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Interacting Domain Between Yeast Chitin Synthase 3 and Chitin Synthase 4 is Involved in Biogenesis of Chitin Ring, but not for Cell Wall Chitin

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Abstract Recently, we identified a domain, termed MIRC3-4, for the protein-protein interaction between yeast chitin synthase 3 (*CHS3*) and chitin synthase 4 (*CHS4*). In this study, the functional roles of MIRC3-4 were examined at the G1 phase and cytokinesis of the cell cycle by Calcofluor staining and FISH. Some mutations in MIRC3-4 resulted in disappearance of the chitin ring in the early G1 phase, but did not affect chitin synthesis in the cell wall at cytokinesis. The chitin distribution in *chs4* mutant cells indicated that *CHS4* was involved in the synthesis of chitin ring in the G1 phase and in the synthesis of cell wall chitin after cytokinesis, suggesting that Chs4p regulates chitin synthase 3 activity differently in G1 and cytokinesis. Absence of the chitin ring could be caused either by delocalization of Chs3p to the bud-neck or by improper interaction with Chs4p. When mutant cells were immunostained with a Chs3p-specific antibody to discriminate between these two alternatives, the mutated Chs3p was found to localize to the bud-neck in all MIRC3-4 mutants. These results strongly indicate that Chs4p regulates Chs3p as an activator but not a recruiter.

Key words: Chitin synthase 3, chitin ring, cell wall chitin, *Saccharomyces cerevisiae*, *ScCHS4*

Chitin synthases (*CHS*) are involved in the biosynthesis of chitin that is an important component in fungal cell walls [12]. *CHS* which exist as isozymes in most fungi, though not found in mammalian cells, became a potential target of antifungal agents [3]. In regard to *Saccharomyces cerevisiae*, extensive studies were performed on the three chitin synthases, named *CHS1*, *CHS2*, and *CHS3* [4, 11, 13, 15]. Since the expressions of *CHS2* and *CHS3* are dependent on the cell cycle, their regulations are important [8]. Chs3p encoded by *CHS3* is responsible for chitin ring formation in the G1 phase, and synthesis of chitin occurs throughout the cell wall at cytokinesis (cell wall chitin, hereafter) [12, 15].

Recently, we identified the domain of *CHS3* involved in the regulation of protein-protein interaction, and observed that mutations in this domain induced reduction of enzymatic activity and chitin contents (Park *et al.*, unpublished data). In the present report, we describe that the same mutations resulted in deficiency of the chitin ring in the G1 phase, however, not in the cell wall chitin at cytokinesis.

Materials and Methods

Strains, Culture Conditions, and Genetic Methods

S. cerevisiae strains used in this study are described in Table 1. Standard methods were used for DNA manipulations [9]. Transformations of yeast, growth media, and other yeast genetic manipulations were carried out according to standard procedures [1].

Construction of Mutated Plasmids for FISH

To confirm the effects of point mutations by exploiting the fluorescent *in-situ* hybridization (FISH), fragments containing mutations were inserted into pHV7-HA to yield pHV7-HA(M2, 3, 5, 7, 11, 14). 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 contains MIRC3-4 was replaced by the corresponding fragments from 14 mutated plasmids to obtain pBII-CHS3(M1-14). For FISH, the *Eco*R1 and *Hin*dIII fragments of 4.1 kb were first removed from pHV7-HA. Into the remaining 8.9 kb fragment, the *Eco*R1 and *Hin*dIII fragments of 2.7 kb from pM2, 3, 5, 7, 11, 14,

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

Strain	Genotype	Source
ECY36-3A	*MATa chs1-23 trp1-1 ura3-52 leu2-2*	a
ECY36-3C	*MATchs1-23 chs2::LEU2 ura3-52 leu2-3,112 trp1-1*	a
ECY36-3D	*MATa chs1-23 cal^{R}1 ura3-52 leu2-3,112 trp1-1*	a
CR4-2	*MATa chs1::URA3 cal2 ura3-52 his4*	a
HPY3	*MATa chs1-23 chs3::TRP1 trp1-1 ura3-52 leu2-3*	b
HPY3 [pM1-14]	*MATa chs1-23 chs3::TRP1 trp1-1 ura3-52 leu2-3* [pM1-14]	b
HPY3 [pCMN] (S621G)	*MATa chs1-23 chs3::TRP1 trp1-1 ura3-52 leu2-3* [pCMN] (S621G)	b
HPY3 [pHV7-HA]	*MATa chs1-23 chs3::TRP1 trp1-1 ura3-52 leu2-3* [pHV7-HA]	This study
HPY3 [pHV7-HA(M2, 3, 5, 7, 11, 14)]	*MATa chs1-23 chs3::TRP1 trp1-1 ura3-52 leu2-3* [pHV7-HA(M2, 3, 5, 7, 11, 14)]	This study

a. The strain was obtained from Dr. D. Cabib (NIH). b. The strain was obtained from Dr. W. Choi (Ewha womans University).

