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Research Article

Maintenance of cell integrity in the *gas1* mutant of *Saccharomyces cerevisiae* requires the Chs3p-targeting and activation pathway and involves an unusual Chs3p localization

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Abstract

Chitin synthase III is essential for the increase in chitin level and for cell integrity in cells lacking Gas1p, a β(1,3)-glucanosyltransferase. In order to discover whether the upregulation of chitin synthesis proceeds through the canonical transport and activation pathway of Chs3p or through an alternative one, here we studied the effects of the inactivation of the *GAS1* and *CHS4-5-6-7* genes. All the double-null mutants showed a temperature-sensitive cell lysis phenotype that could be suppressed by the presence of an osmotic stabilizer. In liquid YEPD at 30 °C, *chs4*Δ *gas1*Δ, *chs5*Δ *gas1*Δ, *chs6*Δ*gas1*Δ and *chs7*Δ*gas1*Δ mutants were unable to grow, whereas they grew very slowly in minimal medium and showed low viability. High osmolarity suppressed the defective phenotype and restored growth. In *chs4 gas1*, *chs5 gas1* and *chs7 gas1*, chitin levels did not increase and were reduced to only 10%, while in *chs6 gas1* the value of *gas1* was reduced to 20–40%. To investigate at which level the upregulation of chitin synthesis could occur, mRNA levels were monitored. The expression of *CHS4-5-6-7* did not change significantly in *gas1*Δ. In strains expressing HA-tagged forms, the localization of Chs3p and Chs5p was examined. In the *gas1* mutant the fluorescence pattern was affected and the proteins appeared abnormally present in the bud. The results indicate that: (a) the function of the *CHS4–7* genes is required for chitin hyperaccumulation in *gas1* mutant and for cell integrity; (b) homologous genes do not replace their function; (c) the regulation of *CHS4–7* genes does not occur at transcriptional level. Control of the position of chitin synthesis could be important in protecting the bud from lysis.

Keywords: **cell wall; chitin; stress**

Introduction

The extracellular matrix of *Saccharomyces cerevisiae* is composed of mannoproteins, β(1,3)-glucans, β(1,6)-glucans and chitin. β(1,3)-glucans constitute the main fibrous matrix and β(1,6)-glucan is a minor component to which glycosylphosphatidylinositol (GPI)-mannoproteins, chitin and β(1,3)-glucans are attached, playing an important cross-linking role (Cabib *et al*., 2001). The enzymes responsible for the cross-linking reactions are still largely unknown. Gas1p is an exocellular GPI-anchored glycoprotein that is homologous to other yeast and fungal proteins, Gel1p of *Aspergillus fumigatus* and Phr1p, Phr2p of *Candida albicans* among those best characterized (Popolo and Vai, 1999). These proteins are β(1,3)-glucanosyltransferases that catalyse the transfer of segments of β(1,3)-glucan to other β(1,3)-glucan chains (Mouyna *et al*., 2000). Lack

of Gas1p in *S. cerevisiae* reduces the cell wall glucan level and causes a marked accumulation of chitin and mannoproteins (Kapteyn *et al*., 1997; Popolo *et al*., 1997; Ram *et al*., 1998). The increase in chitin contents and its cross-links with β(1,6)-glucosylated mannoproteins have been proposed to be a part of a response activated to prevent the excessive release of mannoproteins consequent to the inability to establish cross-links between β(1,6)-glucosylated mannoproteins and β(1,3)-glucans (Fonzi, 1999; Kapteyn *et al*., 1997; Popolo *et al*., 1997; Ram *et al*., 1998). A similar behaviour is also displayed by the *fks1* Δ mutant, which lacks a subunit of β-1,3-glucan synthase and consequently has a reduced β1,3-glucan content (Ram *et al*., 1998).

Chitin normally constitutes only 1–2% of the cell wall dry weight but plays a key role in yeast morphogenesis: it is deposited in a ring at the site of bud emergence and constitutes the primary septum. A tiny amount of chitin is also layered all over the lateral cell wall, especially in the mother cell. Three chitin synthase genes, *CHS1*, *CHS2* and *CHS3*, are responsible for the synthesis of chitin (Cabib *et al*., 1996). Chs1p plays a repair function after cell division and contributes to the successful completion of cytokinesis under certain conditions (Cabib *et al*., 1989). Chs2p is responsible for the deposition of the primary septum and accounts for only about 10% of the total chitin (Shaw *et al*., 1991; Silverman *et al*., 1988). Chs3p is responsible for the deposition of chitin in the ring and lateral cell walls, accounting for more than 90% of cell wall chitin (Shaw *et al*., 1991).

Consistent with a strictly regulated spatial and temporal deposition of chitin during the cell cycle, other genes have been found to be required for the control of Chs3p activity (see Figure 1). *CHS4* encodes a probable activator of the CSIII complex, present in limited amount and required for both *in vitro* and *in vivo* activity, and plays the additional role of anchoring Chs3p at the site of chitin ring formation through interaction with the septin–Bni4p complex (Bulawa, 1993; Trilla *et al*., 1997; De Marini *et al*., 1997). The product of *CHS5* is required for the polarized transport of Chs3p and for *in vitro* and *in vivo* activity. Chs5p co-localizes with Kex2p in the *trans*-Golgi network (TGN) (Santos and Snyder, 1997; Santos *et al*., 1997). Chs6p is involved in the anterograde transport of Chs3p to the plasma membrane and in *in vivo* but not *in vitro* activity (Bulawa, 1992; Ziman *et al*., 1998). Chs7p is an integral membrane protein of the endoplasmic reticulum (ER), promotes the exit of Chs3p from ER, and its absence determines reduced *in vitro* and *in vivo* CSIII activity (Trilla *et al*., 1999). Figure 1 shows a scheme of the Chs3 transport pathway.

