In Schizosaccharomyces pombe, Bgs1/Cps1p is a β(1,3)-glucan synthase required for linear β(1,3)-glucan synthesis and primary septum formation. Here, we have studied the regulation of Bgs1p by Cfh3p/Chr4p, a member of a family of conserved adaptor proteins, which resembles the chitin synthase regulator Chs4p from Saccharomyces cerevisiae and Candida albicans. Cfh3Δ cells showed a genetic interaction with cps1-191 and Cfh3p co-immunoprecipitated with Bgs1p/Cps1p. In the absence of cfh3Δ, cells were more sensitive to digestion by glucanases, and both Calcofluor staining and glucan synthesis were reduced. We found that in a wild-type strain, β(1,3)-glucan synthesis was reduced under stress conditions. In the cfh3Δ, cps1-191, and cfh3Δ cps1-191 strains, β(1,3)-glucan synthesis was further reduced, and growth was impaired under stress conditions, suggesting that Cfh3p and Bgs1p might play a role in ensuring growth in unfavorable environments. In a cfh3Δ mutant, Bgs1p was delocalized when the cells were distressed, but a blockade in endocytosis prevented this delocalization. Finally, we found that the SEL1 repeats are required for Cfh3p function. These results show that Cfh3p is a regulatory protein for Bgs1p and that its function is particularly necessary when the cells are undergoing stress.

In Schizosaccharomyces pombe, the primary septum, composed of linear and branched β(1,3)-glucan, is surrounded by a secondary septum with a composition similar to that of the lateral cell wall (branched β(1,3)-glucan, β(1,6)-glucan, α(1,3)-glucan, and mannoproteins (1, 2)). Bgs1/Cps1p is the β(1,3)-glucan synthase responsible for the synthesis of linear β(1,3)-glucan and the primary septum structure (3). In the absence of this activity, the cells are able to form remedial septa that do not contain linear β(1,3)-glucan. These septa do not stain with low concentrations of Calcofluor and cannot be degraded by glucanases, so the cells remain chained, forming hyphal structures (3). In S. pombe, there are four glucan synthase homologues, bgs1+/cps1+, bgs2+, bgs3+, and bgs4+ (4–9). In the case of the bgs1Δ cells, apical growth takes place in two opposite directions, producing branched cells with a dichotomic growth. This phenotype is not observed in bgs2Δ, bgs3Δ, or bgs4Δ mutants, thus suggesting that Bgs1p might play a specific role in the control of cell growth (3). Finally, Bgs1p is a component of the cytokinesis checkpoint, which coordinates mitosis with actomyosin ring contraction and septum synthesis (10, 11).

The study of Bgs1p regulation should help us to understand the control of cell wall synthesis and cytokinesis in S. pombe. Bgs1p requires an active Septation Initiation Network ( SIN), an assembled contractile actomyosin ring, and the type-V myosin Myo52p to localize properly at the division site (9, 12, 13). Regarding the regulation of biochemical activity, the PKC homologues Pck1p and Pck2p activate β(1,3)-glucan synthesis in an unknown way (14) while the Rho1p GTPase is a direct activator of the β(1,3)-glucan synthase catalytic subunit (15).

The cell wall is a morphogenetic determinant, but also an essential cellular structure that protects the organism against cell lysis in hyposmotic environments. In S. pombe, the mitogen-activated protein (MAP)3 kinase Spm1/Pmk1p pathway (also known as the cell integrity pathway) regulates growth and morphogenesis in response to multiple stresses, including hyper- or hyposmotic conditions, nutrient limitation, and cell wall-damaging compounds (16, 17). Proper growth and morphology under hyposmotic conditions also requires Skb1p and Skb5p, which are regulators of the Shk1/Orb2/Pak1p kinase (18, 19). However, little is known about the role of the cell wall as a protective element against hyposmotic conditions, the presence of high concentrations of chloride ions, or nutrient limitation.

Here we examine the role of the Cfh3/Chr4 protein in cell wall synthesis and response to stress. Cfh3p shares significant similarity with the Chs4 proteins from Saccharomyces cerevisiae and from Candida albicans, which are chitin synthase regulators (20, 21). In S. cerevisiae, regulation of the chitin synthase Chs3p by Chs4p is complex and still not well understood. Chs4p is required for the correct localization of Cfh3p at the bud neck by mediating its anchorage to septins through the adaptor protein Bni4p, but it also acts as a biochemical activator and is required for the stability of Chs3p at the plasma membrane (20, 22–25). In S. pombe, no chitin synthesis occurs during vegetative growth (26–29), glucan being the main cell wall component, there is no Bni4p homologue, and septins are

