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Identification of a Domain in Yeast Chitin Synthase 3 Interacting with Chitin Synthase 4 by Two-Hybrid Analysis

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Abstract It has been proposed that chitin synthase 3 (*CHS3*)-mediated chitin synthesis during the vegetative cell cycle is regulated by chitin synthase 4 (*CHS4*) of *Saccharomyces cerevisiae.* To investigate direct protein-protein interaction between the coding products of these two genes, a domain of Chs3p that is responsible for interaction with Chs4p was identified, using the yeast two-hybrid system. This domain of 54 amino acids, termed MIRC3-4 (*M*aximum *I*nteracting *R*egion of Chs*3*p with Chs*4*p), is well conserved among *CHS3* homologs of various fungi. Some mutations in MIRC3-4 resulted in a decrease in the enzymatic activity and chitin contents. Chs3p carrying those mutations exhibited weak interactions with Chs4p, when assayed by the yeast two-hybrid system. Surprisingly, all the mutants were sensitive to Calcofluor regardless of changes in enzymatic activities or chitin contents. This report deals with a core region in MIRC3-4 that affects the interaction with Chs4p.

Key words: Chitin synthase 3, two-hybrid analysis, *Saccharomyces cerevisiae*, *ScCHS4*

Chitin, the most abundant polysaccharide in nature next to cellulose, is found in a majority of fungal cell walls and septa [12]. In *Saccharomyces cerevisiae*, chitin constitutes a small portion of the cell wall, but is indispensable for cell viability [26]. Its synthesis is catalyzed by chitin synthases that are found as multiple isozymes in many fungi [2]. Three chitin synthase genes (*CHS1*, *CHS2*, and *CHS3*) have been described in *S. cerevisiae* [3, 25, 27, 30]. They share a high structural homology and carry out the same biochemical reactions, however, play distinctive roles throughout the cell cycle; Chs1p encoded by *CHS1* repairs the birth scar after cytokinesis [4, 5], Chs2p encoded by *CHS2* is involved in the formation of the primary septum [26, 27], and Chs3p encoded by *CHS3* is responsible for chitin ring formation in the G1 phase and synthesis of chitin dispersed throughout the cell wall at cytokinesis (cell wall chitin hereafter) [26, 30]. In addition, Chs3p also catalyzes chitin synthesis during mating and sporulation [16]. Accordingly, the activity of each enzyme should be tightly regulated in a spatiotemporal manner so as to exert its functions.

Cell cycle events in *S. cerevisiae* are characterized by several biochemical processes. In terms of septin assembly and septum formation, the cell cycle begins with chitin synthesis by CSIII to form a chitin ring at the base of an emerging bud, next to the microfilament ring and on the mother cell side of it. The primary septum is formed from the chitin ring in a centripetal fashion with the chitin synthesized by Chs2p. Subsequently, two lateral layers of glucan and mannan are added to the primary septum to form secondary septa. After septation, cell wall chitin is generated by CSIII in the daughter cell and is linked to glucan through a β(1,4)-linkage [13]. Cell separation occurs by digestion of the septum through the action of chitinase and the chitin ring is left in the mother cell. Excessive damage to the birth scar of the daughter cell by chitinase is repaired by Chs1p.

Studies on regulation have been focused mainly on *CHS3*, because its multiple regulators have been identified. The *CHS4*, *CHS5*, *CHS6*, and *CHS7* genes are known to be involved only in the functional regulation of *CHS3*. Since *CHS3* is highly homologous to *CHS1* and *CHS2*, it is thought to be a catalytic component of CSIII which is the holoenzyme for chitin synthase 3 activity [26]. *CHS4* may be an activator for CSIII, since a *chs4* mutant is shown to be devoid of most cell wall chitin [20] and of chitin synthase 3 activity [8, 28]. Protein-protein interaction between Chs3p and Chs4p has also been reported, and Chs4p is required for the proper localization of Chs3p

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Table 1. *S. cerevisiae* strains used in this study.

