Prenylation of Saccharomyces cerevisiae Chs4p Affects Chitin Synthase III Activity and Chitin Chain Length

Kariona A. Grabin’ska,* Paula Magnelli, and Phillips W. Robbins

Department of Molecular and Cell Biology, School of Dental Medicine, Boston University,
715 Albany Street, Evans 408, Boston, Massachusetts 02118

Received 27 June 2006/Accepted 2 November 2006

Chs4p (Cal2/Csd4/Skt5) was identified as a protein factor physically interacting with Chs3p, the catalytic subunit of chitin synthase III (CSIII), and is indispensable for its enzymatic activity in vivo. Chs4p contains a putative farnesyl attachment site at the C-terminal end (CVIM motif) conserved in Chs4p of Saccharomyces cerevisiae and other fungi. Several previous reports questioned the role of Chs4p prenylation in chitin biosynthesis. In this study we reinvestigated the function of Chs4p prenylation. We provide evidence that Chs4p is farnesylated by showing that purified Chs4p is recognized by anti-farnesyl antibody and is a substrate for farnesyl transferase (FTase) in vitro and that inactivation of FTase increases the amount of unmodified Chs4p in yeast cells. We demonstrate that abolition of Chs4p prenylation causes a ~60% decrease in CSIII activity, which is correlated with a ~30% decrease in chitin content and with increased resistance to the chitin binding compound calcofluor white. Furthermore, we show that lack of Chs4p prenylation decreases the average chain length of the chitin polymer. Prenylation of Chs4p, however, is not a factor that mediates plasma membrane association of the protein. Our results provide evidence that the prenyl moiety attached to Chs4p is a factor modulating the activity of CSIII both in vivo and in vitro.

Chitin, a linear N-acetylglucosamine (GlcNAc) polymer, is a minor but essential structural component of the yeast cell wall, the organelle responsible for the maintenance of cell shape and osmotic stability (5, 23). The majority (90%) of chitin in the cell wall, including chitin in bud scars and in the lateral wall and the polymer converted to chitosan in spore walls, is synthesized by chitin synthase III (CSIII), encoded by CHS3 gene (35). A number of proteins that are necessary for the proper activity of Chs3p have been identified. Chs7p is required for export of Chs3p from the endoplasmic reticulum (ER), whereas Chs5p and Chs6p are involved in the proper delivery of Chs3p to the plasma membrane (35).

Chs4p (Cal2/Csd4/Skt5) is a regulatory subunit of the CSIII complex, indispensable for its enzymatic activity in vivo in vegetative cells (5, 32, 35, 37, 40). It has been demonstrated that Chs4p interacts directly with Chs3p and is responsible for the localization of Chs3p to the septin ring thorough interaction with the scaffolding protein Bni4p (11). Chs4p contains a possible farnesyl attachment site at the C-terminal end (CaaX motif), which is conserved among Saccharomyces cerevisiae Chs4p and other fungi, including the human pathogens Candida albicans (39) and Cryptococcus neoformans (1). The possibility that Chs4p is prenylated is enhanced by the fact that the CVIM motif is preceded by a lysine-rich amino acid stretch (29). Thus, Chs4p is predicted to be prenylated by the Prenylation Prediction Suite (http://mendel.imp.ac.at/sat/PrePS/). However, the role of prenylation of Chs4p in chitin biosynthesis, or even the occurrence of this modification, has been questioned in several reports (5, 11, 32, 39, 40).

In yeast, 35 proteins, including many important for cell growth, differentiation, morphology, and stress response, require posttranslational modification by covalent attachment of an isoprenoid lipid (prenylation) for proper function (Proteome Bioknowledge Library [http://www.incyte.com]). Prenylated proteins are posttranslationally modified by the formation of cysteine thioethers with the isoprenoid lipid farnesol (C-15) or geranylgeraniol (C-20) at or near the carboxyl terminus. Prenylation is specified by the amino acid sequence motifs CaaX, CC, and CaC at the carboxyl end of the protein, where “a” is an aliphatic amino acid and “X” is any amino acid. The CaaX sequence is a substrate for farnesyl transferase (FTase) (for the known biological substrates, X is S, M, A, or Q) unless X is L, which results in a substrate for geranylgeranyl transferase I (GGTase I). The CC and CaC motifs, present in the Rab family of low-molecular-mass G proteins, are digeranylgeranylated. Typically, prenylation by CaaX protein prenyltransferases is accompanied by further posttranslational processing, most often involving cleavage of the carboxy-terminal tripeptide (-aaX) followed by carboxymethylation of the carboxy terminus (9, 38).

