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Identification of a Domain in Yeast Chitin Synthase 3 Required for Biogenesis of Chitin Ring, But Not Cellular Chitin Synthesis

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Abstract

It hab been proposed that *CHS3*-mediated chitin synthesis during the vegitative cell cycle is regulated by *CHS4*. To investigate direct protein-protein interaction between their coding products, we used yeast two hybrid system and found that a domain of Chs3p was responsible for interaction with Chs4p. This domain, termed MIRC3-4 (maximum interacting region of chs3p with chs4p), spans from 647 to 700 residues. It is well conserved among *CHS3* homologs of various fungi such as *Candida albicans, Emericella nidulans, Neurospora crassa, Magnaporthe grisea, Ustilago maydis, Glomus versiforme, Exophiala dermatitidis, Rhizopus microsporus.* A series of mutaion in the MIRC3-4 resulted in no appearance of chitin ring at the early G1 phase but did not affect chitin synthesis in the cell wall after cytokinesis. Absence of chitin ring could be caused either by delocalization of Chs3p to the septum or by improper interaction with Chs4p. To discriminate those two, not mutually exclusive, alternatives, mutants cells were immunostained with Chs3p-specific antibody. Some exhibited localization of chs3p to the septum, while others failed. These results indicate that simultaneous localization and activation Chs3p by Chs4p is required for chitin ring synthesis.

Introduction

Chitin, the most abundant polymer next to cellulose, is found in a majoity of fungal cell wall and septa. In *Saccharomyces cerevisiae,* chitin constitutes small portion of cell wall, but is indispensible for cell viability (Shaw et al., 1991). Its synthesis is catalyzed by chitin synthases which are found as multiple isozymes in many fungi (Bowen et al., 1992). Three chitin synthase genes (*CHS1*, *CHS2* and *CHS3*) have been described in *S. cerevisiae* (Bulawa et al. 1986; Sburlati and Cabib 1986; Silverman et al., 1988; Valdivieso et al., 1991). They share high structural homology and carry out same biochemical reactions but play distinctive roles throughout the cell cycle. The cell cycle begins with chitin synthesis by CSIII to form chitin ring only at the septin base of the mother cell (Fig.1 A). Primary septum is formed from chitin ring in a centripetal fashion with chitin synthesized by CSII. Subsequently, two lateral layers of glucan and mannan are added to the primary septum to form the secondary septum. After septation, cellular chitin is generated by CSIII in the daughter cell during the cytokinesis (Fig.1 C and D) and is linked to glucan through β(1,4)-linkage (Kollar et al., 1995). Cytokinesis occurs by digestion of septum with the action of chitinase and chitin ring left in the mother cell. Birth scar of daughter cell is repaired by CSI.

Regulation studies have been focused on *CHS3* due to the identification of its multiple regulators. *CHS4*, *CHS5*, *CHS6*, and *CHS7* genes are known to be involved only in functional regulations of *CHS3*, even though *CHS3* is significantly homologous to *CHS1* and *CHS2*. Thus, *CHS3* is thought to be the catalytic component of its activity (Shaw et al., 1992). *CHS4* would be a activator for CSIII as indicated in a couple of reports showing that chs4 mutant is devoid of most of cellular chitin (Roncero et al., 1988) and has abolished CSIII activity (Choi et al. 1994a; Trilla et al., 1997). The protein-protein interaction between Chs3p and Chs4p was also reported and Chs4p is required for the proper localization of Chs3p (DeMarini et al., 1997).

As described above, *CHS3*, in contrast to *CHS1* and *CHS2*, displays its activities in such diverse cellular events, the reason for which may be due to its multiple regulators. Since *CHS5*, *CHS6*, and *CHS7* are currently limited to transport or localization of Chs3p, detailed analysis of Chs3p with its direct activator, Chs4p, would provide important insights to understand how CSIII activity is regulated. Here we report that a brand-new domain of Chs3p is involved in the synthesis of chitin ring by Chs4p.