Strain	Genotype	Source
HPY3	*MATa chs1-23 chs3::TRP1 trp1-1 ura3-52 leu2-3*	This study
HPY3 [pM1-14]	*MATa chs1-23 chs3::TRP1 trp1-1 ura3-52 leu2-3 [pM1-14]*	This study
HPY3 [pCMN] (S621G)	*MATa chs1-23 chs3::TRP1 trp1-1 ura3-52 leu2-3 [pCMN] (S621G)*	This study
SFY526	*MATa ura3-52 his3-200 ade2-101 lys 2-801 trp1-901 leu2-3,112 can' gal4-542 gal80-538 URA3::GAL1-lacZ*	Clonetech Inc.
SFY526 [pGBT-CHS3]	*MATa ura3-52 his3-200 ade2-101 lys 2-801 trp1-901 leu2-3,112 can' gal4-542 gal80-538 URA3::GAL1-lacZ [pGBT-CHS3]*	This study
SFY526 [pGBT-CHS3 (M1-14)]	*MATa ura3-52 his3-200 ade2-101 lys 2-801 trp1-901 leu2-3,112 can' gal4-542 gal80-538 URA3::GAL1-lacZ [pGBT-CHS3(M1-14)]*	This study
SFY526 [pGBT-CHS3 (ΔMIRC)]	*MATa ura3-52 his3-200 ade2-101 lys 2-801 trp1-901 leu2-3,112 can' gal4-542 gal80-538 URA3::GAL1-lacZ [pGBT-CHS3(ΔMIRC)]*	This study

[10]. *CHS5* is required for polarized localization of Chs3p to the bud site and to the mating projection, and for transport of other proteins [23]. *CHS6* is possibly a Chs3p-specific transporter [31], and *CHS7* exports Chs3p from the ER [29].

In contrast to the single roles of *CHS1* and *CHS2*, different functions of *CHS3* in diverse cellular events may explain the need of multiple regulators. Since *CHS5*, *CHS6*, and *CHS7* are apparently involved in the transport and localization of Chs3p, detailed analysis of the interaction of Chs3p with Chs4p may provide important insights into the regulation of chitin synthase 3 activity. In this study, a domain of Chs3p interacting with Chs4p was identified, which is involved in the synthesis of the chitin ring by Chs4p in the G1 phase, but not in the cell wall chitin synthesis after septation.

Materials and Methods

Strains, Culture Conditions, and Genetic Methods

S. cerevisiae strains used in this study are described in Table 1. Calcofluor sensitivity was assayed in SD medium (2% glucose, 0.7% Difco yeast nitrogen base without amino acids, and 0.07% amino acids solid mix) supplemented with 0.1% Calcofluor. Standard methods were used for DNA manipulations [22]. Transformation of yeast, growth media, and other yeast genetic manipulations were carried out according to standard procedures [1, 6].

Construction of Plasmids and Strain

To construct plasmids for two-hybrid analyses, three fragments of the *CHS3* ORF (nucleotide residues 1,500-3,400, 1,937-2,718, and 2,718-3,518) [14] were identified by PCR and cloned into the *Eco*RI site of the DNA binding domain (DBD) vector pGBT9, giving rise to pCHK-B1, pCHK-B2, and pCHK-C, respectively. The entire ORF of *CHS3* and *CHS4* from the genome was amplified by PCR and cloned into the *Eco*RI site of pGBT9 and activation domain (AD) vector pGAD424 to generate pGBT9-CHS3 and pGAD-CHS4, respectively. Each DBD vector was co-transformed with pGAD-CHS4 into yeast strain SFY526.

For mutagenesis, the *CHS3* gene of 3.7 kb that includes promoter and the entire ORF was amplified and was cloned into pRS426, yielding pHP29.

The HPY3 strain (see Table 1) was constructed and the N-terminal region of *CHS3* was replaced by *TRP1*, as follows. The 5.4 kb *Eco*RI-*Sal*I fragment of pHV7-HA [30] (kindly provided by Dr. A. Duran, Spain) was cloned into the pGEM3Zf vector, yielding pHP31. Then, the 1.2 kb *Xho*I-*Xba*I fragment of pHP31 was replaced by the *TRP1* gene that was amplified by PCR using primers TRP1-5 and TRP1-3 (Table 2), yielding pHP32. Subsequently, the 5.3 kb *Eco*RI-*Sal*I fragment of pHP32 containing the *chs3::TRP1* construct was purified and transformed into the strain ECY36-3A to construct HPY3.