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**The Fission Yeast SEL1 Domain Protein Cfh3p**

**A NOVEL REGULATOR OF THE GLUCAN SYNTHASE Bgs1p WHOSE FUNCTION IS MORE RELEVANT UNDER STRESS CONDITIONS**

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involved in cell separation but not in septum synthesis (30, 31). We wanted to investigate whether Cfh3p played any role in cell wall synthesis and/or morphogenesis in the fission yeast. We found that in the absence of cfh3Δ, the cells showed reduced β-glucan synthesis and impaired growth under stress conditions. These phenotypes were aggravated in a double cfh3Δ eps1Δ mutant. Our results suggest that Cfh3p is a regulator of the β-glucan synthase Bgs1p whose presence is more critical when the cells are undergoing environmental stress. Cfh3p belongs to a conserved family of scaffold proteins characterized by the presence of tandem repeats of SEL1 domains, which are involved in signal transduction during different cellular processes (32). Here we found that the SEL1 domains in Cfh3p were required for its function.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—All techniques for S. pombe growth and manipulation have been described previously (33). The relevant genotypes of the strains used are listed in supplemental Table S1. Geneticin (G418, GIBCO Invitrogen) was used at 120 μg/ml.

**Molecular and Genetic Manipulations**—General techniques were according to Ref. 34. The cfh3Δ+ gene was cloned from the genome of a wild-type strain by the Gap repair technique (35). A cfh3::KANMX6 deletion cassette in which the complete cfh3Δ open reading frame had been substituted by the KanMX6 gene, which confers resistance to geneticin (36), was used to transform the strains of interest. Correct integration was always assessed by PCR. Site-directed mutagenesis was used to introduce a NotI restriction site immediately downstream from the initial ATG codon. Three copies of the HA epitope, or the GFP, were introduced downstream from the SEL1 domains, which were eliminated by site-directed mutagenesis, either by introducing an amino acid insertion or a deletion of amino acids, corresponding to the prenylation motif, by a leucine residue and to introduce Pma1Cl restriction sites upstream and downstream from the SEL1 domains, which were eliminated by digestion with this enzyme and plasmid religation. The SEL1(1–2) truncated Cfh3 protein was produced by eliminating amino acids 279–346; the SEL1(3–5) truncation was produced by eliminating amino acids 443–543, and the SEL1(1–5) truncation was produced by deleting amino acids 279–543. The complete amino acid sequence of the modified proteins is shown in the supplemental Fig. S1. The accuracy of the constructions was assessed by DNA sequencing. These constructs, in which the cfh3Δ+ gene was under the control of its own promoter, were integrated at the leu1+ locus. Double mutants were obtained by tetrad analysis. Combination of mutated alleles with HA- , GFP-, or RFP-tagged proteins was performed either by plasmid transformation or by “random spore” selection from genetic crosses (33).

**Protein Techniques**—For co-immunoprecipitation, cell extracts were obtained in extraction buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 0.5% IGEPAL CA-630) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride; 1 μg/ml aprotinin, leupeptin, and pepstatin). 5 mg of protein from each extract were brought up to 330 μl with extraction buffer. Then, 30 μl from each sample was boiled in sample buffer and used to perform a Western blot. 300 μl of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 2% Triton X-100) with protease inhibitors were added to the remaining 300 μl of the extracts. 2.5 μl of rabbit anti-GFP antibody (Invitrogen, anti-GFP, serum) was added to the samples, which were incubated for 2 h at 4 °C in a tube rotator. Then, 50 μl of protein A-Sepharose CL-4B beads (Amersham Biosciences, 0.1 g/ml in IP buffer) were added to the samples, and the mixture was incubated overnight at 4 °C. The beads were washed three times using IP buffer and once with phosphate-buffered saline, after which the beads were boiled in a final volume of 50 μl with Laemmli sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 143 mM β-mercaptoethanol, 10% glycerol) and centrifuged. Each supernatant was used to load two 6.5% polyacrylamide gels to be developed either with monoclonal anti-HA (12C5A, Roche, 1:4000) or with monoclonal anti-GFP (JL8, BD Biosciences, 1:1000). Secondary goat anti-mouse (Bio-Rad) or anti-rabbit (Amersham Biosciences) antibodies were used at 1:10,000 dilution, and ECL (Amersham Biosciences) was used to develop the blots. For Western blot, cells were broken in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Triton X-100 supplemented with protease inhibitors. 100 μg of protein from each sample were loaded in a 6.5% SDS-polyacrylamide gel and decorated with monoclonal anti-HA, anti-GFP, or anti-α-tubulin (clone B-5–1-2, Sigma; 1:10,000) antibodies. In the case of the Bgs1 protein, the extracts were incubated in 1.6 M urea at 4 °C for 3 h before boiling in sample buffer.

