The yeast Chs4 protein stimulates the trypsin-sensitive activity of chitin synthase 3 through an apparent protein–protein interaction

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Inducible overexpression of the *CHS4* gene under the control of the *GAL1* promoter increased Chs3p (chitin synthase 3) activity in *Saccharomyces cerevisiae* several fold. Approximately half of the Chs3p activity in the membranes of cells overexpressing Chs4p was extracted using CHAPS and cholesteryl hemisuccinate. The detergent-extractable Chs3p activity appeared to be non-zymogenic because incubation with trypsin decreased enzyme activity in both the presence and absence of the substrate, UDP-*N*-acetylglucosamine. Western blotting confirmed that Chs3p was extracted from membranes by CHAPS and cholesteryl hemisuccinate and revealed that Chs4p was also solubilized using these detergents. Yeast two-hybrid analysis with truncated Chs4p demonstrated that the region of Chs4p between amino acids 269 and 563 is indispensable not only for eliciting the non-zymogenic activity of Chs3p but also for binding of Chs4p to Chs3p. Neither the EF-hand motif nor a possible prenylation site in Chs4p was required for these activities. Thus, it was demonstrated that stimulation of non-zymogenic Chs3p activity by Chs4p requires the amino acid region from 269 to 563 of Chs4p, and it seems that Chs4p activates Chs3p through protein–protein interaction.

Keywords: yeast, chitin synthase 3, overexpression, protein–protein interaction

INTRODUCTION

In *Saccharomyces cerevisiae*, chitin deposition at the bud neck is important for the normal budding process, and inhibition of chitin synthesis causes a defect in septum formation. Three yeast chitin synthases, Chs1p, Chs2p and Chs3p, have been characterized by generating mutants affected in these proteins. Chs1p repairs damaged chitin, counter-balancing the activities of chitinase (Bulawa *et al.*, 1986; Cabib *et al.*, 1989, 1992), and Chs2p is responsible for formation of the primary septum (Sburlati & Cabib, 1986; Shaw *et al.*, 1991; Silverman, 1989; Silverman *et al.*, 1988). Chs3p synthesizes most of the cell-wall chitin and also links chitin to the non-reducing end of 1,3- β -glucan by a β -1,4-linkage (Kollar *et al.*, 1995; Valdivieso *et al.*, 1991). Disruption of *CHS1* did not result in any significant morphological alterations, but simultaneous disruption of *CHS2* and *CHS3* is lethal (Shaw *et al.*, 1991), indicating the importance of chitin for viability in yeast. The level of Chs2p is regulated during the cell cycle. Chs2p is localized in the bud neck at the end of mitosis,

and then degraded in a Pep4p-dependent manner (Chuang & Schekman, 1996). Chs3p is a metabolically stable protein that appears at a ring on the cell surface at bud emergence and then accumulates in the mother-bud neck of small-budded cells (DeMarini *et al.*, 1997; Santos & Snyder, 1997; Ziman *et al.*, 1996). Chs3p function requires several cellular factors, including the products of the genes *CHS4*, *CHS5*, *CHS6*, *MYO2* and *TIG1* (Bulawa, 1992; DeMarini *et al.*, 1997; Holthuis *et al.*, 1998; Santos & Snyder, 1997; Santos *et al.*, 1997; Trilla *et al.*, 1997; Ziman *et al.*, 1998). *CHS4* encodes a protein that directs the correct localization of Chs3p at the mother-bud neck by binding Bni4p as well as one of the septins, Cdc10p (DeMarini *et al.*, 1997). Chs5p, Myo2p and Tig1p play important roles in the polarized distribution of Chs3p (Holthuis *et al.*, 1998; Santos & Snyder, 1997), and Chs6p mediates the translocation of Chs3p from the chitosome to the cell surface (Ziman *et al.*, 1998). Among these genes, mutation in either *CHS4* or *CHS5* decreases Chs3p activity and cell-wall chitin content (Bulawa, 1992; Santos *et al.*, 1997; Trilla *et al.*, 1997).

