Mutation of the *SPS1*-encoded protein kinase of *Saccharomyces cerevisiae* leads to defects in transcription and morphology during spore formation

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During sporulation of *Saccharomyces cerevisiae*, meiosis is followed by encapsulation of haploid nuclei within multilayered spore walls. Completion of the late events of the sporulation program requires the *SPS1* gene. This developmentally regulated gene, which is expressed as cells are nearing the end of meiosis, encodes a protein with homology to serine/threonine protein kinases. The catalytic domain of Sps1 is 44% identical to the kinase domain of yeast Ste20, a protein involved in the pheromone-induced signal transduction pathway. Cells of a *MATa/MATα sps1/sps1* strain arrest after meiosis and fail to activate genes that are normally expressed at a late time of sporulation. The mutant cells do not form refractile spores as assessed by phase-contrast microscopy and do not display the natural fluorescence and ether resistance that is characteristic of mature spores. Examination by electron microscopy reveals, however, that prospore-like compartments form in some of the mutant cells. These immature spores lack the cross-linked surface layer that surrounds wild-type spores and are more variable in size and number than are the spores of wild-type cells. Despite their inability to complete spore formation, *sps1*-arrested cells are able to resume mitotic growth on transfer to rich medium, generating haploid progeny. Our results suggest that the developmentally regulated Sps1 kinase is required for normal progression of transcriptional, biochemical, and morphological events during the later portion of the sporulation program.

[Key Words: *Saccharomyces cerevisiae*; kinase; sporulation; spore wall]

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Sporulation is initiated in the yeast *Saccharomyces cerevisiae* when *MATa/MATα* diploid cells are starved for nitrogen in the presence of a nonfermentable carbon source such as acetate (for review, see Esposito and Klapholz 1981). A single round of DNA replication is followed by a lengthy prophase during which homologous chromosomes pair and undergo a high level of genetic recombination. The homologous chromosomes segregate to opposite poles of the nucleus in the meiosis I reductional division. This is followed rapidly by the mitosis-like meiosis II division in which sister chromatids segregate from each other. In yeast, the nucleus remains intact throughout meiosis. The intranuclear segregation of the chromosomes generates four bulges in the nucleus, one beside each spindle pole body (for review, see Byers 1981). Prospore walls begin to form at the spindle pole bodies and expand to encapsulate the haploid meiotic products present in each nuclear lobe. Maturation of

the spore walls then generates the four spores that are retained within the ascus.

A number of different approaches have been used to identify genes involved in sporulation-specific events. Various genetic screens have led to the isolation of mutants that are defective in meiotic recombination, homolog pairing, segregation of chromosomes, and spore formation (for examples, see Esposito and Esposito 1969, 1974; Tsuboi 1983; Rockmill and Roeder 1988; Hollingsworth and Byers 1989; Briza et al. 1990b; Malone et al. 1991). Cloning and characterization of a large number of the genes identified in these studies have almost invariably revealed that they are expressed preferentially in sporulating cells (for review, see Mitchell 1994). In contrast, many of the sporulation-specific genes that were identified on the basis of their expression pattern (Clancy et al. 1983; Percival-Smith and Segall 1984; Gottlin-Ninfa and Kaback 1986; Law and Segall 1988; Bishop et al. 1992; Coe et al. 1992) do not appear to have an essential role in the sporulation program (Garber and Segall 1986; Gottlin-Ninfa and Kaback 1986; Percival-

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Smith and Segall 1986; Law and Segall 1988; Kao et al. 1989; Kallal et al. 1990; Muthukumar et al. 1993). *SPS1* is one of the few sporulation-specific genes isolated in a differential hybridization screen (Percival-Smith and Segall 1984) that was found to be required for spore formation (Percival-Smith and Segall 1986). As reported here, determination of the sequence of *SPS1* revealed that the gene encodes a protein with homology to serine/threonine protein kinases. Characterization of the phenotype of *sps1/sps1* cells indicates that the *SPS1*-encoded kinase is required for spore wall formation.

Results

Sps1 is a putative serine/threonine protein kinase

The identification of *SPS1* as a gene that is preferentially expressed midway through the sporulation program of *S. cerevisiae* (Percival-Smith and Segall 1984) suggested that its product might participate in a sporulation-specific event. We found previously that mutation of *SPS1* blocks spore formation (Percival-Smith and Segall 1986). To determine whether the deduced amino acid sequence of Sps1 might provide some clue as to its role, we determined the nucleotide sequence of the gene. The sequence revealed a single long open reading frame (ORF) of 1470 bp (Fig. 1). The predicted 490-amino-acid protein encoded by this ORF has a calculated molecular weight of 55,668. A comparison of the predicted Sps1 protein with proteins in the National Center for Biotechnology Information data base revealed that the Sps1 sequence from residue 18 to 272 was similar to the catalytic domain of protein kinases. Sps1 contained all of the invariant amino acids identified by Hanks (1991) in a comparison of 100 serine/threonine protein kinases. The amino acid sequences of subdomains VIb and VIII, which appear to determine the specificity of the kinase, suggested that Sps1 was a serine/threonine protein kinase (Hanks et al. 1988). The carboxy-terminal nonkinase domain of Sps1 had no significant similarity to other proteins in the data base. A BLAST search (Altschul et al. 1990) of the National Center for Biotechnology Information data base revealed that the proteins with the highest sequence identity with the kinase domain of Sps1 were the yeast kinase Ste20, which is a member of the mitogen-activated protein (MAP) kinase pathway involved in pheromone response in yeast; p65^{PAK}, a kinase from rat brain that is activated by the GTP-bound form of a small Ras-related protein and that is postulated to be involved in response of the cytoskeletal network to extracellular signals (Manser et al. 1994); a human B lymphocyte protein kinase (Katz et al. 1994); and an uncharacterized yeast kinase (GenBank accession number X69322). The primary sequence of the kinase domain of Sps1 was 44% and 42% identical with the sequence of the kinase domains of Ste20 and p65^{PAK}, respectively (Fig. 2). The similarity in sequence between Sps1 and a member of a MAP kinase activation pathway raises the possibility that Sps1 might be involved in a signal transduction event during sporulation.

