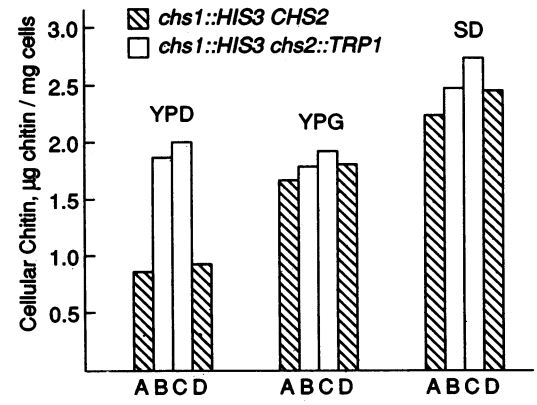


FIG. 3. Quantitation of chitin in cells from tetrad 42.1. Cells were grown in the indicated medium at 30°C and the amount of chitin *in vivo* was determined. A, B, C, and D represent strains 42.1A, 42.1B, 42.1C, and 42.1D, respectively.

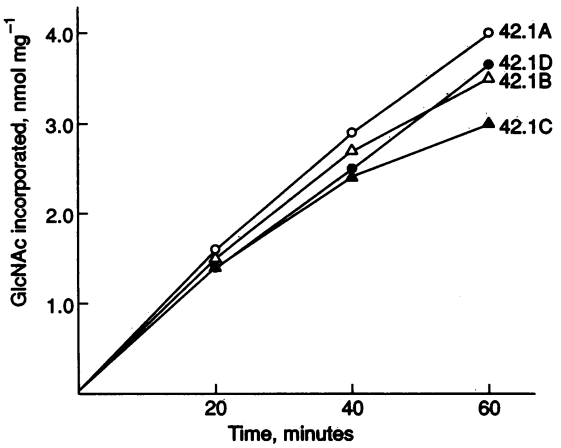


FIG. 4. Quantitation of trypsin-independent chitin synthase activity in membrane preparations from four sister strains. Logarithmically growing cells in YPD were harvested, and membranes were prepared and assayed for chitin synthase activity. The genotypes are 42.1A and 42.1D (*chs1::HIS3 CHS2*) and 42.1B and 42.1C (*chs1::HIS3 chs2::TRP1*).

We characterized the effect of proteolysis on the activity in each strain of tetrad 42.1. Membranes were preincubated alone or with the indicated amount of trypsin for 10 min at 30°C, proteolysis was terminated with trypsin inhibitor, and chitin synthase activity was measured. When membranes were preincubated without trypsin, about 40% of the activity was lost; this was observed with all four strains (Fig. 5 *Inset*). This instability was previously reported by Orlean (13). When trypsin was included, a stimulation in chitin synthesis was observed in the *chs1::HIS3 CHS2* membranes (Fig. 5), as expected. In contrast, the activity in the double mutant was inhibited over the entire range of trypsin concentrations. We conclude that chitin synthase III, unlike the *CHS1* and *CHS2* gene products, is not stimulated by trypsin.