Site-Directed Mutagenesis

Mutated plasmids pM1-14 were derived from pHP29 by using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) and primers listed in Table 2. Positions of mutagenized amino acids are shown in Table 3.

To confirm the effects of point mutation by exploiting the yeast two-hybrid system, the fragments containing mutation were inserted into the pGBT9-CHS3 to obtain pGBT9-CHS3(M1-M14), as follows: the *CHS3* ORF (3,498 bp) was amplified by PCR, and cloned into the *Eco*RI site of pBluescriptII (pBII-CHS3). The 1.5 kb *Hin*cII/*Hin*dIII fragment of pBII-CHS3 that contained MIRC3-4 was replaced by the corresponding fragments from 14 mutated plasmids to obtain pBII-CHS3(M1-14). The *Eco*RI fragments from the pBII plasmids above were cloned into pGBT9 to generate pGBT-CHS3 and pGBT-CHS3(M1-14). As a negative control, MIRC3-4 was deleted as follows. The 800 bp fragment (from 1,579 to 2,400) containing MIRC3-4 was removed from pBII-CHS3 by digesting with *Eco*RV and *Pst*I, filling with Klenow, and ligated. The above *Eco*RI-digested 2.7 kb fragment of plasmid was subcloned into the *Eco*RI site of pGBT9 to obtain pGBT-CHS3 (ΔMIRC).

T ble 2. Oligonucleotide primers used for PCR.

N me	Sequence	Name	Sequence
C IS3A-5'[1]	5' CTCGAGTATTTTTGGTGTAGG 3'	M6-3	3' GACTCCTACTTCTCAGAAATTCTTGGTGAAATCTG 5'
C IS3A-3'[1]	3' CTGTAATTGCCTTAGCCACGT 5'	M7-5	5' GAAGAGGGTTTAGAAACCACTTTAGAC 3'
C IS3B-5'[2]	5' GAATTCATGACCGGCTTGAATGGAGAT 3'	M7-3	3' CTTCTCCCAAATCTTTGGTGAAATCTG 5'
C IS3B-3'[2]	3' GAATTCTGCAACGAAGGAGTCACTTTC 3'	M8-5	5' AGAACCACTTTAAACTCTCTTTCTACC 3'
C IS3C-5'[3]	5' GAGCATATGAATCACCTGGAT 3'	M8-3	3' TCTTGGTGAAATTTGAGAGAAAGATGG 5'
C IS3C-3'[3]	3' GAATTCCCAACGGAAGTAGCA 5'	M9-5	5' TTAGACTCTCTTGCTACCACAGATTAT 3'
T .P1-5	5' CTCGAGCCCAGGTTCGGATGTTCA 3'	M9-3	3' AATCTGAGAGAACGATGGTGTCTAATA 5'
T .P1-3	3' ATACGGGAGTACTCTGTTGAGCTC 5'	M10-5	5' TCTACCACAGATCTTCCAAATTCCCAT 3'
N 1-5	5' CCATACGGGTTCCCAGAGATTCATACTATCTG 3'	M10-3	3' AGATGGTGTCTAGAAGGTTTAAGGGTA 5'
N 1-3	3' GGTATGCCCAAGGGTCTCTAAGTATGATAGAC 5'	M11-5	5' CTGATGGTTGTTGCTGATGGTTTAATT 3'
N 2-5	5' GGGTTCCCATTGATTTTTACTATCTGTTTTGTTAC 3'	M11-3	3' AATTAAACCATCAGCAACAACCATCAG 3'
N 2-3	3' CCCAAGGGTAACTAAAAATGATAGACAAAACAATG 5'	M12-5	5' ATGGTTGTTTGTAATGGTTTAATTAAG 3'
N 3-5	5' CTATCTGTTTTGTTGATTGTTATTCTGAGGATG 3'	M12-3	3' TACCAACAAACATTAACCAATTAATTC 5'
N 3-3	3' GA TAGACAAAACAACTAACAATAAGACTCCTAC 5'	M13-5	5' GTTGTTTGTGATAGTTTAATTAAGGGC 3'
N 4-5	5' GTTTTGTTACTTGTTATGCTGAGGATGAAGAGGG 3'	M13-3	3' CAACAAACACTATCAAATTAATTCCCG 5'
N 4-3	3' CAAAACAATGAACAATACGACTCCTACTTCTCCC 5'	M14-5	5' ATTAAGGGCTCGAGCAACGATAAGACT 3'
N 5-5	5' GTTACTTGTTATTCTATGGATGAAGAGGGTTTAAG 3'	M14-3	3' TAATTCCCGCGATCGTTGCTATTCTGA 5'
N 5-3	3' CAATGAACAATAAGATACCTACTTCTCCCAAATTC 5'	CMN-5	5' TCTCCTGTACCAGGAGGATCGCTGATTCAG 3'
N 6-5	5' TGAGGATGAAGAGTCTTTAAGAACCACTTTAGAC 3'	CMN-3	3' AGAGGACATGGTCCTCCTAGCGACTAAGTC 5'

