Chitin Synthesis in a *gas1* Mutant of *Saccharomyces cerevisiae*

M-HENAR VALDIVIESO,¹ LAURA FERRARIO,² MARINA VAI,^{2†} ANGEL DURAN,¹
AND LAURA POPOLO^{2*}

Departamento de Microbiología y Genética/Instituto de Microbiología Bioquímica, Universidad de Salamanca/CSIC, Campus Miguel de Unamuno, 37007 Salamanca, Spain,¹ and Dipartimento di Fisiologia e Biochimica Generali, Università degli Studi di Milano, 20133 Milano, Italy²

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The existence of a compensatory mechanism in response to cell wall damage has been proposed in yeast cells. The increase of chitin accumulation is part of this response. In order to study the mechanism of the stress-related chitin synthesis, we tested chitin synthase I (CSI), CSII, and CSIII in vitro activities in the cell-wall-defective mutant *gas1Δ*. CSI activity increased twofold with respect to the control, a finding in agreement with an increase in the expression of the *CHS1* gene. However, deletion of the *CHS1* gene did not affect the phenotype of the *gas1Δ* mutant and only slightly reduced the chitin content. Interestingly, in *chs1 gas1* double mutants the lysed-bud phenotype, typical of *chs1* null mutant, was suppressed, although in *gas1* cells there was no reduction in chitinase activity. *CHS3* expression was not affected in the *gas1* mutant. Deletion of the *CHS3* gene severely compromised the phenotype of *gas1* cells, despite the fact that CSIII activity, assayed in membrane fractions, did not change. Furthermore, in *chs3 gas1* cells the chitin level was about 10% that of *gas1* cells. Thus, CSIII is the enzyme responsible for the hyperaccumulation of chitin in response to cell wall stress. However, the level of enzyme or the in vitro CSIII activity does not change. This result suggests that an interaction with a regulatory molecule or a posttranslational modification, which is not preserved during membrane fractionation, could be essential in vivo for the stress-induced synthesis of chitin.

Yeast cells are surrounded by a matrix composed of $\beta(1,3)/(1,6)$ -glucans and mannoproteins as major components and chitin as a minor one (21). Chitin constitutes only 1 to 2% of the cell wall dry weight, but it plays a key role in yeast morphogenesis and is essential for the viability of yeast and fungal cells. During vegetative growth chitin is deposited at the site of bud emergence, forms a ring that surrounds the neck between the mother and daughter cells, and constitutes the primary septum. On the surface of mother cells a chitin ring is still recognizable after cell division, the so-called bud scar, and in the corresponding site on the daughter surface a birth scar is present. A tiny amount of chitin is also layered over the whole of the lateral cell wall, and this occurs in the mother cell.

Three chitin synthase (CS) activities, CSI, CSII, and CSIII, are responsible for the deposition of cell wall chitin. The three isoenzymes differ in certain properties, such as the optimum pH, metal specificity, and susceptibility to inhibitors (6). CSI and CSII activities are determined only by the product of *CHS1* and *CHS2* genes, respectively, which encode the polypeptides containing the catalytic domain of each chitin synthase. Chs1p is responsible for the synthesis of chitin after cell separation. It plays a repair function, since it counterbalances the acid-induced increase in the chitinase activity that hydrolyzes the chitin present in the primary septum at the end of cytokinesis (3–5, 17, 18). CSI represents about 90% of the in vitro measurable chitin synthase activity, but its contribution to the production of chitin in vivo is negligible. Chs2p is responsible for deposition of the primary septum and is thus necessary for cell division (33, 34). CSIII activity is responsible for the deposition of chitin in the ring and lateral cell walls and

contributes to the synthesis of most cell wall chitin during vegetative growth (33). During cell cycle progression the Chs3p level remains constant (10, 38), but its localization changes (10, 31). A complex regulation of synthesis and transport determines the spatial and temporal control of chitin deposition by Chs3p, the catalytic component. The *CHS4* to *CHS7* genes are involved in this regulation (19, 32, 35, 36, 39). Additionally, CS activities exhibit in vitro zymogenic properties, suggesting that they are regulated at a posttranslational level (6, 8, 9).