Homologues of *CHS4* and *CHS6* genes are present in the yeast genome: the product of *SHC1* and YDL203c show, respectively, 43% and 28% identity with Chs4p (Sanz *et al*., 2002) and YKR027w presents 43% identity with Chs6p. By contrast, Chs5p shares with YEL043w only the presence of a fibronectin type III domain fold (Bateman and Chothia, 1996).

The Chs3p transport pathway also involves the recycling of Chs3p from the plasma membrane to targeted areas, such as the site of incipient bud formation and the lateral wall, through an endocytic route in vesicles containing the syntaxin Tlg1p (Holthius *et al*., 1998, Madden and Snyder, 1998). Sbe2 and Sbe22, two Golgi proteins, are also required for correct Chs3p localization (Santos and Snyder, 2000).

It has been shown that, in *fks1* Δ mutant which lacks the catalytic subunit of the β-1,3-glucan

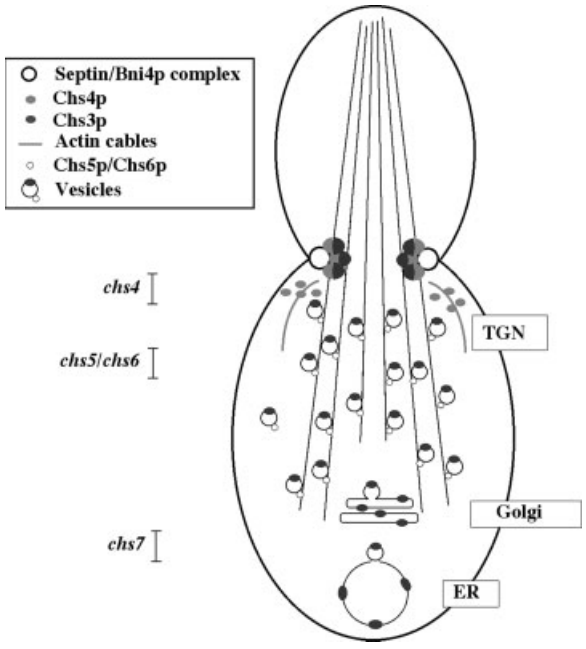

Figure 1. Anterograde transport of Chs3p to the plasma membrane

synthase, chitin increase is mediated by Chs3p and by the function of *CHS4-5* and *7* gene products, whereas the function of *CHS6* is dispensable, suggesting a possible alternative route for stress-related chitin accumulation (Osmond *et al*., 1999). In a previous work we demonstrated that CSIII is the enzyme responsible for the hyperaccumulation of chitin in *gas1* Δ cells (Valdivieso *et al*., 2000). Here we investigated the role of *CHS4-5-6-7* genes in the increased deposition of chitin in a *gas1* mutant observing that they are all required to maintain a high level of chitin and that they are essential for cell integrity. No transcriptional regulation was detected for these genes. Moreover, the localization of Chs3p and Chs5p was found to be affected in the mutant.

Materials and methods

Strains, growth conditions and genetic methods

The yeast strains used 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 minimal medium (SD) (Difco yeast nitrogen base without amino acids 6.7 g/l, containing 2% glucose and the required supplements: 50 mg for the amino acids and uracil, 100 mg/l adenine) or in YEPD (1% yeast extract, 2% Bacto Peptone, 2% dextrose). For solid media, 2% agar was added (SDA or YEPDA). As an osmotic stabilizer 1 M sorbitol (+S) was used. Diploids were sporulated in New Sporulation Medium (8.2 g/l sodium acetate, 1.9 g/l KCl, 0.35 g/l $MgSO_4$, 1.2 g/l NaCl, 15 g/l agar) at 24 °C. Spore germination was carried out at 24 °C or 30 °C on YEPDAT plates (YEPD, 2% agar, 100 mg/l adenine and 50 mg/l tryptophan). The osmolarity of the liquid media was measured by an Osmomat 30 (Gonotec) osmometer.

Growth was monitored as the increase in cell number determined on mildly sonicated cells by use of a Coulter counter. The rate constant (k, h^{-1}) of exponential growth was determined by linear regression on a semilogarithmic plot and duplication time (T) was calculated from the expression $T\ (\mathrm{h}) = ln2/K$.

Table 1. *Saccharomyces cerevisiae* strains

Strain	Genotype	Source
Y604	MAT**a** *ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200*	Santos and Snyder, 1997
Y1306	Y604 *CHS3::3XHA*	Santos and Snyder, 1997
Y1306ΔG	Y1306 *gas1::HIS3*	M-H. Valdivieso *et al*., 2000
Y1303	Y604 *CHS5::3XHA*	Santos and Snyder, 1997
Y1303ΔG	Y1303 *gas1::HIS3*	This work
W303-1A	MAT**a** *ade2-1 his3-11,15 trp1-1 ura3-1 leu2-3,112 can1-100*	P. P. Slominski
W303-1B	MATα *ade2-1 his3-11,15 trp1-1 ura3-1 leu2-3,112 can1-100*	P. P. Slominski
WB2d	W303-1B *gas1::LEU2*	Vai *et al*., 1991
WA1d	W303-1A *gas1::LEU2*	Vai *et al*., 1991
W303-*fks1*Δ	W303-1A *fks1::URA3*	C. Roncero
W303-*chs4*Δ	W303-1A *chs4::URA3*	Trilla *et al*., 1999
W303-*chs5*Δ	W303-1B *chs5::ADE2*	Trilla *et al*., 1999
W303-*chs6*Δ	W303-1A *chs6::URA3*	Trilla *et al*., 1999
W303-*chs6*Δ	W303-1B *chs6::HIS3*	C. Roncero
W303-*chs7*Δ	W303-1A *chs7::HIS3*	Trilla *et al*., 1999
LF7	$W303^2$ *gas1::LEU2/GAS1 chs4::URA3/CHS4*	This work
LF8	$W303^2$ *gas1::LEU2/GAS1 chs5::ADE2/CHS5*	This work
LF9	$W303^2$ *gas1::LEU2/GAS1 chs6::URA3/CHS6*	This work
LF10	$W303^2$ *gas1::LEU2/GAS1 chs7::HIS3/CHS7*	This work
LF11	$W303^2$ *fks1::URA3/FKS1 chs6::HIS3/CHS6*	This work
BJ5464	MATα *ura3-52 leu2Δ trp1 his3Δ2000 pep4::HIS2 prb1Δ1,6R can1*	M. Molina
BJ5464-*slt2*Δ	(Derived from BJ5464)*slt2::URA3*	M. Molina