SPS1 is expressed as meiosis is nearing completion

To determine the time at which Sps1 serves its role in sporulation, we correlated the time of expression of the *SPS1* gene in our standard wild-type strain with the major events of sporulation. The temporal pattern of expression of *SPS1* was determined by Northern blot analysis of RNA prepared from cells that had been harvested at various times after transfer to sporulation medium (Fig. 3A). Completion of the meiotic divisions was assessed by examination of aliquots of 4', 6'-diamidino-2 phenylindole (DAPI)-stained cells; in this procedure, the fluorescent visualization of DNA allows chromosome segregation to be monitored (Fig. 3B). The appearance of binucleate cells and tetranucleate cells is considered to mark the completion of meiosis I and meiosis II, respectively. Spore formation was monitored by phase-contrast microscopy (Fig. 3B,D). This analysis indicated that *SPS1* transcripts were first detectable 6 hr after transfer of cells to sporulation medium (Fig. 3A). Accumulation of transcript was maximal at 8–10 hr of sporulation (Fig. 3A); at this time the sporulating cells were beginning to complete meiosis I as assessed by the appearance of binucleate cells (Fig. 3B). As tetranucleate cells began to accumulate between 10 and 13 hr, the level of *SPS1* transcripts was beginning to decline (Fig. 3A,B). The level of *SPS1* transcripts continued to decline during the period of spore formation between 12 and 16 hr (Fig. 3A,B).

We also compared the time of expression of *SPS1* with the time of expression of two other sporulation-specific genes, *HOP1* and *DIT1*. *HOP1*, which is required for pairing of homologous chromosomes (Hollingsworth and Byers 1989; Hollingsworth et al. 1990), is expressed coincidentally with a large number of other meiotic genes such as *SPO11* (Atcheson et al. 1987), *RED1* (Thompson and Roeder 1989), and *REC114* (Pittman et al. 1993), which encode products required for successful completion of meiosis (for review, see Mitchell 1994). *DIT1*, which is expressed just prior to spore formation, is required for deposition of the outermost layer of the spore wall (Briza et al. 1990b). We found that *HOP1*, *SPS1*, and *DIT1* were turned on sequentially (Fig. 3A); expression of *HOP1* preceded expression of *SPS1* by ~2 hr, and expression of *DIT1* began 2 hr after the *SPS1* gene had been turned on. This sequential pattern of gene expression was consistent with previous observations (Briza et al. 1990b; Mitchell et al. 1990; Smith et al. 1990).

SPS1 is not essential for meiosis

The observation that *SPS1* was expressed as meiosis was nearing completion suggested that *SPS1* was required for a postmeiotic event. We found that cells of a *MATa/MATa Δsps1/Δsps1* strain successfully completed the meiotic divisions. Generation of the *Δsps1* allele for this strain was achieved by replacement of the 5' region of *SPS1* with a DNA fragment containing *TRP1* (see Materials and methods); no *SPS1*-derived transcripts could be