[1]I imers for amplifying *CHS* gene to construct pHP29.
[2]I imers for amplifying *CHS* ORF for two-hybrid analysis.
[3]I imers for amplifying *CHS* ORF for mutagenesis.

I reparation of Membrane and Determination of Chitin S ynthase Activity

T ie total cell membranes were prepared, and chitin s nthase 3 activity was measured according to the method p eviously described [7, 8, 30].

M leasurement of β-Galactosidase Activity

β -Galactosidase activity was measured with crude extracts, a previously described [17, 18, 21].

M leasurement of Chitin Contents

T he amount of chitin in the cell wall was measured as p eviously described [19].

RESULTS

Identification of a Domain in Chs3p Interacting with Chs4p

Based on sequence comparison, the C-terminal region of Chs3p has been suggested to be essential for chitin synthase 3 activity [11]. Since chitin synthase 3 activity is regulated by Chs4p that may act as a post-translational activator for Chs3p [8], it was of interest to study direct protein-protein interaction between these two proteins by yeast two-hybrid analyses. Assay for β-galactosidase of three fusion constructs, covering the C-terminal two-thirds of Chs3p, showed that the region of nucleotide residues 1,937–2,718 interacted with Chs4p. A

T able 3. Substitutions of amino acid in MIRC3-4.

Strain	Substitution
I PY3 [pHP29]	P L I H T I C F V T C Y S E D E E G L R T T L D S L S T T D Y P N S H K L L M V V C D G L I K G S G
I PY3 [pM1] (L651E)	-E--
I PY3 [pM2] (H653P)	---P--
I PY3 [pM3] (T659D)	---------D--
I PY3 [pM4] (S662A)	------------A-------------------------------------
I PY3 [pM5] (E663M)	-------------M------------------------------------
I PY3 [pM6] (G667S)	-----------------S--------------------------------
I PY3 [pM7] (R669E)	-------------------E------------------------------
I PY3 [pM8] (D673N)	-----------------------N--------------------------
I PY3 [pM9] (S676A)	--------------------------A-----------------------
I PY3 [pM10] (Y680L)	------------------------------L-------------------
I PY3 [pM11] (C691A)	---A--------
I PY3 [pM12] (D692N)	--N-------
I PY3 [pM13] (G693S)	---S------
I PY3 [pM14] (G699S)	---S

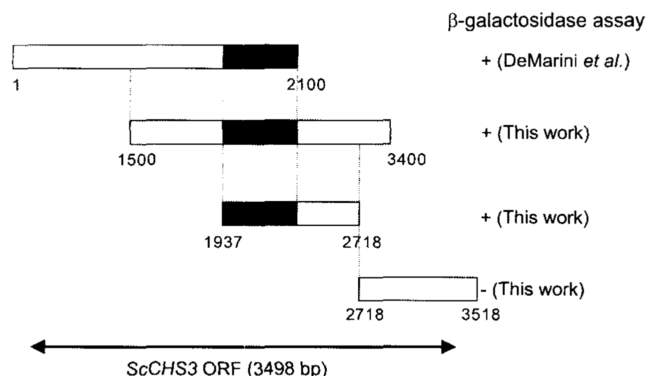

Fig. 1. Identification of *M*aximum *I*nteracting *R*egion of *C*hs*3*p with Chs*4*p (MIRC3-4) by two-hybrid analyses.
Protein-protein interactions were tested by β-galactosidase assay. Based on previously published data [8], three fusion constructs of Chs3p narrowed down the interacting region to 164 bp (nucleotide residues 1,937–2,100). + indicates positive (blue color) and - indicates negative (white color), respectively, in β-galactosidase filter assay.

