Homologous Subunits of 1,3-Beta-Glucan Synthase Are Important for Spore Wall Assembly in Saccharomyces cerevisiae

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During sporulation in Saccharomyces cerevisiae, the four haploid nuclei are encapsulated within multilayered spore walls. Glucan, the major constituent of the spore wall, is synthesized by 1,3-β-glucan synthase, which is composed of a putative catalytic subunit encoded by FKS1 and FKS2. Although another homolog, encoded by FKS3, was identified by homology searching, its function is unknown. In this report, we show that FKS2 and FKS3 are required for spore wall assembly. The ascospores of fks2 and fks3 mutants were enveloped by an abnormal spore wall with reduced resistance to diethyl ether, elevated temperatures, and ethanol. However, deletion of the FKS1 gene did not result in a defective spore wall. The construction of fusion genes that expressed Fks1p and Fks2p under the control of the FKS2 promoter revealed that ascis transformed with FKS2p-driven Fks1p and Fks2p were resistant to elevated temperatures, which suggests that the expression of FKS2 plays an important role in spore wall assembly. The expression of FKS1p-driven Fks3p during vegetative growth did not affect 1,3-β-glucan synthase activity in vitro but effectively suppressed the growth defect of the temperature-sensitive fks1 mutant by stabilizing Rho1p, which is a regulatory subunit of glucan synthase. Based on these results, we propose that FKS2 encodes the primary 1,3-β-glucan synthase in sporulation and that FKS3 is required for normal spore wall formation because it affects the upstream regulation of 1,3-β-glucan synthase.

Sporulation in the budding yeast Saccharomyces cerevisiae provides a model system for studying the developmental processes of many eukaryotic cells. Sporulation in the a/a diploid cells is triggered by carbon starvation and is followed by meiosis and the formation of ascI that contain four haploid spores encapsulated within a spore wall (25, 33). The spore wall, which consists of four distinct layers, has been shown to play a central role in protecting the cell from environmental damage. The inner two layers consist of β-glucan and mannan (4), components that are similar to those found in the vegetative cell wall. In contrast, the outer layer consists of chitosan, a polymer of β-1,4-linked glucosamine, and the outermost layer consists of dityrosine, both of which are specific to the spore wall (3, 4, 5, 6, 43). Previous investigations of spore wall formation have focused on the specific components of the spore wall, such as chitosan and dityrosine. The mechanism of assembly of spore walls, including the synthesis of the inner two layers, remains unclear. We speculate that β-glucan is also largely responsible for spore resistance to environmental damage, since glucan is the major constituent of the spore wall (4) and provides rigidity to the cell wall during vegetative growth (36).

In yeast, glucan is constituted predominantly by 1,3-β-glucan, which is synthesized by 1,3-β-glucan synthase (GS), which in turn consists of a catalytic and a regulatory subunit. Two genes for the putative catalytic subunit of GS in budding yeast have been identified: FKS1, which synthesizes 1,3-β-glucan, the main structural component of the cell wall (12, 17), and FKS2, which was identified by virtue of its cross-hybridization with FKS1 (31). The regulatory subunit copurifies with glucan synthase and is encoded by RHO1 (13, 38). Rho1p acts as a molecular switch that monitors and receives upstream signals of cell morphogenesis. A third Fks1p homolog protein, Fks3p (Ymr306wp), was found by homology searching and shares 56% identity with Fks1p and Fks2p (31). FKS1 and FKS2 encode a pair of integral membrane proteins with 16 predicted transmembrane domains that share 88% identity. Deletion of FKS1 leads to a decrease in the level of glucan and an increase in the chitin and mannanprotein levels in the cell wall (27). Deletion of FKS2 causes no obvious cell wall defect, although the fks1 fks2 double mutant is nonviable (17, 31), which suggests that in vegetative growth Fks1p and Fks2p are alternative subunits with essentially overlapping functions. However, the role of FKS3 has not been clarified. The fks3-null mutant has no apparent cell wall defects and there are no genetic interactions between FKS3 and FKS1 or FKS2 (11). A synthetic genetic array analysis revealed no synthetic interactions with the fks3-null strain (27). In the fission yeast Schizosaccharomyces pombe, four genes have been reported for the putative GS catalytic subunits, i.e., bgs1~/cps1~, bgs2~, bgs3~, and bgs4~, the sequences of which share high-level homology with those of FKS1 and FKS2 (9, 10, 18, 29, 30). The bgs1~, bgs3~, and bgs4~ genes are essential for vegetative growth and have been shown to be localized to specific sites of cell wall growth (9, 10, 18, 30). The bgs2~ gene is not
expressed during vegetative growth but is essential for spore wall formation. GS activity is diminished in sporulating bgs2Δ diploids (29). Bgs2p localizes to the ascospore periphery and is required for appropriate spore wall maturation (28, 29).

In this study, we analyzed the roles of glucan and glucan synthase in sporulation by yeast. It has been reported that fks2 mutants form aberrant spores (31). Recently, Huang et al. (16) have reported that Smk1p, which is a mitogen-activated protein kinase, binds to the 1,3-β-glucan synthase Fks2p and that Fks2p activity is increased in the smk1 mutant. It has been suggested that Fks2p plays an important role in spore wall assembly through protein kinases. However, nothing is known about the function of FKS3 in spore wall formation. Our investigation of the roles of two glucan synthase genes and a homologous gene at meiosis and sporulation provides novel information on spore wall assembly.