1
 GAGCTCCATT CCTAAGTCTT CTCAAAATTT GATGTCGCCA ACAAACTTAT TAACAAATGCA TAAGTCTTTA
 71
 AAATGCGCCA AAAAGGACAA AAAATAACAT ATATGATAAT TTAACGTGAT TTTTTCCTCT TTCTCAATAC
 141
 TTTTCTCTCA AGTTATATTT CGGAGCTGTC CCAGGTTGCG AACAACTGCG CATCAGGGAT AAAAAAAAT
 211
 GACACATAAA AACGAAAAG ACACAAATTC CATTTTTTTT TTTTGAGCGG AAGAATGAC ACTACTTCT
 281
 TCTATTGTAA CCAATTCCTAA CATCGAATTT TTAATTGTTA GTTCAGATAT ATACTAAGA GTCACTTAAT
 351
 ATACTGTTTT TGTATTATAT CTGGTGTCCA ACTGCTTCAT TTAATATTTT CTATCCAAA AAAGCAGCT
 421
 AAATCTCTTC TTTTCTTTC TTAATTTGCC ATTTTCAATA TATCGAATTT AATGAACCC TGGTAGTCA
 491
 GTTGTCTTAG CAACATTTTC TCAAAAGAAA GCAAACGAGC ATTTGCTTTT TAAATTTAGT TTTTCTACTA
 561
 GCTAATTCAT TTAATAACA ACAAAAGAAA ATAGACAAA
 601/1
 ATG GAA AGC AAA GAA ATA TCA ATT AGG TCA AGA ACC OCT CCA TCG AAG CTA TAT TCC ATC
 Met Glu Ser Lys Glu Ile Ser Ile Arg Ser Arg Thr Pro Ser Lys Leu Tyr Ser Ile
 661/21
 CAA TCA TGT ATT GGC AGA GGT AAC TTT GGT GAT GTG TAT AAA GCA GTG GAT AGA GTT ACG
 Gln Ser Cys Ile Gly Arg Gly Asn Phe Gly Asp Val Tyr Lys Ala Val Asp Arg Val Thr
 721/41
 CAA GAA ATT GTG GCA ATT AAG GTG GTC AAC CTG GAG CAT TCC GAC GAA GAG ATT GAA CTG
 Gln Glu Ile Val Ala Ile Lys Val Val Asn Leu Glu His Ser Asp Glu Asp Ile Glu Leu
 781/61
 TTA GCA CAG GAA ATT TTT TTT CTG GCG GAA TTG AAA TCT CCT CTT ATT ACA AAC TAT ATT
 Leu Ala Gln Glu Ile Phe Phe Leu Ala Glu Leu Lys Ser Pro Leu Ile Thr Asn Tyr Ile
 841/81
 GCA ACA ATG TTA GAA GAT GTC TCT ATG TTG AIT GTC ATG GAA TAT TGC GGC GGA GGA TCA
 Ala Thr Met Leu Glu Asp Val Ser Met Trp Ile Val Met Glu Tyr Cys Gly Gly Ser
 901/101
 TGT TCA GAT TTA CTG AAG CGA AGT TAC GTC AAC GGT TTG CCT GAA GAA AAA GTT TCC TTC
 Cys Ser Asp Leu Leu Lys Arg Ser Tyr Val Asn Gly Leu Pro Glu Glu Lys Val Ser Phe
 961/121
 ATT ATT CAT GAA GTC ACC TTG GGT TTG AAA TAC CTG CAT GAG CAA AGG AAA ATT CAT GGT
 Ile Ile His Glu Val Thr Leu Gly Leu Lys Tyr Leu His Glu Gln Arg Lys Ile His Arg
 1021/141
GAT ATC AAA GCA GCT AAT ATT TTA CTA AAT GAA GAA GGT ATG GTT AAG TTG GGT GAT TTC
 Asp Ile Lys Ala Ala Asn Ile Leu Leu Asn Glu Glu Gly Met Val Lys Leu Gly Asp Phe
 1081/161
 GGA TGA AGT GGC CAC CAT GTC TCC ATG TTG AAA AGG GAC ACT TTT GTA GGA ACG CCG TAT
 Gly Val Ser Gly His Ile Arg Ser Thr Leu Lys Arg Asp Thr Phe Val Gly Thr Pro Tyr
 1141/181
 TGG ATG GCT CCG GAA GTT GTT TGC TGC GAA GTT GAT GGG TAT AAT GAG AAA GCA GAT ATA
 Trp Met Ala Pro Glu Val Val Cys Cys Glu Val Asp Gly Tyr Asn Glu Lys Ala Asp Ile
 1201/201
 TGG TCC TTG GGT ATC ACC ACC TAT GAG TTA CTC AAG GGC TTA CCC CCA TTA TCT AAA TAT
 Trp Ser Leu Gly Ile Thr Thr Tyr Glu Leu Leu Lys Gly Leu Pro Pro Leu Ser Lys Tyr
 1261/221
 GAT CCT ATG AAA GTT ATG ACT AAC TTA CCA AAA AGG AAG CCT CCA AAA TTA CAA GGT CCC
 Asp Pro Met Lys Val Met Thr Asn Leu Pro Lys Arg Lys Pro Pro Lys Leu Gln Gly Pro
 1321/241
 Phe Ser Asp Ala Ala Lys Asp Phe Val Ala Gly Cys Leu Val Lys Thr Pro Ala Asp Arg
 TTT TCG GAT GCA GCA AAG GAT TTC GTA GCT GGT TGT CTT GTT AAA ACA CCT GCT GAT CCG
 1381/261
 CCA TCG GCA TAT AAC TTG TTA TCA TTT GAA TTT GTT AAG AAT ATC ACC ATA ACG AAC TTG
 Pro Ser Ala Tyr Asn Leu Leu Ser Phe Glu Phe Val Lys Asn Ile Thr Ile Thr Asn Leu
 1441/281
 AAA AGT GAT GTT GAT CTA ATT AAA CAG AAA AAG GTT CAG GAG AGA TAT ACA AAA GTT CCC
 Lys Ser Asp Val Asp Leu Ile Lys Gln Lys Lys Val Gln Glu Arg Tyr Thr Lys Val Pro
 1501/301
 AAA TAC CCT CTT CAA AAT ACC CCG TTG TAT AAA AAC AGC AAC ACA GTG ACG GGA AAG GAA TTT
 Lys Tyr Pro Leu Gln Asn Arg Leu Tyr Lys Asn Ser Asn Thr Val Arg Gly Lys Glu Phe
 1561/321
 TGG AAT TTT GAA TCG ACA AGA CTA AGC ACA ACA CAA ATT TCC AAA GAG GAA TTA TCT CCC
 Trp Asn Phe Glu Ser Thr Arg Leu Ser Thr Thr Gln Ile Ser Lys Glu Glu Leu Ser Pro
 1621/341
 Ile Thr Gln Asp Ser Pro Thr Ser Ser Leu Asn Met Glu Ser Pro Tyr Leu Leu His Gly
 ATT ACA CAA GAC TCT CCA ACA TCA TCT TTA AAT ATG GAA AGT CCT TAT GTA CTT CAT GGG
 1681/361
 CAA ACT GTA ACG CCG ATA ACC AAC CCA AGT TCT TCA TCA TTT AGA AAA TGT ACG CAA CCA
 Gln Thr Val Thr Pro Ile Thr Asn Pro Ser Ser Ser Ser Phe Arg Lys Cys Thr Gln Pro
 1741/381
 GTT TTC GAG CTT GAT TCA GGA ATG GAT ATA GAT TCA GGC TGC CCA AAT GCT CAA GCA GAG
 Val Phe Glu Leu Asp Ser Gly Met Asp Ile Asp Ser Gly Cys Pro Asn Ala Gln Ala Glu
 1801/401
 Thr Glu Ile Val Pro Leu Ser Asn His Asn Lys Lys His Lys Lys Asn Asp Ile Gln Ala
 ACC GAA ATA GTT CTT CTT TCT AAT CAT AAT AAG AAG CAT AAA AAG AAC GAT ATT CAG CCG
 1861/421
 TTG AAG ATA GAA AAG TTT GAT TCG AAA AAC ATT GEA TCT CAT ATT CTC AAC AGA ATG
 Leu Lys Ile Glu Lys Phe Asp Tyr Leu Lys Asn Ile Val Ser His Ile Leu Asn Arg Met
 1921/441
 TAT GAT CGT GCG CCG CAC GAT GAA ACA AGA AAA TAC GEA AAT GAA ATG TTA AAG CAA TTC
 Tyr Asp Arg Ala Arg Asp Asp Thr Thr Arg Lys Tyr Val Asn Glu Met Leu Lys Gln Phe
 1981/461
 ATT AAA ACT GAG GCA AAC GTT CCT GGA TTT AAT GAG GTT TTT ATA GAA GAG ATC TCA CTA
 Ile Lys Thr Glu Ala Asn Val Pro Gly Phe Asn Glu Val Phe Ile Glu Glu Ile Ser Leu
 2041/481
 AGA ATT GAA GCA ATA AAG AAA GGA TTC GTT TAA
 Arg Ile Glu Ala Ile Lys Lys Gly Phe Val TER