Previously, we used mutation analyses to demonstrate the residues important for Chs2p activity. Chs2p contains two regions called con1 and con2, which are highly conserved among various chitin synthases, in the middle and at the C-terminus of the protein. Region con1 contains two amino acid sequence motifs, Glu-Asp-Arg and Gln-Arg-Arg-Arg-Trp, and con2 has one motif, His-Asp-X-X-Trp-X-Thr, where X represents variations. Because enzyme activity was severely impaired by mutations of the conserved amino acids in these motifs, they are presumed to contribute to the active site of the enzyme (Nagahashi *et al.*, 1995; Yabe *et al.*, 1998). In fact, mutations of some of these conserved amino acids in Chs3p also diminished the activity and functionality of the enzyme (Cos *et al.*, 1998).

In contrast to the sequence conservation in the probable active sites among the three yeast chitin synthases, Chs3p activity is regulated by a mechanism different from those for Chs1p and Chs2p. Firstly, overexpression in yeast of *CHS1* or *CHS2* led to an increase in chitin synthase activity, whereas overexpression of *CHS3* alone did not (Bulawa *et al.*, 1986; Nagahashi *et al.*, 1995; Valdivieso *et al.*, 1991). Introduction into yeast of additional copies of *CHS4*, however, enhances Chs3p activity and increases the amount of cell-wall chitin (Bulawa 1993; Trilla *et al.*, 1997), suggesting that Chs3p activity requires *CHS4* as a positive regulator. Secondly, two types of Chs3p activities, trypsin-dependent (zymogenic) and trypsin-sensitive (non-zymogenic), are present in membranes (Choi *et al.*, 1994), although both Chs1p and Chs2p are known only as zymogens (Cabib *et al.*, 1996). The zymogenic activity of Chs3p is not detergent-extractable, and Chs3p needs to be in complex with the substrate during activation by trypsin (Choi *et al.*, 1994). Zymogenic Chs3p activity was detected only after the membranes were treated with detergents, and a certain level of detergent-extractable non-zymogenic Chs3p activity might be expected to be present in the membranes (Choi *et al.*, 1994). Such non-zymogenic Chs3p activity, however, has never been recovered and confirmed.

In this paper we show that the non-zymogenic activity of Chs3p can be extracted with CHAPS and cholesteryl hemisuccinate from membranes prepared from cells overexpressing *CHS4*, and that the association of Chs3p and Chs4p is important for non-zymogenic Chs3p activity. In addition, the region of Chs4p required for the activity of, and binding to, Chs3p is demonstrated by truncation of *CHS4* and yeast two-hybrid analysis.

METHODS

Plasmids and yeast strains. The entire ORFs of *S. cerevisiae* *CHS3* and *CHS4* were amplified by PCR and cloned at the *Xba*I site (*CHS3*) and between the *Bam*HI and *Xba*I sites (*CHS4*) located downstream of the *GAL1* promoter of pYES2, which carries *URA3* as a selection marker (Invitrogen), creating pYES-CHS3 and pYES-CHS4, respectively. To detect Chs4p, the triple c-Myc-sequence tag was inserted just after the first ATG codon within the *CHS4* ORF. The primers used