Strain constructions

In order to obtain the *gas1::HIS3* mutation, PCR was used to synthesize a linear fragment which lacked almost the whole of the *GAS1* coding sequence and YEp6 was used as a template (Valdivieso *et al*., 2000). Correct integration was tested by PCR analysis and immunoblot analysis further confirmed the absence of the *GAS1* gene product in the PCR-positive transformants. Deletion of *CHS4-5-6-7* genes was performed as described by Trilla *et al*. (1999) and that of *FKS1* as in Garcia-Rodriguez *et al*. (2000).

Measurement of chitin level

Total cellular chitin was measured by an enzymatic method as described by Bulawa *et al*. (1992). Washed cells (about 100 mg wet weight) were resuspended in 1 ml 6% KOH and incubated at 80 °C for 90 min. After cooling to room temperature, 100 µl glacial acetic acid was added and the centrifugation supernatant was removed. Insoluble material was sequentially treated with *Serratia marcescens* chitinase (Sigma) and *Helix pomatia* β-glucoronidase and then N-acetylglucosamine content was assayed. A chemical method was also used and is described in Popolo *et al*. (1997).

Microscopy techniques

Indirect immunofluorescence was performed as outlined by Pringle *et al*. (1991) and Santos and Snyder (1997). Cells growing exponentially at 30 °C in SD were fixed in 3.7% formaldehyde and 0.1 M potassium phosphate buffer, pH 6.5, for 60 min and then washed three times with 0.1 M potassium phosphate buffer, pH 6.5, and once with digestion buffer (1.2 M sorbitol, 120 mM potassium phosphate, pH 5.9, and 33 mM citric acid). Spheroplasts were prepared by incubating cells in digestion buffer containing 50 µg/ml Zymolyase 100T and 0.2% β-mercaptoethanol at 37 °C for 45 min. Spheroplasts were washed and allowed to settle onto polylysine-coated slides. They were then washed once with 130 mM NaCl, 0.2 mM EDTA, 3.3 mM NaH_2PO_4 and 6.7 mM Na_2HPO_4, pH 7.2, 0.1% BSA (PBS/BSA), twice with PBS/BSA containing 0.1% NP-40 and once with PBS/BSA. For the detection of HA-tagged protein, primary antibody incubations were performed using the HA.11 mouse anti-HA monoclonal antibody (Babco, Richmond, CA), and the Rhodamine-(TRITC)-conjugated anti-mouse $F(ab')_2$ fragment (Jackson Immunoresearch Laboratories, West Grove, PA.) was used as secondary antibody. Washes before and after the secondary antibody were carried out with PBS/BSA or only PBS.

Chitin was visualized by fluorescence microscopy after being stained with 2 mg/l of Calcofluor White or by wheatgerm agglutinin–fluorescein isothiocyanate (WGA–FITC) (Popolo *et al*., 1997; Roncero *et al*., 1988). Determination of cell viability by the methylene blue method was performed as described by Iida *et al*., 1990.

Northern analysis

CHS4 and *CHS6* probes were obtained using the TOPO TA cloning kit Dual Promoter (Invitrogen). Plasmids pCHS4 and pCHS6 were constructed by inserting the following PCR fragments into the pCRII-TOPO vector: a 1 kbp fragment for *CHS4* (nucleotides 119–1098 from ATG) and a 1 kbp fragment for *CHS6* (nucleotides 675–1727 from ATG). pCHS7 was obtained by cloning a 1.2 kbp *NdeI* fragment from the *CHS7* gene into pGEM5 linearized with *NdeI*. The *ACT1* probe was prepared from the pACT plasmid (Valdivieso *et al*., 2000).

Immunoblot analysis

Total cell extracts, electrophoresis and blotting were performed as previously described (Valdivieso *et al*., 2000). For the experiment of detection of the phosphorylated form of Slt2p a cocktail of phosphatase inhibitors was added during extract preparation. Its composition, together with the immunoblot analysis, are described in Martin *et al*. (2000). Anti-phospho p44/p42 MAPK antibody (New England Biolabs) was used to detect the dually phosphorylated form of Slt2p. After stripping off the antibodies, the blot was treated again with anti-Slt2p (1 : 1000 dilution) kindly provided by Professor Maria Molina. Secondary anti-rabbit horseradish peroxidase-conjugated antibodies and an ECL detection system were used to detect the binding of the primary antibodies.