Figure 1. Nucleotide sequence of the *SPS1* gene. The nucleotide sequence of the nontranscribed strand of the *SPS1* gene is given with the predicted translation product indicated below the DNA sequence. Throughout the translated region, the first number refers to the nucleotide position relative to a *SacI* site 600 bp upstream of the initiator ATG; the second number refers to the position of the codon relative to the predicted initiator ATG. The *Clal* recognition site beginning at nucleotide 302 and the *EcoRV* recognition site beginning at nucleotide 1021, which were used in the construction of the Δ *sps1::TRP1* allele, are doubly underlined. The *BglIII* site beginning at nucleotide 2030, which was used in the construction of the *sps1::URA3* allele, is underlined. The sequence beyond this *BglIII* site is from Percival-Smith and Segall (1986).

detected in the *MATa/MAT α Δ sps1/ Δ sps1* strain in sporulation medium (data not shown). Examination of DAPI-stained cells by fluorescence microscopy indicated that both meiosis I and meiosis II occurred at approximately the same times in the mutant cells as in wild-type cells with the majority of multinucleate cells appearing between 8 and 12 hr (Fig. 3B,C). In various experiments, we found that meiosis I occurred in the mutant cells with an efficiency of 80–100% of that in wild-type cells (Fig. 3B,C; data not shown) and meiosis II occurred with an efficiency of 60–75% of that in wild-type cells (Fig. 3B,C; data not shown). Although the *SPS1* mutant cells reproducibly showed a reduction in the efficiency of the second meiotic division, the majority of the mutant cells that had completed meiosis I went on to complete meiosis II but were then unable to form spores as assessed by phase-contrast microscopy (Fig. 3C,E; Percival-Smith and Segall 1986). We found that a portion of the mutant cells that had been in sporulation medium were unusually large (Fig. 3E). The mutant cells that had been in sporulation medium also lacked the fluorescence and ether resistance that is attributed to deposition of a dityrosine-containing macromolecule on the outer surface of the spore (data not shown; Briza et al. 1990a,b). We therefore concluded that the major effect of the absence of the *SPS1* gene product was to prevent proper packaging of spores.

Haploid progeny can be generated from sps1-arrested cells

As mentioned above, DAPI is a fluorescent dye allowing visualization of DNA; thus the segregation of homologous chromosomes to opposite poles during meiosis I leads to the appearance of cells that are referred to as binucleate, and the subsequent segregation of sister chromatids to opposite poles during meiosis II leads to the appearance of cells that are referred to as tetranucleate. The nuclear envelope, however, remains intact during yeast meiosis; at the end of meiosis II, the extended and lobulated nucleus of the so-called tetranucleate cell contains a haploid complement of chromosomes in each nuclear lobe. Compartmentalization of the haploid genomes is thought to be dependent on formation of prospore walls around the nuclear lobes. The prospore envelope, which pinches off the haploid nuclei and traps some cytoplasmic material, then serves as the scaffold for deposition of spore wall-specific material (Moens 1971; Moens and Rapport 1971). Upon germination, the four spores present within the ascus are released and the growth of the individual spores gives rise to haploid progeny.