that contains promoter and two thirds of *CHS3* ORF, were cloned to yield pHV7-HA(M2, 3, 5, 7, 11, 14).

Calcofluor Staining

Calcofluor was added to the washed mid-log cells at the concentration of 0.01% and stained for 10 min. The fluorescence was observed with an LSM 510 confocal microscope at 365 nm (Carl Zeiss, Zottinzen, Germany).

Indirect Immunofluorescence Microscopy

Indirect immunofluorescence was performed, as described previously, with minor modifications [2]. Cells were fixed in 2% formaldehyde for 1 h, washed three times with 1× KS buffer (1 M sorbitol, 0.1 M potassium phosphate, pH 7.0), and resuspended in 1× KS buffer. Spheroplasts were prepared by incubating cells in KS buffer, containing 50 μg/ml Zymolase-20T (Seikagaku, Tokyo, Japan) and 0.2%

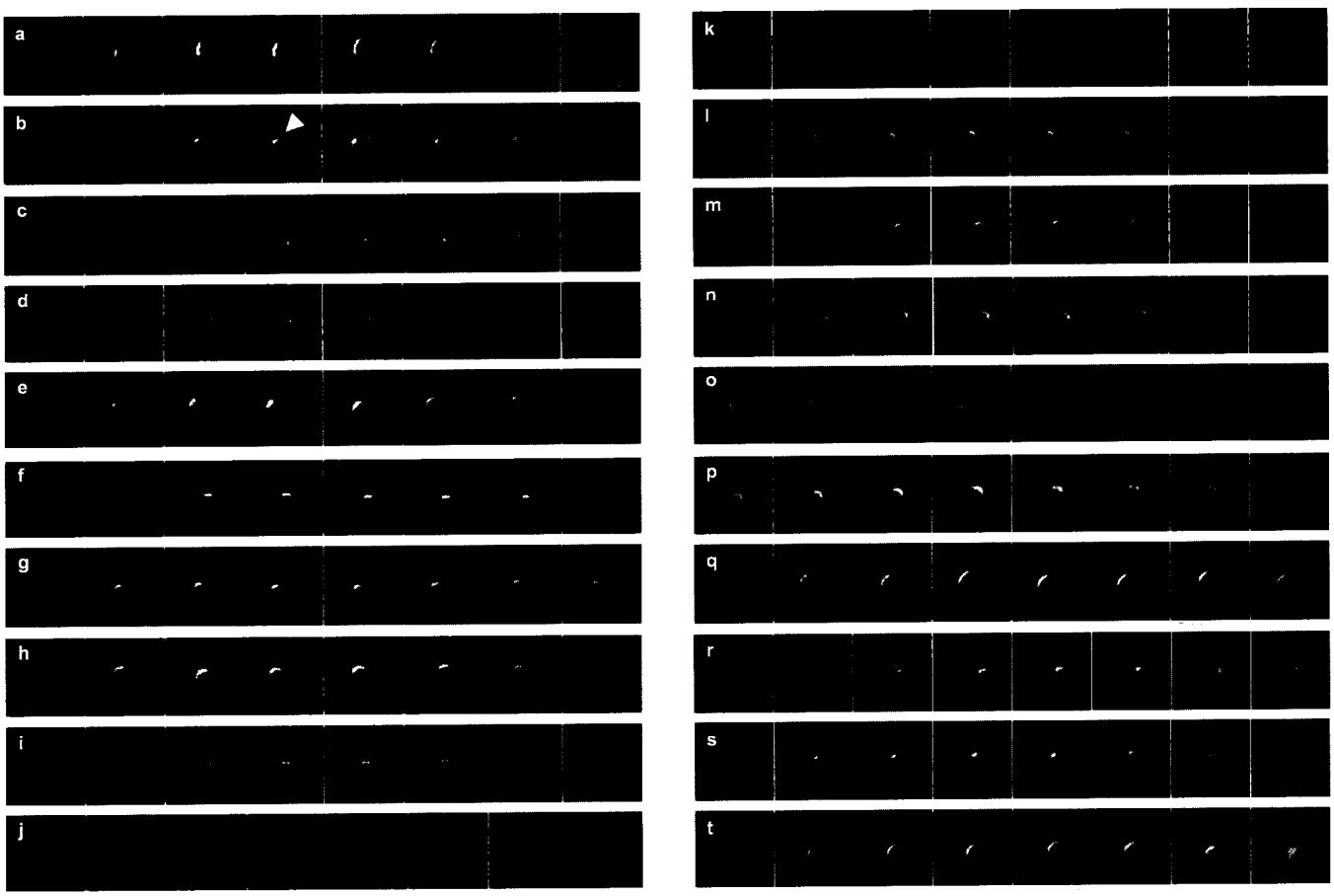

Fig. 1. Presence of chitin ring and septum in MIRC3-4 mutants.
Fourteen mutants in MIRC3-4 were stained with Calcofluor white and analyzed by serial dissection with a confocal microscope. a, ECY36-3A (*CHS2* and *CHS3*); b, ECY36-3C (*chs2* and *CHS3*); c, ECY36-3D (*CHS2* and *chs3*); d, HPY3 (*chs3::TRP1*); e, HPY3[pHP29] (multi-copy *CHS3*); f, HPY3[pCMN] (internal control); g-t, MIRC3-4 mutants, HPY3[pM1-14]. The chitin ring appears as a crescent in a, b, e-h, p, q, t, while the septum appears as a dot in the neck in c, d, i-o, r, s.

β-mercaptoethanol, at 37°C for 30–60 min. Spheroplasts were washed, and additional methanol/acetone fixation was carried out. Cells were settled onto poly-L-lysine-coated slides. For detection of Chs3p, HA antibody (provided by Dr. K. Lee, Ewha Womans Univ., Seoul, Korea) was used at a 1:20 or 1:50 dilution for 2 h at 25°C. Rhodamine conjugated goat anti-mouse IgG (Molecular Probes Inc. Eugene, OR, U.S.A.) was used at a 1:1,000 dilution for 60 min at 25°C. Samples were examined at 545 nm for rhodamine and at 365 nm for DAPI staining with an LSM 510 confocal microscope (Carl Zeiss, Zottinzen, Germany).

RESULTS

MIRC3-4 is Required for the Biogenesis of the Chitin Ring, but not for Cell Wall Chitin Synthesis