Like other lipid modifications, prenylation has been viewed as a mechanism for posttranslational attachment of proteins to membranes. However, it now appears that lipid modification by protein prenyltransferases has a more complex role: for example, the farnesyl and geranylgeranyl moieties are directly involved in protein-protein interactions as well as in protein-membrane interactions (28, 38).

Since clinical studies in progress are exploring the antitumor activity of FTase inhibitors as potential therapeutic agents (3), prenylation attracts the attention of many laboratories. In order to decrease the costs associated with de novo drug design and accelerate the development of new chemotherapeutics, FTase inhibitors are currently being investigated as agents for...
protozoan pathogens (13). Since deletion of the FTase catalytic subunit (RAM1) is lethal in the pathogenic fungus Cryptococcus neoformans, in contrast to the case in Saccharomyces cerevisiae (44), FTase inhibitors may be suitable as antifungal drugs.

In this study we have reinvestigated the function of Chs4p farnesylation and shown that prenylation of Chs4p does not affect membrane anchoring of Chs4p; however, it does affect the catalytic properties of CSIII.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Strains and plasmids used in this study are listed in Table 1, and oligonucleotide primers are listed in Table 2. To generate the mutation leading to the abolition of Chs4p farnesylation (a cysteine 693 to serine substitution) directly at the chromosomal locus, we adapted the cysteine 693 to serine substitution) directly at the chromosomal locus, we used the marker gene was removed by expression of recombinase Cre.

In order to construct the trp1 auxotrophic KG101B yeast strain, we decided to delete only the first 312 nucleotides of the TRP1 open reading frame to leave intact the putative YDR080C open reading frame located on the complementing strand of DNA. Deletion was accomplished by the method described by Gueldener et al. (16). The deletion cassette was amplified with primers DF-TRP1 and DR-TRP1 from the pUG27 plasmid used as a template and then transformed into BY4741 yeast cells. Transformants able to grow on medium lacking histidine and requiring tryptophan were isolated, and correct insertion of the cassette was verified by PCR with the TRP1UP and KanB primers. Finally, the marker gene was removed by expression of recombinase Cre.

In order to construct a yeast strain expressing TAP-Chs4p (33, 34), KG101B cells were transformed with a PCR cassette amplified with plasmid pBS1761 as a template and primers TAP1-CHS4 and TAP2-CHS4. Transformants able to grow on medium lacking tryptophan were isolated, and correct insertion of the cassette was verified by PCR with primers CHS4-C693S and F1-CBP. The marker gene TRP1 was removed by expression of recombinase Cre.

TAPCHS4B5 and TAPCHS4B6 were obtained by mating TAPCHS4B3 with KG101B strain. Sporulation of the diploid cells and tetrad dissection was done by standard yeast genetic methods. Yeast cells were cultured in 2% (wt/vol) Bacto peptone and 1% (wt/vol) yeast extract glucose supplemented with auxotrophic requirements. For solid media, agar (Difco) was added to YPD or SD at a 2% (wt/vol) final concentration. Bacterial cells carrying plasmid pET30a were grown in LB medium (1% [wt/vol] Bacto tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl) supplemented with kanamycin (25 mg/liter) and chloramphenicol (34 mg/liter).
Heterologous expression and purification of Chs4p from Escherichia coli.

Wild-type (wt) CHS4 and CHS4 alleles mutated at the prenylation box (C09/3S) were amplified by PCR using yeast genomic DNA as a template, CHS4BamHF and CHS4

| Primer/Clone | Sequence |
|-------------|----------|
| TAP1-CHS4 | ACCAGTTCGCTCCTTTTGGTTGATAGGTAAGTTAAAAAAAGGAT1AAAAGAACAAAAGGTTGGCATC |
| TAP2-CHS4 | TGATGACCTGACGCTTGTAATGAGTCATAGGGTTGCTTTTTTGGTGATAA |
| CHS4-290R | ACAAGAAGGTAAAAAGACAGTGTAATTATGTAACAG |
| CHS4xwt | AAAGACTGTTAATGTGAAACAGTCAGTTGAGGACAG |
| DF-TRP1 | TATTGAGCACGTGAGTATACGTGATTAA |
| CHS4CtoS | CHS4wt |
| CHS4-1920 | CHS4wt |
| TRP1UP | TRP1UP |
| CHS4-BamHI | CHS4-BamHI |
| CHS4-1920 | CHS4-1920 |
| TRP1UP | TRP1UP |

Chitin content measurement.

