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

Kariona A. Grabińska,* 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. Preynylation 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 thiethers 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...
TABLE 1. Strains and plasmids

<table>
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protozoan pathogens (13). Since deletion of the FTase catalytic subunit (RAML) 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 _loxP_Crc-based disruption system (16). The homologous recombination cassette was amplified by PCR using oligonucleotides comprising the 3’ region complementary to the sequence in the terminator region of the _CHS4_ gene, did not reveal any differences.

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 _YDR008C_ open reading frame located on the complementary 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 _Crc_.

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 _CHS4-290R_ and _F1-CBP_. The marker gene _TRP1_ was removed by expression of recombinase _Crc_.

TAPCHS4B5 and TAPCHS4B6 were obtained by mating TAPCHS4B3 with BY4741ram1Δ. Sporulation of the diploid 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 supplemented with 2% glucose (wt/vol) (YPD). Synthetic minimal media (SD) were made of 0.67% (wt/vol) yeast nitrogen base and 2% (wt/vol) glucose, supplemented with auxotrophic requirements. For solid media, agar (Difco) was added to YPD or SD at 2% glucose (wt/vol) (YPD).
Heterologous expression and purification of Chs4p from Escherichia coli. Wild-type (wt) CHS4 and CHS4 alleles mutated at the prenylation box (C693S) were amplified by PCR using yeast genomic DNA as a template, CHS4::BamHI as a forward primer, and Chs4-R or Chs4-C693S-R as a reverse primer. PCR product sequences were cloned into pGEM-T Easy vector (Promega), and a BamHI NotI insert was subcloned into the pET30a vector (Novagen) in such a way that a poly-His sequence and an N-terminal tag were added to the N-terminus of Chs4p. pET30a-CHS4 and pET30a-cha-C693S were each transformed into the Rosetta 2 strain of E. coli. E. coli cell pellets in the logarithmic growth phase were induced to express Chs4p by incubation with 1 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside) for 4 h at 30°C. The harvested cells were lysed by sonication. wt and mutated Chs4p were purified by means of N-terminal poly-His with a nickel column according to the Invitrogen protocol and checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using monocular mouse immunoglobulin G (IgG) raised against the tag (Novagen).

Miscellaneous methods. Protein levels on microtiter plates were determined by the Bradford method (Bio-Rad Laboratories). Protein samples were resolved on an SDS-sodium dodecyl sulfate (SDS)-PAGE (Pierce) under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes, blocked in 5% milk, and probed with antibodies. Chs3p was recognized by polyclonal rabbit antibodies (25), plasma membrane marker Gaslp1 with polyclonal rabbit antibodies (25), membrane marker Dpm1p with monoclonal 5C5 mouse antibody (Molecular Probes), and TAPtag-fused proteins with peroxidase–anti-peroxidase soluble complex (PAP) rabbit IgG coupled with horseradish peroxidase (Sigma) or anti-Chs3p IgG. Farnesylated purified CBP-Chs4p was detected with rabbit anti-farnesyl antiserum (Sigma). As secondary antibodies, horseradish peroxidase-conjugated anti-mouse antibodies or anti-rabbit antibodies (Promega) were used. Binding was visualized with the Western Lightning chemiluminescence reagent (Perkin-Elmer) according to the manufacturer’s instructions.

Prenylation of Chs4p in vivo. In order to purify Chs4p, a modified TAPtag purification method was applied (33). Yeast cells expressing TAP-Chs3p (TAPCHS43B) or TAP-Chs4p C693S (TAPCHS4B) were grown overnight in YPD medium to late logarithmic phase (optical density at 600 nm [OD600] 2 to 3 units/mL). Cells were collected by centrifugation, washed with water, and then disrupted in IPP1000 buffer (10 mM Tris-HCl [pH 8], 1 M NaCl, 0.5% nonylphenol-polyethylene glycol [NP-40], Sigma), 2 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma) by vortexing with glass beads. Lysates were clarified by 15 min of centrifugation at 1,500 × g and were then centrifuged at 100,000 × g for 90 min at 4°C.

The supernatant was incubated overnight at 4°C on a rotating platform with 50 μl of IgG-Sepharose 6 Fast-Flow (Amersham Biosciences). The IgG-Sepharose beads were collected by centrifugation, washed extensively with IPP1000 buffer, equilibrated with TEV cleavage buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl, 0.1% NP-40, 0.5 EDTA, 1 mM dithiothreitol), and suspended in 50 μl of TEV cleavage buffer containing 5 μl (30 units) of TEV protease (Innogenetics). The mixture was incubated for 3 h at 16°C on a rotating platform in order to cleave the CBP-Chs4p fusion protein from the PROTAg tag. The mixture was centrifuged (20,000 × g) in order to separate the upper-phase eluate containing CBP-Chs4p fusion protein from the IgG-Sepharose beads. Protein samples were separated by protein electrophoresis and analyzed by immunoblotting.