Recently, the region of Chs3p interacting with Chs4p was identified by two-hybrid analysis and 14 mutants in MIRC3-4 were generated by PCR-directed mutagenesis (Park *et al.*, unpublished data). Since it is known that *CHS3* functions in the chitin ring formation in the G1 phase and cell wall chitin synthesis after septation, chitin distribution in all 14 mutants was examined by Calcofluor staining. Chitin is present in the chitin ring that surrounds the neck and in the septum that crosses the neck. To distinguish such subcellular localizations of chitin, confocal microscopic sectioning of the neck region from the surface to the equatorial layer of the cell was performed. Since the chitin primary septum is formed at cytokinesis in a very short period of time (10 min; personal communication from Dr. E. Cabib, U.S.A.), it should be observed only in big-budded cells but not in small- or medium-budded cells. When several control strains were examined, the combination of septum and chitin ring was represented as a crescent shape with fluorescence intensity diminishing from the surface in ECY36-3A, which contains Chs2p and Chs3p (Fig. 1a). In ECY 36-3C (*chs2*), the chitin ring was seen only in the mother side of the neck as a crescent (Fig. 1b), and there seemed to be some gap between mother and daughter cells, probably due to the lack of a septum (arrow in Fig. 1b). In ECY 36-3D (*chs3*), the septum appeared as a spot on the middle of the neck (Fig. 1c). Septum spots suspected to be artifacts were occasionally observed even in small- or medium-budded cells (data not shown). The staining pattern of HPY3 (*chs3::TRP1*) was the same as that of ECY36-3D (Fig. 1d), whereas HPY3 that was transformed with wild-type *CHS3* (HPY3[pHP29]) restored the chitin ring shape shown in Fig. 1e). Fourteen mutants in MIRC3-4 were divided into two groups, depending on the presence of a chitin ring (Figs. 1g-t): one group with a chitin ring (Figs. 1g, 1h, 1p, 1q, and 1t for pM1, 2, 10, 11, and pM14, respectively), and the other without a chitin ring (Figs. 1i-1l, 1r, and 1s for pM3-9, pM12, and pM13, respectively).