Chitin content was measured by an assay adapted for microtiter plates as described previously (6) with minor modifications. Chitin polymer was digested with chitinase c (Interspect) in McIlvaine's buffer (pH 6.0) and were then centrifuged at 100,000 × g for 90 min at 4°C. The clear cell lysate was mixed with an equal volume of 200 mM sodium carbonate or HOCl containing 1 M NaCl, 2% Triton X-100, or 2% SDS. After incubation for 1 h on ice, samples were centrifuged at 200,000 × g for 1 h to separate soluble and particulate fractions. The pellet was then resuspended in HOCl in the same volume as the corresponding soluble fraction; 5 μl of each fraction was subjected to protein electrophoresis and immunoblotting.

CSIII activity.

CSIII activity was measured by the colorimetric assay adapted for microtiter plates as described previously (25). The enzyme source was prepared as follows. Cells were harvested by centrifugation, washed once with water, then resuspended in 20 mM Tris-HCl (pH 8.0) buffer containing 2 mM PMSE and protease inhibitor cocktail (Sigma) and broken by agitation with glass beads. Lysates were cleared by 5 min of centrifugation at 50,000 × g for 30 min at 4°C. The supernatants were mixed with an equal volume of 200 mM sodium carbonate or HOCl containing 1 M NaCl, 2% Triton X-100, or 2% SDS. After incubation for 1 h on ice, samples were centrifuged at 200,000 × g for 1 h to separate soluble and particulate fractions. The pellet was then resuspended in HOCl in the same volume as the corresponding soluble fraction; 5 μl of each fraction was subjected to protein electrophoresis and immunoblotting.

Degree of polymerization of chitin.

Chain lengths were estimated according to the method of Kang et al. (22). To obtain pure chitin, β-1,3-glucan and mannan
hexosaminidase activity, digests chitin to free N\textsubscript{\textalpha}-(Sigma) for 16 h at 37°C. This crude endochitinase preparation, containing GlcNAc under the conditions described above. The degree of polymerization was calculated as micromoles of GlcNAc/micromoles of total alditols.

To determine the amount of terminal residues, the supernatant were removed by digesting isolated cell walls with 2 mg/ml of Zymolyase X100 (Seikagaku). In order to remove the remaining \beta-L-glucan attached to chitin chains, the washed pellet was later digested with 4 units/ml of \beta-L-endo-glucanase (27). The chitin pellet was reduced in 100 \mu l of 0.1 M NaOH with 500 \mu Ci of Na\textsubscript{\textbeta}H\textsubscript{\textalpha} (100 mCi/mmol) (NEN) for 6 h at 25°C. The reaction was stopped with 200 \mu l of 0.5 M acetic acid. After extensive washing, the reduced chitin was digested in 0.2 ml of 0.1 M KPO\textsubscript{4} (pH 6.0) with 0.015 units of P4 chromatography after reducing GlcNAc under the conditions described above. The degree of polymerization was calculated as micromoles of GlcNAc/micromoles of total alditois.

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RESULTS

Chs4p is prenylated. Although Chs4p contains a possible farnesyl attachment site at the C-terminal end (the CVIM motif) which is conserved between Chs4p of S. cerevisiae and a number of other fungi, the role of farnesylation of Chs4p in chitin biosynthesis has been questioned by several reports (5, 11, 32, 39, 40). Since previously wt or mutated Chs4p was expressed from a plasmid, we suspected that the effect of the mutation might have been masked by variations in the protein expression level; therefore, in this study we used only yeast strains expressing Chs4p from the genome. The lack of the phenotype in previous studies may also have been due to the fact that in some cases the chitin level was measured by CFW or wheat germ agglutinin coupled with a fluorescein isothiocyanate binding assay (11, 40). These methods may not be accurate enough to observe small differences in chitin levels. We confirmed that when purified from a bacterial source, Chs4p is a substrate for yeast farnesyltransferase in vitro (Fig. 1A). To reinvestigate whether Chs4p is prenylated in vivo, we constructed yeast strains expressing Chs4p with an N-terminal TAPtag (ProtA-protease TEV cleavage site-CBP) mutated at the C-terminal CVIM farnesylation motif (C693S substitution) (33). Affinity purification on IgG-Sepharose from yeast extracts gave TAP-Chs4p and TAP-Chs4p\textsubscript{C693S} with the same efficiency (Fig.1Bi). CBP-tagged forms of the proteins were then released into solution from the ProtA bound to the IgG-Sepharose by treatment with protease TEV. Antibodies raised against N-acetyl-S-farnesy-l-cysteine, which recognize farnesyl or, with less specificity, geranylgeranylated-modified proteins (2, 24), were able to recognize wt Chs4p but not the mutated protein (Fig.1Bi).