previous report showing that the N-terminal region (nucleotide residues 1–2,100) of Chs3p interacted with Chs4p [10] enabled us to narrow down the interacting region to 164 bp (nucleotide residues 1,937–2,100), designated the *M*aximum *I*nteracting *R*egion of *C*hs*3*p with Chs*4*p (MIRC3-4) (Fig. 1). This region was found to be strictly conserved among chitin synthase 3 homologs (Fig. 2A), however, not among chitin synthase 1 or 2 of *S. cerevisiae* and *Candida albicans CHS1* (Fig. 2B). Thus, it is highly likely that MIRC3-4 is a functionally fundamental region specific to Chs3p. Protein-protein interaction is usually mediated by various defined motifs, but no such interaction motif was found in MIRC3-4.

Biochemical Characterization of MIRC3-4

The effects of MIRC3-4 on the function of *ScCHS3* were examined by individually substituting fourteen conserved residues using a site-specific mutagenesis technique with pHP29 as a template. All substitutions were designed to affect the wildtype MIRC3-4 as much as possible, for example, by converting a basic residue to an acidic one. When the mutated and wildtype plasmids were independently transformed into the *CHS3*-deletion strain, HPY3, the growth rate of cells harboring mutated plasmid decreased by some degree compared with the wildtype (data not shown).

Chs3p shows dual functions during the cell cycle; it is involved in the synthesis of the chitin ring at the G1 phase and of cell wall chitin at the cytokinesis [26]. To address the function of MIRC3-4, enzymatic activity, chitin contents,