**Cell Wall Analysis**—Analysis of cell wall composition was performed as described (39). Briefly, exponentially growing cultures were supplemented with 0.5 μCi/ml of d-[U-14C]glucose (281 mCi/mmol, Amersham Biosciences CFB96) and incubated for 6 h at 25 °C. Total glucose incorporation was monitored by measuring the radioactivity in trichloroacetic acid-insoluble material. 100-μl aliquots of total cell walls were incubated with 100 units of Zymolase 100T (Seikagaku Kogyo Co. Ltd) or Quanzyme (recombinant β(1,3)-glucanase; Quantum Bio-technologies Inc.) for 36 h at 28 °C. The samples were centrifuged, and the supernatants and washed pellets were counted separately. The precipitate from the incubation with Zymolase 100T corresponded to the α-D-glucan, and the supernatant corresponded to β-glucan plus galactomannan. The precipitate from the incubation with Quanzyme corresponded to α-glucan plus galactomannan, and the supernatant corresponded to β(1,3)-glucan. The difference between the precipitates of Quanzyme and Zymolase 100T corresponded to galactomannan. The method did not distinguish β(1,6)-glucan, which is a minor component of the S. pombe cell wall (1).

**Enzyme Preparation and β(1,3)-Glucan Synthase Assay**—Cell extracts and glucan synthase assays were essentially as described previously (9). Early logarithmic phase cells grown at 25 °C in the presence or the absence of 1 M KCl were washed

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4 M. Valdivieso, unpublished results.
Cfh3p Regulates Bgs1p

with buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM β-mercaptoethanol), suspended in 100 μl of buffer A supplemented with 1 μl of 10 mM GTPγS and broken with glass beads in a FastPrep FP120 apparatus (Savant; BIO 101. Three 15-s pulses at a speed of 5.0). Broken material was diluted with buffer A, the cell debris was removed by low speed centrifugation (800 × g for 5 min), and the supernatant was centrifuged at 48,000 × g for 30 min at 4 °C. The pellet was resuspended in buffer A containing 33% glycerol and GTPγS. Standard glucan assay mixture contained 5 mM UDP-[U-14C]glucose (CFB102, Amersham; 4 × 10^4 cpm/200 mol) and enzyme (15 μg of protein) in a total volume of 40 μl. Reaction mixtures were incubated at 30 °C for 30 min, stopped by adding 2.5 ml of 10% trichloroacetic acid, and incubated at 4 °C for at least 30 min before filtering. Radioactivity was measured using a scintillation counter. All reactions were carried out in duplicate, and data for each strain represent the average value from five independent cultures. The enzymatic unit was defined as the amount of enzyme that catalyzes the incorporation of 1 μmol of glucose per min at 30 °C.

Digestion with Glucanases—Sensitivity to digestion with glucanases was performed as described (39). Briefly, cells were resuspended in 2 ml of 50 mM citrate/phosphate buffer, pH 5.6, supplemented with different amounts of glucanases (Zymolase 100T, Seikagaku, or Novozyme 234, Novo Industries) and incubated at 28 °C in a tube rotator. The incubation time, and the data were analyzed.

Microscopy—The observation of GFP-tagged proteins was performed on cells collected by filtration. Images were captured with a Leica DM RXA microscope equipped with a Photometrics Sensys CCD camera, using the Qfish 2.3 program. Images were processed with Adobe Photoshop.

RESULTS

Cfh3Δ Mutants Show a Genetic Interaction with Mutants Defective in Septum Synthesis—Deletion of the cfh3Δ/chr4 Δ gene did not lead to any apparent defect in cell morphology or in growth (38). To gain further information about the proteins functionally related to Cfh3p, we constructed double mutants involving cfh3Δ and the following mutants defective in different steps of cytokinesis: cdc4-8, myo2-E1, myo3Δ, and myo2-E1 myo3Δ (carrying mutations in type II-myosin components); cdc11-119, and cdc14-118 (carrying mutations in genes from the Septation Initiation Network, which controls septum synthesis); spn1::ura4Δ, spn2::ura4Δ, spn3::ura4Δ, and spn4::ura4Δ (deleted for septins), cbs2Δ (defective in a chitin synthase-like protein), and cps1-191. The WT strain and the mutants carrying single or double mutations were streaked onto YES plates and incubated at different temperatures (from 22 to 37 °C). Only in the case of the cfh3Δ cdc14-118 and the cfh3Δ cps1-191 strains, did the double mutants show an enhanced thermosensitivity with respect to the WT and the corresponding single mutants (Fig. 1A and results not shown). 1.2 M sorbitol supported the growth of the double mutants at the restrictive temperature (Fig. 1A), suggesting that those strains had a defect in the cell wall.