Results and discussion

To study the genetic interactions between *GAS1* and elements of the Chs3p transport and activation

pathway, heterozygous diploids (LF7, LF8, LF9 and LF10) were obtained by crossing *gas1* null mutants (Wb2d and WA1d) with *chs4* Δ, *chs5* Δ, *ch6* Δ and *chs7* Δ mutants (Table 1). After sporulation and isolation of the haploid progeny, germination was allowed to proceed at 24 °C or 30 °C. At 24 °C, *chs4* Δ*gas1* Δ, *chs5* Δ*gas1* Δ, and *chs7* Δ*gas1* Δ mutants were under-represented with respect to the expected values whereas *chs6* Δ *gas1* Δ spores appeared at the expected frequency (Figure 2A). At 30 °C all the double mutant spores were unviable with the exception of *chs6* Δ*gas1* Δ, 8 out 10 spores of which generated microcolonies. In the presence of 1 M sorbitol at 30 °C all the double mutant spores appeared at the expected frequency (data not shown). Figure 2B shows an example of representative tetratype tetrads from the *chs4* Δ*gas1* Δ heterozygous diploid. The *chs4* Δ*gas1* Δ mutant produced a microcolony at 24 °C and was unable to produce a visible colony at 30 °C, but the inclusion of 1 M sorbitol in the plate completely suppressed the synthetic lethality. Microscopic examination of the dissection plate revealed that, at 30 °C and in the absence of sorbitol, *chs4* Δ*gas1* Δ cells germinated but stopped growing after 3–4 cell divisions and appeared as cells with a thin cell wall (Figure 2C, D). These results suggest that the *chs4 gas1* double mutant is not defective in germination but afterwards becomes susceptible to lysis, and hence it fails to form a visible colony. A similar phenotype was also detected for the other double mutants (data not shown).

chs4gas1, *chs5gas1*, *chs6gas1* and *chs7gas1* double null mutants were also tested simultaneously for susceptibility to lysis. From a plate at 24 °C, cells were streaked onto fresh plates and incubated again at 24 °C or at 37 °C. The single *chs* mutants and *gas1* were able to grow at the higher temperature, whereas the double mutants were all non-viable. For simplicity, in Figure 3 the single *chs5* mutant is representative of all the single *chs* mutants. Inclusion of 1 M sorbitol restored the growth of all the double mutants. A freshly isolated *chs3gas1* double mutant was included as a control and showed the same behaviour as the other double mutants.

In order to study the effect of the double mutations during vegetative growth in liquid culture, spores were inoculated in liquid rich (YEPD) or minimal (SD) medium at 30 °C. In liquid YEPD all the double mutants, including *chs3 gas1*, which has not been studied in this condition before, were unable to grow (Table 2). In particular, all the double mutant cells appeared clumped, enlarged, budded and with large vacuoles and a granular

A

DIPLOID GENOTYPE	°C	N° TETRADS	SPORE GENOTYPE			
			wt	*chs4::URA3*	*gas1::LEU2*	*chs4::URA3 gas1::LEU2*
chs4::URA3/CHS4 gas1::LEU2/GAS1	24	14	13	15	12	4 (13)
	30	14	17	11	10	0 (17)
			wt	*chs5::ADE2*	*gas1::LEU2*	*chs5::ADE2 gas1::LEU2*
chs5::ADE2/CHS5 gas1::LEU2/GAS1	24	9	12	6	6	10 (12)
	30	13	13	13	12	1 (13)
			wt	*chs6::URA3*	*gas1::LEU2*	*chs6::URA3 gas1::LEU2*
chs6::URA3/CHS6 gas1::LEU2/GAS1	24	10	12	8	8	12 (12)
	30	10	9	10	9	8 (10)
			wt	*chs7::HIS3*	*gas1::LEU2*	*chs7::HIS3 gas1::LEU2*
chs7::HIS3/CHS7 gas1::LEU2/GAS1	24	12	17	7	7	8 (17)
	30	14	14	13	13	0 (14)
			wt	*chs6::HIS3*	*fks1::URA3*	*chs6::HIS3 fks1::URA3*
chs6::HIS3/CHS6 fks1::URA3/FKS1	24	13	16	10	8	10 (16)
	30	13	12	11	14	6 (12)

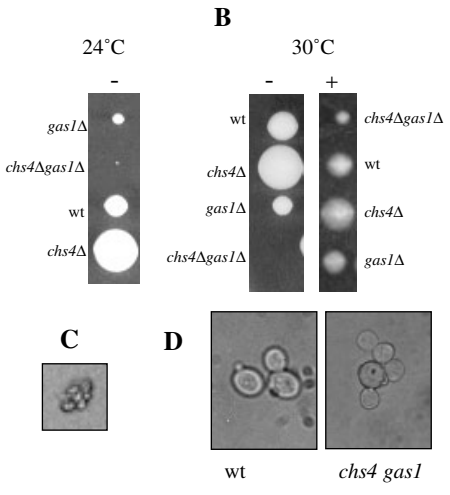

Figure 2. (A) Analysis of viable spores from heterozygous diploids germinated on YEPDAT at 24 °C and 30 °C. The number of spores of each genotype is indicated. The expected values are indicated in parentheses. (B) Representative tetratype tetrads from a *chs4 gas1* heterozygous diploid and effect of temperature and of inclusion (+) of sorbitol in the dissection plate. (C) Microcolony morphology of a *chs4 gas1* spore germinated on YEPDAT at 30 °C. (D) Morphology of wt and *chs4* Δ *gas1* Δ cells in the spore colony