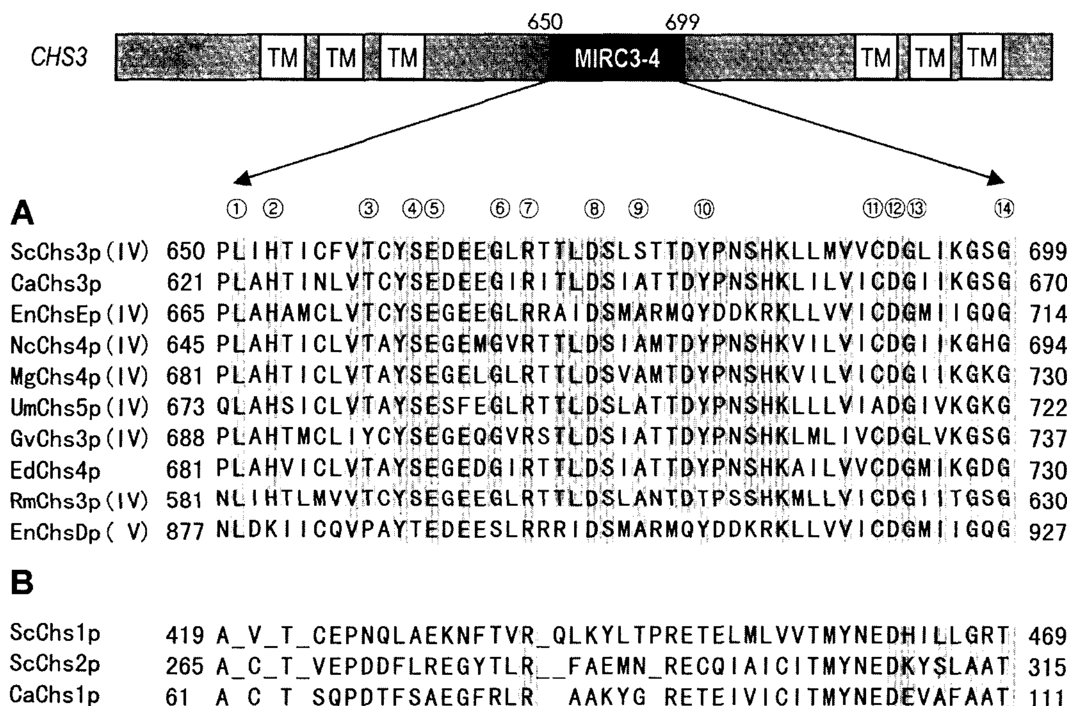

Fig. 2. Comparison of amino acids sequences in MIRC3-4 among various fungal chitin synthases.
(A) Comparison among various fungal chitin synthase 3 homologues. (B) Comparison between ScChs3p and its isozymes, ScChs1p, ScChs2p, and CaChs1p. Amino acid similarity was determined by BLAST search [15]. Highly conserved amino acids are shaded. Circled numbers indicate mutagenized residues. *Sc*, *Saccharomyces cerevisiae*; *Ca*, *Candida albicans*; *En*, *Emericella nidulans*; *Nc*, *Neurospora crassa*; *Mg*, *Magnaporthe grisea*; *Um*, *Ustilago maydis*; *Gv*, *Glomus versiforme*; *Ed*, *Exophiala dermatitidis*; *Rm*, *Rhizopus microsporus*. The accession numbers of the sequences are A23944 for *ScCHS1*, M23865 for *ScCHS2*, S45879 for *ScCHS3*, S11808 for *CaCHS1*, P30593 for *CaChs3*, U52362 for *EnCHSE*, U25097 for *NcCHS4*, O13353 for *MgCHS4*, O1394 for *UmCHS5*, AJ009630 for *GvCHS3*, AAD28744 for *EdCHS4*, T30202 for *RmCHS3*, and P78611 for *EnCHSD*.