MATERIALS AND METHODS

Plasmids. The plasmids used in this study are listed in Table 1. For DNA manipulations, standard techniques were used. The 6.0-kb BglII-XhoI fragment that includes FKS3 (derived from the Yepl13 genomic bank) (44) was cloned into the integrating vector pRS305 (42) to generate pYO2279. The 6.0-kb BglII-XhoI fragment that includes FKS3 was cloned into pBluescript to generate pYO2729. The plasmids pYO22730, pYO2731, and pYO2733 contain the FKS2, FKS2, and FKS3 genes, respectively, cloned into the pUpl site of pYO326, and pYO2732 contains a fragment that has the FKS3 gene cloned into the pUpl site of pYO326. We constructed the FKS1p-FKS2-HA fusion (hemaggulitin-tagged FKS2 gene driven by the FKS1 promoter) and the FKS2p-FKS1-HA fusion, and as controls, we constructed the FKS3p-FKS1-HA and FKS3p-FKS2-HA fusions as follows. The FKS1 promoter and the FKS2-2HA (two tandem repeats of the hemaggulitin tag) gene fusion were fused at the first ATG codon of the open reading frame (ORF) by using PCR, and the fusion was cloned into the pRS314 plasmid to generate pYO2736. The same procedure was used for the FKS1 promoter and the FKS2-2HA gene fusion, the FKS2 promoter and the FKS1-2HA gene fusion, and the FKS2 promoter and the FKS2-2HA gene fusion to generate pYO2737, pYO2738, and pYO2739, respectively. The fused FKS1 promoter and FKS3-2HA gene fusion were cloned into pRS316 and pYO326 to generate pYO2740 and pYO2742, respectively. The pYO2764 plasmid is a pRS305 derivative that contains the FKS3-2HA gene fusion, the stop codon of which was replaced with sequences that encode the 2× HA tag.

Strains. Escherichia coli strains CEN and JMM10 were used as plasmid hosts. The yeast strains used in this study are listed as derivatives of the rapidly sporulating strain SKI (Table 2) (22) and as derivatives of YPH (Table 3) (22). Strains were maintained using standard techniques and other genetic procedures (21). The strains constructed in this study will be deposited in the Yeast Genetic Resource Center Japan (http://yeast.lab.nig.ac.jp/nig/english/index.html or http://bio3.tokyo.jst.go.jp/jst/english/).

The S799 and S800 derivatives of SKI (Table 2) were constructed by M. Lichtén (National Institutes of Health) and provided by K. Ohita (Riken). NKY899, NKY900, NKY486, and NKY487 were provided by N. Kclekner (Harvard University). The FKS1 genes in YCO406C and YCO406D were replaced with the fls1::URA3 allele in plasmid pYO1753, and the FKS1 genes in YCO406C and YCO406D were replaced with the fls1::LEU2 allele in plasmid pYO494. YOC406C, YOC406D, YOC4070, YOC4071, YOC4114, and YOC4115 were constructed by using PCR-mediated gene disruptions as previously described (40). Primers were used to amplify the CgLEU2 (in plasmid pYO2241) or CgURS3 (in plasmid pYO2244) gene of Candida glabrata, together with flanking sequences derived from the upstream and downstream regions of the FKS2 and FKS3 genes. YOC4070 was crossed with YCO4062, and the resulting diploid was sporulated and subjected to tetrad analysis to generate YOC4078 and YOC4079. The various FKS1 and FKS2 expression plasmids were introduced into fls1 Δ bks2 double-null mutants that harbored the URA3-borne FKS1 plasmid, and this plasmid was subsequently eliminated by treatment with 5′-fluoroorotic acid. In the resultant cell line, either the FKS1 or FKS2 gene was expressed in the absence of endogenous Fks1p and Fks2p under the control of the FKS1 or FKS2 promoter. Thus, we established the strains YOC4084, YOC4085, YOC4086, and YOC4087.

For the derivatives of YPH (Table 3), the detailed constructions of YCO1001 (FKS1 and YCO1087 (fsk1-1154 FKS2) are described elsewhere (41). Briefly, YCO1087 is a temperature-sensitive strain with a deletion of both FKS1 and FKS2 and a mutant allele of fks1 (fsk1-1154) that is integrated at the ADE3 locus. YCO1001 is the wild-type control for YCO1087, with FKS1 and FKS2 deleted and the wild-type FKS1 gene integrated at the ADE3 locus.

Media and synchronous sporulation. Yeasts were grown vegetatively in YPD (1% yeast extract, 2% peptone, 2% glucose) and SD (0.67% yeast nitrogen base without amino acids and 2% glucose) supplemented with the appropriate amino acids (1% yeast extract, 2% peptone, 2% glucose) and SD (0.67% yeast nitrogen base without ammonium sulfate and amino acids, 0.05 M potassium phosphate, pH 7.4, containing 50 mM EDTA, 2% sodium dodecyl sulfate, and 40 mM mercaptoethanol) and extensively washed with water. The alkali-soluble (AS) and alkali-insoluble (AI) fractions were extracted with 1 N NaOH solution. Borohydride was removed after neutralization with acetic acid and evaporation in the presence of methanol.

Immunoelectron microscopic analysis. Thin sections of the yeast cells were prepared by the freeze-substituted fixation method as described previously (19), except that HPM010 (BALT-TEC AG) or EM wee (Leica, Solms, Germany) was used for cell freezing and EMAFS (Leica) was used for warming. For 1,3-glucan immunolabeling, a mouse monoclonal antibody (MAb) against 1,3-glucan was used for cell freezing and EMAFS (Leica) was used for warming. For 1,3-glucan immunolabeling, a mouse monoclonal antibody (MAb) against 1,3-glucan was used for cell freezing and EMAFS (Leica) was used for warming.