for PCR were 5'-GATCGCGGATCCGCGATGGAACAA-AAGCTTATTTCTGAAGAAGACTTGGCAAGTTTCAC-CGCAGGTACATCCA-3' and 5'-ATCTAGTGCTCTAGATTACATAATTACACAGTC-3' for *CHS4*, and 5'-GAACACGCAGCTAGCATGGAACAAAAGCTTATTTCTGAAGAAGACTTGAATCACCTGGATGTTTTACC-3' and 5'-GACCGTTCAGCTAGCCTATGCAACGAAGGAGTCACTTTC-3' for *CHS3*. These plasmids were transfected into the haploid *S. cerevisiae* strain YMS120 (*MATa ade2 trp1 leu2 ura3 his3 chs1Δ::ADE2 chs2Δ::HIS3*), in which the 2.1 kb *Nco*I-*Nco*I region of the chromosomal *CHS1* and the 1.1 kb *Bgl*II-*Bgl*II region of the chromosomal *CHS2* were replaced by *ADE2* and *HIS3*, respectively, by the lithium acetate method (Ito *et al.*, 1983). For the simultaneous overexpression of *CHS3* and *CHS4*, YpL-CHS3, where the *CHS3* ORF was cloned at the *Xba*I site (downstream of the *GAL1* promoter) of YpLX harbouring *LEU2* (Nagahashi *et al.*, 1995), was also created, and was transfected into YMS120 along with pYES-CHS4. The *Ura*⁺ (for the pYES2-based plasmids), *Leu*⁺ (for the YpLX-based plasmids) and *Ura*⁺ *Leu*⁺ (for double transfection) transformants were cultured at 30 °C in yeast nitrogen base (Difco) supplemented with glucose and necessary amino acids, and the expression of Chs3p and Chs4p was induced in the same medium, but containing galactose instead of glucose, at 30 °C for 12 h. A series of truncated *CHS4* genes, which would express N-terminally and/or C-terminally truncated Chs4 proteins, was also created by PCR and cloned between the *Bam*HI and *Xba*I sites of pYES2.

To examine the functionality of *CHS4*, the haploid *S. cerevisiae* strain YMS1 (*MATa ade2 trp1 leu2 ura3 his3 chs4Δ::ADE2*), in which the 4.2 kb *Afl*II-*Nhe*I region of the chromosomal *CHS4* was replaced by *ADE2*, was transformed by pYES-CHS4 carrying the wild-type or a truncated *CHS4* and selected by uracil auxotrophy in the presence of galactose. The resulting *Ura*⁺ transformants were collected and spread on plates containing galactose and 2 mg Calcofluor white ml⁻¹.

Membrane preparation and chitin synthase assay. Total membranes were prepared from yeast cells as described previously (Nagahashi *et al.*, 1995). To extract Chs3p, 20 mg protein from total membranes was suspended in 2 ml of a buffer containing 20 mM Tris/HCl (pH 7.6), 160 mM NaCl, 20 μM GTP-γS, 5 mM dithiothreitol, 160 mM NaCl, 33% (v/v) glycerol, 0.5% (v/v) CHAPS, and 0.1% (w/v) cholesteryl hemisuccinate, and left on ice for 30 min. After centrifugation at 200 000 g for 30 min, the supernatants were collected and used as the membrane extracts (S100). The precipitates were also suspended in an equal volume of the same buffer and used as the extracted membranes (P100). In some experiments, the total membranes and membrane extracts were incubated with the indicated concentrations of trypsin in the presence or absence of 1 mM UDP-N-acetylglucosamine (UDP-GlcNAc). After incubation at 30 °C for 15 min, trypsin was inactivated by the addition of soybean trypsin inhibitor at a concentration twice that of the trypsin added to the reaction mixture.

Unless otherwise specified, chitin synthase assays were performed in a standard 100 μl reaction mixture containing 30 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 32 mM GlcNAc, 1.0 mM [³H]UDP-GlcNAc (specific activity 10 000 d.p.m. nmol⁻¹), the indicated amount of total membranes or membrane extract at 30 °C for 60 min, and acid-insoluble radioactivity was measured using a scintillation counter.