In the present study we investigated the increase in chitin accumulation which appears to be part of the responses that a yeast cell activates to counteract cell wall damage. The *fks1Δ* mutant, which lost a subunit of the $\beta(1,3)$ -glucan synthase, has a reduced level of $\beta(1,3)$ -glucan and exhibits an induction of chitin accumulation (15, 28). A similar response is present in *gas1* cells which lack a $\beta(1,3)$ -glucosyltransferase activity (20) that is important for the correct incorporation of glucan and mannoproteins (see references 15, 24, 28, and 27 for a review). Moreover, the loss of Fks1p or Gas1p induces also the expression of Fks2p, the alternative subunit of the $\beta(1,3)$ -glucan synthase, a 20-fold increase in the cross-links between cell wall mannoproteins and chitin, and a 3-fold increase in *CWP1* expression (15, 24, 28). The increase in chitin deposition could be included in a compensation mechanism that mutants defective in cell wall synthesis or assembly activate to maintain cell integrity. In our study we focused on the role of CS activities in the possible mechanism of increased chitin synthesis in the *gas1Δ* mutant.

MATERIALS AND METHODS

Strains, growth conditions, and genetic methods. The yeast strains used here are listed in Table 1. Standard techniques were used for diploid construction, sporulation, and tetrad dissection. Cells were grown in batches at 30°C in YNB-glucose (Difco yeast nitrogen base without amino acids at 6.7 g/liter, 2% glucose, and the required supplements) or in YEPD (1% yeast extract, 2% Bacto-Peptone, 2% dextrose). For solid media, 2% agar was added. Diploids were sporulated in New Sporulation Medium (8.2 g of sodium acetate, 1.9 g of KCl, 0.35 g of MgSO₄, 1.2 g of NaCl, and 15 g of agar per liter) at 24°C. Spore germination

* Corresponding author. Mailing address: Università degli Studi di Milano, Dipartimento di Fisiologia e Biochimica Generali, Via Celoria 26, 20133 Milano, Italy. Phone: 39(02)70644808. Fax: 39(02)70632811. E-mail: Laura.Popolo@unimi.it.

† Present address: Università degli Studi di Milano-Bicocca, Dipartimento di Biotecnologie e Bioscienze, 20126 Milano, Italy.

TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source or reference
W303-1A	<i>MATa ade 2-1 his3-11,15 trp1-1 ura3-1 leu2-3,112 can1-100</i>	P. P. Slominski
W303-1B	<i>MATa ade2-1 his3-11,15 trp1-1 ura3-1 leu2-3,112 can1-100</i>	P. P. Slominski
WB2d	<i>MATa gas1::LEU2</i> ; as W303-1B	37
WAH	<i>MATa gas1::HIS3</i> ; as W303-1A	This study
W303- <i>chs3</i> Δ	<i>MATa call (chs3)::LEU2</i> ; as W303-1B	A. Duran
W303- <i>chs1</i> Δ	<i>MATa chs1::URA3</i> ; as W303-1A	This study
LF3	<i>MATa/α CHS3/chs3::LEU2 GAS1/gas1::HIS3; ade2-1/ade2-1 his3-11,15/his3-11,15 trp1-1/trp1-1 ura3-1/ura3-1 leu2,3-112/leu2,3-112 can1-100/can1-100</i>	This study
LF4	<i>MATa/α CHS1/chs1::URA3 GAS1/gas1::LEU2; ade2-1/ade2-1 his3-11,15/his3-11,15 trp1-1/trp1-1 ura3-1/ura3-1 leu2,3-112/leu2,3-112 can1-100/can1-100</i>	This study
Y604	<i>MATa ura3-52 lys2-801ade2-101 trp1-901 his3-Δ200</i>	31
Y1306	<i>MATa CHS3::3XHA; ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200</i>	31
Y1306ΔG	<i>MATa CHS3::3XHA gas1::HIS3</i> ; as Y1306	This study
LF5	<i>MATa/α chs1::URA3 chs3::LEU2 gas1::HIS3; ade2-1/ade2-1 his3-11,15/his3-11,15 trp1-1/trp1-1 ura3-1/ura3-1 leu2,3-112 leu2,3-112 can1-100/can1-100</i>	This study

was carried out at 24°C on YEPDAT plates (YEPD, 2% agar, and 100 mg of adenine and 50 mg of tryptophan per liter) containing 0.5 M KCl.