In the subsequent cell line, the 1,3-glucan determinations.

1,3-β-Glucan and 1,6-β-glucan determinations. Collection of spores by using a Percoll gradient was performed as described previously (4). For cell wall 1,3- and 1,6-β-glucan determinations, spores were boiled twice in 50 mM Tris-HCl, pH 7.4, containing 50 mM EDTA, 2% sodium dodecyl sulfate, and 40 mM β-mercaptoethanol and extensively washed with water. The alkali-soluble (AS) and alkali-insoluble (AI) fractions were extracted with 1 N NaOH solution. Borohydride was removed after neutralization with acetic acid and evaporation in the presence of methanol.

TABLE 1. Plasmids used in this study

| Name Description Reference | Reference |
|----------------------------|-----------|
| pRS305 Yeast integration vector marked with LEU2 | a |
| pRS306 Yeast integration vector marked with URA3 | a |
| pRS314 Yeast-E. coli shuttle vector (CEN) with TRP1 | a |
| pRS315 Yeast-E. coli shuttle vector (CEN) with LEU2 | a |
| pRS316 Yeast-E. coli shuttle vector (CEN) with URA3 | a |
| pBluescript SK E. coli high-copy-number vector | b |
| pYO325 Yeast-E. coli shuttle vector (2μm) with LEU2 | c |
| pYO327 Yeast-E. coli shuttle vector (2μm) with URA3 | c |
| pYO901 FKS1p-loc2 fusion | c |
| pYO902 FKS2p-loc2 fusion | c |
| pYO946 Δfks1::LEU2 | c |
| pYO965 Δfks2::LEU2 | c |
| pYO991 Δfks3::LEU2 | c |
| pYO1751 Δfks1::URA3 | c |
| pYO1752 Δfks2::URA3 | c |
| pYO1753 Δfks3::URA3 | c |
| pYO2241 pBS-CgLEU2 | f |
| pYO2244 pBS-CgURA3 | f |
| pYO2367 ROM2 in pRS316 | d |
| pYO2728 FKS3 in pYES305 | g |
| pYO2739 FKS3 in pBluescript SK | g |
| pYO2730 FKS1 in pRS326 | g |
| pYO2731 FKS2 in pRS326 | g |
| pYO2732 FKS3 in pRS325 | g |
| pYO2739 FKS3 in pRS326 | g |
| pYO2736 FKS1p-FKS1-HA fusion in pRS314 | g |
| pYO2737 FKS1p-FKS2-HA fusion in pRS314 | g |
| pYO2738 FKS2p-FKS1-HA fusion in pRS314 | g |
| pYO2739 FKS2p-FKS2-HA fusion in pRS314 | g |
| pYO2740 FKS1p-FKS3-HA fusion in pRS314 | g |
| pYO2742 FKS1p-FKS3-HA fusion in pRS326 | g |
| pYO2764 FKS3-HA fusion in pRS305 | g |

a, a. Sikorski and Hieter (42); b, Stratagene; c, Qudota et al. (38); d, Sekiya-Kawasaki et al. (41); e, Inoue et al. (17); f, Sakamoto et al. (40); g, this study.
Determination of the total hexose content was performed using the phenol-sulfuric acid procedure with glucose as the standard (14). The amount of 1,3-β- and 1,6-β-glucans in the AI and AS fractions digested by 1,3-β- or 1,6-β-glucanases was quantified by measuring the release of reducing sugars after digestion by the p-amino-phenyl-benzoic acid hydrazide method, as described previously (15). For 1,3-β-glucanase digestion, incubation was carried out with recombinant Thermotoga neapolitana LamA (9 ng/25 to 50 μg) bearing a plasmid provided by Vladimir Zverlov (Institute of Molecular Genetics, Moscow, Russia) (45), in sodium phosphate-citrate buffer (50 mM, pH 6.2) for 24 h at 80°C. For 1,6-β-glucanase (2) in pichia pas-1154, the culture supernatant was collected by centrifugation (4,000 g for 5 min), concentrated 20-fold on Amicon-Ultra (Millipore), and prepared for hydrophobic interaction chromatography as described previously (2). Hydrophobic interaction chromatography was carried out on a Phenyl Superose HR 5/5 column (Pharmacia) as described previously (2). Fractions containing 1,6-β-glucanase activity (2) were pooled, dialyzed, and concentrated sixfold on Amicon.

Spore viability of ascospores at elevated temperatures and in the presence of diethyl ether and ethanol. Assays for measuring the resistance of spores to ether and heat are described elsewhere (5,26). Sporulated cultures were tested for cell viability after exposure to 50% diethyl ether, a temperature of 55°C, and 25% ethanol for the times indicated in the figures. Cells were sampled at the times indicated in the figures, diluted to between 300 and 500 colonies per plate, and plated onto YPD plates. The cell viability for each mutant was estimated by counting the viable colonies on YPD plates and comparing the numbers to the numbers of colonies at 0 min. The results are expressed as means ± standard deviations.