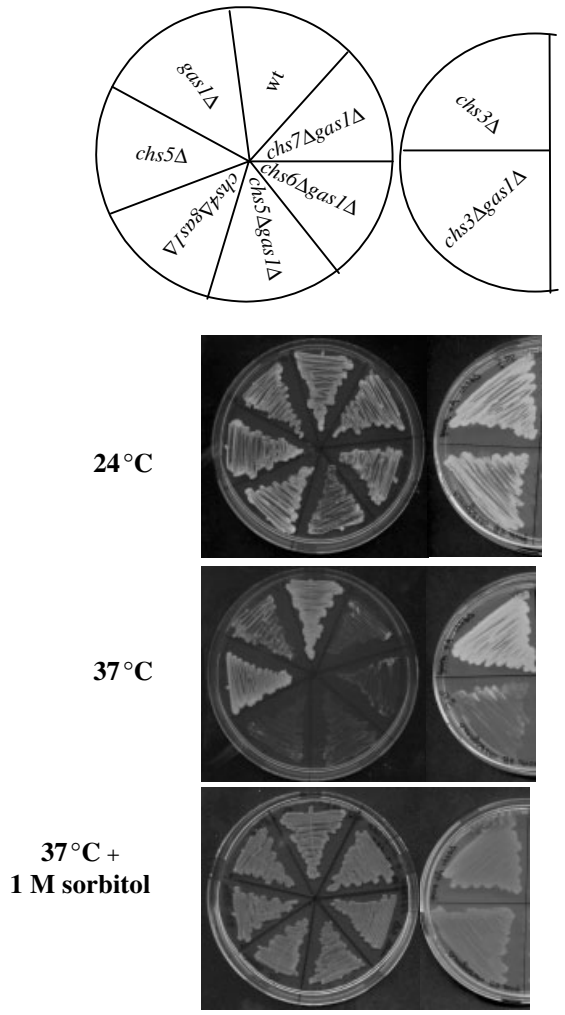

Figure 3. Lysis phenotype of *chs3-7 gas1* double null mutants. Cells were streaked on YEPDA plates in the absence or presence of 1 M sorbitol. Cells were incubated at the indicated temperatures for 2 days

cytoplasm. Cells were particularly susceptible to mild sonication, after which they appeared as refractile ghosts leaking cytoplasm from the bases or tips of the buds. An example is shown in Figure 4A. Growth in YEPD was rescued by the presence of an osmotic stabilizer that restored a pattern of growth kinetics similar to that of the single *gas1* mutant (Table 2) and also a more normal morphology.

In minimal medium (SD), the double mutants exhibited slow growth rates with duplication times of 7–12 h (Table 2), a high percentage of unseparated cells with a large neck between mother and daughter cells (Figure 4B) and a high percentage of methylene-blue stained cells, indicating low viability. All these defects were alleviated or completely suppressed in osmotically stabilized SD media (Table 2) or by using 24 °C as the growth temperature (data not shown).

In SD the activation of the cell integrity pathway was also examined in a representative tetrad from the *chs4 gas1* heterozygous diploid. The phosphorylated form of Slt2p was used as a reporter of the activation of the pathway and the *gas1* mutation was found to induce a high level of Slt2p phosphorylation, together with a doubling of the protein itself with respect to wild-type (Figure 4C), in agreement with reported data (de Nobel *et al.*, 2000). In the single *chs4* Δ mutant the level of phosphorylated Slt2p was very low, indicating that the lack of most of the chitin does not induce a severe enough cell wall damage to activate the pathway. By contrast, the double *chs4 gas1* mutant showed a high basal level of Slt2p phosphorylation that was strongly reduced by the presence of 1 M sorbitol.

Total cellular chitin was measured by an enzymatic method. As shown in Table 3, in SD the *gas1* mutant showed a three-fold higher level than the control. In *chs5 gas1*, and *chs7 gas1* double mutants, chitin was reduced to about 90% whereas in *chs6 gas1* to about 80% with respect to the single *gas1* mutant. These results were confirmed by a method based on chemical hydrolysis.

Chitin was also measured in SD and in YEPD in the presence of sorbitol. Whereas all the double mutants showed a 90% reduction in chitin levels with respect to *gas1*, in *chs6 gas1* the reduction was 60–80%. The effect of the *chs6* null mutation in the cell wall mutant *fks1* was also analysed. A double heterozygous diploid (LF11) was obtained by crossing *chs6* and *fks1* null mutants obtained in W303 (see Table 1). The results of tetrad dissections are shown in Figure 2 and were similar to those obtained for *chs6 gas1*. The inclusion of sorbitol in the dissection plates allowed all the expected spores at 30 °C to be viable (data not shown). The slow growth rate on YEPD (Table 2) appears to be a consequence of cell lysis, since about 50% of the cells were refractile under phase-contrast microscope examination. The *fks1* mutant was found to have from three to about six times more chitin than the wild-type. In the presence of the *chs6* null mutation the high level of chitin

Table 2. Duplication times (h) of *gas1*Δ *chsx*Δ mutants growing at 30 °C

Spore genotype	YEPD	YEPD + S	SD	SD + S
wt (W303)	2	1.5	2.5	2.5
*gas1*Δ	5	2.3	4.5	2.5
*chs3gas1*Δ	No growth	3.6	Slow growth	2.8
*chs4gas1*Δ	No growth	3.1	Slow growth	2.7
*chs5gas1*Δ	No growth	3.1	Slow growth	4.8
*chs6gas1*Δ	No growth	3	Slow growth	2.7
*chs7gas1*Δ	No growth	2.9	Slow growth	4.9
single *chs3/4/5/6/7*Δ	2	1.5	2.5	N.D.
*fks1*Δ	2.2	2.3	2.4	2.8
*chs6fks1*Δ	6	2.3	3.3	2.8

Cells from a YEPD + S plate were inoculated in the indicated liquid media in the presence (+S) or absence of 1 M sorbitol. Growth was monitored as the increase in cell numbers. Data are mean of a least two different experiments.
No growth = cell density per ml did not change from inoculation over a 3 days time span.
Slow growth = corresponds to duplication times in the range of approximately 7–12 h.
N.D. = not determined.