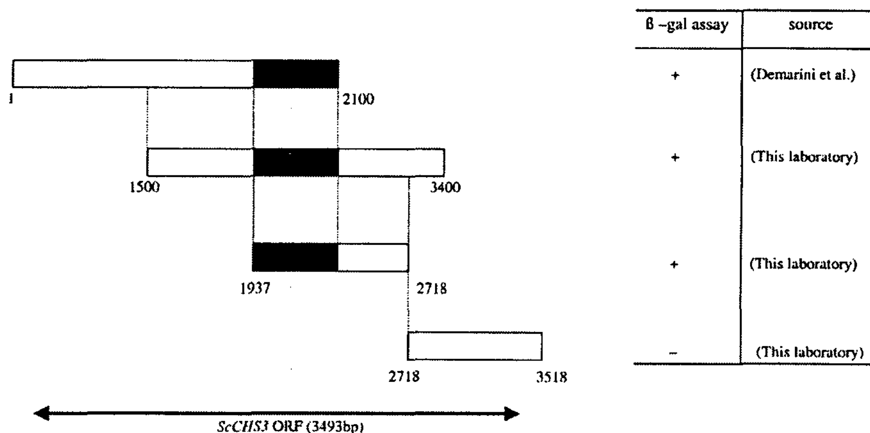

Figure 1. Identification of maximum interacting region of chitin synthase3 with chitin synthase4 (MIRC3-4) by two-hybrid analysis.

Results and Discussion

Identification of a domain in Chs3p interacting with Chs4p

Based on the sequence comparison data, the C-terminal region of Chs3p was speculated to be essential for CSIII activity (Ford et al., 1996). Since CSIII activity is regulated by Chs4p which would act as a post-translational activator for Chs3p (Choi et al., 1994), we were interested in proving direct protein-protein interaction between those two proteins by using the yeast two-hybrid system. β-gal assay of three fusion constructs covering C-terminal two thirds of Chs3p showed that the region of nucleotide residues 1937-2718 interacted with Chs4p. The subsequent publication in which the N-terminal region (nucleotide residues 1-2100) of Chs3p interacted with Chs4p under the yeast two-hybrid system (DeMarini et al., 1997) enabled us to narrow down the interacting region to 164 bp (nucleotide residues 1937-2100), designated maximum interacting region of Chs3p with Chs4p (MIRC3-4) (Fig. 2). The MIRC3-4 is located near the proximal part of C-terminal region which shows significant homology among various chitin synthases (Ford et al., 1996). Protein-protein interaction is usually mediated by various but defined motifs, but there was no recognizable protein-protein interaction motif in the MIRC3-4.

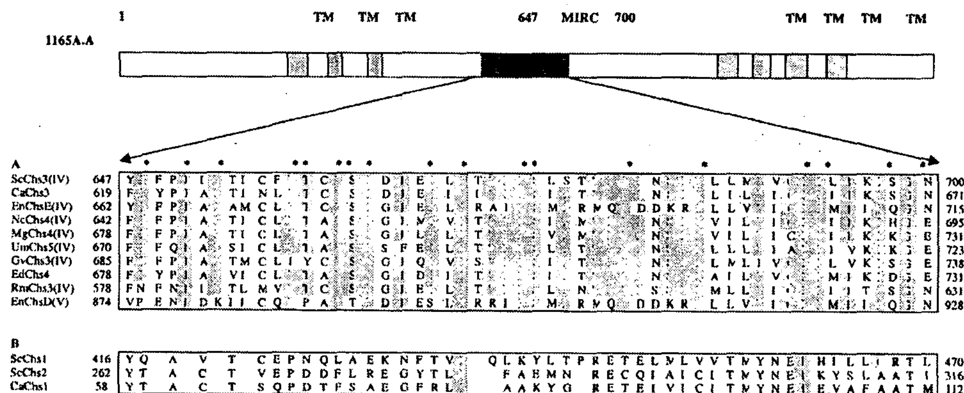

Figure 2. Comparison of amino acids sequences in MIRC3-4 among various fungal chitin synthase