The following *Escherichia coli* strains were used: JM101 [Δ (*lac-proAB*) *thrA supE endA sbcB hsdR* (*F'* *traD36 proAB laqI*⁺ *lacZ*ΔM15)], DH5α [*F'* *endA1 hsdR17(r_K⁺m_K⁺) supE44 thi-1 recA1 gyrA[NaI]⁺ relA1 Δ(*lacZYA-orig*)F⁺U169 *deoR*(ϕ 80 *dlacZ*Δ(*lacZ*)M15)], and TOP10 [*F'* *mcrA* Δ(*mrr hsdRMS mcrBC*) Φ 80*lacZ*ΔM15 *ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL* (*Str*^r) *endA1 nupG*] (Invitrogen, Carlsbad, Calif.).*

DNA manipulations. Recombinant DNA manipulations were performed using standard techniques (30). Transformation of yeast cells was carried out using the lithium acetate procedure (14) or the *Saccharomyces cerevisiae* EasyComp Transformation Kit (Invitrogen).

Plasmid and strain construction. The *S. cerevisiae* haploid strain WB2d was generated from the wild-type strain W303-1B by gene replacement (37). In order to obtain the *gas1::HIS3* mutation we used PCR to synthesize a linear fragment which lacked almost the whole of the *GAS1* open reading frame (ORF). The upstream primer (5'-TGC GGA CGA TGT TCC AGC GAT TGA AGT TGT TGG TAA TAA GGT CCT GTT CCC TAG CAT GTA-) was designed to join 40 bp corresponding to the region from nucleotides 63 to 102 of the *GAS1* ORF with the 5' end from nucleotides 66 to 83 of *HIS3*. The downstream primer (5'-AGA CTT GGA AGA AGA CCC CGA AGC GTT AGA AGA GGC AGT ACT TGC CAC CTA TCA CCA CCA-) was designed to join 40 bp corresponding to the region from nucleotides 1470 to 1509 of the *GAS1* ORF with the 3' end from nucleotides 1446 to 1426 of *HIS3*. These primers were used to amplify a ~1.2-kbp fragment from YEp6. This PCR product was transformed into W303-1A and Y1306. His⁺ transformants were selected, and correct substitution was tested by PCR analysis. Immunoblot analysis further confirmed the absence of the *GAS1* gene product.

To construct W303-*chs1*Δ and WAH-*chs1*Δ, the *Bam*HI/*Bgl*II fragment (~2.4 kbp) of pHV149 (32), which carries the *chs1::URA3* allele, was used to transform strains W303-1A and WAH. Correct substitution at the *CHS1* locus was verified by PCR analysis and the absence of CSI activity.

CHS1 and *CHS2* radiolabeled RNA probes were obtained using the TOPO TA Cloning Kit Dual Promoter (Invitrogen). Plasmids pCHS1 and pCHS2 were constructed by inserting the following PCR fragments, covering the ORF regions, into the pCRII-TOPO vector: a 1.3-kbp fragment from pMS1 (3) for *CHS1* (from nucleotides 531 to 1821 from ATG), and a 1.5-kbp fragment from pUC19-CHS2 (M. H. Valdivieso, unpublished data) for *CHS2* (nucleotides 787 to 2253 from ATG). *ACT1* and *CHS3* probes were obtained from pACT and pCAL1 plasmids. pACT was obtained by cloning the 1.5-kbp *Hind*III-*Bam*HI fragment of the *ACT1* gene into the *Hind*III- and *Bam*HI-digested pGEM-3Zf(+). Plasmid pCAL1 was constructed by inserting the *Bgl*II-*Hind*III fragment of ca. 0.9 kbp of the *CHS3* gene into the pGEM-Blue plasmid cut with *Bam*HI and *Hind*III.

RNA extraction and Northern analysis. Total RNA was prepared according to the method of selective precipitation with LiCl (12). Northern analysis was performed as previously described, using nonradioactive or ³²P-radiolabeled single-stranded RNA probes generated by *in vitro* transcription (25). After hybridization at 50°C, blots were twice washed at 50°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min, once in 1× SSC-0.1% sodium dodecyl sulfate (SDS) for 30 min, and twice in 0.1× SSC-0.1% SDS for 30 min. Two final washes were carried out at 68°C in 0.2× SSC-0.1% SDS for 15 min and 0.2× SSC for 2 min. Densitometric quantification of mRNA was performed by using a computer program. mRNA loading was normalized using the hybridization signal of *ACT1*.