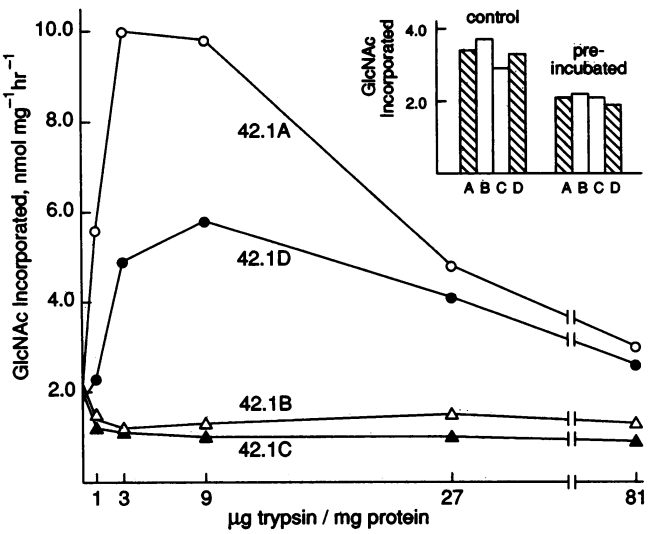


FIG. 5. Chitin synthesis in *chs1::HIS3 chs2::TRP1* membranes is inhibited by trypsin. Chitin synthase activity was determined as described in the legend to Fig. 4 except that the membranes were preincubated with the indicated amount of trypsin. The genotypes are 42.1A and 42.1D (*chs1::HIS3 CHS2*) and 42.1B and 42.1C (*chs1::HIS3 chs2::TRP1*). (*Inset*) Membranes were preincubated without trypsin for 10 min at 30°C (preincubated) or were kept on ice (control). All samples were then assayed for chitin synthase activity. GlcNAc incorporation is in nmol·mg^{-1}·hr^{-1}. The abbreviations and shading are defined in Fig. 3 and its legend.

The *chs1::HIS3 chs2::TRP1* Mutant Has Defects in Morphology, Chitin Localization, and Nuclear Migration. When grown in YPD, the *chs1::HIS3 chs2::TRP1* mutant displays several morphological abnormalities, including distortion of shape, variation in size, and widening of the neck region (Fig. 6*C*). Most of the cells are found in large aggregates (not shown) that are only partially dispersed by sonication. Despite these defects, the cells grow at about the same rate as the *chs1::HIS3 CHS2* control. A *CHS1 chs2::TRP1* mutant was indistinguishable from the double mutant (data not shown).

We examined the localization of chitin in *chs1::HIS3 chs2::TRP1*cells with the fluorescent dye Calcofluor. This dye binds specifically to bud scars (14), chitin-rich structures that remain on the mother cell after division. Strain 42.1D (*chs1::HIS3 CHS2*) exhibits the typical pattern for haploid cells, a series of adjacent rings with little nonspecific staining of the wall (Fig. 6*B*). In contrast, the periphery of the *chs1::HIS3 chs2::TRP1* mutant stains brightly and, in most cases, the outline of the cell is clearly visible. Rings can still be observed (Fig. 6*D*), but their size and position are more random than in the wild type.

We tested whether nuclear migration is perturbed by staining cells with DAPI. To eliminate interference from