Quantitative 1,3-β-glucan measurements. The amount of 1,3-β-glucan per cell was measured using aniline blue as described previously (41). The fluorescence of dye-bound 1,3-β-glucan was quantified using a spectrofluorophotometer (RF-5300PC; Shimadzu, Kyoto, Japan) with an excitation wavelength of 400 nm/slit width of 3 nm and an emission wavelength of 460 nm/slit width of 3 nm. It was measured using aniline blue as described previously (41). The fluorescence of dye-bound 1,3-β-glucan was quantified using a spectrofluorophotometer (RF-5300PC; Shimadzu, Kyoto, Japan) with an excitation wavelength of 400 nm/slit width of 3 nm and an emission wavelength of 460 nm/slit width of 3 nm.
FIG. 1. Electron microscopic analysis of the asc of the fks2 and fks3 mutants. Electron microscopic images of the wild-type (WT) (A and D) and fks2 (B and E) and fks3 (C and F) mutant cells at a late stage of sporulation are shown. Panel D presents an enlarged image of the spore wall shown in panel A. Panel E shows an enlarged image of the abnormal outer wall (arrow) shown in panel B. Panel F shows an enlarged image of the abnormal outer membrane (arrow) shown in panel C. (G to I) Immunoelectron micrographs of the wild type (G) and fks2 (H) and fks3 (I) mutants using an anti-1,3-β-glucan antibody. (J to M) A set of thin serial sections of an fks2 mutant spore. The abnormal structures are inside the spore. These structures are continuous across the sections. Abnormal structures (arrows), spore walls (SW), nuclei (N), and mother cell walls (CW) are indicated.
cells. After centrifugation at 100,000 \( \times g \) for 30 min, the pellet was suspended in a buffer that contained 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 33% glycerol, and this suspension was used as the membrane fraction. GS activity was measured according to a previously described procedure (17) using an excess of GTP-Gs.

**Western blotting.** Protein samples for Western blotting analysis were prepared by disrupting yeast cells with glass beads. The crude lysate was centrifuged at 1,500 \( \times g \) for 5 min to sediment the cell debris and unbroken cells. The supernatant was centrifuged at 100,000 \( \times g \) for 30 min in the model RPS/T rotor (Hitachi) with Himac CP 65 (Hitachi). The resultant pellet was subjected to SDS-PAGE, Western blot analysis using a polyclonal antibody against Rho1p (38).

**Pull-down assay for active Rho1p.** The pull-down assay for active Rho1p was performed as previously described for mammalian RhoA and yeast Rho1p (23; K. Kono, M. Abe, S. Nogami, M. Nishizawa, S. Morishita, D. Pellman, and Y. Ohya, submitted for publication) with some modifications. The region that encodes the Pkc1p Rho1p-binding domain (PRBD) was cloned into pGEX-3X and expressed, purified, and bound to glutathione-Sepharose 4B beads (GE Healthcare, Milwaukee, WI) according to the manufacturer’s protocol.

**RESULTS**

**FKS2 and FKS3 are essential for spore wall formation.** To investigate the sporulation defect in mutants with deletions of the *FKS* homologous genes, we constructed *fks1*, *fks2*, and *fks3* deletion strains in the SK1 genetic background (22). These homozygous diploid mutants grew as well as the wild-type cells during vegetative growth and produced viable ascis as efficiently as the parental strain upon transfer to sporulation medium (data not shown). To obtain further insight into the nature of sporulation in these mutants, we employed the freeze-substituted fixation method to observe the ascis of wild-type cells and *fks1*, *fks2*, and *fks3* mutant diploid cells by electron microscopy (EM). A typical wild-type ascus and spore wall with several layers are shown in Fig. 1A and D. After maturation of the spore wall, most *fks2* mutant spores were enveloped by an abnormal spore wall, which contained abnormal structures (Fig. 1B and E). We observed sets of serial sections with the same structures at the edge of the *fks2* mutant spore (Fig. 1J to M). The abnormal structures were observed on the inside of the spore wall. Careful examination of serial sections revealed that these structures were continuous across the sections rather than being separated structures. Observations of the serial sections of the structures in the middle of the *fks2* mutant spore walls revealed that the small circular structures were also continuous across the sections (data not shown). These morphological observations suggest that the abnormal structures are tubelike. To investigate the distribution of glucan, immunoelectron microscopy (immunoEM) was carried out using an anti-1,3-\( \beta \)-glucan antibody. In a typical wild-type ascus, signals for glucan were detected only in the innermost layers of the mature spore wall (Fig. 1G). However, in the spore walls of the *fks2* mutants, the signals for glucan were detected only in the innermost layers (Fig. 1H). We were unable to stain the tubular structures with the anti-glucan antibody (Fig. 1H). These morphological observations suggest that the *FKS* genes may perform essential functions related to the formation of the layers of the spore walls and are consistent with previous observations of the *fks2* mutants (16).

Most of the *fks3* mutant spores were enveloped by abnormal spore walls, which were of uneven thickness and in some cases included cytoplasm between the inner and outer layers (Fig. 1C...
and F). By using immunoEM, the signals for glucan were found to be delocalized between the inner and outer layers of the spore walls of the fks3 mutants. Finally, we observed the spore walls of the fks2 fks3 mutant diploid cells by using EM. Most of the fks2 fks3 mutant spores were enveloped by abnormal spore walls of uneven thickness similar to the fks3 mutant spore walls. By using immunoEM, the signals for glucan were found to be delocalized in the spore walls of the fks2 fks3 mutants, similar to those in the fks3 mutant spore walls (data not shown). These morphological observations suggest that the FKS2 and FKS3 genes perform essential functions in the formation of the layers of the spore wall.