Preparation of total extracts and membrane fractionation. For total extracts, 2 × 10⁸ cells were collected by filtration, washed, and resuspended in ice-cold deionized water supplemented with a Protease Inhibitor Cocktail (Boehringer

Mannheim), one capsule in 25 ml, and 1 mM phenylmethylsulfonyl fluoride. After a 2-min centrifugation at 4°C, the pellets were frozen in dry ice-acetone and stored at -80°C. After thawing, 400 μl of SB-minus buffer (0.0625 M Tris-HCl pH 6.8; 5% SDS), supplemented with the protease inhibitors, was added to each pellet. After the addition of glass beads, cells were broken by shaking on a vortex for four cycles of 1 min alternated with 1 min in ice. After 5 min of centrifugation, the clarified lysate was withdrawn, quickly frozen, and stored at -80°C until use. For the determination of protein concentration, 15-μl aliquots of lysates in duplicate and the DC Protein Assay (Bio-Rad) were used. For SDS-polyacrylamide gel electrophoresis (PAGE) analysis, appropriate amounts of a concentrated solution were added to the lysate in order to bring the samples to a final concentration of 10% glycerol, 5% β-mercaptoethanol, and 0.002% bromophenol blue (BFB). Before the loading, samples were denatured at 100°C for 2 min.

Membranes were prepared as described by Orlean (22), except that the protease inhibitors were present during the whole procedure. Then, 2 × 10⁹ cells were collected by centrifugation, washed once with cold distilled water and then again with TM buffer (50 mM Tris-HCl, pH 7.5; 2.5 mM MgCl₂), and finally resuspended in 1.5 ml of TM buffer. After mechanical breakage, a pooled cell-wall-free extract was obtained as described above and then centrifuged at 60,000 × g for 45 min at 4°C, yielding supernatant (S) and pellet (P) fractions. The P fraction was resuspended in 200 to 400 μl of SB-minus buffer containing protease inhibitors, whereas the S fraction was concentrated with Centricon-10. After we determined the protein concentrations, aliquots of the P fraction were supplemented with the appropriate amounts of glycerol, β-mercaptoethanol, and BFB, whereas an equal volume of double-strength SDS sample buffer (0.0625 M Tris-HCl, pH 6.8; 2.3% SDS; 5% β-mercaptoethanol; 10% glycerol) was added to aliquots of the S fraction. Samples were denatured at 100°C for 2 min.

Electrophoresis and immunoblotting. Proteins were resolved by SDS-PAGE on 7 or 8% polyacrylamide slab gels. Immunodecoration was carried out as previously described (26). Mouse anti-hemagglutinin (HA) monoclonal HA.11 antibodies (Babco) were used at a 1:1,000 dilution in TBS (0.01 M Tris-0.9% NaCl, pH 7.4) containing 5% bovine serum albumin (BSA) and 0.5% Tween 20 and anti-Gas1p rabbit polyclonal antibodies at a 1:3,000 dilution in TBS, 5% BSA, and 0.1% Tween 20. Horseradish peroxidase-conjugated anti-mouse antibodies (Amersham) or anti-rabbit antibodies (Zymed) were diluted to 1:5,000 and 1:10,000, respectively, in TBS-5% BSA-0.2% Tween 20. Binding was visualized with the ECL Western Blotting Detection Reagent (Amersham) according to the manufacturer's instructions.

Measurement of chitin levels. Pellets corresponding to 5 × 10⁹ cells were collected, resuspended in 4.5 ml of H₂O, and divided into three equal aliquots (one was used for the determination of dry weight, and the other two were centrifuged), and the pellets stored at -20°C until use. After three extractions with 3% NaOH at 75°C, the alkali-insoluble pellet was neutralized and treated for 16 h with 4 mg of Zymolyase 100T per ml at 37°C. The chitin present in the indigestible material of the alkali-insoluble fraction was measured as described previously (24). The micrograms of glucosamine were normalized to the milligrams of dry weight.

Measurement of CS and chitinase activities. For CS activity measurements, cell extracts were obtained according to the protocol described previously (2). CS assays were performed in Tris (pH 7.5) in the presence of Mg²⁺, Co²⁺, or Co²⁺ plus Ni²⁺ in order to discriminate among the three different activities, as described earlier (7). For CSI activity, MES buffer at pH 6.3 was also used (7). The determination of CS activity in cells permeabilized with digitonin was accomplished as described previously (13), except that CSIII activity was measured in the presence of 50 mM Tris (pH 7.5), Co²⁺, and Ni²⁺. Chitinase activity assays were performed as described previously (17).