Ftase activity. To determine FTase activity, 1.5 μg of Chs3p (wt or mutated at the farnesylation box) purified from bacterial cells was incubated with 4 μM farnesyl pyrophosphate-(1-3H-N) (15 Ci/mmol; American Radiolabeled Chemicals) in a 30-μl reaction mixture containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 5 μM ZnCl2, 0.1 mM PMSF, and 0.2 μg of recombinant S. cerevisiae farnesyltransferase (Sigma). The reaction mixture was incubated at 30°C for 60 min, stopped by the addition 1 ml of TEV buffer, and concentrated with an Amicon ultra-filter device (Millipore), and proteins were separated by SDS-PAGE and blotted onto a PVDF membrane. Farnesylated protein was visualized by autoradiography.

In order to assess the level of prenylation in the wt background and in cells lacking the catalytic subunit of FTase (Ram1p), yeast strains expressing TAP versions of wt and mutated forms of Chs4p in the RAM1 knockout genetic background were constructed. Cells were disrupted in IPP1000 buffer, and the protein extract (100,000 × g supernatant) was incubated for 3 h with 4°C on a rotating platform with IgG-Sepharose 6 Fast-Flow (10 μl of Chs4p was incubated with extract made from 0.25 g of cell pellets to ensure saturation of the resin with the protein). The IgG-Sepharose beads were collected by centrifugation, washed extensively with IPP1000 buffer, and equilibrated with FTase buffer lacking farnesyl pyrophosphate; FTase and 10 μl of resin with immobilized protein was used for the FTase assay in a 60-μl reaction mixture as described previously. After the reaction, the resin was washed five times with 1 ml of TEV buffer, and the radioactivity was measured by scintillation counting.

Subcellular fractionations. The analysis of organelles by differential centrifugation was performed according to methods described previously (21, 42) with modifications.

To study the membrane association of Chs4p, 0.6 g of yeast cells in mid-log phase (OD600 0.5 to 0.8) were harvested, washed with ice-cold 10 mM NaCl, 10 mM KF, 50 mM Tris-HCl (pH 7.5) buffer, and then with 30 mM Tris-HCl (pH 7.5) buffer containing 100 mM EDTA buffer. Samples were suspended in 1 ml of TEV cleavage buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride [PMSF], and protease inhibitor cocktail [Sigma]) and broken by agitation with glass beads. Lysates were clarified by 5 min of centrifugation at 500 × g. The cleared cell lysate was mixed with an equal volume of 200 mM sodium carbonate or HOB 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 HOB in the same volume as the corresponding soluble fraction; 5 μl of each fraction was subjected to protein electrophoresis and immunoblotting.

Chtin 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 (Interspex) in McIlvaine’s buffer, pH 6.0, for 3 h.

CSII activity. CSII 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, resuspended in 20 mM Tris-HCl (pH 8.0) buffer containing 2 mM PMSF and protease inhibitor cocktail (Sigma), and broken by vortexing with 425- to 600-μm-diameter glass beads. The cell extract was clarified by 15 min of centrifugation (1,500 × g) and then centrifuged 1 h at 100,000 × g. The membrane fraction was then incubated in 20 mM Tris-HCl (pH 8.0) buffer supplemented with protease inhibitors. Trypsin pretreatment of the enzyme source was done according to the method described for measurement of chitin synthase II activity (25).

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
were removed by digesting isolated cell walls with 2 mg/ml of Zymolyase X100 (Seikagaku). In order to remove the remaining β-1,6-glucan attached to chitin chains, the washed pellet was later digested with 4 units/ml of β-1,6-endoglucanase (27). The chitin pellet was reduced in 100 μl of 0.1 M NaOH with 500 μCi of NaBH₄ (100 mCi/mmol) (NEN) for 6 h at 25°C. The reaction was stopped with 200 μl of 0.5 M acetic acid. After extensive washing, the reduced chitin was digested in 0.2 ml of 0.1 M KPO₄ (pH 6.0) with 500 μCi of NaBH₄ (Sigma) for 16 h at 37°C. This crude endochitinase preparation, containing hexosaminidase activity, digests chitin to free N-acetylglucosamine (GlcNAc) (27). After digestion, the total GlcNAc was estimated by the Morgan Elson assay, as described above. To determine the amount of terminal residues, the supernatants were chromatographed in a P4 Biogel column (1 by 120 cm). Three fractions were collected: (i) a major fraction containing high molecular weight GlcNAc and GlcNAc-glucitol (residual glucan stubs attached to the chitin reducing end) 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).