bgs1/cps1-191 cells show a dichotomic growth and a multiseptation phenotype when they are kept alive in the presence of the osmotic stabilizer sorbitol (3). Accordingly, we wanted to study the morphology of the cfh3Δ/cps1-191 mutants with respect to that of the cps1-191 strain in the presence and the absence of sorbitol. In YES medium at 32 °C, cps1-191 cells were roundish or pear-shaped (Fig. 1B). In the presence of 1.2 M sorbitol, cps1-191 cells were rod-shaped, although some exhibited more than one septum (arrowheads in Fig. 1B), as has been described for other bgs1 mutants (3). In YES and in sorbitol-supplemented YES media, the cfh3Δ cps1-191 mutant exhibited long, multi- septated, branched cells with a dichotomic growth pattern. These cells were frequently dead, showing that this strain had a stronger defect than the single mutants, and that osmotic protection was not sufficient to correct this defect (Fig. 1B). The double mutant exhibited this phenotype even at 25 °C (not shown). Thus, cfh3Δ showed a genetic interaction with some mutants affected in genes required for septum synthesis. Additionally, we found that a multicopy plasmid carrying the cfh3Δ+ gene under the control of its own promoter was able to improve the reduced growth of the cps1-191 mutant at 32 °C (Fig. 1C). All these results strongly suggest a functional relationship between Cfh3p and Bgs1p.

Cfh3Δ Mutants Show a Defect in Glucan Synthesis—Because the cfh3Δ mutant showed a genetic interaction with cps1-191, a mutant defective in the Bgs1p glucan synthase, we decided to determine whether the absence of the cfh3Δ+ gene might lead to a defect in glucan synthesis. We analyzed the growth of the WT, cfh3Δ, cps1-191, or cfh3Δ cps1-191 strains in the presence of the glucan synthase inhibitors Caspofungin and Enfumafungin (40, 41). We found that the cfh3Δ, cps1-191 and cfh3Δ cps1-191 cells were more sensitive than the WT at 2 μg/ml of Caspofungin and that the double mutant was the most sensitive of all the strains because it did not grow at 1.5 μg/ml, a concentration at which the single mutants were only partially sensitive. The
mutants were also hypersensitive to 7 μg/ml of Enfumafungin (Fig. 2A). We next measured the β(1,3)-glucan synthase activity in membrane extracts from the WT and the mutant strains incubated at 30 °C. As shown in Fig. 2B, the specific activity was reduced to 85 and 84% in the cfh3Δ and the cps1-191 strains, respectively, and to 71% in the double mutant. Although the differences in the specific activity between the strains were small, the tendency was the same in the five experiments that were performed, in which the mutants always exhibited a lower activity than the WT strain.

We then wanted to investigate whether the in vitro defect in the activity correlated with an in vivo defect in the synthesis of β(1,3)-glucan. To do so, the sensitivity to digestion by glucanases of the WT cells was compared with that of the cfh3Δ, cps1-191, or cfh3Δ cps1-191 cells. We found that the cfh3Δ cells lysed faster than the WT cells when they were incubated in the presence of 50 μg/ml of Novozyme; that the cps1-191 cells lysed faster than the cfh3Δ cells, and that the cfh3Δ cps1-191 double mutant cells were the most sensitive to digestion by the enzyme mixture (Fig. 2C). Similar results were obtained when 5 μg/ml of Zymolyase 100T was used (results not shown). These results indicated that the cell wall composition was altered in all the mutant strains, probably because of a defect in β-glucan.

To confirm this, we analyzed the incorporation of radioactive [14C]glucose into the cell wall polysaccharides of different strains incubated at 25 °C for 6 h. As shown in Fig. 2D, the percentage of glucose incorporation into β-glucan was 22.7% ± 1.91, 20% ± 2.15, 19.0% ± 1.86, and 13.7% ± 1.27 for the WT, cfh3Δ, cps1-191, and cfh3Δ cps1-191 strains, respectively. Thus, β-glucan was reduced by 10% in the cfh3Δ strain; by 16% in the cps1-191 strain, and by 39% in the double mutant. In the case of the cps1-191 and cfh3Δ cps1-191 cells, this decrease was compensated by an increased synthesis of mannan and, more significantly, by an increased synthesis of α-glucan. Accordingly, these two strains have more cell wall material than the WT and the cfh3Δ strains.