The presence of wt and mutated CBP-Chs4p in the eluate was confirmed by mass spectrometry analysis of the purified proteins (data not shown). This result confirmed that Chs4p is prenylated in vivo.

Since prenylation is an irreversible process, we assume that if Chs4p is preferentially farnesylated, inactivation of endogenous FTase will increase the amount of unmodified Chs4p available as a substrate for reaction in vitro, even if cross-specificity between FTase and GGTase I are observed (29, 31, 41). To prove the involvement of FTase in modification of Chs4p, we immobilized TAP-Chs4p expressed in wt yeast cells or cells lacking the catalytic subunit of FTase (Ram1p) on IgG-Sepharose beads and used as a substrate for the FTase assay in vitro. To measure background, the resin carrying the TAP-Chs4p C693S mutant was used.

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FIG. 1. Chs4p is prenylated. (A) Chs4p is a substrate for farnesyl transferase in vitro. Chs4p, wt and mutated at the CaaX prenylation box and purified from E. coli, was used as an acceptor for the H\textsubscript{\textalpha}-labeled farnesyl group in an in vitro reaction with FTase. The proteins, after the reaction, were separated by bis-Tris SDS-NuPAGE and blotted onto a PVDF membrane, and the position of prenylated protein was visualized by autoradiography. (B) Chs4p is prenylated in vivo. Chs4p, wt (left lane) or mutated at the farnesylation site (right lane) and tagged with TAP (ProtA-TEV-CBP) epitope on the N terminus, was purified from yeast cells and immobilized on IgG-Sepharose beads. Then, CBP-Chs4p (~82 kDa) was removed from TAP-Chs4p (~97 kDa) by cleavage with protease TEV. IgG-Sepharose bead suspension samples (before [i] and after TEV cleavage) and a final eluate containing purified wt or mutated CBP-Chs4p were analyzed by bis-Tris SDS-NuPAGE and immunoblotting. TAP-tagged proteins were recognized with PAP antibodies (i) and prenylated CBP-Chs4p protein was recognized with anti-farnesyl antibodies (ii). (C) Inactivation of FTase increases the level of unmodified Chs4p in cells. TAP-Chs4p expressed in wt yeast cells or cells lacking the catalytic subunit of FTase (Ram1p) was immobilized on IgG-Sepharose beads and used as a substrate for the FTase assay in vitro. To measure background, the resin carrying the TAP-Chs4p C693S mutant was used.
Prenylation of Chs4p is not essential for its plasma membrane localization or membrane association. All known prenylated proteins are found, at least to some extent, bound to cellular membranes, and prenylation has often been viewed as a mechanism for posttranslational attachment of proteins to membranes (38). Chs4p is also known to be a membrane protein, and so we determined whether farnesylation of Chs4p influences its membrane association or plasma membrane localization.

To test membrane association of Chs4p, protein extracts from cells expressing TAP-Chs4p and TAP-Chs4p C693S were fractionated into supernatant (soluble) and pellet (membrane-associated) portions by centrifugation. TAP-Chs4p and TAP-Chs4pC693S were found in the pellet fraction (Fig. 2A). To test whether lack of prenylation changes the membrane association of Chs4p, protein extracts were treated with sodium chloride, sodium carbonate, or SDS to disrupt integral membrane association. TAP-Chs4p and TAP-Chs4pC693S were solubilized to similar extents by treatment with sodium chloride, sodium carbonate, or SDS, but Triton X-100 (a widely used nonionic surfactant for recovery of membrane components under mild nondenaturing conditions) had no effect in either case, indicating that the membrane association of Chs4p is independent of farnesylation.