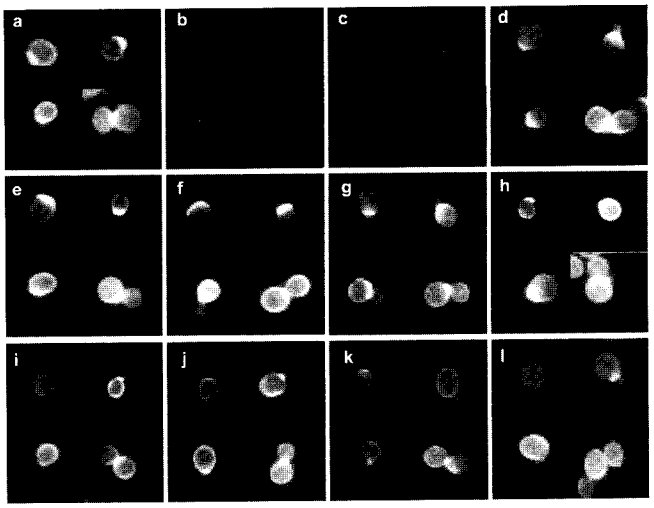

Fig. 2. Presence of cell wall chitin in several MIRC3-4 mutants. Seven mutants with or without chitin ring were stained with Calcofluor to visualize cell wall chitin. Depending on the cell cycle stage, each mutant was categorized into single cell (after cytokinesis), small-budded (G1), medium-budded (S and G2), and large-budded (M and cytokinesis) states. Differential staining between mother and daughter cells is clear in small-budded and medium-budded cells, while equal staining is apparent in large-budded cells. a, ECY36-3A (*CHS2* and *CHS3*); b, HPY3 (*chs3::TRP1*); c, CR4-2 (*chs4*); d, HPY3[pHP29] (multi-copy *CHS3*); e, HPY3[pCMN] (internal control); f, HPY3[pM2]; g, HPY3[pM11]; h, HPY3[pM14]; i, HPY3[pM3]; j, HPY3[pM5]; k, HPY3[pM8]; l, HPY3[pM12]. f, g, h; with chitin ring. i, j, k, l; without chitin ring.

As mentioned earlier, Chs3p is also involved in cell wall chitin synthesis after cytokinesis through its regulator, Chs4p. Cell wall chitin is present in the mother cells but appears in the daughter cells only after septum formation. It was curious to find the presence of cell wall chitin in MIRC3-4 mutants. Surprisingly, cell wall chitin was observed in a stage-specific manner in all 14 mutants, regardless of the presence of a chitin ring, similar to a wild-type. Figure 2 shows the results from 7 selected mutants.

Taken together, the above results show that several core amino acids in MIRC3-4, including pM3-9 and pM12-13, were involved only in chitin ring synthesis, but not in cell wall chitin synthesis. This result is in good agreement with the previous result that mutations in MIRC3-4 caused the decrease in Chs3p activity and chitin content of the cell and resulted in the lack of a chitin ring. This result also provides an explanation of why 14 mutants were sensitive to Calcofluor. The cell wall chitin synthesis at cytokinesis was not affected by the mutations in MIRC3-4, in contrast to the effect on the chitin ring in the G1 phase. All the mutants were sensitive to Calcofluor due to the presence of cell wall chitin, which is the target of Calcofluor.

Chs4p as an Activator for Chitin Ring Biogenesis

In previous reports [6, 14], Chs4p was proposed to function in localizing Chs3p to the bud base and in

activating the CSIII complex. Absence of a chitin ring due to MIRC3-4 mutations in Chs3p can arise either from loss of proper localization capability or from lack of enzymatic activity caused by aberrant interaction with a regulator(s), despite proper localization. As shown in Fig. 3, these two alternatives were distinguished by immunostaining of Chs3p in mutant cells. Chs3p is known to undergo dynamic localization during the cell cycle; Chs3p is present in the incipient bud site of unbudded cells and in the neck of small or big budded cells [10]. When mutated pHV7-HA(M2, 3, 5, 7, 11, 14) was immunostained and compared with wild-type Chs3p, the Chs3p of all mutants examined was correctly localized in the mother-bud neck of small or big budded cells, even in the absence of chitin ring synthesis in the G1 phase. Yeast two-hybrid analyses for the interaction between Chs3p and Chs4p provide legitimacy for the co-localization of Chs3p and Chs4p to the bud base [6]. These results indicate that Chs4p functions as an activator for the CSIII complex.