It has been shown that in S. pombe Calcofluor binds linear β-glucan, which is present mainly in the primary septum, and that bgs1Δ/cps1Δ cells do not synthesize this structure, so the cells do not stain with low concentrations of this dye (3). We stained cells from the WT and the cfh3Δ strains with 0.125 μg/ml of Calcofluor, and we found that at this concentration the dye was able to stain the septa of the WT cells efficiently, while in the cfh3Δ cells, the septa only exhibited a faint staining (Fig. 2E). These results show that the Bgs1p activity is compromised in the cfh3Δ cells.

The Growth of the cfh3Δ, cps1-191, and cfh3Δ cps1-191 Strains Is Impaired under Certain Stress Conditions—During this work, we realized that cfh3Δ cps1-191 cells showed the strongest morphological phenotype when the cells were entering the stationary phase, or exiting it, and that most of the cells died if they were kept on plates at 25 °C for more than 4 days. To capture this observation, we incubated the same number of cells from the WT, cfh3Δ, cps1-191, and cfh3Δ cps1-191 strains in liquid YES medium at 25 °C. Samples were collected at 4 or 7 days of incubation and 3 × 10⁴ cells and serial 1:4 dilutions were spotted onto YES plates and incubated at 25 °C for 3 days before photography. As shown in Fig. 3A, the survival of the cfh3Δ cps1-191 cells was very low after they had been in the stationary phase, and that of the single cps1-191 cells was also reduced with respect to that of the WT strain. This result suggested that the cps1-191 and the cfh3Δ cps1-191 cells were sensitive to nutritional stress. We therefore analyzed whether the cfh3, cps1-191, and cfh3Δ cps1-191 strains were sensitive to other sources of stress. We included a cwg1-1 mutant, which carries a point mutation in the bgs4 Δ glucan synthase homologue (4) and a spm1Δ mutant, defective in the Spm1/Pmk1 MAP kinase pathway, which controls morphogenesis and stress (17). As shown in Fig. 3B, at the permissive temperature (25 °C) the cfh3Δ cps1-191 and the spm1Δ cells were...
hosphobic to 7 mM caffeine; the cfh3Δ, the cps1-191 and the double mutant were hypersensitive to 0.2 mM MgCl$_2$, and the cfh3Δ, the cps1-191, the double mutant, and the spm1Δ strains were sensitive to 1.4 mM KCl. In all conditions, the cfh3Δ cps1-191 cells were more sensitive to stress than the corresponding single mutants (Fig. 3B). The cwg1-1 cells behaved as the WT did (Fig. 3B), suggesting that a defect in bgs1$^{+}$, but not in bgs4$^{+}$, renders the cells sensitive to stress.

We next analyzed the growth of the single cfh3Δ mutant and the spm1Δ strain on YES plates supplemented with different amounts of KCl, NaCl, MgCl$_2$, caffeine, and H$_2$O$_2$, and incubated at 32 °C. We found that the cfh3Δ and the spm1Δ cells were more sensitive than the WT cells to the presence of 1.2 mM KCl and 6 mM caffeine; the cfh3Δ and the spm1Δ cells were slightly resistant to 0.1 mM NaCl, and the cfh3Δ cells were hypersensitive to 0.1 mM MgCl$_2$ (see Fig. 3C). We did not find differences in the growth of the WT and cfh3Δ strains when they were incubated in the presence of different concentrations of hydrogen peroxide (Fig. 3C and results not shown), suggesting that caffeine and osmotic stress, but not oxidative stress, affects the growth of cfh3Δ cells.

**Impaired Growth of the cfh3Δ, cps1-191, and cfh3Δcps1-191 Strains Under Stress Conditions Is Concomitant with a Reduction in Glucan Synthesis**—It is known that mutants in the Spm1/Pmk1 MAP kinase pathway show a strong defect in cytokinesis when incubated under stress conditions (17). We observed the morphology of WT, cfh3Δ, cps1-191, and cfh3Δ cps1-191 cells that had been growing on solid or in liquid YES medium or in these media supplemented with 1.2 mM sorbitol or 1.0 mM KCl incubated at 25 or 32 °C for different times. For each strain, the morphology of the cells grown under stress conditions was similar to that observed when the cells were grown in YES medium. The morphology of a cfh3Δ spm1Δ double mutant was similar to that of the single spm1Δ mutant under all conditions (supplemental Fig. S2 and results not shown). These results showed that the sensitivity of the cfh3Δ and the cps1-191 cells to stress was not due to a defect in cytokinesis.