To test for compartmentalization of the meiotic products in *sps1*-arrested cells, we assessed the ploidy of progeny derived from these cells. If the meiotic nucleus generated in an *sps1/sps1* cell did undergo compartmentalization, we anticipated that it would be possible to recover haploid progeny. First, we determined whether *sps1*-arrested cells could resume mitotic growth (Fig. 4). We found that the viability of *sps1/sps1* cells that had been in sporulation medium for 24 hr was ~80% that of wild-type sporulating cells (Fig. 4). We then assessed the

Figure 2. Alignment of the kinase domain of Sps1 with the corresponding domains of Ste20 and p65^{PAK}. The deduced sequences of the kinase domains of Sps1, Ste20 (Leberer et al. 1992; Ramer and Davis 1993), and p65^{PAK} (Manser et al. 1994) have been aligned by eye, guided by the alignments generated by the BLAST program (Altschul et al. 1990). The positions of gaps, introduced to maintain alignment and denoted by dashes, were guided by the comparisons of Hanks (1991). The sequences of the kinase domains of Sps1, Ste20, and p65^{PAK} begin at residues 18, 620, and 269, respectively. Amino acids that are identical between Sps1 and Ste20, between Sps1 and p65^{PAK}, or among all three proteins are

highlighted. The top line denotes amino acids that are invariant (uppercase) or almost invariant (lowercase) in a comparison of the catalytic domains of 100 serine-threonine protein kinases (Hanks 1991). Roman numerals indicate the 12 kinase subdomains identified by Hanks and Quinn (1991).

DNA content of the progeny derived from sporulating cells by flow cytometry. FACS analyses were first performed on exponentially growing cultures of haploid *SPS1* cells, diploid *SPS1/SPS1* cells, and diploid *sps1/sps1* cells. These control scans showed a typical distribution of cells with a greater portion of the population being in G₂ than in G₁ (Fig. 5A–C). Similar scans were then performed on cells that had been incubated in sporulation medium for 21 hr, diluted, and grown in liquid rich medium for 21 hr. In the scan of wild-type diploid cells subjected to this regimen, germination and growth of haploid spores were visualized by the appearance of a peak of G₁ cells with a 1N DNA content (Fig. 5D). The peak of G₂ cells with a 4N DNA content in this scan indicated that a portion of the cells in this population were diploids. Most of these diploid cells were presumably derived from cells that did not enter the meiotic pathway on transfer to sporulation medium. It is possible that these nonsporulating cells resumed growth prior to germination of spores, accounting for the relatively low portion of haploid cells in the population. FACS analysis of a culture derived from *sps1*-arrested cells returned to growth medium revealed a minor population of haploid cells, approximately one-fifth to one-third the portion present in the population derived from wild-type sporulating cells (Fig. 5E; data not shown). On the basis of this analysis we concluded that the *sps1*-derived cells could generate haploid progeny, although less efficiently than did wild-type cells. The reduced level of haploidization can be accounted for in part by the fact that the mutant cells completed meiosis less efficiently than did wild-type cells.

As an alternative approach for detecting haploid progeny of *sps1*-arrested cells, we tested for segregation of recessive drug-resistance markers. In this experiment, *SPS1/SPS1 CAN⁸¹/can¹ CYH²/cyh²* and *sps1/sps1 CAN⁸¹/can¹ CYH²/cyh²* cells, which are sensitive to canavanine and cycloheximide, were incubated in sporulation medium for 21 hr and then plated on rich medium

and tested for resistance to cycloheximide and canavanine. One-half of the haploid progeny of wild-type cells would be expected to be resistant to each drug, as we were testing colonies derived from asci, each colony would be expected, in the absence of sib-matings, to contain drug-resistant cells. We found that 53% and 57% of the colonies derived from wild-type cells that had been in sporulation medium contained cells that were resistant to cycloheximide and canavanine, respectively. Nineteen percent and 15% of the colonies recovered from *sps1/sps1* cells that had been in sporulation medium were resistant to cycloheximide and canavanine, respectively. The *sps1 cyh²* and *sps1 can¹* cells appeared to be derived by haploidization, as most of the drug-resistant cells were also maters (data not shown). In summary, both this genetic analysis and analysis of DNA content by flow cytometry indicated that *sps1*-arrested cells could generate haploid progeny on resumption of growth, although less efficiently than did wild-type sporulating cells.