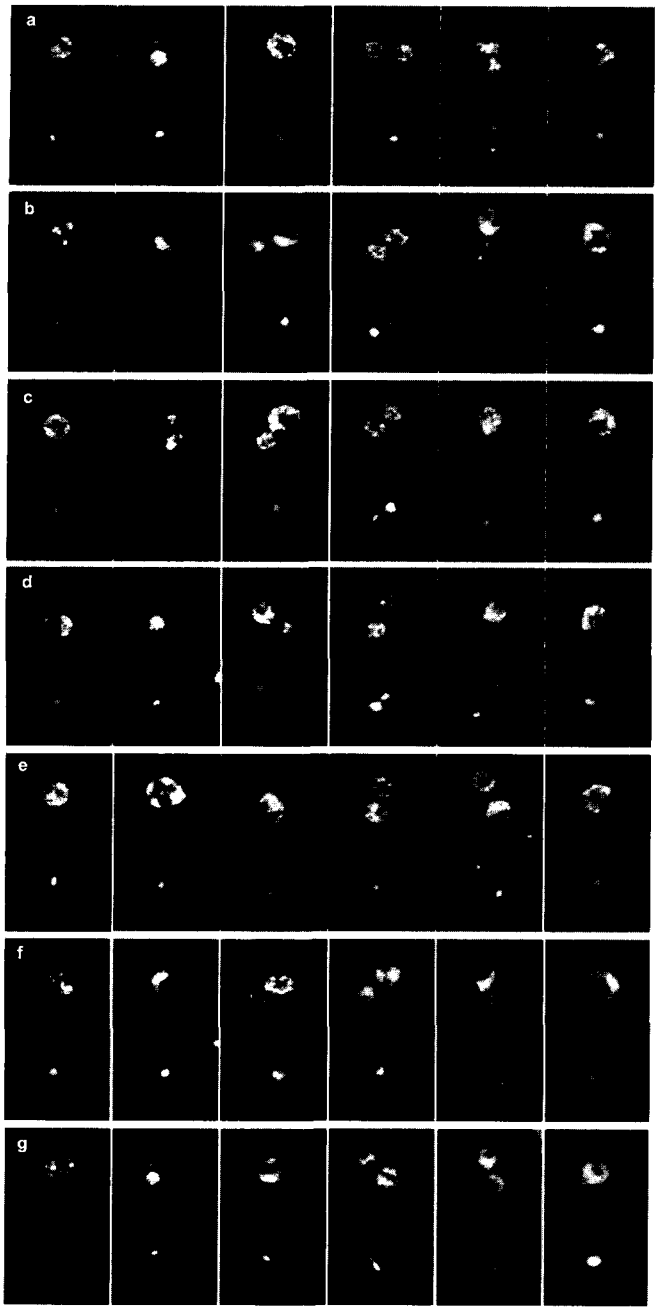

Fig. 3. Localization of Chs3p.
Wild-type and MIRC3-4 mutants expressing Chs3p-HA fusion protein were hybridized *in situ* with HA-specific antibody. Each cell was categorized into single cell (after cytokinesis), small-budded (G1), medium-budded (S and G2), and large-budded (M and cytokinesis) depending on the stage of cell cycle. The DNA status was confirmed by DAPI staining. a, HPY3 [pHV7-HA(CHS3)]; b, HPY3 [pHV7-HA(M2)]; c, HPY3 [pHV7-HA(M3)]; d, HPY3 [pHV7-HA(M5)]; e, HPY3 [pHV7-HA(M7)]; f, HPY3 [pHV7-HA(M11)]; g, HPY3 [pHV7-HA(M14)].

The next issue was whether Chs4p is necessary for the biogenesis of cell wall chitin. As shown in Fig. 2c, Calcofluor staining of mutated Chs4p exhibited neither a chitin ring nor cell wall chitin, indicating that Chs4p is necessary for both cell wall chitin synthesis and chitin ring formation. Taking this result together with the above findings, it was concluded that overall chitin synthesis requires Chs4p, however, Chs4p has different roles at the two locations, i.e., the bud base and cell wall.