Data obtained from genome-wide microarray studies indicate that the amount of FKS1 mRNA declines to background levels during meiosis, that the level of FKS2 mRNA increases moderately during meiosis, and that FKS3 is expressed in a meiosis-specific fashion as a middle gene (7). To verify these data, we prepared total-RNA samples from cells at different stages of sporulation and investigated the presence of FKS1, FKS2, and FKS3 mRNAs by Northern analysis. Consistent with the microarray data, the levels of FKS1 transcripts decreased according to the progression of sporulation whereas the levels of FKS2 transcripts increased severalfold under the same conditions. The FKS3 transcripts were strongly induced during the first 2 h after transfer to sporulation medium, with maximal accumulation at 4 h (data not shown). Our results confirm the genome-wide results and indicate that FKS2 and FKS3 genes are responsible for the formation of spore walls.

The AI fraction of the ascospore cell wall that represents the skeleton of the cell wall is composed mostly of glucan and chitosan. In contrast, the AS fraction of the ascospore cell wall that is the amorphous cement is composed mainly of mannan and glucan. High ratios of the hexose concentrations in the AI fractions to the total hexose concentrations in the AS and AI fractions may indicate a tight structural organization of the cell wall. Accordingly, Fig. 2A suggests that the ascospore cell walls of the fks2 fks3 and fks2 mutants were less structured than the cell walls of the ascospores of the wild type and the fks1 and fks3 mutants. Figure 2B shows that the ascospore cell walls of all wild-type and mutant strains contained a significant amount of 1,3-β-glucans. The concentrations of 1,3-β-glucans varied, however, among the different mutants and the AS and AI fractions. The most striking cell wall perturbations were seen in the fks2 fks3 mutant, where the largest amount of 1,3-β-glucan was released from the AI fraction by the 1,3-β-glucanase. This result was in agreement with EM data and suggested that the cell walls of the ascospores of this mutant were the least organized. In addition, the amounts of 1,3-β-glucan in the AS fractions of the fks2 fks3 and fks2 mutants were the smallest. These variations in 1,3-β-glucans were not compensated for by a modification of 1,6-β-glucans since the degradation of the AI and AS fractions by the 1,6-β-glucanase never exceeded 3% of the total hexose concentration in the AI and AS fractions (data not shown).

**Sensitivities of the asci of fks2 and fks3 mutants to diethyl ether, heat, and ethanol.** It has been shown that the asci of several mutants that have defects in spore wall production are more sensitive to certain stresses, including exposure to diethyl ether, heat, and ethanol, than those of the wild type (5, 6, 8, 26). We tested the resistance of the asci of the wild type and the fks1, fks2, fks3, and fks2 fks3 mutant diploids to diethyl ether. The asci of the fks2, fks3, and fks2 fks3 mutants were 200- to 500-fold more sensitive to a 9-min exposure to diethyl ether than the asci of the wild type (Fig. 3A), although the asci of the fks1 mutant had a survival rate that was similar to that of the asci of the wild type (Fig. 3A). Next, to confirm the stress-sensitive phenotype, we tested all of the mutants for resistance to heat and ethanol. We examined the thermotolerance of the wild-type and mutant spores after exposure to 55°C for various time periods. As shown in Fig. 3B, the asci of the fks2, fks3, and fks2 fks3 mutants were more sensitive than the asci of the wild type to the elevated temperature. Again, the asci of the fks1 mutants had a survival rate that was similar to that of the asci of the wild type. Finally, we examined the viability of the wild-type and mutant spores after exposure to 25% ethanol for various periods of time. The asci of the fks2, fks3, and fks2 fks3 mutants were more sensitive to ethanol than those of the wild type, whereas the asci of the fks1 mutants had a survival rate that was similar to that of the asci of the wild type (data not shown). The correlation of these phenotypic effects in the fks2 and fks3 mutants showing mutational effects on spore wall structure suggests that spore wall formation is linked to spore resistance to stresses, which include exposure...
to diethyl ether, heat shock, and ethanol. In addition, the sensitivities of the asci of the fks2 fks3 mutants to these stresses (based on survival rates) were the same as those of the fks3 mutants, which suggests that the fks2 mutation confers no effect additional to that of the fks3 mutation.

Expression of FKS2 under the control of the FKS2 promoter is important for spore wall assembly. Since Fks1p and Fks2p are highly homologous, they may have overlapping functions in sporulation. Therefore, we investigated whether the overexpression of Fks1p or Fks3p suppressed the spore defect of fks2 mutants. Multiple copies of the FKS1 gene partially suppressed the heat-sensitive phenotype of the fks2 mutant asci (Fig. 4). We postulated that this partial suppression might be due to the difference in the expression levels of Fks1p and Fks2p. Therefore, we constructed plasmids that contained FKS1p-driven FKS1, FKS1p-driven FKS2, FKS2p-driven FKS1, and FKS2p-driven FKS2 (Fig. 5A), and each of these plasmids was introduced into the fks1 fks2 double-null mutants. All of the strains (YOC4084, YOC4085, YOC4086, and YOC4087) were viable and produced asci of which more than 80% had four spores. Western blotting analysis with an anti-HA antibody revealed that these proteins were present in all the transformed strains (data not shown). We examined the asci of the wild type and the transformed mutants following exposure to 55°C for various periods of time. The asci of the fks1 fks2 mutant cells transformed with the FKS1p-driven Fks1p and Fks2p plasmids showed heat sensitivities similar to those of the fks2 mutant asci, whereas the asci of the fks1 fks2 mutant cells transformed with the FKS2p-driven Fks1p and Fks2p plasmids showed heat sensitivities similar to those of the wild-type asci (Fig. 5B). These results suggest that the molecular function of Fks2p in spore wall assembly resembles that of Fks1p and that the induction of FKS2 serves an important role in spore wall assembly. In contrast, multiple copies of the FKS3 gene did not suppress the heat-sensitive phenotype of the fks2 mutant spores (Fig. 4). This result and the expression data on the FKS3 gene expressed at sporulation suggest that Fks3p does not have any function overlapping with that of Fks2p in sporulation.