In vitro mutagenesis of MIRC3-4

Site-specific mutagenesis is one of commonly used methods for identification of amino acid residues responsible for protein function. Interspecies sequence comparison often furnishes valuable information on which residue(s) is subject to mutagenesis. When the amino acid sequence of MIRC3-4 were compared among different fungal Chs3ps, a number of residues were perfectly or properly conserved among species examined (Fig. 3). After each of twenty residues selected was substituted site-specifically using pHV8 which contains the full *CHS3* ORF and regulatory sequences (Valdivieso et al., 1991), mutated plasmids as well as the wild type palsmid were independently transformed into the *CHS3*-deletion strain, HPY3. When CSIII activity, chitin content, osmotic stability, and sensitivity to Calcofluor white as an indicator of *in vivo* cellular chitin synthesis were measured in all mutants, CSIII activity and chitin content were decreased to some extent compared to those of the wild type (Table 3). The lowest CSIII activity of M8 (Glu663Met) suggests that Glu663 might be a core residue for interaction between Chs3p

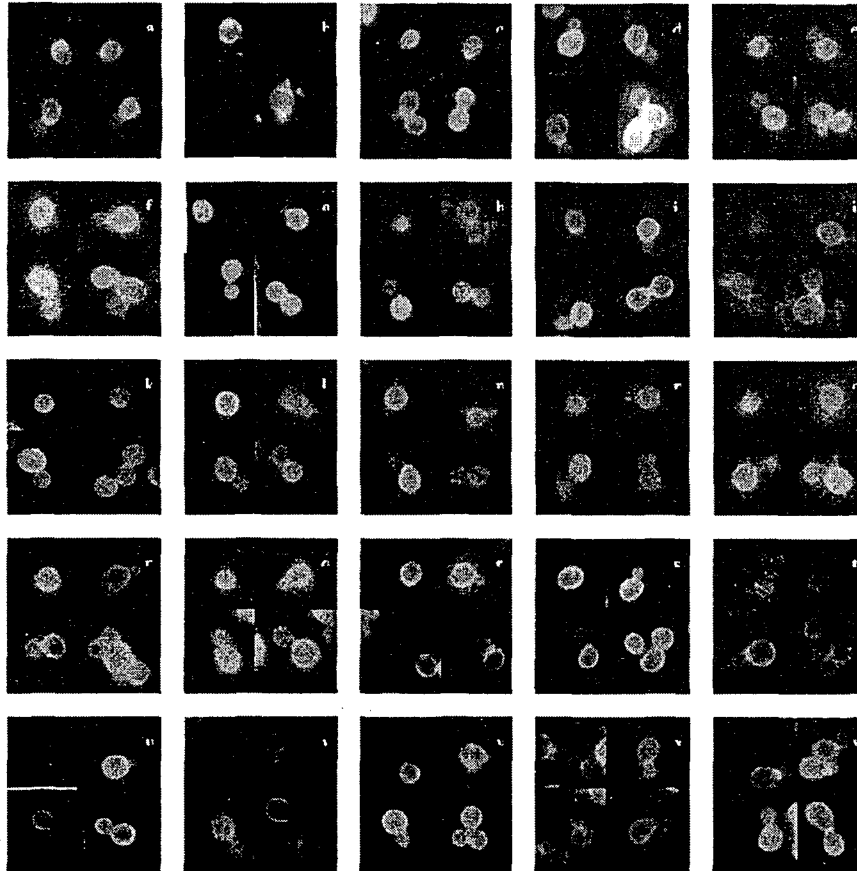

Figure 3. Calcofluor staining of mutants in MIRC3-4

and Chs4p. Meanwhile, other physiological properties such as Calcofluor sensitivity, osmotic stability, and actin distribution were normal (Table 3 with no actin distribution data shown). However, no close relationship between CSIII activity and chitin content was observed, indicating that minimum amount of Chs3p and chitin is sufficient for maintenance of physiological condition To test the general applicability of these results, one mutant outside the MIRC3-4, designated M-1 in which Ser617 was changed to Gly, was constructed and examined for above biochemical and physiological properties. As shown in Table 3, M-1 functioned normally as the wild type. Taken together, no mutation in MIRC3-4 caused significant changes in cellular chitin synthesis, resulting in restoration of Calcofluor sensitivity and osmotic stability.