Aberrant spore formation in sps1/sps1 cells

To assess the sporulation defect of *sps1/sps1* cells in more detail, we used electron microscopy to examine cells that had been in sporulation medium for 21 hr. A typical wild-type ascus is depicted in Figure 6A. The outermost surface of the spore wall (Fig. 6C), which appears as a very thin, osmiophilic layer, consists of a cross-linked insoluble macromolecule containing a large amount of dityrosine (Briza et al. 1990a,b). This layer, which is responsible for the resistance of spores to degradative enzymes and organic solvents (Briza et al. 1990b) is closely associated, perhaps by covalent linkages, with an underlying chitosan layer (Briza et al. 1988), which appears as a more diffuse osmiophilic layer. The innermost layers of the spore wall are similar in composition to the vegetative cell wall and often appear

Figure 3. The *SPS1* gene is expressed at the end of meiosis and is not required for completion of the meiotic divisions. (A) RNA was prepared from aliquots of wild-type cells (LP-HT) taken at various times after transfer to sporulation medium. The RNA was denatured, separated by electrophoresis through a 1.5% agarose-formaldehyde gel, and transferred to a nylon membrane. The blot was hybridized sequentially with radioactively labeled probes containing the *HOP1* gene, the *SPS1* gene, the *DIT1* gene, and the *PYK1* gene (see Materials and methods). Hybridization was quantified by PhosphorImager, and the relative amount of each sporulation-specific RNA is given as a ratio of the amount of that RNA to the amount of *PYK1* RNA at each time point. This ratio was normalized by giving the highest ratio for each sporulation-specific RNA a value of 1. (□) *HOP1*; (■) *SPS1*; (○) *DIT1*. (B) Samples of cells from the same sporulating culture that was analyzed in A were fixed, stained with DAPI, and examined by fluorescence microscopy to determine the percentage of cells that had completed meiosis I (○) or meiosis II (●). Cells that appeared binucleate, trinucleate, or tetranucleate by DAPI staining were considered to have completed meiosis I. Cells that appeared trinucleate or tetranucleate were considered to have completed meiosis II. Cells were examined by phase-contrast microscopy to monitor ascus formation (□). (C) Samples of *MATa/MATα Δsps1/Δsps1* cells (Y26) were examined as described for B. (D) Photograph of wild-type sporulated cells (Y30) viewed by phase-contrast microscopy. A high proportion of cells have formed asci with a triad or tetrad of spores visible. (E) Photograph of *sps1/sps1* cells (Y26) viewed by phase-contrast microscopy. No asci are visible.

of development, consisting of a nucleus surrounded by a double membrane. Other compartments had the appearance of prospores arrested just prior to spore wall maturation; at this stage, electron-transparent spore wall material has been deposited within the double membrane

Figure 4. Viability of *MATa/MATα Δsps1/Δsps1* cells in sporulation medium. Samples of *MATa/MATα SPS1/SPS1* cells (Y30, □) and *MATa/MATα Δsps1/Δsps1* cells (Y26, ●) were taken at various times after transfer to sporulation medium. The aliquots were vortexed, diluted, and plated on YPD. For each strain, the number of viable cells is expressed as a ratio of the number of colony-forming units (cfu) at a given time after transfer of cells to sporulation medium to the number of cfu at the time of transfer to sporulation medium. In this experiment, the efficiency of ascus formation was 54% for strain Y30. Strain Y26 generated no asci.

as a single electron-transparent layer (Katohda et al. 1984; Briza et al. 1988).

Examination of *sps1/sps1* cells that had been in sporulation medium for 21 hr revealed that many of the mutant cells could elaborate prospore-like compartments. There were generally only one or two of these compartments per cell, and they were generally smaller than were wild-type spores. Some of the compartments were very similar in appearance to a prospore at an early stage

Figure 5. FACS analysis reveals that *sps1*-arrested cells generate haploid progeny. Cells were fixed and stained with propidium iodide prior to analysis in a Becton Dickinson FACScan. (A) Scan of haploid *SPS1* cells (W3031B) grown in YPD. The peaks of cells marked 1N and 2N represent cells in G_1 and G_2 of the cell cycle, respectively. (B) Scan of diploid *SPS1/SPS1* cells (Y30) grown in YPD; (C) scan of diploid *sps1/sps1* cells (Y26) grown in YPD. The peaks of cells marked 2N and 4N represent cells in G_1 and G_2 of the cell cycle, respectively. (D) Scan of *SPS1/SPS1* cells (Y30) that had been incubated in sporulation medium for 21 hr and then grown in YPD for 21 hr. The efficiency of sporulation of these cells was ~60%. The peak of cells marked 1N represents haploid cells in G_1 . These cells were derived from spores that had germinated and had begun to grow vegetatively. The peak of cells marked 2N represents both haploid cells in G_2 and diploid cells in G_1 . The diploid cells are cells that failed to sporulate. The peak of cells marked 4N represent diploid cells in G_2 . (E) Scan of *sps1/sps1* cells (Y26) that had been incubated in sporulation medium for 21 hr and then grown in YPD for 21 hr. No asci were seen in these cells after 21 hr in sporulation medium. The graphs depict relative DNA content (x-axis) vs. cell number (y-axis).

oped nuclei, could be found within a single cell. In some instances, it was not clear whether the compartment contained a nucleus.