Enrichment with Fks3p is not observed during purification of GS in vegetative growth. To determine if Fks3p is incorporated into a component of the GS complex, we monitored the amount of Fks3p during the purification of GS. We constructed a plasmid that expressed HA-tagged Fks3p under the control of the FKS1 promoter (FKS1p-FKS3) (Fig. 6A). Western blot analysis with an anti-HA antibody revealed that this protein was present in the membrane fractions of transformed strains (Fig. 6B). We measured the in vitro GS activities of the membrane fraction and the purified GS fraction of cells that expressed FKS1p-FKS3. Endogenous Fks1p was enriched in the partially purified GS fractions (Fig. 6C), and the specific activity of GS increased approximately 200-fold (Fig. 6E). However, FKS1p-driven Fks3p-HA was not detected with the anti-HA antibody (Fig. 6D). These results suggest that Fks3p is
lost during the preparation of the purified GS fraction and that Fks3p is not a tightly bound component of the GS complex.

The in vivo glucan synthesis defects are rescued by the expression of Fks3p. To determine the molecular function of Fks3p in 1,3-β-glucan synthase, we examined whether the expression of the FKS1p-FKS3 allele could suppress the growth defect of the fks1-1154 Δfks2 mutant, a temperature-sensitive mutant of GS (hereinafter referred to as the fks1-1154 mutant). Recently, Sekiya-Kawasaki et al. (41) have identified seven multicopy suppressors of the fks1-1154 mutation as positive regulators of GS. Therefore, we examined the growth phenotype of the fks1-1154 mutant cells, which were able to grow at 25°C but failed to grow at temperatures above 35°C. Figure 7A shows the growth at various temperatures (33 to 37°C). Robust growth was observed for fks1-1154 mutant cells transformed with multiple copies of FKS1p-FKS3, which indicates that the FKS1p-FKS3 allele expressed from a high-copy-number plasmid is able to suppress the fks1-1154 mutation.

To examine the possible mechanisms of suppression whereby multiple copies of FKS1p-FKS3 restore the defect in 1,3-β-glucan synthesis, we tested in vivo glucan synthesis in fks1-1154 mutant cells that expressed Fks3p under the control of the FKS1 promoter. First, we stained the mutant cells with aniline blue, which is a fluorescent dye that interacts preferentially with 1,3-β-glucan. As reported previously (41), most of the fks1-1154 mutant cells appeared to lose their staining signal specifically in the bud at the restrictive temperature (Fig. 7B, panel a). The fks1-1154 mutant cells that were transformed with multiple copies of FKS1 and the FKS1p-FKS3 mutant cells exhibited uniform staining of the cell walls on the cell surface similar to the wild type (Fig. 7B, panels c and e). We quantified the populations of cells that exhibited lower levels of 1,3-β-glucan staining in the bud. Four hours after the shift to 35°C,
more than 70% of the fks1-1154 mutant cells with vector alone exhibited a loss of glucan staining in the bud. In contrast, the overexpression of FKS1 and FKS1p-FKS3 removed the defects in glucan staining in the fks1-1154 mutant cells (Fig. 7C). Next, we quantified the total amount of 1,3-β-glucan in fks1-1154 mutant cells with FKS1 and FKS1p-FKS3 by using a fluorescence spectrophotometer with aniline blue staining. The fks1-1154 mutant cells that were transformed with multiple copies of FKS1p-FKS3 showed increased levels of glucan (Fig. 7D). From these results, we conclude that multiple copies of FKS1p-FKS3 have a positive effect on the in vivo GS of fks1-1154 mutant cells.

To examine the positive effect of Fks3p in vitro, we measured the in vitro GS activity of the membrane fraction from the fks1-1154 mutant cells that overexpressed FKS1p-FKS3, which we found to be as low as that from cells with the vector alone (Fig. 7E). This result was similar to that of ROM2, a multicopy suppressor of the fks1-1154 mutation. The overexpression of Rom2p, the GDP-GTP exchange factor of Rho1p (37), increased in vivo GS activity by shifting the equilibrium of Rho1p to the GTP-bound state. Since in vitro GS activity was assayed in the presence of an excess amount of GTPγS, which is a nonhydrolyzable analog of GTP (17), all of the Rho1p was assumed to be in the active state in this in vitro GS assay. These results support the idea that the expression of FKS1p-FKS3 increases in vivo GS activity by shifting the equilibrium of Rho1p to the GTP-bound state.