TABLE 2. CS activities in the *gas1* null mutant and its isogenic strain

Strain	CS activity (mU/mg of protein) ^a					
	CSI		CSII		CSIII	
	– trypsin	+ trypsin	– trypsin	+ trypsin	– trypsin	+ trypsin
W303-1B	38.5 ± 7.9	266.5 ± 26.5	2.7 ± 0.1	6.3 ± 0.3	8.7 ± 2	14.2 ± 1.7
WB2d	75.2 ± 2.7	428.0 ± 37	2.9 ± 0.1	6.1 ± 0.5	7.7 ± 0.8	14.8 ± 1.8

^a Assays were performed on crude membrane preparations in the presence of 50 mM Tris (pH 7.5) with (+) or without (–) trypsin.

Microscopic techniques. Chitin was visualized by fluorescence microscopy after being stained with 2 mg of Calcofluor White (CF) per ml (24).

RESULTS

CSI activity and *CHS1* mRNA increases are not responsible for the hyperaccumulation of chitin in *gas1Δ* cells. CS activities were measured in the wild-type (W303-1B) and *gas1Δ* (WB2d) strains in the presence or absence of trypsin. The results (Table 2) reveal that in the absence of trypsin, the CSI activity doubles in the *gas1Δ* mutant with respect to the isogenic strain and that trypsin treatment elicits a six- to sevenfold increase in CSI activity in both strains, a finding in agreement with the zymogenic properties of this enzyme. On the contrary, the CSII and CSIII activities were similar in both strains.

We then analyzed the expression of the *CHS1*, -2, and -3 genes in the *gas1Δ* mutant. The level of *CHS1* mRNA undergoes an increase of about 70% in *gas1Δ* cells (Fig. 1A), whereas the expression of the *CHS3* and *CHS2* genes was unchanged (data not shown). The increase in the *CHS1* mRNA level is in good agreement with the increase of CSI activity in the *gas1Δ* mutant.

In order to determine whether the induction of CSI activity

is involved in the increase in cell wall chitin levels in the *gas1Δ* mutant, we analyzed the tetrads obtained after sporulation of the *chs1Δ gas1Δ* heterozygous diploid LF4 (see Table 1). The spores obtained after dissection of 19 asci germinated normally, and all of them were viable. Two tetratype tetrads were analyzed in greater depth. No additional detrimental effect on the growth rate or morphological modification of *gas1Δ* cells were brought about by the *chs1Δ* mutation. Staining with CF revealed that the surface fluorescence of *gas1Δ* cells was very intense and was not changed by introduction of the *chs1* null mutation (data not shown).

The chitin present in the zymolyase-undigestible material of the alkali-insoluble fraction was measured. The amounts of chitin were 3.2 ± 0.5 , 2.8 ± 0.15 , 35 ± 5.8 , and 28 ± 4.7 μ g of glucosamine/mg (dry weight) of cells for the wild-type, *chs1Δ*, *gas1Δ*, and *gas1Δ chs1Δ* spores, respectively. In the *gas1Δ chs1Δ* mutant the chitin level was about 20% lower than in *gas1Δ*, but it was still 10-fold higher than in *chs1Δ*.

This result and the genetic analysis indicate that despite the increase in the in vitro CSI activity, Chs1p is not the major enzyme responsible for in vivo chitin synthesis in the *gas1* null mutant.

The *gas1* null mutation suppresses the lysed-bud phenotype of *chs1* null mutants. When *chs1Δ* cells are grown in unbuffered minimal medium, numerous small refractile buds can be observed by phase-contrast microscopy (3, 4). The refractile cells have been shown to be lysed cells because of the lack of the Chs1p repair function, which does not counterbalance the acid-induced chitinase activity after cell separation (4, 5). Surprisingly, in the *chs1Δ gas1Δ* double mutant this phenotype could not be observed (Fig. 1B).

We wondered whether the suppression in the lysed-bud phenotype of the *chs1 gas1* null mutant might be a consequence of a decrease in chitinase activity induced by the *gas1Δ* mutation. Thus, we measured chitinase activity in the spores of a tetrad. Unexpectedly, the secreted chitinase activity was stimulated in the presence of the *gas1Δ* mutation, with activities of 0.12 ± 0.01 nmol/10⁷ cells/min for the wild-type spores and 0.18 ± 0.01 , 0.4 ± 0.15 , and 0.32 ± 0.12 nmol/10⁷ cells/min for the *chs1Δ*, *gas1Δ*, and *chs1Δ gas1Δ* spores, respectively. Thus, suppression of the lysis-bud phenotype cannot be due to a decrease in chitinase activity. Moreover, this result strongly points to the notion that the increase in chitin accumulation in *gas1Δ* cells must be due to an increase of the synthesis of this polymer and not to a reduction in its degradation.