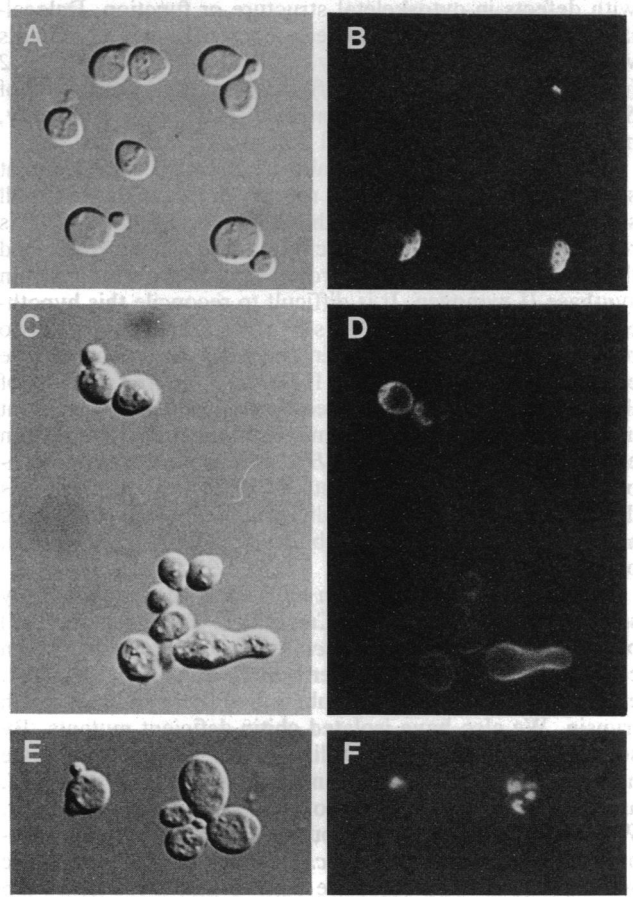


FIG. 6. Calcofluor and 4′,6-diamidino-2-phenylindole (DAPI) staining of strains 42.1C (*chs1::HIS3 chs2::TRP1*) and 42.1D (*chs1::HIS3 CHS2*). Cells of 42.1C and 42.1D were grown in YPD to A_{600} of 1–2. (*A–D*) Cells were fixed with formaldehyde, sonicated, stained with Calcofluor, and examined by differential interference contrast (DIC) and fluorescence microscopy. (*A*) DIC image of 42.1D. (*B*) Same field as in *A* except viewed by fluorescence microscopy. (*C*) DIC image of 42.1C. (*D*) Same field as in *C* except viewed by fluorescence microscopy. (*E* and *F*) Cells were fixed with 70% ethanol, sonicated, stained with DAPI, and examined by DIC and fluorescence microscopy. (*E*) DIC image of 42.1C. (*F*) Same field as in *E* except viewed by fluorescence microscopy.

mitochondrial DNA (14), ρ^0 derivatives of 42.1C and 42.1D were used. In the *chs1::HIS3 chs2::TRP1* culture, >20% of the cells either were anucleate or contained multiple DAPI-staining regions; the number of abnormal cells was 50-fold higher than in the *chs1::HIS3 CHS2* culture. Fig. 6 *E* and *F* show a cell that continued to bud in the absence of normal nuclear migration. These data show that CHS2 is required for normal morphology, division, and chitin localization.

DISCUSSION

We have shown that *CHS2* is nonessential for growth and for chitin synthesis in the strain YPH274. The viability of *chs1 chs2* spores is influenced by germination conditions. Using the strain SGY1046, Baymiller and McCullough* have recently obtained viable *chs2* haploids and have identified a second gene that permits growth in the absence of *CHS2*.

We have used the *chs1::HIS3 chs2::TRP1* mutant to demonstrate the presence of a third chitin synthase, which, unlike the *CHS1* and *CHS2* gene products, is not stimulated by trypsin. That *chs1* membranes contain a trypsin-independent chitin synthase was suggested by Orlean (13).

The *CHS2* gene is required for normal growth and division. The *chs2::TRP1* mutant is phenotypically similar to mutants with defects in cytoskeletal structure or function. Delocalized Calcofluor staining has been reported in various mutants with defects in actin assembly such as *act1* (actin) (17), *cap2* (capping protein) (18), and *pfy* (profilin) (19). Disruption of *SPA1* (20), a putative component of the spindle pole body, produces cells with abnormal numbers of nuclei and buds.

The morphological abnormalities of the *chs2::TRP1* mutant suggest that *CHS2* plays a role in determining cell wall structure. Cabib *et al.* (2) have proposed that *CHS2* is required for synthesis of the chitin-rich division septum and that septation is initiated by proteolyic activation of the chitin synthase II zymogen. It is difficult to reconcile this hypothesis with results from the present study. First, there was no reduction in chitin *in vivo* in the *chs1 chs2* mutant. We estimate that chitin synthase II makes no more than 10% of the chitin in a wild-type cell (see discussion of chitin-deficient mutants below). Second, when membrane preparations from exponentially growing *chs1 CHS2* strains were assayed without trypsin, little, if any, active *CHS2* gene product was detected; to date, there is no direct evidence that proteolytic activation, similar to that obtained *in vitro* with trypsin, occurs in the cell (2).

Mutants that lack chitin *in vivo* have been isolated. By selecting cells resistant to Calcofluor, Roncero *et al.* (7) obtained strains, designated *cal*, with a 9-fold reduction in cellular chitin. However, no enzymatic defect was detected, either with or without preincubation of the membranes with trypsin. We also have isolated chitin-deficient mutants. By screening for defects in the utilization of [^{3}H]glucosamine, we have identified three complementation groups, *csd2*, *csd3*, and *csd4*, that contain <10% of the wild-type level of chitin. When assayed directly, without trypsin pretreatment, membranes from the *csd2* and *csd4* mutants lack enzymatic activity (8), and therefore we believe that these genes are required for chitin synthase III activity. Partial DNA sequencing of *CSD2* indicates regions of similarity between the CSD2 gene product and the CHS gene products, suggesting that the *CSD2* gene encodes the catalytic portion of chitin synthase III (C.E.B., unpublished results).