In order to determine whether prenylation affects Chs4p subcellular distribution, the cells were converted to spheroplasts and lysed by osmotic shock, and membranes were separated by differential centrifugation at 100,000 × g for 10 min to obtain a Golgi/endosome-rich fraction (S) and a plasma membrane (PM)/ER-rich fraction (P). As can be seen, prenylation did not change the distribution of Chs4p between the S and P fractions. Chs4p populated both fractions. However, in contrast to the catalytic subunit of CSIII (Chs3p), it is present to a greater extent in the Golgi/endosome-rich fraction (Fig. 2B).

We also did not observe a difference in sedimentation of wt and mutated Chs4p on a step sucrose/EDTA density gradient (data not shown). These results confirm that abolition of Chs4p prenylation does not prevent trafficking to the plasma membrane.

Mutation of the farnesylation site confers resistance to CFW. Since CFW is a fluorescent dye that intercalates with nascent chitin chains, preventing microfibril assembly and inhibiting growth of yeast strains, sensitivity to CFW is often an indicator of changes in cellular chitin levels (14, 36). Expecting that prenylation of Chs4p could influence chitin biosynthesis, we compared the growth rates of chs4 mutants and the corresponding wt yeast strain on medium supplemented with CFW. The results in Fig. 3 show that chs4-C693S yeast cells harboring the nonprenylated version of Chs4p are more resistant to CFW than are wt cells and less resistant than are chs4 yeast cells.

To confirm the role of prenylation of Chs4p, we also constructed a chs4-I695L,M696L yeast strain expressing Chs4p with the C-terminal CVLL motif (the CVLL motif present in yeast Rho1p was proven experimentally to be a substrate for geranylgeranyl transferase type I [31]). Geranylgeranylation of Chs4p only partially restores the sensitivity to CFW.

Farnesylation of Chs4p affects CSIII activity in vitro and chitin content. Next, we examined the effects of mutagenesis of the Chs4p farnesylation site on the cellular chitin content and CSIII activity under different growth conditions. As shown in Table 3, abolishing farnesylation causes approximately a 30 to 40% decrease in chitin content under various conditions of growth (CFW or glucosamine supplementation) or in various
genetic backgrounds, including those that induce the cell wall stress response (deletion of the HSK1 or GAS1 gene [15, 43]). However, the rates of increase in chitin levels produced by CFW addition in wt and mutated yeast cells were very similar. In both cases, maximal levels of chitin were achieved after 4 to 6 h of exposure to CFW and half of this value after 2 h (data not shown). Since the kinetics of response to CFW treatment are similar in yeast strains bearing a wt or mutated CHS4 gene, we can assume that prenylation of Chs4p does not contribute directly to the mechanism of the cell wall stress response.

Changes reported here in the cellular chitin content are well correlated with the observed 60% decrease in CSIII specific activity (Fig. 4A). Restoration of prenylation by introducing the CVLL geranylgeranylation motif is able only to partially restore the wt phenotype. Treatment of the enzyme source with trypsin is able to increase CSIII in chs4Δ mutants to the level measured in the parental strain (Fig. 3B), which indicates that proteolytic treatment activates CSIII in a way that is independent of Chs4p function.

Chs4p prenylation affects chitin chain length. We have noticed that cells carrying nonprenylated Chs4p are much more resistant to CFW (Fig. 3) than expected from a 30% reduction in chitin content (Table 3). CFW binds to the insoluble chitin microfibrils in the cell wall. If Chs4p somehow determines chitin chain length, its partial loss of function will affect not only total chitin content but also the structure of the microfibrils. To confirm our supposition, we measured the average chitin chain length. For chitin isolated from wt cells, the degree of polymerization was 60 units, while from chs4Δ, it was 45 (Table 4). Mutations (fps1Δ and gas1Δ) inducing cell wall stress responses lead to a two- to fourfold increase in length. Nevertheless, the effect of chs4Δ-C693S on chitin structure is observed in these genetic backgrounds as well. We also observed that a CFW-induced cell wall stress response leads to a fourfold increase in chitin length. However, treatment with glucosamine, which activates chitin synthesis without induction of the cell wall integrity pathway (7), has only a minor effect on the length of the polymer.