**Relationship between Fks3p and Rho1p.** To examine whether the expression of FKS1p-FKS3 increases in vivo GS activity through Rho1p activation, fks1-1154 mutant cells with FKS1p-FKS3 were transformed with a plasmid that expressed the active form of Rho1p. If the expression of FKS1p-FKS3 activated GS by converting Rho1p into the active form, no additional effect on GS activity should be observed. We quantified colorimetrically the total amount of 1,3-β-glucan in fks1-1154 mutant cells with plasmids by using aniline blue staining. No additional effect on GS activity was observed, which suggests that Fks3p activates GS by converting Rho1p into the active form (Fig. 8A). Next, to confirm Rho1p activation by Fks3p in vivo, we measured the amount of the active form of Rho1p. The state of Rho1p was monitored in a pull-down assay using the GST-fused Rho1p-binding domain of Pkc1p, since the Rho1p-binding domain of Pkc1p binds specifically to the active form of Rho1p (34). Our established system specifically pulls down the active form of Rho1p (GTP-fixed Rho1p), while the inactive form (GDP-fixed Rho1p) is not pulled down (K. Kono, M. Abe, S. Nogami, M. Nishizawa, S. Morishita, D. Pellman, and Y. Ohya, submitted for publication). As a control, we performed the pull-down assay for active Rho1p with fks1-1154 mutant cells and with fks1-1154 mutant cells transformed with the ROM2 gene. The active-Rho1p level was higher in the fks1-1154 mutant cells transformed with the ROM2 gene than in the untransformed fks1-1154 mutant cells (Fig. 8B), which is consistent with the previous results. Next, we performed the same pull-down assay using fks1-1154 mutant cells that overexpressed Fks3p under the control of the FKS1 promoter. The active-Rho1p level was higher in fks1-1154 mutant cells that overexpressed Fks3p than in fks1-1154 mutant cells without Fks3p overexpression (Fig. 8B). These results suggest that the overexpression of FKS1p-FKS3 has a positive effect on GS activity through the activation of Rho1p in vivo.

**Fks1p and Fks2p do not suppress the phenotypes of fks3 mutants.** To examine whether Fks3p has a role distinct from Fks1p and Fks2p at sporulation and whether FKS1 or FKS2 acts in the same pathway as FKS3 at sporulation, we introduced plasmids that overexpressed Fks1p, Fks2p, and Fks3p into fks3 mutant cells and examined the phenotypes of their
**A**

| Temperature (°C) | 25 | 34 | 35 | 36 | 37 |
|------------------|----|----|----|----|----|
| **FKS1 Δfks2**   | ![Bar Graph](image1.png) | ![Bar Graph](image2.png) | ![Bar Graph](image3.png) | ![Bar Graph](image4.png) | ![Bar Graph](image5.png) |
| **fks1-1154 Δfks2** | ![Bar Graph](image6.png) | ![Bar Graph](image7.png) | ![Bar Graph](image8.png) | ![Bar Graph](image9.png) | ![Bar Graph](image10.png) |
| **FKS1p-FKS3 (m/c)** | ![Bar Graph](image11.png) | ![Bar Graph](image12.png) | ![Bar Graph](image13.png) | ![Bar Graph](image14.png) | ![Bar Graph](image15.png) |
| **FKS1p-FKS3 (s/c)** | ![Bar Graph](image16.png) | ![Bar Graph](image17.png) | ![Bar Graph](image18.png) | ![Bar Graph](image19.png) | ![Bar Graph](image20.png) |
| **ROM2** | ![Bar Graph](image21.png) | ![Bar Graph](image22.png) | ![Bar Graph](image23.png) | ![Bar Graph](image24.png) | ![Bar Graph](image25.png) |
| **RHO1 (Q68L)** | ![Bar Graph](image26.png) | ![Bar Graph](image27.png) | ![Bar Graph](image28.png) | ![Bar Graph](image29.png) | ![Bar Graph](image30.png) |

**B**

- **fks1-1154 Δfks2**
- **fks1-1154 Δfks2 +p[FKS1]**
- **fks1-1154 Δfks2 +p[FKS1p-FKS3]**

**C**

**% cells with 1,3-β-glucan staining**

- **Wild type + vector**
- **vector**
- **FKS1**
- **FKS1p-FKS3H4**

**D**

**Fluorescence intensity (relative units)**

- **Wild type + vector**
- **vector**
- **FKS1**
- **FKS1p-FKS3H4**

**E**

**GS activity (nmol/min/mg)**

- **Wild type + vector**
- **vector**
- **FKS1**
- **ROM2**
- **FKS1p-FKS3H4**
spores. After exposure to 55°C, the asci of the fks3 mutant cells transformed with multiple copies of FKS3 had a survival rate similar to that of the wild-type asci (Fig. 9). However, multiple copies of the FKS1 or FKS2 gene did not restore the heat-sensitive phenotype of the fks3 mutant asci (Fig. 9). Next, we constructed plasmids that contained FKS3p-driven FKS1 and FKS3p-driven FKS2, both of which were introduced into the fks3 mutant cells. The asci of the fks3 mutant cells transformed with FKS3p-driven Fks1p and the Fks2p plasmid showed heat sensitivities similar to that of the fks3 mutant asci, which suggests that these genes do not restore the heat-sensitive phenotype (data not shown). Our findings suggest that Fks3p exerts a distinct effect on GS during spore wall maturation.

**DISCUSSION**

In budding yeast, the spore wall has been shown to play a central role in protecting the cell from environmental damage. In this paper, we demonstrate that Fks2p, the putative catalytic subunit of the glucan synthase, is required for spore wall maturation, consistent with the results reported by Huang et al. (16), and that Fks3p, which shares homology with the catalytic subunits, is also required for spore wall maturation. In addition, we found that the lack of the FKS2 gene and the FKS3 gene causes assembly defects in the 1,3-β-glucan layers and reduces the resistance to certain stresses.

The fks2 mutant shows abnormal morphology under EM. In addition, the abnormal features (a low ratio of the hexose concentration in the AI fraction to the total hexose concentration in the AI and AS fractions and a small amount of alkali-soluble 1,3-β-glucan) may represent a less organized cell wall in this mutant. In spite of abnormal spore wall morphology, the fks3 mutant shows a normal ratio of the hexose concentration in the AI fraction to the total hexose concentration in the AI and AS fractions. However, fine structural modifications could exist but could not be investigated with our enzymatic methodology since in contrast to those of the vegetative cells, the ascospore cell wall is very resistant to 1,3-glucan.