Using polymerase chain reaction, Chen-Wu and Robbins† have detected *CHS*-related proteins in several other yeasts and fungi. A detailed mutational analysis of the *CHS2* gene in *S. cerevisiae* should identify functionally important regions of chitin synthase II that may be conserved throughout the *CHS* protein family.

Note Added in Proof. At a recent meeting, Valdivieso and Durán (21) reported that *CAL1* encodes a protein of 124 kDa that has regions of homology to chitin synthase I and chitin synthase II.

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1. Cabib, E., Roberts, R. & Bowers, B. (1982) *Annu. Rev. Biochem.* **51**, 763–793.
2. Cabib, E., Silverman, S. J., Sburlati, A. & Slater, M. L. (1990) in *Biochemistry of Cell Walls and Membranes in Fungi*, eds. Kuhn, P. J., Trinci, A. P. J., Jung, M. J., Goosey, M. W. & Copping, L. G. (Springer, Berlin), pp. 31–41.
3. Bulawa, C. E., Slater, M., Cabib, E., Au-Young, J., Sburlati, A., Adair, W. L., Jr., & Robbins, P. W. (1986) *Cell* **46**, 213–225.
4. Silverman, S. J., Sburlati, A., Slater, M. L. & Cabib, E. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4735–4739.
5. Silverman, S. J. (1989) *Yeast* **5**, 459–467.
6. Cabib, E., Sburlati, A., Bowers, B. & Silverman, S. J. (1989) *J. Cell Biol.* **108**, 1665–1672.
7. Roncero, C., Valdivieso, M. H., Ribas, J. C. & Duran, A. (1988) *J. Bacteriol.* **170**, 1950–1954.
8. Bulawa, C. E. & Robbins, P. W. (1989) *J. Cell. Biochem.* Suppl. **13E**, p. 27.
9. Sikorski, R. S. & Hieter, P. (1989) *Genetics* **122**, 19–27.
10. Tabor, S. & Struhl, K. (1988) in *Current Protocols in Molecular Biology*, eds. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (Wiley, New York).
11. Hill, J. E., Myers, A. M., Koerner, T. J. & Tzagoloff, A. (1986) *Yeast* **2**, 163–167.
12. Sherman, F., Fink, J. R. & Hicks, J. B. (1986) *Methods in Yeast Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
13. Orlean, P. (1987) *J. Biol. Chem.* **262**, 5732–5739.
14. Pringle, J. R., Preston, R. A., Adams, A. E. M., Stearns, T., Drubin, D. G., Harrer, B. K. & Jones, E. W. (1989) *Methods Cell Biol.* **31**, 357–435.
15. Sburlati, A. & Cabib, E. (1986) *J. Biol. Chem.* **261**, 15147–15152.
16. Gooday, G. W. (1990) in *Biochemistry of Cell Walls and Membranes in Fungi*, eds. Kuhn, P. J., Trinci, A. P. J., Jung, M. J., Goosey, M. W. & Copping, L. G. (Springer, Berlin), pp. 61–79.
17. Novick, P. & Botstein, D. (1985) *Cell* **40**, 405–416.
18. Armatruda, J. R., Cannon, J. F., Tatchell, K., Hug, C. & Cooper, J. A. (1990) *Nature (London)* **344**, 352–354.
19. Haarer, B. K., Lillie, S. H., Adams, A. E. M., Magdolen, V., Bandlow, W. & Brown, S. S. (1990) *J. Cell Biol.* **110**, 105–114.
20. Synder, M. & Davis, R. W. (1988) *Cell* **54**, 743–754.
21. Valdivieso, M. H. & Durán, A. (1990) *Yeast* **6**, abstr. 04-55B, S189.

*Baymiller, J. & McCullough, J., 90th Annual Meeting of the Society for Microbiology, May 13–17, 1990, Anaheim, CA, abstr. H-133, p. 176.

†Chen-Wu, J. & Robbins, P. W., Second Conference on Candida and Candidiasis: Biology, Pathogenesis and Management, April 1–4, 1990, Philadelphia, abstr. S14, p. 3.