Chs3p is responsible for the increase in cell wall chitin levels in the *gas1* null mutant. We checked whether deletion of the *CHS3* gene might have any effect on the chitin synthesis process in the *gas1Δ* mutant. A modification of the *CHS3* locus by plasmid targeting had provided the first indication that the phenotype of *gas1Δ* cells is severely affected in the double mutant (24). In order to avoid any residual activity of Chs3p, we used a construct in which a large portion of the *CHS3* gene had been replaced by the *LEU2* gene. A heterozygous *chs3Δ*

FIG. 1. (A) Northern blot analysis of *CHS1* expression in *gas1Δ* cells. Total RNA was extracted from the *gas1Δ* mutant (WB2d) and its isogenic strain (W303-1B) when cells in YNB–glucose at 30°C reached a density of about 8×10^6 to 10^7 cells/ml. Ca. 8 μ g of RNA was loaded into each lane. Hybridization was performed with ³²P-labeled *CHS1* or *ACT1* antisense RNA probes. The autoradiograms were quantitated by densitometry. (B) Suppression of the lysis bud phenotype on the *chs1Δ gas1Δ* mutant. Cells were examined by phase-contrast microscopy. The arrows indicate the refractile cells.

isogenic strain (Fig. 3A). Upon overexposure of the blots additional smeared fragments of about 66 and 60 kDa were detected, and one of about 47 kDa was present only in the mutant (Fig. 3B). The extent of recovery of these fragments was not always the same across a series of experiments, but the pattern was qualitatively reproducible. These results suggest that Ha-tagged Chs3p is more susceptible to proteolytic degradation in the *gas1Δ* mutant than in the wild type.

Membrane and supernatant fractions were obtained with the same protocol used to determine the CSIII activity. In the membrane fraction (P), the 150-kDa polypeptide was the prominent band and its level did not show any appreciable differences between wild-type and *gas1Δ* strains (Fig. 3A). No enrichment of the 47- or 60- to 66-kDa polypeptides was observed, suggesting that they are probably unstable. Analysis of the supernatant fractions did not reveal any relevant species recognized by the antibodies used (data not shown).

CSIII activity increases in *gas1* mutant cells permeabilized with digitonin. In order to measure CSIII activity under more physiological conditions, whole cells permeabilized with digitonin were used (13). To avoid the high CSI activity level detected under these conditions, we constructed a set of strains that were deleted of the *CHS1* gene and were either wild type or mutant for the *CHS3* and/or *GAS1* genes. A heterozygous diploid (LF5) was constructed by crossing *chs1Δ* cells with the suppressed LF3-13D spore (*chs3Δ gas1Δ*) and then sporulated. Using this method we were able to detect an increase in CSIII activity in *gas1Δ* cells (30.4 ± 5.2 mU/ 10^7 cells in the *chs1Δ gas1Δ* mutant spore compared to 9.6 ± 2.3 mU/ 10^7 cells in the *chs1Δ* spore). No activity was detected in the *chs1Δ chs3Δ* or the *chs1Δ chs3Δ gas1Δ* control strains.

DISCUSSION

The aim of this study was to establish the role of the different CSs present in *S. cerevisiae* in the increase in chitin accumulation in the *gas1* mutant. Of the three CS activities, only CSI activity was affected by the presence of the *gas1Δ* mutation. The basal activity (measured without adding trypsin) doubled in the *gas1Δ* mutant. In contrast to this, the phenotype of the *chs1Δ gas1Δ* double mutant was indistinguishable from the single *gas1Δ* mutant in growth properties and morphology. Furthermore, the chitin level was only slightly decreased. We can therefore exclude the possibility that the increase in CSI would be implicated in the chitin response of the mutant. The induction of CSI could be explained in terms of the need to counterbalance the increase in chitinase activity that was unexpectedly found in the mutant. CSI is involved in the repair of damage to the cell wall caused by excessive chitinase activity at an acidic pH. The *gas1* null mutant could require a higher chitinase activity at the time of cytokinesis due to the higher chitin content in the cell wall. The increase in CSI could subsequently compensate for this increase but would not be essential since the *chs1 gas1* double mutant does not show any worsening of the phenotype of *gas1Δ* cells. In addition, it is relevant to note that in α -factor-treated cells, a well-known condition in which an increase in chitin occurs, *CHS1* mRNA was also found to be induced (1), and the expression of myc-Chs1p is approximately threefold higher than in untreated cells (38). Nevertheless, no direct participation of Chs1p in shmoo formation or mating was found, suggesting secondary roles for these changes in the conjugation process (32).