Table 3. Chitin levels in *gas1chs4/5/6/7* double mutants

	Chitin* (μg/mg)		
Spore genotype	**SD**	**SD + S**	**YEPD + S**
wt (W303)	7.1 ± 1.9	3.8 ± 0.3	3 ± 0.1
*chs3*Δ	0.6 ± 0.2		
*chs4*Δ	0.5 ± 0.2		
*chs5*Δ	2 ± 0.3		
*chs6*Δ	1.4 ± 0.1		
*chs7*Δ	0.5 ± 0.2		
*gas1*Δ	23.2 ± 4.0	14.2 ± 2.0	35 ± 3.0
*chs3gas1*Δ	1.6 ± 0.2	1.1 ± 0.1	0.7 ± 0.1
*chs4gas1*Δ	N.D.	0.8 ± 0.2	2.1 ± 0.2
*chs5gas1*Δ	2.3 ± 0.1	2.8 ± 0.3	2 ± 0.3
*chs6gas1*Δ	4.2 ± 0.3	6.1 ± 0.3	5.8 ± 0.4
*chs7gas1*Δ	1.5 ± 0.5	2.2 ± 0.2	1.1 ± 0.1
*fks1*Δ	17 ± 2.5	14.6 ± 2.0	17 ± 3.0
*chs6 fks1*Δ	1.3 ± 0.1	2.5 ± 0.3	3.7 ± 0.5

*μg N-acetylglucosamine/mg cell dry weight. Values are mean ± SD of at least three different assays on spores from different tetratype tetrads.

decreased (Table 3). Thus, in the genetic background of W303, Chs6p is required for the increase in chitin in both *gas1* and *fks1* mutants.

We examined whether the expression of the *CHS4-5-6-7* genes was affected by the *gas1* null mutation. The level of *CHS4*, *CHS6* and *CHS7* mRNA was monitored by Northern blot analysis, as previously described (Valdivieso *et al*., 2000). The level of *CHS4*, *CHS6* and *CHS7* mRNA did not change in the *gas1* mutant growing in SD at 30 °C (Figure 5A). The level of Chs5p was evaluated by immunoblot using anti-HA antibodies. Chs5p appeared as a polypeptide band of about 150 kDa, in agreement with previous reported data (Santos and Snyder, 1997) whose level was not affected by the presence of the *gas1* null mutation (Figure 5B). The upregulation of chitin synthesis in *gas1* does not appear to be controlled at transcriptional level.

Localization of Chs3p

To analyse whether the subcellular distribution of Chs3p was affected in the *gas1* mutant, the Y1306 strain harbouring a *CHS*3 :: 3 × *HA* fusion integrated in the chromosome and its derivative Y1306ΔG (*gas1*Δ) were used (Table 1). Cells growing exponentially at 30 °C in SD were processed for indirect immunofluorescence.

As shown in Figure 6, in the wild-type strain Chs3p was detected in cytoplasmic patches, at the incipient bud sites and occasionally at the neck region of mother cells with large buds, in agreement with the previous data, indicating that the Chs3p level remains constant but that its localization changes, coinciding with chitin deposition (Chuang and Schekman, 1996). In *gas1*Δ cells, Chs3p was still present at the incipient bud site, in agreement with the presence of normal chitin rings in these mutants. However, the fluorescence pattern was less punctuate than in the wild-type and Chs3p was also detected at the bud plasma membrane in budded cells, either as a crescent at the edge of the buds or diffused throughout the bud. This pattern

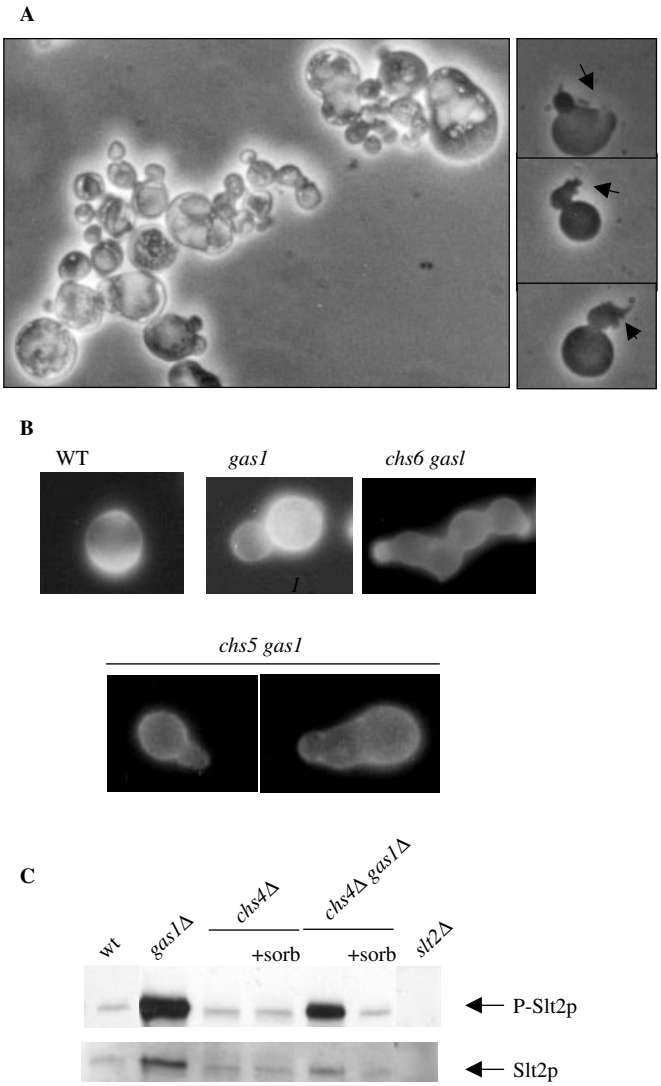

Figure 4. (A) Terminal phenotype of *chs3 gas1* mutant cells in YEPD at 30 °C, examined at phase-contrast microscopy before (left panel) or after a sonication (right small panels) of two 6 s cycles. (B) Morphology of cells growing in SD and stained with Calcofluor white. The bright fluorescence of *gas1* and the abnormal morphology and the lack of chitin rings in the double mutants are clearly visible. (C) Activation of the cell integrity pathway in cells growing in SD and SD + sorbitol at 30 °C. Total extracts were subjected to immunoblot analysis as described in Materials and methods

was not seen in the control cells. No staining was detected in the Y604 isogenic untagged strain (data not shown).