Electron micrographs of representative *sps1*-arrested cells are presented in Figure 6. The cells in Figure 6, E and F, contained very immature prospore-like compartments enclosed by a double membrane. In one case, the prospore delimiting membranes had not yet enveloped a presumptive nucleus completely (Fig. 6G). The cells presented in Figure 6, H and I, contained more mature prospore-like compartments. Cell wall material had been deposited between the double membrane leading to an electron-transparent layer. The two innermost layers of the wall could be seen in the prospore shown in Figure 6J. As has been noted previously for cells in the early stages of spore formation, the mutant cells contained many small electron-transparent vesicles (Beckett et al. 1973; Illingworth et al. 1973) and the prospore-like compartments were surrounded by endoplasmic reticulum (Lynn and Magee 1970) (Fig. 6G,J). Figure 6B presents an example of a mutant cell containing one of the more mature spore-like compartments that we observed in the mutant population. The spore wall appeared to lack the outer chitosan and the dityrosine-containing layers present in wild-type spores (Fig. 6D). In summary, *SPS1* mutant cells were able to divide their postmeiotic nucleus into distinct compartments. In some instances, the early stages of spore wall development appeared normal but spore wall maturation did not occur.

SPS1 is required for normal expression of late sporulation-specific genes

Several classes of temporally distinct sporulation-specific genes, referred to as early, middle, mid-late and late, are sequentially expressed as the sporulation program proceeds (for review, see Mitchell 1994). Expression of middle *SPS* genes, which include *SPS1*, *SPS2*, and *SPS4* (Percival-Smith and Segall 1984, 1986; Garber and Segall 1986), is followed by expression of the mid-late genes, *DIT1* and *DIT2*, which are essential for formation of the

(for descriptions of spore wall development, see Lynn and Magee 1970; Moens 1971; Guth et al. 1972; Beckett et al. 1973; Illingworth et al. 1973). Spore-like compartments that differed in appearance, as well as unenvel-

Figure 6. Electron microscopy of sporulated *SPS1/SPS1* and *sps1/sps1* cells. (A) A typical ascus showing three spores is depicted for the wild-type strain Y30 (*SPS1/SPS1*). Note that electron micrographs of four-spored asci rarely show more than three spores within one plane as a result of the tetrahedral arrangement of the spores. (B) A sporulated cell from strain Y26 (*sps1/sps1*) showing one relatively mature prospore-like compartment (top), one immature prospore-like compartment (middle), and one unencapsulated nucleus (bottom). (C) A portion of the wall of two of the spores present in the ascus of A is depicted at higher magnification. The arrowheads refer to d, the outermost, very thin and osmiophilic dityrosine-containing layer of the spore wall; c, the underlying, diffuse, and osmiophilic chitosan layer; and i, the innermost, electron transparent layers. (D) Higher magnification view of the boxed region of the mutant *sps1/sps1* cell depicted in B showing the envelope surrounding the prospore-like compartments. Note the absence of a well-defined outer membrane and the absence of the chitosan and dityrosine-containing layers. (E,F) Examples of *sps1/sps1* cells containing relatively immature-looking prospore-like compartments. (G) Higher magnification view of the prospore-like compartment seen at the top, left of the cell shown in F. The spore-delimiting membrane has not completely enclosed its contents. (H,I) Examples of *sps1/sps1* cells containing relatively mature-looking prospore-like compartments. (J) Higher magnification view of the prospore-like compartment seen in the cell of I. (V) vacuole; (N) unencapsulated nucleus; (arrowheads) prospore-like compartments. Bars, 400 nm.

outermost layer of the spore wall (Briza et al. 1990b). The late *SPS* genes *SPS100* and *SPS101* are activated ~4 hr after the *DIT* genes are turned on. The *SPS100* gene has been shown to contribute to the development of the ether resistance that is a characteristic of mature spores (Law and Segall 1988). To determine whether mutation of the *SPS1* gene affected the pattern of sporulation-specific gene expression, we monitored accumulation of transcripts of various genes in wild-type cells and in *sps1/sps1* cells in sporulation medium by Northern blot analysis. The expression of *SPS2* and *SPS4*, which coincides with expression of *SPS1*, was similar in both strains (Fig. 7A,B). *DIT1* transcripts, however, accumulated to a much higher level in the *sps1/sps1* strain than in the wild-type strain (Fig. 7C), suggesting that the turning off of the *DIT1* gene was delayed in the absence of

Sps1. It is also possible that the *DIT1* promoter was more active or that *DIT1* transcripts were more stable in the absence of *Sps1*. Another dramatic effect of mutation of *SPS1* was seen on examining expression of the *SPS100* and *SPS101* genes. In wild-type cells, transcripts corresponding to these genes accumulated to a high level between 11 and 15 hr of sporulation and remained at a high level until 24 hr of sporulation (Fig. 7E,F; Law and Segall 1988). Accumulation of *SPS100* transcripts was both delayed and reduced in the *sps1/sps1* strain in sporulation medium (Fig. 7E), and *SPS101* transcripts failed to accumulate to a significant extent at any time in the mutant strain (Fig. 7F). Rehybridization of the blots of Figure 7 with radioactively labeled *PYK1* DNA confirmed that approximately equal amounts of RNA had been loaded in each lane of the gels (Fig. 7D,G; data not shown).

Figure 7. Expression of sporulation-specific genes in *SPS1*/*SPS1* and *sps1/sps1* cells. Accumulation of transcripts was monitored for the following genes: (A) *SPS2*; (B) *SPS4*; (C) *DIT1*; (E) *SPS100*; (F) *SPS101* as denoted in the column titled probe. RNA was purified from wild-type cells (strain LP-HT; denoted *SPS1* in the column titled strain) and from *sps1/sps1* cells (strain YRL1; denoted *sps1* in the column titled strain) at the indicated times after transfer to sporulation medium. The RNA was denatured, separated by electrophoresis through a 1.5% agarose-formaldehyde gel, and transferred to a nylon membrane. The RNA blots were hybridized with the indicated radioactively labeled gene-specific probes (see Materials and methods). After autoradiography, the hybridized DNA was removed and all blots were rehybridized with a radioactively labeled probe containing the *PYK1* gene to test for equal loading of RNA in all lanes. (D,G) The control hybridizations with the *PYK1* probe for the filters of C and F, respectively. Only the portions of the autoradiographs that revealed hybridization with the probes are shown.