Because the mutants under study showed a defect in cell wall synthesis, we wondered whether this defect was responsible for their sensitivity to stress. To address this issue, we analyzed the cell wall composition of the strains of interest in cells that had been incubated for 6 h in the presence of 0.6 mM KCl. As seen in Fig. 4A, we found that all the strains showed a reduced incorporation of [14C]glucose into the cell wall as compared with the data obtained when the cells had been growing in YES medium (the dashed line in Fig. 4A represents the value of incorporation for the WT strain grown in YES medium. Compare Figs. 4A and 2D). Regarding the β-glucan content, the percentage of glucose incorporation into this polymer when the cells were grown in...
YES with 0.6 M KCl was 19.8% ± 1.74, 17.2% ± 1.66, 14.7% ± 1.14, and 12.3% ± 1.65 for the WT, cfh3Δ, cps1-191, and cfh3Δ cps1-191 strains, respectively. Thus, in the WT strain grown under stress conditions the level of β-glucan was reduced by 12% with respect to the amount of this polymer in the cells grown in YES medium; this reduction was 24% in the cfh3Δ strain, 35% in the cps1-191 strain, and 45% in the double mutant strain. The dotted line in Fig. 4A represents the level of β-glucan in the WT cells grown in YES medium.

To rule out the possibility that this defect in glucan synthesis under stress conditions was due to a defect in the accessibility of the substrate to the enzyme, we measured the β-glucan synthase activity in membrane extracts from the WT and the mutant strains that had been incubated in the presence of 1 M KCl-supplemented YES medium (Fig. 4B). As happened when the cells had been incubated in YES medium, the activity values for the mutants strains were reproducibly lower than that obtained for the control strain (Figs. 4B and 2B).

To determine whether the effect on the cell wall synthesis was specific for KCl or whether it was a general response to growth in a stressing condition or in a medium of higher osmolarity than the standard one, we analyzed cell sensitivity to digestion with glucanases. We incubated cells from the WT, cfh3Δ, cps1-191, and cfh3Δ cps1-191 strains for 6 h in YES or YES supplemented with 0.6 M KCl, 1.2 M sorbitol, or 0.125 M MgCl2, and then we treated them with 50 μg/ml Novozyome or with 5 μg/ml Zymolyase-100 T. We found that in all cases the cells lysed faster when they had been incubated under stress conditions than when they had been incubated in YES medium (Fig. 4C and results not shown). These results show that cell wall synthesis in general, and β-glucan synthesis in particular, is significantly reduced when the cells are undergoing stress, and that the impaired growth of the cfh3Δ, cps1-191, and cfh3Δ cps1-191 strains in the presence of stressing compounds is correlated with the low β-glucan content in their cell walls.

Cfh3p Regulates Bgs1p Localization at the Plasma Membrane—All the above results suggested that Cfh3p was a regulator of Bgs1p and that this regulation was more needed when the cells were undergoing some stress. To understand the nature of this regulation, we performed several experiments. First, we used Western blotting to determine the level of Bgs1p in WT and cfh3Δ cells that had been treated with YES medium alone or supplemented with 1 M KCl for 15 min. The Golgi protein Cfr1p (42) was used as a loading control. As shown in Fig. 5A, there was no difference in the amount or the mobility of Bgs1p in the WT or the mutants cells regardless of whether they had suffered osmotic shock or not. This result showed that Cfh3p was acting at a post-translational level. Next, we wanted to know whether Cfh3p formed a complex with the glucan synthase machinery. To elucidate this, we constructed a strain that carried GFP-Bgs1- and RFP-Cfh3-tagged proteins and observed it under the fluorescence microscope. We found that both proteins colocalized (not shown), suggesting that they could be in close contact. To confirm this, we performed a co-immunoprecipitation experiment using strains that carried GFP-Bgs1, HA-Cfh3, or both tagged proteins. HA-Cfh3 was detected in the anti-GFP immunoprecipitates from the strain bearing both tagged proteins, but not from the control strains. Bgs1p and Cfh3p also co-immunoprecipitated when the cells had been incubated in the presence of 1 M KCl for 15 min (Fig. 5B).

We also analyzed co-immunoprecipitation between Cfh3p and the Rho1p GTPase, which regulates glucan synthase activity (15). In this case, we used strains carrying GFP-tagged Cfh3p, HA-tagged Rho1p, or both proteins. As shown in Fig. 5C, we detected co-immunoprecipitation between GFP-Cfh3p and HA-Rho1p when the cells had been incubated either in YES medium or in YES medium supplemented with 1 M KCl for 15 min (Fig. 5C). As a control, we analyzed co-immunoprecipitation between GFP-Cfh3p and HA-Cdc42p (a membrane-associated Rho family GTPase) using the same conditions and failed to observe any association between these proteins (not shown). All these results strongly suggested that Cfh3p would interact with the Bgs1 glucan synthase complex. To see whether Cfh3p was regulating the activation of Bgs1p by Rho1p, we determined the level of active Rho1p using the Rheotekin binding assay (43). We found that the levels of active Rho1p were similar in both the WT and the mutant strain (results not shown).