Fig. 1. Increase in Chs3p activity following overexpression of Chs4p. (a) Yeast cells, carrying pYES-CHS3 or pYES-CHS4, were grown to mid-exponential phase, then further cultured for 12 h in medium containing glucose (–) or galactose (+). Approximately 100 μg membrane protein was fractionated by SDS-PAGE and transferred to a PVDF membrane. For cells harbouring pYES-CHS3, blots were incubated with anti-Chs3p antibody (Chs3p); for cells harbouring pYES-CHS4 they were incubated with anti-c-Myc antibody (Chs4p). (b) Chs3p activity in cells overexpressing Chs3p or Chs4p. Yeast cells in mid-exponential phase were further cultured in medium containing galactose for 12 h before harvest. Chs3p activity was determined in membranes from YMS120 cells that had been transformed with pYES2 (■), pYES-CHS3 (●) or pYES-CHS4 (▲). (c) Effect of trypsin on Chs3p activity. Chs3p activity was determined with approximately 80 μg protein from membranes of YMS120 cells that had been transformed with pYES-CHS4. The yeast cells were cultured as described in (b). Before the assay, the membranes were treated with the indicated concentrations of trypsin in the absence (◇) or the presence (□) of the substrate. The error bars represent mean $\pm$ SD for three independent experiments.

Western blotting. The indicated amounts of total membranes or membrane extract were suspended in a loading buffer for SDS-PAGE (Laemmli, 1970) and heated at 90 °C for 2 min. The proteins were then fractionated by SDS-PAGE (Laemmli, 1970), transferred electrophoretically to a PVDF membrane (Sambrook *et al.*, 1989), and reacted with an anti-c-Myc monoclonal antibody (clone 9E10, Oncogene Science) or an anti-Chs3p polyclonal antibody, and then with horseradish-peroxidase conjugated protein A (Amersham). The proteins which hybridized with the anti-c-Myc antibody or the anti-Chs3p antibody were detected by using an ECL-protein detection kit (Amersham). To generate the anti-Chs3p antibody, the Chs3p fragment that encompasses amino acids 255–434 was expressed in *Escherichia coli* as a fusion with glutathione *S*-transferase (GST). Rabbits were immunized with the purified protein, and the IgG fractions that contained the anti-Chs3p antibody were purified by affinity column chromatography using protein A Sepharose CL-4B (Pharmacia). To remove anti-GST antibodies from the IgG fractions, the purified IgG fractions were repeatedly loaded onto GST columns in which GST was bound to glutathione Sepharose beads, and the flow-through fractions, which contained no detectable anti-GST antibody, were pooled and used as the anti-Chs3p antibody preparation.

Yeast two-hybrid analysis. The entire ORF of *CHS3* was cloned between the *EcoRI* and *BamHI* sites of pGAD424, generating a plasmid that would express Chs3p as a fusion protein with the transactivation domain of Gal4p. Regions of the *CHS4* ORF encoding truncated Chs4 proteins were also cloned between the *BamHI* and *Sall* sites of pGBT9 (Clontech) to express these products as fusion proteins with the DNA-binding domain of Gal4p. The resulting plasmids were then transformed into *S. cerevisiae* strain HF7c (MATa *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-*

538 LYS2::GAL1-HIS3 URA3::(GAL4 17-MERS)₃-CYC1-LACZ), where *HIS3* gene expression was dependent on both the DNA-binding and transactivation domains of Gal4p (Feilottter *et al.*, 1994). After the transformation of HF7c, the Leu⁺ Trp⁺ transformants were collected and tested for their ability to grow in the absence of histidine.

RESULTS

Involvement of *CHS4* in Chs3p activity

It has been shown previously that introduction into yeast of additional copies of *CHS4* increases Chs3p activity and the total amount of chitin synthesized (Trilla *et al.*, 1997). We sought to establish whether the inducible overexpression of *CHS4* by the *GAL1* promoter would enhance Chs3p activity. We used YMS120 cells, which lack functional *CHS1* and *CHS2* alleles, as the host, to eliminate Chs1p and Chs2p activities during the assay for Chs3p activity. To confirm the induced expression of Chs3p and Chs4p, an anti-Chs3p polyclonal antibody was generated, and Chs4p was N-terminally fused with the c-Myc-sequence tag. There were no detectable amounts of Chs3p and Chs4p when YMS120 cells bearing either pYES-CHS3 or pYES-CHS4 were cultured in medium containing glucose (Fig. 1a). However, cultivation of these cells in galactose medium strongly increased the levels of the Chs3 and Chs4 proteins (Fig. 1a). The sizes of Chs3p and Chs4p detected by Western blotting were larger than would have been expected from their amino acid sequences (132 kDa for Chs3p, 77 kDa for Chs4p) (Fig. 1a). These larger sizes are presumably due to glycosylation of Chs3p and Chs4p, because endoglycosidase-H treatment