**DISCUSSION**

Previous studies suggested two roles for Chs4p in chitin synthesis. One of its proposed functions is activation of Chs3p catalytic activity (5, 11, 32, 40). Two-hybrid analysis indicates that this process depends on direct interaction between the catalytic subunit, Chs3p, and Chs4p (5, 11, 32). DeMarini and coworkers (11) revealed the second role of Chs4p, anchoring Chs3p to the septum via Bin4p, which confers septum localization to CSIII. Two facts indicate that the two roles are separable: (i) delocalized chitin is present in bni4Δ but not in a bni4Δ chs4Δ double mutant, and (ii) it is known that a truncated version of Chs4p allows chitin synthesis but does not localize Chs3p to the septum (11). Whether Chs4p has a function other than stimulation of CSIII activity at the lateral wall (for example, recruiting Chs3p to the specific scaffolding proteins) remains to be established.

In previous reports, no phenotype related to chitin synthesis was attributed to loss of the potential prenylation site (CVIM) in Chs4p (5, 11, 32, 39, 40). Two reasons led us to reinvestigate the role of Chs4p farnesylation. First, a prenylation motif is present in a number of Chs4p homologues, and second, we realized that C-terminal tagging of the CHS4 gene in the genomic locus affects the chitin content (data not shown). We demonstrate here for the first time that the intact prenylation motif of Chs4p is indispensable for full activity of CSIII. Lack of the farnesylation consensus sequence causes an approximately 60% decrease in CSIII activity, which leads to a substantial lowering in chitin content and partial resistance to CFW. Reduction in CSIII activity is also correlated with an approximately 20% decrease in average chitin chain length.

We used several approaches to prove that Chs4p is prenylated. We have shown that unprenylated recombinant Chs4p is a substrate for FTase in vitro. The protein isolated from yeast cells reacts with serum raised against N-acetyl-S-farnesyl-

![Table 3. Chitin content](image-url)

**TABLE 3. Chitin content**

| CHS4 allele | YPD | YPD + GlcNH2 | YPD + CFW | fps1Δ | gas1Δ |
|-------------|-----|--------------|----------|-------|-------|
| wt          | 5.47 ± 0.23 | 12.99 ± 0.42 | 22.23 ± 3.11 | 18.37 ± 1.37 | 17.03 ± 1.01 |
| chs4-C693S  | 3.39 ± 0.28 | 6.79 ± 0.40 | 12.64 ± 2.14 | 11.33 ± 1.31 | 13.7 ± 1.24 |
| chs4-I695L  | 4.19 ± 0.40 | 8.9 ± 0.8 | 17.47 ± 1.77 | |
| M696L       | |
| chs4Δ       | 1.55 ± 0.15 | |

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FIG. 3. Mutation in the CVIM motif of Chs4p leads to resistance to CFW. Substitution C693S blocks farnesylation of Chs4p. Substitution M696L I695L changes the CVIM farnesylation sequence to the CVLL geranylgeranylation (present in Rho1p) motif. Cells grown overnight in liquid YPD medium were diluted to a final concentration of 0.8 OD units per 1 ml in water, and then 3 µl of each suspension and three subsequent 10-fold serial dilutions were each spotted onto YPD, YPD plus 50 µg/ml CFW, or YPD plus 200 µg/ml CFW plates. Cells were incubated at 30°C for 3 days.

![Image of graph showing data](image-url)
L-cysteine, which recognizes farnesyl or, with less specificity, geranylgeranyl-modified proteins. This confirms the supposition that Chs4p is prenylated in vivo. The occurrence of farnesylation but not geranylgeranylation is strongly supported by two facts. First, deletion of the gene encoding the catalytic subunit of FTase increases the amount of Chs4p, which upon purification is the substrate for the enzyme in vitro. Second, changing the CVIM farnesylation site to the known CVLL geranylgeranylation motif (31) corrects only partially the phenotype caused by the mutation abolishing Chs4p prenylation. At this point, however, we cannot tell whether the phenotype induced by the CVIM-to-CVLL motif substitution is due to the difference in structure of the attached prenyl group or to lower efficiency of prenylation by geranylgeranyl transferase I.