Finally, we wanted to know whether Cfh3p was regulating Bgs1p localization. To check this, we analyzed GFP-Bgs1 localization in the WT or the cfh3Δ strain grown in YES or in 1 M KCl-supplemented YES for 15 min. The localization of Bgs1p was similar in both strains when they were incubated in YES medium (Fig. 5D), although the signal in the cfh3Δ cells seemed to fade faster. When the cells were incubated in the presence of KCl, the GFP-Bgs1p signal localized at the poles and the septum in the WT strain, although in some cells the septal area seemed to be deformed and the signal seemed to spread along the new cell pole (see arrows in Fig. 5D). By contrast, in about 80% of the cfh3Δ cells Bgs1p could not be detected either at the septum or at the poles (Fig. 5D) after the osmotic shock. A similar result was obtained upon exposing the cells to 1.2 M sorbitol for 15 min (not shown). Then, we wanted to investigate whether the different behavior of the GFP-Bgs1 protein in the WT and the mutant strains was a consequence of a different behavior of actin in response to the osmotic insult. To address this question, we observed the distribution of coronin (Ref. 44; a protein that associated with the actin patches. Crn1-GFP in Fig. 5E) in the WT and the cfh3Δ cells that had been exposed to 1 M KCl for 15 min. We found that in both strains the distribution of the actin patches was similar, mostly observed at the poles and the medial region of the cells. In both cases it was possible to detect some defect in the morphology of the septal area (arrow in Fig. 5E) as was observed in the WT strain carrying the GFP-Bgs1 protein (Fig. 5D). We then wished to know whether the absence of the GFP-Bgs1 signal in the cfh3Δ cells incubated with KCl was due to a defect in the delivery of the protein to the cell membrane or to its rapid endocytosis. As shown in Fig. 5F, the GFP-Bgs1 fluorescent signal was observed at the poles and mid-
zone of the\textit{cfh3}\Delta cells when they had been incubated in the presence of both 1 M KCl and the actin-depolymerizing drug Latrunculin A for 15 min, suggesting that a blockade in endocytosis was able to compensate for the absence of Cfh3p. This result was confirmed by analyzing the localization of Bgs1p in \textit{cfh3}\Delta end4\Delta cells exposed to KCl for 15 min (Fig. 5F). To know whether Cfh3p regulated the stability of other proteins at the plasma membrane, we analyzed the localization of Bgs4p and Chs2p in the WT or the \textit{cfh3}\Delta strains that had been incubated in YES or YES supplemented with 1 M KCl for 15 min. We found that Bgs4p delocalized from the plasma membrane in the presence of KCl in both the WT and the \textit{cfh3}\Delta strains (supplemental
The SEL1 Repeats but Not the Prenylation Signal Are Required for Cfh3p Function—Cfh3p is a member of a family of adaptor proteins that is present through the biological scale and is characterized by the presence of several copies of SEL1 domains, which are a subfamily of the Tetratricho Peptide Repeat (TPR) domains (45). Additionally, Cfh3p has a C-terminal prenylation motif (CIIS, see Fig. 6A for a scheme of the structure of Cfh3p). We eliminated several or all of the SEL1 domains, and the CIIS prenylation motif, to see whether they were relevant for the function of Cfh3p. We analyzed the stability of the truncated forms of Cfh3p by performing Western blotting of the GFP-fused proteins. As shown in Fig. 6B, all the Cfh3p variants were stable. Then, we observed under the fluorescence microscope cells that carried the full-length Cfh3 protein, Cfh3 proteins in which all or several SEL1 domains had been deleted, or the Cfh3 protein in which the prenylation sequence had been eliminated, all fused to the GFP. We found that all these proteins localized to the cell poles and medial zone (Fig. 6C, and results not shown). Finally, we checked the relevance of these domains for Cfh3p function by analyzing the growth capacity of cells carrying the untagged mutated forms of Cfh3p on minimal medium plates supplemented with 0.2 M MgCl₂ incubated at 34 °C. As shown in Fig. 6D, elimination of some or all of the SEL1 domains abrogated function while the protein lacking the CIIS motif was able to support growth in the presence of MgCl₂ (Fig. 6D).