Fig. 2. Extraction of active Chs3p from membranes. Yeast cells that had been transformed with YpL-CHS3 and pYES-CHS4 were grown to mid-exponential phase then further cultured for 12 h in medium containing galactose to induce the expression of Chs3p and Chs4p. (a) Membranes were prepared, incubated in a buffer containing CHAPS and cholesteryl hemisuccinate and centrifuged as described in Methods. Twenty-five microlitres of the S100 (sup) and P100 (ppt) fractions of the membrane extracts were fractionated by SDS-PAGE, transferred to a PVDF membrane and hybridized with the anti-Chs3p antibody (Chs3p) or the anti-c-Myc antibody (Chs4p). (b) Effect of trypsin on Chs3p activity. Chs3p activity in P100 (ppt) and S100 (sup) fractions of membrane extracts was determined for 80 μ l of each fraction; the values plotted are nmol GlcNAc incorporated h⁻¹. Before the assay, S100 and P100 fractions were untreated (dotted bars) or treated with trypsin in the absence (white bars) or the presence (black bars) of the substrate. For trypsin treatment, 1.0 μ g trypsin was added per μ g protein of each fraction. The error bars represent mean $\pm$ SD for three independent experiments.

gave rise to faster-migrating bands (data not shown). Consistent with a previous report (Trilla *et al.*, 1997), overexpression of Chs4p alone led to a several-fold increase in Chs3p activity in the total membranes, whereas overexpression of Chs3p led to a much smaller increase in Chs3p activity (Fig. 1b). This confirms that there is a limiting factor for Chs3p expression and that Chs4p positively regulates enzyme activity.

There are two types of Chs3p activities, zymogenic and non-zymogenic (Choi *et al.*, 1994). Therefore, we examined whether the increased Chs3p activity caused by the overexpression of Chs4p is further increased by trypsin treatment. When the total membranes prepared from the cells overexpressing Chs4p were treated with trypsin, the Chs3p activity declined as trypsin concentration increased. Moreover, addition of UDP-GlcNAc during the trypsin treatment did not protect the

enzyme from trypsin (Fig. 1c). This suggests that, most, if not all, of the Chs4p-elicited Chs3p activity is non-zymogenic. According to Choi *et al.* (1994), non-zymogenic Chs3p activity can be released from membranes with detergents, such as CHAPS, though such detergent-extractable Chs3p activity has never been confirmed by others. We also looked for detergents that could extract Chs3p activity from membranes and found that about half of the Chs3p activity in membranes from cells that contained an increased level of Chs4p was extracted from the membranes by the combination of CHAPS and cholesteryl hemisuccinate (Fig. 2). Although some degraded protein bands appeared after the membranes were treated with the detergents (Fig. 2a), the detergent-extracted Chs3p retained enzyme activity (Fig. 2b). Consistent with the non-zymogenic property of Chs4p-induced Chs3p activity in membranes, the detergent-extracted Chs3p activity was trypsin-sensitive even in the presence of UDP-GlcNAc (Fig. 2b). Together, these results demonstrate that the Chs4p-elicited Chs3p activity is non-zymogenic.