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 be not 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(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-farnesyl-t-cysteine, which recognize farnesyl or, with less specificity, geranylgeranyl-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 (Ramlp) 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-Chs4p C693S 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 and sodium carbonate to disrupt peripheral or protein-protein associations or with Triton X-100 or SDS to disrupt integral membrane association. TAP-Chs4p and TAP-Chs4p C693S 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 (13,000 × g for 10 min) into a PM/ER fraction (P) and a Golgi/endosome-rich fraction (S). The protein compositions of fractions obtained from differential centrifugations and sucrose gradients were analyzed by SDS-NuPAGE (PM markers, Gas1p and Chs3p; ER membrane marker, Dpm1p; soluble fraction marker, Pgk1p).

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/hs4 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

![FIG. 2. Prenylation does not affect the subcellular distribution of Chs4p. (A) Extraction of TAP-Chs4p. The total homogenate prepared in HOB was treated with 100 mM Na2CO3, 0.5 NaCl, or 1% Triton X-100 (TX100), and the soluble and insoluble fractions were separated by centrifugation at 200,000 × g for 1 h. Treatment with 4% Triton X-100 also does not solubilize Chs3p or Chs4p (not shown). (B) Chs4p and Chs3p fractionation by differential centrifugation of membranes from wt and mutated (Chs4p C693S) cells. Total membranes from concanavalin A-coated spheroplasts were separated by differential centrifugation (13,000 × g for 10 min) into a PM/ER fraction (P) and a Golgi/endosome-rich fraction (S). The protein compositions of fractions obtained from differential centrifugations and sucrose gradients were analyzed by SDS-NuPAGE (PM markers, Gas1p and Chs3p; ER membrane marker, Dpm1p; soluble fraction marker, Pgk1p).](http://ec.asm.org/doi/pdf/10.1128/EC.01630-17)
The effect of chs4-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 septins via Bin4p, which confers septum localization to CSIII. Two facts indicate that the two roles are separable: (i) delocalized chitin is present in bni4 but not in a bni4 chs4 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 unfarnesylated recombinant Chs4p is a substrate for FTase in vitro. The protein isolated from yeast cells reacts with serum raised against N-acetyl-S-farnesyl-
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 chs4Δ yeast strains with the chitin binding dye CFW did not reveal chitin delocalization in the mutant cells (not shown). However, we could not exclude more subtle changes in Chs4p localization that might be difficult to detect by standard molecular biology methods. For example, prenylation may be involved in efficient loading of Chs4p to the plasma membrane. Unfortunately, in contrast to Chs3p trafficking, which has been subject of numerous studies (35, 42, 43), little is known about Chs4p trafficking apart from the fact that both Chs3p and Chs4p relocalize in response to stress conditions, leading to chitin deposition in the lateral wall (8, 15). It will be of interest to study the route of Chs4p trafficking in greater detail. Such information may be crucial to a final understanding of the role of Chs4p prenylation.

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

### TABLE 4. Degree of polymerization of chitin

<table>
<thead>
<tr>
<th>Genetic background and growth conditions</th>
<th>Average chitin chain length for indicated CHS4 allele (yeast strain)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>$59 \pm 6.1^<em>$ (KG103B) 46 $1.7^</em>$ (KG102B)</td>
</tr>
<tr>
<td>wt + glucosamine</td>
<td>68 (KG103B)</td>
</tr>
<tr>
<td>wt + CFW</td>
<td>206 (KG103B)</td>
</tr>
<tr>
<td>fks1Δ</td>
<td>114 (KG106B) 86 (KG104B)</td>
</tr>
<tr>
<td>gas1Δ</td>
<td>180 (KG107B) 151 (KG105B)</td>
</tr>
</tbody>
</table>

*a Chitin chain lengths were estimated as described in Materials and Methods.

*b, data point is an average of four independent experiments.
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, it 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 propo-
sition 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 can-
didates is Bni4p. However, we did not observe that inactivation
of BN14 enhances or suppresses the CFW resistance of the
chs4-C693S 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 inde-
pendent of the localization of chitin synthase and the induc-
tion of the cell wall integrity pathway. Also, Chs3p requires
Shc1p during sporulation as an alternative to the Chs4p acti-
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other factors that may produce increased chitin synthesis in-
clude moderately increased transcription of CHS4 and CHS3
(6, 18, 20, 40) and alterations in the localization of the enzyme
(8, 15, 42). However, this attractive hypothesis needs to be
proved. Our data strongly encourage reconstruction in vitro of
the active CSIII complex so that the role of Chs4p may finally
be understood and so that the function of its prenylation may
be explained.

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