However, morphological differences between cells harboring wild type and mutated plamids were noticed; all mutants exhibited protuberances, abnormal budding, or clumping, characteristics resulting from a lack of chitin ring as in the *CHS3*-deletion strain HPY3. These data strongly suggest that mutations in MIRC3-4 might affect chitin mother-bud neck should be different between bud emergence and late in the cell cycle. Our results confirms this hypothesis and visualizes the roles of Chs4p early and late in the cell cycle near the mother-bud neck. The MIRC3-4 domain could be involved in the interaction with Chs4p during bud emergence and chitin ring is synthesized. This initial complex should be disappeared at cytokinesis because the transcript of CHS4 was not occurred during the cell cycle (Park and Choi, unpublished observation). But Chs3p reformed with Chs4p during cytokinesis with different arrangement using different domain(s) for the biosynthesis of cellular chitin in the daughter cell.

Table I. Substitutions of amino acid in MIRC and functional analysis.

Strain	Substitution	Enzymatic activity(%)	Chitin content(%)	Calcofluor Resistancy	Osmotic stability
ECY36-3A	YGFPLIHTICFVTCYSEDEEGLRTTLDSLSTTDYPNSHKLLMVVCDGLIKGSGN	100	100	+	++
M1 (G648S)	-S--	71.4	73.8( )	+	+
M2 (L651E)	----E---	69.7	75.9(30)	+	+
M3 (H653P)	------P---	80	71.6(26.9)	+	++
M4 (V658S)	-----------S--	65.4	85.1(24.7)	+	+
M5 (T659D)	------------D---	77.1	77.3(21.4)	+	++
M6 (Y661L)	--------------L---------------------------------------	82.9	75.9(19.1)	+	++
M7 (S662A)	---------------A--------------------------------------	73.1	85.8(30.5)	+	+
M8 (E663M)	----------------M-------------------------------------	45.7	71.6(19.3)	+	++
M9 (G667S)	--------------------S---------------------------------	91.4	80.1()	+	+
M10 (R669E)	----------------------E-------------------------------	68.3	95(30.5)	+	ND
M11 (D673N)	--------------------------N---------------------------	66	86.5(25.5)	+	ND
M12 (S674A)	---------------------------A--------------------------	82.6	85.1(23.4)	+	ND
M13 (S676A)	-----------------------------A------------------------	71.1	85.1(23.4)	+	ND
M14 (Y680L)	---------------------------------L--------------------	62	74.5((24.2)	+	ND
M15 (K685E)	--------------------------------------E---------------	76	71.6()	+	ND
M16 (C691A)	--A---------	82.6	104.2()	+	ND
M17 (D692N)	---N--------	52.6	79.4(21.9)	+	ND
M18 (G693S)	--S-------	50	85.8()	+	ND
M19 (G697S)	--S---	89.7	71.6()		ND
M20 (G699S)	--S-	91.1	80.1()		ND
M-1 (S621G)			(20.4)		
HPY3	*chs3::TRP1*(Δ1- 479 a.a.)	28.6	(21)		

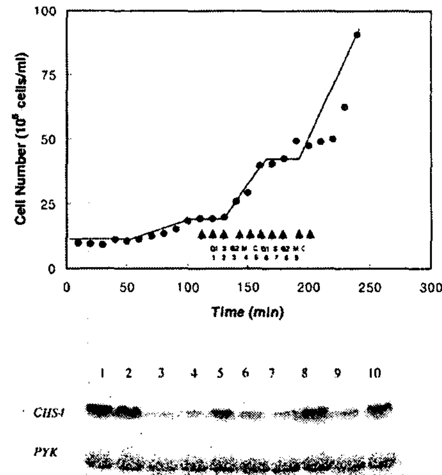

Figure 4 .Northern analysis of CHS3 during cell vegetative cycle

During the bud emergence, chitin ring biogenesis occurred asymmetrically in the mother-cell side, while late in the cell cycle cellular chitin is formed in the daughter cell by CSIII complex . Therefore CSIII complex could be different in two situations.
Identification of the domain involved only in chitin ring synthesis was surprising, since such broad region was involved in protein-protein interaction with Cha4p and all 20 residues examined were independently essential. In general, the length of protein-protein interaction motif is around 20 amino acids at most. Capacity of MIRC3-4 for anchoring another molecule in addition to Chs4p needs to be investigated

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