Region of Chs4p essential for Chs3p activity

The pathogenic fungus *Candida albicans* also harbours a *CHS4* homologue (GenBank accession number AB003310). The amino acid sequence of *S. cerevisiae* Chs4p is highly homologous to that of *C. albicans* Chs4p in the C-terminal two-thirds, but the N-terminal one-third is rather divergent (Sudoh *et al.*, 1999). This prompted us to determine the region of Chs4p that is important for Chs3p activity. A series of truncated *CHS4* genes was created and expressed in YMS120 cells (Fig. 3). As shown in Fig. 4, deletion of either the N-terminal 268 amino acids or the C-terminal 134 amino acids only partially diminished the ability of Chs4p to elicit the Chs3p activity in the total membranes of YMS120 cells. Neither the EF-hand motif nor the potential prenylation site of Chs4p was essential to stimulate Chs3p activity, because Chs4(269–563)p still significantly enhanced Chs3p activity (Fig. 4a). Further deletion from the C-terminus, however, nearly completely abolished the ability of Chs4p to elicit Chs3p activity; Chs4(269–445)p was no longer capable of increasing the Chs3p activity in YMS120 cells (Fig. 4a).

We also examined the effects of the N-terminal and C-terminal truncations of *CHS4* on the sensitivity of yeast cells to Calcofluor white. Cells of YMS1, which carries no functional *CHS4* allele, were resistant to Calcofluor white, because of a decreased level of cell-wall chitin (Sudoh *et al.*, 1999). Introduction of the wild-type *CHS4* into the YMS1 cells restored the sensitivity to Calcofluor white (Fig. 4b). Deletion of the C-terminal 252 amino acids of Chs4p completely abolished the ability of the protein to confer Calcofluor-white-sensitive growth on YMS1 cells, while deletion of the N-terminal 268 amino acids and C-terminal 134 amino acids did not (Fig. 4b). From these results, we concluded that the region of Chs4p that is essential for the elicitation of Chs3p activity resides in the amino acid positions between 269 and 563.

Fig. 3. Truncated *CHS4* genes encoding the N-terminally and C-terminally truncated Chs4 proteins used in this study. Each mutant was created by PCR and expressed under the control of the *GAL1* promoter in pYES2. The mutant genes were designated according to the corresponding amino acid positions in the wild-type Chs4p. CHS4(1-697)/WT represents the wild-type *CHS4*, and the predicted domain structure of the wild-type Chs4p is shown at the top.

Region of Chs4p that is essential for binding to Chs3p

It has been shown that Chs4p is physically associated with Chs3p and Bni4p and that this interaction is necessary for the recruitment of Chs3p to the plasma membrane (DeMarini *et al.*, 1997). Therefore, we wondered whether the association of Chs3p and Chs4p is also important for the stimulation of Chs3p activity. The ability of Chs4p to interact with Chs3p was confirmed by yeast two-hybrid analysis using the truncated *CHS4* genes listed in Fig. 3. As shown in Fig. 5, the ability of the Chs4p fragments to interact with Chs3p correlated well with their capacity to stimulate Chs3p activity in the YMS120 cells and to confer Calcofluor sensitivity on the YMS1 cells (Fig. 4). Thus, Chs4(214-445)p and Chs4(269-445)p, which failed to enhance Chs3p activity and restore Calcofluor-white-sensitive growth, were unable to directly interact with Chs3p (Fig. 5). In contrast, all of the other fragments of Chs4p which elicited these responses interacted directly with Chs3p. These results strongly support the idea that Chs4p stimulates the non-zymogenic activity of Chs3p by binding with it.

DISCUSSION

In this paper, we demonstrate that Chs4p stimulates the non-zymogenic activity of Chs3p which can be extracted from membranes using CHAPS and cholesteryl hemisuccinate. In addition, we have shown that the region of Chs4p which is necessary for the stimulation of non-zymogenic Chs3p activity resides between amino acids 269 and 563. Only this region was required for binding of Chs3p to Chs4p, and taken together these data

suggest that the formation of a complex between Chs3p and Chs4p leads to activation of Chs3p. About half of the Chs3p activity remained in membranes after the detergent treatment and could not be easily extracted even by repeated incubation of membranes with detergent. Thus, two different types of non-zymogenic Chs3p, CHAPS-extractable and non-CHAPS-extractable, may exist. As mentioned before, Choi *et al.* (1994) demonstrated that a certain level of Chs3p activity in membranes is attributable to the zymogenic activity of Chs3p, and that such zymogenic activity is not extracted by detergents. The ratio and physiological significance of zymogenic and non-zymogenic Chs3p activities, however, remain elusive.