Based on these observations and previous studies, functional differences among glucan synthase subunits and their homologous proteins and their roles in glucan synthesis during the sporulation process are discussed.

**Differences among 1,3-β-glucan synthase subunits involved in spore wall assembly.** The assembly of the 1,3-β-glucan layers of spores requires the 1,3-β-glucan synthase, which is located in the spore plasma membrane. Two genes in *S. cerevisiae* encode putative catalytic subunits of the synthase. FKS1 encodes the subunit that is primary during vegetative growth and FKS2 appears to encode the subunit predominant during sporulation (17, 31). It has been shown that the expression of these genes is controlled differentially. FKS1 is regulated in the cell cycle and predominates during growth on glucose, whereas FKS2 is expressed in the absence of glucose, especially during sporulation (31). It has also been shown that Fks1p and Fks2p exhibit different sensitivities to some 1,3-β-glucan synthase inhibitors, such as L-773,560 and aerothricin (12, 24). In the present study, we show that Fks1p is functional in spore wall assembly and is able to compensate for the sporulation defect in fks2 ascis when expressed under the control of the FKS2 promoter. This finding suggests that there are few functional differences between Fks1p and Fks2p and that the regulation of expression of the glucan synthase gene plays an important role in spore wall assembly (Fig. 5B). Although the expression of the FKS2 gene is important for spore wall formation, the FKS1 gene may have a role in this process because, even in the fks2 mutant, the spore wall is formed and contains 1,3-β-glucan (Fig. 1H and 2). A residual amount of Fks1p expressed from its authentic promoter can substitute functionally for Fks2p in spore wall assembly, although it may not be sufficient for complete formation of the normal spore wall. In fission yeast, it has been reported that bgs1Δ, bgs3Δ, and bgs4Δ gene products under the control of the bgs2p promoter are unable to compensate for the sporulation defect in bgs2Δ (29), which suggests that each bgs gene product has a different role in sporulation, in contrast to the case for budding yeast.

**Function of FKS3 during spore wall assembly.** In this study, we investigated the molecular functions of Fks3p. We found that the overexpression of FKS1p-FKS3 effectively suppressed the growth defect of the fks1Δ-1154 mutant (Fig. 7A), which suggests that FKS1p-FKS3 has a positive effect on in vivo GS of fks1Δ-1154 cells. However, the overexpression of FKS1p-FKS3 did not increase in vitro GS activity (Fig. 6 and 7E). Therefore, we postulate that Fks3p has distinct functions for GS and may
regulate GS through the activation of a GS effector. We found that the active-Rho1p level increased in fks1-1154 mutant cells transformed with FKS1p-FKS3 relative to that in fks1-1154 mutant cells (Fig. 8), which suggests that the fks1-1154 mutant has a weak glucan synthase activity that can be stimulated by the activation of Rho1p by Fks3p and that the overexpression of FKS1p-FKS3 acts positively on GS through the activation of Rho1p in vivo. If Fks3p activates Rho1p during spore wall assembly, the active form of Rho1p (RHO1 [Q68L]) should complement the spore wall defect of the fks3 mutant. However, the asci of the fks3 mutant cells transformed with the RHO1 (Q68L) plasmid showed heat sensitivities similar to those of the fks3 mutant asci, which indicates that these genes do not restore the heat-sensitive phenotype (data not shown). In addition, the spore wall defects in the fks3 mutant were not restored when both FKS1 and FKS2 were introduced via an expression plasmid (Fig. 9), which suggests that the relationship between Rho1p and Fks1/2p at sporulation is different from that at vegetative growth. Our results support the idea that Fks3p has distinct functions for GS and also regulates GS during spore wall assembly. We speculate that Fks3p interacts with accessory components of GS that modify GS activity and increase GS activity during glucan layer assembly. However, it is unclear whether Rho1p is the only target of Fks3p. It has recently been reported that in fission yeast, the Rho5p GTPase, which is highly homologous to Rho1p, is required for spore wall formation. The asci of the rho5Δ strain are less resistant to heat than the asci of the wild-type (39). It is possible that a similar regulation mechanism mediated by Rho1p or other homologous proteins operates during spore wall assembly in budding yeast. In addition, protein kinases Smk1p and Sps1p play roles in the trafficking of both Fks2p and the Chs3p chitin synthase to the spore plasma membrane (16, 20). Both Fks2p and Chs3p localize to the prospore membrane and spore wall (20). We have determined by microscopic observation that Fks3p also localizes to the prospore membrane and spore wall.
(data not shown). Fks3p may be required for the trafficking, localization, and activation of Fks2p through interactions with accessory components of GS.

Spore wall resistance is dependent upon glucan assembly. The low organization of the cell wall of the fks2, fks3, and fks2 fks3 mutants as shown by EM, ImmunoEM analysis using the anti-1,3-β-glucan antibody, and chemical cell wall analysis (Fig. 1 and 2) is the direct cause of the reduced resistance of the ascospores of these mutants to diethyl ether, elevated temperatures, and ethanol. Therefore, the assembly of the glucan layer and subsequent assembly of the spore wall may be essential for spore wall resistance to environmental damage. In summary, glucan is a key assembly component of the spore wall, and its synthesis and assembly are regulated by a subunit of glucan synthase, Fks2p, and its regulatory homolog Fks3p. Glucan layer assembly is essential for spore wall formation and confers resistance to environmental damage.

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