**DISCUSSION**

In this work we have characterized the function of the Cfh3 protein. Matsuo et al. (38) suggested that Cfh3p/Chr4p would be a regulator of Chs2p, a chitin synthase-like protein that lacks such activity and is required for the stability of the contractile actomyosin ring during its contraction (46, 47), because they found that in a cha4Δ mutant Chs2p could be observed at the ring and in some cytoplasmic dots. The following results are inconsistent with this conclusion. (i) In our previous work (46, 47) and in this work we observed that Chs2p localized at internal vesicles in both the WT and the cfh3Δ strain (supplemental Fig. S4), depending on the culture conditions. (ii) We did not find any genetic interaction between the cfh3Δ and the chs2Δ mutants. (iii) The chs2Δ mutant showed a genetic interaction with the myosin mutants (47) but the cfh3Δ mutant did not (this work). (iv) The localization of Chs2p under stress conditions was the same in the WT and the cfh3Δ mutant (supplemental Fig. S3). (v) The cellsAΔ cells were not sensitive to stress conditions (supplemental Fig. S5).

Our results point to a role of Cfh3p as a protein required in glucan synthesis, whose absence is more detrimental for growth under certain stress conditions. We found that in a cfh3Δ mutant there was a small reduction in the glucan synthase activity and in the amount of β-glucan. This reduction was similar to that found in the glucan synthase-mutant cps1-191 (Fig. 2, B and D). Although we cannot rule out some regulation of other glucan synthases, the facts that a mutant in the bgslΔ glucan synthase was not sensitive to stress, that a low concentration of Calcofluor could not stain the septa in the cfh3Δ mutant, and that the morphology of the cfh3Δ cps1-191 cells was similar to that of the bgslΔ cells maintained alive with an osmotic support (3) suggest that the defect in the β-glucan synthesis in the cfh3Δ cells would be due to a defect in the activity of Bgs1p. A defect in this glucan synthase is expected to result in a small defect in the β-glucan content since Bgs1p is responsi-
Cfh3p Regulates Bgs1p

The effect of stress was abolished when endocytosis was pre-observed at the cell periphery when Cfh3p was absent (Fig. 5).

Cfh3p carries out a hitherto undescribed regulation of the synthesis of this polymer.

The most relevant feature in the Cfh3p molecule is the presence of five SEL1 repeats. In S. pombe, there are only seven proteins that bear SEL1 domains. Four of these proteins (Cfh1p to Cfh4p, Ref. 38) share the highest similarity with the chitin synthase regulators ScChs4p and CaChs4p (20, 21), which also bear SEL1 domains (see Fig. 6). Proteins bearing SEL1 domains share a modular structure with several α-helices, which gives them a solenoid structure, although normally share low levels of similarity in their primary sequences (32). Thus, it is not surprising that Cfh3p is 26 and 28% identical to ScChs4p and CaChs4p, respectively, and that these identity values only increase to 29 and 30%, respectively, when the comparison is restricted to the SEL1 domains. Our results show that Cfh3p plays a role in the regulation of glucan synthesis, in agreement with the idea that in S. pombe Chs-like proteins have maintained a general function not related to chitin synthesis, as reported for Chs2p and Cfr1p (42, 46, 47). In this case, the function would be to ensure the stabilization of enzymes involved in cell wall synthesis at the plasma membrane. It will be interesting to determine whether each of the other Cfh proteins regulates the Bgs2p, Bgs3p, and Bgs4p glucan synthases by stabilizing them at the plasma membrane or if any of the other Cfh proteins play some role in cell separation through an interaction with septins.

Cfh3p, ScChs4p, and CaChs4p also share the presence of a prenylation site. We found that the prenylation motif was not essential for Cfh3p function. In the case of S. cerevisiae, a Chs4 protein lacking the prenylation site was also found to be able to complement a chs4Δ mutant (22, 25), although this domain seems to be required for a robust chitin synthase III activity (23). Deletion of the SEL1 domains in Cfh3p resulted in a loss of function. In S. cerevisiae, the region of Chs4p containing the SEL1 domains is able to complement a chs4Δ mutant (22, 24, 25). The region of the C. albicans Chs4 protein that contains the SEL1 domains is able to complement the S. cerevisiae chs4Δ mutant (21). These results show that these domains are relevant for the function of this family of proteins. It is known that there are SEL1 domain proteins acting as adaptors in multiprotein complexes involved in different cellular processes such as cell cycle control, ER-associated protein degradation or bacterial virulence (32). Our results, together with those found in S. cerevisiae and C. albicans, show that protein complexes involving SEL1-domain proteins participate in cell wall synthesis and morphogenesis in different organisms.

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REFERENCES

1. Humbel, B. M., Konomi, M., Takagi, T., Kamasawa, N., Ishijima, S. A., and Osumi, M. (2001) Yeast 18, 433–444
2. Durán, A., and Pérez, P. (2004) in The Molecular Biology of Schizosaccharomyces pombe (Egel, R., ed), pp. 269–279, Springer, Heidelberg