Chs4p contains the amino acid sequence motifs characteristic of EF-hand calcium-binding sites and prenylation sites in the middle and at the C-terminus, respectively (Kawamoto *et al.*, 1992). Experiments involving the truncation of Chs4p revealed that neither the EF-hand motif nor the prenylation site of Chs4p was essential for Chs3p-binding and stimulation of non-zymogenic Chs3p activity. The real roles of these sequence motifs in Chs4p function remain to be elucidated; they may be required for other roles of Chs4p such as resistance to *Kluyveromyces lactis* toxin (Kawamoto *et al.*, 1992; Takita & Castilho-Valvicius, 1993).

DeMarini *et al.* (1997) also demonstrated the physical association of Chs3p and Chs4p by yeast two-hybrid analysis. According to their results, the C-terminal 86 amino acids of Chs4p play an important role in binding to Chs3p because Chs4p lacking the C-terminal 86 amino acids interacted only weakly with Chs3p (DeMarini *et al.*, 1997). It was also demonstrated that the C-terminal region of Chs4p was crucial for the association

Fig. 4. Chs3p activity of cells overexpressing the truncated Chs4 proteins. (a) YMS120 cells that had been transformed with pYES2 carrying the indicated *CHS4* genes were grown to mid-exponential phase then further cultured for 12 h in medium containing galactose to induce the expression of the truncated Chs4 proteins. Chs3p activity was determined using approximately 80 µg membrane protein. The error bars represent mean $\pm$ SD for three independent experiments. (b) YMS1 cells that had been transformed with pYES2 carrying the indicated *CHS4* genes were spread on agar plates containing galactose. Two independent colonies from each transformation were further cultured for 3 d in the absence (–CW) or presence (+CW) of 2 mg Calcofluor white ml⁻¹.

with Bni4p, which directs the correct localization of Chs3p at the bud neck through interaction with Cdc10p (DeMarini *et al.*, 1997). In this study, we have shown that the C-terminal 134 amino acids of Chs4p are not essential for Chs3p activity, cellular chitin synthesis or binding to Chs3p. Because we judged association of the Chs4p fragments with Chs3p based on the histidine

Fig. 5. Yeast two-hybrid analysis of the interaction of Chs3p with Chs4p. Yeast HF7c cells that were transformed with pGBT9 bearing the indicated *CHS4* genes were further transfected with pGAD424 carrying *CHS3*. Two independent colonies from each transformation were collected and further cultured on agar plates with (+His) or without (–His) histidine for 3 d. The capacity of transformed strains to grow in the absence of histidine was dependent upon the interaction of Chs3p with Chs4p.

auxotrophy of transformants, it was difficult to determine the binding affinities of the truncated Chs4 proteins with Chs3p quantitatively. Nevertheless, our results and those of DeMarini *et al.* (1997) imply that a weak association of Chs4p with Chs3p is sufficient to elicit Chs3p activity.

The mechanism underlying the stimulation of Chs3p activity by Chs4p remains unclear. One possibility is that Chs4p stabilizes and/or increases the amount of Chs3p, although this is rather unlikely for the following reasons. Firstly, Chs3p is a stable protein with a half-life in excess of 10 h *in vivo* (Chuang & Schekman, 1996). Secondly, overexpression of *CHS3* alone increased the amount of Chs3p in membranes, yet Chs3p activity remained very low. Another possibility is that Chs4p affects the conformation of Chs3p and converts an inactive form of Chs3p into an active form. This seems more likely, because the simultaneous overexpression of *CHS3* and *CHS4* did not significantly increase the chitin synthase activity detected as compared to that of cells overexpressing *CHS4* alone (data not shown). Thus, the expression of non-zymogenic Chs3p activity would appear to depend directly on the amount of Chs4p present.

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