DISCUSSION

Functional Analysis of MIRC3-4

The effect of MIRC3-4 on the functions of Chs3p was examined. Together with the results from a previous study (Park *et al.*, unpublished data), it was concluded that MIRC3-4 is involved in the biogenesis of the chitin ring in the G1 phase through interaction with Chs4p, but not in the synthesis of cell wall chitin at cytokinesis. Considering that the *chs4* mutant is completely devoid of a chitin ring and cell wall chitin (Fig. 2c), the mechanism by which Chs3p is regulated by Chs4p might be different between G1 phase and cytokinesis.

A Domain for Chs3p Localization

The polarization and dispersion of Chs3p during the cell cycle [ref. 10 and Fig. 3 of this study] imply that localization of Chs3p at proper times may be crucial for temporal syntheses of the chitin ring and cell wall chitin. Accordingly, it is highly probable that molecules exist that localize Chs3p to the bud neck where chitin ring synthesis occurs and to the daughter cell wall. Since it is shown that Chs4p is responsible for localization of Chs3p to the bud neck [6], Chs4p is a primary candidate that directs Chs3p to the bud neck. However, localization of Chs3p to the bud neck and the cell wall is not dependent on the presence of MIRC3-4, as shown in Fig. 3. There seems to be three different functional domains in Chs3p: a catalytic domain (CD) that

performs chitin synthesis, a regulatory domain (RD), i.e., MIRC3-4, responsible only for the regulation of chitin ring synthesis, and an unidentified localizing domain (LD) that directs proper localization. For these three domains to function normally, Chs4p is indispensable, meaning that Chs4p is a multifunctional, not simply bifunctional, protein. Of these, MIRC3-4 is the only domain that could be identified by yeast two-hybrid analyses. The reason for the failure of identification of CD and LD by yeast two-hybrid analyses may be either that they have conformational domains or that additional molecules are necessary to link them to Chs4p. The latter possibility is more likely, especially in the case of LD, because the exploiting of 'mediators' is more effective for spatiotemporal localization of a protein than changing the conformation in a different cell cycle. It would be interesting to identify such mediators for Chs3p and Chs4p.

Recent molecular analysis of Chs3p revealed two C-terminal domains responsible for *in vivo* function and chitin synthase 3 activity: one (QRRRW, amino acid residues 992-996) for catalysis and the other (amino acid residues 1109-1163) for hypothetical Chs3p-specific regulation [5]. These two domains are apart from MIRC3-4, which suggests that at least three distinct domains participate in the regulation of chitin synthase 3 activity. If residues of QRRRW do not contribute to LD, there would be four domains in Chs3p for cellular chitin synthesis.

Analyses of MIRC3-4

One of the conclusions to be drawn from this study is that MIRC3-4 is a domain of Chs3p that is specifically required for the activation of CSIII by Chs4p for chitin ring synthesis. All mutations in MIRC3-4 affected chitin synthase 3 activity, indicating that MIRC3-4 is a genuine functional domain through which chitin synthase 3 activity is regulated (Park *et al.*, unpublished data). In general, severely reduced chitin synthase 3 activity resulted in the absence of a chitin ring and low chitin content, as expected from the previous finding that 80% of cellular chitin is derived from the chitin ring [7]. There are some exceptions, such as in the case of D673N, where there was over 50% activity but low chitin content and an absence of a chitin ring, whereas in contrast, L651E and G699S had one third of wild-type activity but a rather high chitin content and the presence of a chitin ring. Among mutants with the same degree of chitin synthase 3 activity (approximately 30 %), the presence of a chitin ring was not correlated, as in the cases of pM1 (L651E), pM6 (G667S), pM12 (D692N), and pM14 (G699S); pM6 and pM12 lacked a chitin ring, whereas pM1 and pM14 did not. Thus, a routine guideline cannot be given as to how much chitin synthase 3 activity is sufficient for the synthesis of detectable amounts of chitin ring and cell wall chitin. It is suspected that the extent to which a specific residue affects chitin synthase 3 activity and chitin ring synthesis might not be necessarily interdependent. A rather complicated regulation of chitin synthase 3 activity, as discussed above, suggests that such effects of particular residues may be attributed to three-dimensional conformation of Chs3p that occurs for optimal chitin synthase 3 activity and chitin ring synthesis.

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