In addition to the lack of any compromise of the growth rate in the *chs1Δ gas1Δ* mutant, we observed a clear suppression of the small-bud-lysis phenotype typical of *chs1Δ* cells. Since this phenotypic trait has been ascribed to the loss of the repair

function of Chs1p at the birth scar, we interpret this result as being a consequence of the presence of an increased chitin deposition also found in the daughter cells, which could reduce the detrimental effects of the lack of CSI activity after cell division.

Biochemical analysis revealed that CSII and CSIII activities do not change in the mutant. However, we analyzed in detail the phenotypes of yeast cells carrying deletions in the *GAS1* and *CHS3* genes. The double-mutant cells were severely affected in germination, and the double mutation led to detrimental effects in liquid medium. The progressive adaptation of the cells suggested that suppressors were selected, as was also described for other double null mutants in cell-wall-related genes (for example, *kre6 skn1* [29]). Analysis of the chitin content of double-mutant cells clearly demonstrated that the bulk of chitin induced by the presence of the *gas1Δ* mutation is produced by CSIII. Since chitinase activity is increased, it can be ruled out that inhibition of degradation of this polysaccharide would contribute to the increase in its accumulation.

We attempted to determine at which level chitin synthesis is regulated in the *gas1* mutant. The *CHS3* mRNA level did not change in the *gas1Δ* mutant, excluding the idea that a transcriptional regulation would be involved. The protein level in total membrane did not change significantly between the mutant and the control. The presence of Chs3p-derived polypeptides of lower mobility in total protein extracts from *gas1* cells indicates an increased turnover of the Chs3p full-length protein. For the time being, it is not possible to say whether this effect is associated with a possible activation of the protein, with increased mobilization of the protein through the endocytotic pathway, or whether it might simply be an indirect consequence of pleiotropic effects of the mutation. Preliminary experiments have indicated that the same proteolytic fragments are detectable when the HA-tagged Chs3p is overproduced by the *GAL1-GAL10* promoter, indicating that they probably represent endogenous products of proteolysis, which for some reason are slightly stimulated in the mutant.

It is well known that also under other conditions of increase in chitin levels, such as treatment with CF or sporulation, no increase in the Chs3p level is found. By contrast, with α -factor treatment of the Chs3p level is sixfold higher, although this does not change the *in vitro* activity (9, 11). Thus, there is no correlation between the levels of protein and chitin synthesis, probably because other factors, such as posttranslational modifications, mobilization of the enzyme, or interaction with proteins limit the enzymatic activity. It is relevant to note here that a recent study has proposed that the stress-related chitin synthesis probably has a unique targeting and activation mechanism (23). Interestingly, the deposition of chitin by Chs3p in a *fks1Δ* mutant was found to be independent from Chs6p (23). Moreover, a putative cell wall sensor protein, Mid2, which functions upstream of the cell integrity pathway, appears to specifically modulate the accumulation of chitin in response to cell wall stress (16).

We measured CSIII activity in cells permeabilized with digitonin. This method was described to measure CSI activity (13); however, the facts that no activity was detected in the *chs3Δ* strains and that we obtained reproducible results confirm that it can also be used to detect CSIII activity. Although it is possible that the twofold increase in CSIII activity, detected under these conditions, could be due to different effects of digitonin in the control and the *gas1* strains because of their difference in cell wall structure, the results obtained are in agreement with genetic data demonstrating a requirement for CSIII activity in chitin accumulation in the *gas1* mutant. The ability to detect such an increase is lost in membrane fractions.

This suggests that in the case of the *gas1* null mutant a post-translational modification, an interaction with a regulatory molecule, or a specific ion requirement is not preserved in the membrane preparations used for testing CSIII activity according to the method currently available. In order to understand this posttranslational regulation, experiments to determine the role of other genes involved in the regulation of cell wall biosynthesis in the increase in chitin levels in the *gas1Δ* mutant are in progress.

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