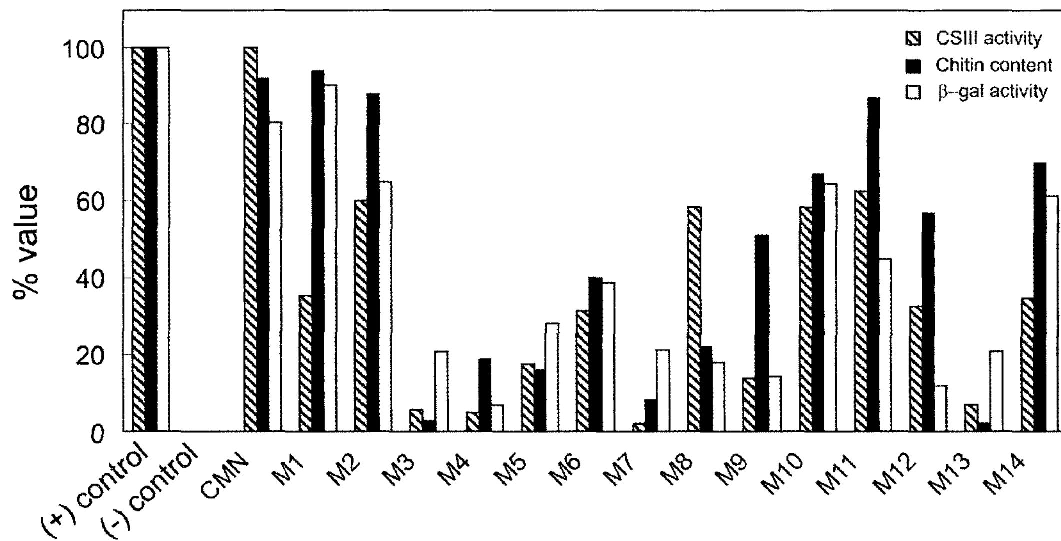

Fig. 3. Functional analyses of 14 MIRC 3-4 mutants.
Chitin synthase 3 activity (dotted bars) and chitin contents (hatched bars) were measured from 14 MIRC3-4 mutants, (HPY3[pM1-14]), and β-galactosidase assays (open bar) were performed with SFY526 yeast cells co-transformed with pGAD-CHS4 and pGBT9-CHS3 or MIRC3-4 mutated plasmids [pGBT-CHS3(ΔMIRC), pGBT-CHS3(M1-14)]. Chitin synthase 3 activity was measured using Ni^{++} to inhibit the chitin synthase 2 activity. Results were expressed as a percentage between 100% positive (HPY3[pHP29] for chitin synthase 3 activity and chitin contents and pGBT9-CHS3 for β-gal assays) and 0% negative (HPY3 for enzymatic activity and chitin contents and pGBT-CHS3(ΔMIRC) for β-gal assays) controls. Results of enzymatic activity and chitin content are the average of six independent experiments, and β-galactosidase assays were repeated four times. M1 (L651E), M2 (H653P), M3 (T659D), M4 (S662A), M5 (L663M), M6 (G667S), M7 (R669E), M8 (D673N), M9 (S676A), M10 (Y680L), M11 (C691A), M12 (D692N), M13 (G693S), M14 (G699S), CMN (S621G).

and sensitivity to Calcofluor were examined in the 14 mutants. As shown in Fig. 3, the degree of enzymatic activity and chitin contents correlated with each other; the mutants with high enzymatic activity exhibited high chitin contents, while mutants with low enzymatic activity exhibited low chitin contents. pM3, 4, 5, 6, 7, and 13 showed low enzymatic activity as well as low chitin content, indicating that a core region for the function of Chs3p existed. pM2, 10, 11, 12, and 14 represented high enzymatic activity as well as high chitin contents, indicating that these mutations were barely affected. Meanwhile, enzymatic activity and chitin contents of three mutants (p1, 8, 9) were found to not correlate. When sensitivity to calcofluor was examined, all the mutants were surprisingly sensitive (data not shown).

For further confirmation of these results, one mutant outside the MIRC3-4, designated HPY3[pCMN] in which Ser617 was changed to Gly, was examined for the above biochemical and physiological properties. As shown in Fig. 3, HPY3[pCMN] functioned as normally as the wildtype.

Interaction of MIRC 3-4 Mutants with Chs4p

In conjunction with the above results, the effects of MIRC3-4 on the interaction with *ScCHS4* were examined. After 14 mutated MIRC3-4 sequences (M1-14) were placed into the pGBT9 vector (see Materials and Methods) and transformed into SFY526 containing the pGAD-CHS4, β-gal assays were performed. The intact *CHS3* ORF, and MIRC3-4-deleted *CHS3* ORF, were used as positive and negative controls, respectively.

As shown in Fig. 3, cells harboring pGBT9-CHS3 (M1, 2, 10, 11, 14) exhibited high intensity, and cells harboring pGBT9-CHS3 (M3-9, M12-13) exhibited very low intensity. The mutants (pM 3-7, pM12-13) with low enzymatic activity and low chitin content also exhibited weak interaction with Chs4p. In the case of pM8 and pM9, three biochemical characteristics did not completely match with each other. These results indicate that two subregions were required for the interaction with Chs4p.

DISCUSSION

In this study, the interacting region between Chs3p and Chs4p, the only regulator of Chs3p activity known to date, was identified. Together with results from earlier studies [9, 11], this shows that there exist at least two functional domains in Chs3p. One is located at the C-terminal of Chs3p that has been suggested to be likely a catalytic domain, in light of the high similarity among its isozymes from various fungi. The other is MIRC3-4 that interacts with Chs4p, as shown in this study. MIRC3-4 is well conserved among *CHS3* homologues of various fungi, but not among *CHS3* isozymes (Fig. 2), suggesting a regulatory domain necessary for chitin synthase 3 activity. Since the syntheses of chitin ring and cell wall chitin occur in the G1 phase and after cytokinesis, a rather complex regulation by differential localization of Chs3p is thought to be required for Chs3p function [24]. Syntheses of chitin ring and cell wall chitin by Chs3p are strictly

governed by Chs4p; deletion of *CHS4* leads to lack of chitin synthase 3 activity [28]. Based on the observation that some mutations in MIRC3-4 resulted in a decrease of enzymatic activity, chitin contents and β-galactosidase activities (Fig. 3), it was concluded that *CHS4* regulates chitin ring synthesis only by interaction with Chs3p through MIRC3-4. In addition to the biochemical characterizaion of MIRC3-4, a further functional analysis is necessary to examine its role in the regulation of chitin synthase 3.

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