It has previously been shown that the localization of Chs3p at the incipient bud site or at the neck requires Chs5p, and that Chs5p and Chs3p colocalize in cytoplasmic patches (Santos and Snyder, 1997). To study the localization of Chs5p in the *gas1* Δ mutant, the *gas1* null mutation was introduced in the Y1303 strain harbouring the gene expressing an HA-tagged form of Chs5p integrated in the chromosome (Table 1). In wild-type cells, Chs5p localized to cytoplasmic patches that appeared uniformly distributed in mother and daughter cells, in agreement with the reported data (Santos and Snyder, 1997), (Figure 6). In the *gas1* Δ mutant, the cytoplasmic patches appeared brighter than those in the control strain and accumulated in the bud. This pattern of staining, not seen in wild-type cells, indicates an unusual

polarization of Chs5p toward the growing bud and is consistent with the localization observed for Chs3p. Consistent with these observations, staining by wheatgerm agglutinin (WGA)–fluorescein isothiocyanate (FITC) complex previously detected the presence of chitin in *gas1* both in the lateral wall of the mother cell and of the bud, whereas in the wild-type no staining was present in the buds, as expected (Roncero *et al*., 1988). In addition, electron microscopic analysis revealed an increased labelling with WGA–gold all over the lateral cell wall of the bud in the *gas1* null mutant, as compared to the wild-type (data not shown).

In this work we were interested in knowing whether the hyperaccumulation of chitin proceeds through the normal pathway of transport and activation of Chs3p, or whether it occurs through an alternative pathway involving genes homologous to *CHS4* (*SHC1* and YDL203c) or *CHS6* (YKR027w) that are not required in chitin synthesis under normal conditions. Recently, *SHC1* has been found to activate chitin synthesis during sporulation (Sanz *et al*., 2002). Here we have shown that the loss of any elements, Chs4 to Chs7p, of the Chs3p pathway results in a conditionally lethal phenotype if the *gas1* null mutation is present; the deletions inhibit the hyperaccumulation of chitin typical of the *gas1* mutant. In another cell wall mutant, *fks1* Δ, *CHS6* deletion did not decrease the high level of chitin (Osmond *et al*., 1999). In order to compare the behaviour of *chs6 gas1* with that of *fks1 chs6*, we isolated the double-null mutant in

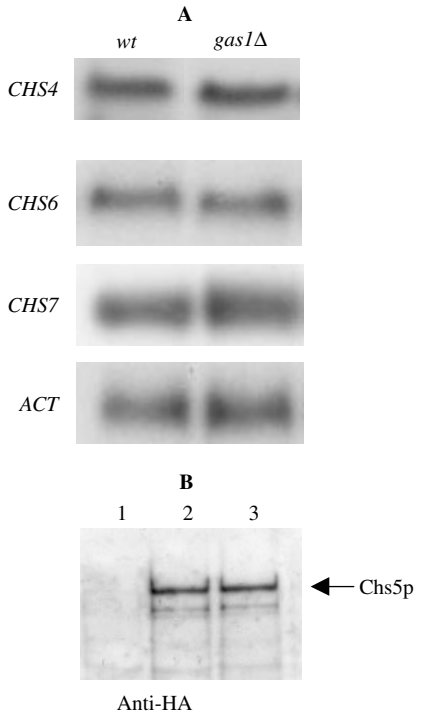

Figure 5. Expression of *CHS* genes in *gas1* mutant. (A) Northern blot performed with RNA obtained from wt (W303-1B) and *gas1*Δ (WB2d) strains. In all cases, densitometric analysis of autoradiographic images was performed and the relative amounts of *CHS4*, *CHS6* and *CHS7* mRNA were referred to ACT mRNA (data not shown). (B) Western blot analysis of Chs5p levels: 1, Y604 strain (untagged); 2, Y1303 (*CHS5::3 x HA*) and its derivative strain Y1303ΔG (*gas1*Δ). About 30 μg proteins was loaded in each lane and immunoblotting was performed using anti-HA antibodies and ECL detection kit (Amersham)