In almost all instances, prenylated proteins are membrane associated, and protein prenylation is often viewed as a modification that serves to increase protein hydrophobicity, producing membrane association for proteins that otherwise lack membrane affinity. Since Chs4p lacks any predicted transmembrane domain, one might expect that membrane association of Chs4p would be at least to a certain extent prenylation-dependent. This prediction was not borne out, as wt and nonprenylated versions of Chs4p behave similarly in membrane association experiments and both are partially solubilized by 100 mM sodium carbonate or 0.5 NaCl, as is typical for peripheral membrane proteins. Further, like the catalytic subunit of CSIII, Chs3p, Chs4p is not sensitive to Triton X-100 treatment (Fig. 2A). However, it is completely solubilized by the anionic detergent SDS. This result is compatible with the results of DeMarini and coworkers (11), which show that localization of Chs4p to the septum depends on interaction with Bni4p and Chs3p in a manner independent of the presence of the CaaX box. It also suggests that localization of Chs4p to the lateral wall depends on the interaction with Chs3p, and perhaps also with other proteins. Separation of membranes by differential centrifugation (Fig. 2B) or on sucrose density gradients (data not shown) indicates that prenylation of Chs4p does not affect its endomembrane trafficking. Furthermore, staining of wt and chs4-C693S yeast strains with the chitin binding dye CFW did not reveal chitin delocalization in the mutant cells (not shown).

Although protein prenylation may facilitate anchoring of proteins to lipid membranes, data suggesting its role in protein interaction and activation are accumulating (12, 17, 19, 28, 30, 38). Our data support the proposition of Magee and Seabra (26), which stresses the role of prenyl groups in protein-protein interaction and activation. Figure 4 shows that mutation of the CVIM motif of Chs4p affects CSIII activity. Substitution C693S blocks farnesylation of Chs4p. Substitution M696L changes the CVIM farnesylation motif to the CVLL geranylgeranylation motif. (A) CS III activity was measured by the nonradioactive method, as described previously (25). As an enzyme source, the total membrane fractions from yeast cells grown for 6 h at 30°C in YPD liquid medium, YPD supplemented with glucosamine (15 mmol), or YPD supplemented with CFW (25 μg/ml) were used. Cultures for CSIII assay were inoculated with a saturated overnight culture and grown to mid-log phase. (B) Influence of trypsinization on CSIII activity. As an enzyme source, the total membrane fractions from yeast cells grown for 6 h at 30°C in YPD liquid medium and pretreated in the presence (+Trypsin) or absence (–Trypsin) of protease were used.
interaction in addition to its role in membrane binding. Since prenylation does not affect membrane association of Chs4p but clearly affects CSIII enzymatic activity and alters chitin chain length, is possible that the farnesyl group attached to Chs4p interacts with a hydrophobic pocket in Chs3p, modifying the structure of the CSIII complex and in turn influencing the disengagement of the nascent polymer from the enzymatic complex. This hypothesis is in good agreement with the proposition that Chs4p is a direct activator of Chs3p (5, 11, 32, 40).

Other possible explanations for the described phenotype should be also taken into account. The farnesyl group may be necessary, for example, for interactions with protein factors other than Chs3p or with the membrane bilayer during the assembly of the CSIII complex. Here, one of the obvious candidates is Bni4p. However, we did not observe that inactivation of BNI4 enhances or suppresses the CFW resistance of the chs4-6 chs3 mutant in comparison to the wt background (data not shown).

There does not seem to be a specific role of farnesylation of Chs4p in the cell wall stress response. As mentioned before, a defect in prenylation affects chitin synthesis in a manner independent of the localization of chitin synthesis and the induction of the cell wall integrity pathway. Also, Chs3p requires Shc1p during sporulation as an alternative to the Chs4p activating subunit (37). However, Shc1p does not possess a prenylation motif. In spore cell walls, chitin is a substrate for chitin deacetylase, which forms chitosan (10). Thus, the different structural requirement for the final product of Chs3p under different conditions may not require prenylation of Shc1p.

Cell wall composition changes during growth, budding, mating, sporulation, and stress response, and these dynamic processes require synthesis of new sets of proteins as well as remodeling of the cross-linking of β-1,3- and β-1,6-glucans (23). The CSIII synthesized polymer is attached to different acceptors (β-1,3-glucan at the bud neck and β-1,6-glucan in the lateral wall) depending on the deposition site. However, both types have the same polydisperse size profile (7). In this study, we observed a new characteristic feature of cell wall remodeling during the stress response, an increase in chitin content coupled with an increase in average chitin chain length (Table 4). This increase in chain length is clearly associated with an increase in the rate of chitin synthesis. Our suggestion that changes in chitin chain length are one of the features of the cell wall stress response is confirmed by the fact that treatment with glucosamine, which stimulates chitin synthesis in vivo, results in an anti-Gas1p antibody and to Martin Steffen, Boston University Medical School, for mass spectrometry analysis.

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