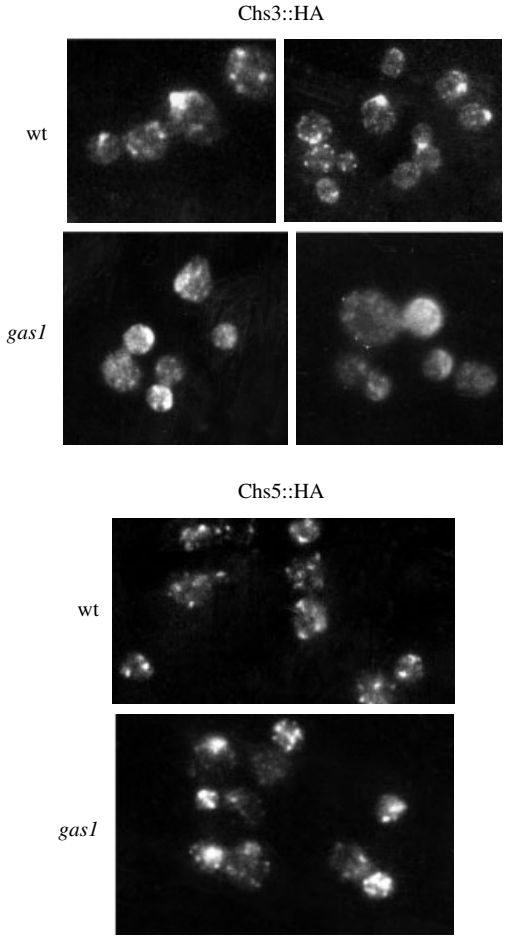

Figure 6. Localization of epitope-tagged Chs3p and Chs5p in *gas1*Δ cells. Y1306 wild-type (*CHS3::3XHA*), Y1306ΔG (*CHS3::3XHA gas1*Δ), Y1303 (*3XHA::CHS5*) and Y1303ΔG (*3XHA::CHS5 gas1*Δ) cells, growing exponentially in SD at 30 °C, were fixed and stained with anti-HA antibodies

the same congenic strain. The high level of chitin present in the *fks1* null mutant was greatly reduced in the presence of the *CHS6* deletion. Thus, at least in the genetic background of W303, *CHS6* is required for chitin hyperaccumulation in both the *gas1* and *fks1* mutants.

The effects of the double deletions are particularly detrimental for cells growing in rich medium and temperature higher than 24 °C. By contrast, in SD medium cells can grow slowly. This is not due to any difference in osmolarity between the two media, since osmolarity was found to be about 270 mOSM for YEPD and 220 mOSM for SD. In minimal medium, cells can survive and also progressively adapt, as indicated by the reduction in their duplication time (data not shown). The adaptation in SD of a previously described *chs3 gas1* null mutant did not occur through restoration of chitin synthesis (Valdivieso *et al*., 2000). Further experiments are currently under way to study the remodelling of the cell wall of this mutant.

The subcellular localization of Chs3p is affected in *gas1*. The diffuse fluorescence pattern observed here indicates that vesicle transport could be affected. Moreover, the anomalous presence of Chs3p in the bud can be interpreted in terms of the notion that the lack of the cross-linking activity of Gas1p could elicit a more severe weakening in the growing bud, where the cell wall is more plastic. This defect could direct the deposition of chitin to protect the bud from lysis. Several observations support the possible fragility of the bud cell wall in *gas1* cells: (a) treatment with Calcofluor white blocks wild-type cells in different stages of the cell cycle, whereas it arrests *gas1* cells after they have formed small buds, suggesting that Gas1p function is crucial at this stage (Ram *et al*., 1995); (b) the lysed-bud phenotype typical of *chs1*Δ cells is suppressed by the presence of *GAS1* deletion, indicating that the chitin deposited into the bud could preserve its integrity from excessive chitinase action after cell division (Valdivieso *et al*., 2000); (c) the synthesis of Gas1p is maximal at the G_1/S boundary, suggesting a possible requirement of this protein for bud emergence (Popolo *et al*., 1993; Ram *et al*., 1995). A similar partial delocalization of Chs3p has also been observed in *fks1* mutant (Garcia-Rodriguez *et al*., 2000).

The finding that Chs5p accumulates in cytoplasmic patches in the bud, suggests that it could be involved in the transport of vesicles containing Chs3p from the Golgi apparatus toward the bud. Chs5p is assumed to be a protein that associates with the outside of vesicles deriving from the TGN (Madden and Snyder, 1998; Ziman *et al*., 1996). Also, it could interact with components of the transport machinery to deliver Chs3p to the bud. An attractive hypothesis is that the alteration in the transport of Chs3p could be mediated by the organization of the actin/Myo2p cytoskeleton through a specific control by Rho1p, a key regulator of yeast morphogenesis and secretion (Guo *et al*., 2001). Rho1p is activated in *gas1* cells by the increase in the activity of the exchange factor Rom2 (Bickle *et al*., 1998). This could lead not only to the activation of the Pkc1–MAP kinase pathway, but also to that of a recently proposed Pkc1p-activated branch, which seems to be responsible for the reorganization of the actin cytoskeleton in response to cell wall stresses independently of Bni1p or to a direct interaction of Rho1p with Sec3p (Delley and Hall, 1999; Guo *et al*., 2001). In any case it is clear that in *gas1*Δ cells there is a specific hyperpolarization of Chs3p, such that the restriction of chitin synthesis at the bud–cell boundary is overcome.

Finally, we found that the increased synthesis of chitin that occurs in *gas1* does not seem to be related to differences in the expression of *CHS* genes. In a previous work, no appreciable differences were found in the levels of *CHS3* mRNA or of mature Chs3p in asynchronous populations of *gas1* cells (Valdivieso *et al*., 2000). A recent large-scale analysis of transcript profiles in a *fks1* mutant revealed only a modest increase in the *CHS3* transcript level and no modulation in the other *CHS4-5-6-7* transcript levels, in good agreement with the present results (Terashima *et al*., 2000). No increase in Chs3p and Chs4p levels was detected in *fks1*, whereas a remarkable increase in Chs7p levels was found, although the physiological significance of this is unclear (Garcia-Rodriguez *et al*., 2000).

It is not yet clear how the synthesis of chitin is upregulated in cells with constitutive cell wall defects. A specific hyperpolarization of Chs3p and a so far unknown mechanism of post-translational regulation of CSIII activity seem to be the processes involved in the increased chitin synthesis observed in the *gas1* null mutant.

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