Discussion

Mutation of SPS1 blocks spore wall development and late sporulation-specific gene expression

The finding that *SPS1* encodes a putative protein kinase prompted us to investigate the role of Sps1 in the sporulation program of *S. cerevisiae* in greater detail. As might be expected from the observation that maximal expression of *SPS1* occurs as meiosis is nearing completion, we found that *sps1/sps1* cells could complete meiosis I and meiosis II as assayed by DAPI staining of nuclear material. The mutant cells, which did not form spores as assessed by phase-contrast microscopy, were able to re-enter the mitotic cycle on transfer to rich medium as has been shown for several other meiotically arrested mutants (Davidow and Byers 1984; Shuster and Byers 1989; Bishop et al. 1992; Honigberg et al. 1992; Rose and Holm 1993; Sym et al. 1993; Honigberg and Esposito 1994). The recovery of haploid progeny from *sps1*-arrested cells suggested that postmeiotic nuclear compartmentalization had occurred in a portion of the mutant cells. Electron microscopy revealed the formation of prospore-like compartments, usually one or two, in some of the *sps1*/

sps1 cells. These compartments, which were more variable in size than were wild-type spores, had the appearance of prospores that had been arrested at various stages of development prior to spore wall maturation. The observation that spore-like compartments that were blocked at different stages of development as well as unenveloped nuclei could be found within the same cell suggests some degree of independence in the development of sister spores.

The finding that some *sps1/sps1* cells appeared to contain separated nuclei that had not been enveloped by prospore membranes contrasts with the observation that in wild-type cells meiotic nuclear division occurs concomitantly with prospore wall formation (Moens 1971). However, nuclear division occurring independently of spore wall formation has been observed previously in *spo1/spo1*, *spo2/spo2*, and *spo3/spo3* mutant cells (Moens et al. 1974). Because some *sps1/sps1* cells contained more than four compartments, including prospore-like compartments and unenveloped nuclei, it would appear that occasionally only part of a nucleus was encapsulated or that an anucleate spore-like compartment formed. This is reminiscent of the lack of coordination between nuclear division and prospore wall formation seen in *spo2* and *spo3* diploids (Moens et al. 1974).

The mature wild-type spore wall is multilayered with the two outermost layers being spore specific (Kreger-van Rij 1978; Briza et al. 1988, 1990a). The outermost layer, which contains a dityrosine-containing molecule, is deposited over a chitosan-containing layer (Briza et al. 1988, 1990a). These two outer layers appeared to be absent from the *sps1* spore-like compartments. It should be noted that the absence of these layers alone does not prevent visualization of spores by phase-contrast microscopy (Briza et al. 1990a; Pammer et al. 1992). We therefore infer that there is an additional structural defect in *sps1* prospore walls. The chitosan layer appears to be essential for incorporation of dityrosine into the spore wall (Briza et al. 1990b, 1994; Pammer et al. 1992). Because we have found that at least one of the genes required for formation of the dityrosine-containing layer (Briza et al. 1990b, 1994) is expressed in the mutant cells, we therefore suggest that the absence of the chitosan layer might prevent deposition of the dityrosine-containing layer around the *sps1* spore-like compartments.

Because the viability of *sps1*-arrested cells was not as high as was the viability of wild-type sporulating cells, it is possible that completion of meiosis was lethal to some of the mutant cells. The recovery of a population of haploid progeny from *sps1*-arrested cells indicates, however, that some of the postmeiotic cells were viable. The origin of the haploid progeny that were derived from *sps1*-arrested cells is unclear. It is possible that a haploid nucleus that failed to be encapsulated in an immature spore migrated into a daughter bud on resumption of growth, as has been shown recently for *spo14*-arrested cells (Honigberg et al. 1992; Honigberg and Esposito 1994). Another possibility is that the *sps1* prospores were released from the ascus and began to grow.

In addition to preventing spore wall maturation, mutation of *SPS1* also perturbed the pattern of sporulation-specific gene expression during the later portion of the sporulation program. *DIT1* transcripts accumulated to a higher level in *SPS1* mutant cells than in wild-type cells. Expression of *SPS100* was delayed and reduced, and expression of *SPS101* was almost undetectable in *SPS1* mutant cells. Interestingly, this aberrant pattern of late gene expression is similar to that seen in cells compromised for efficient sporulation attributable to high-copy effects of *SPS2* (Percival-Smith and Segall 1987).

Possible targets of the SPS1-encoded protein kinase

In contemplating a possible role for Sps1 kinase in sporulation, not only do we wish to account for the defect in spore packaging in the mutant cells but also for the observation that the mutant cells are less efficient than are wild-type cells in completing meiosis II. A structure that could contribute both to efficient completion of meiosis and to spore packaging is the spindle pole body. During the second meiotic division, an outer plaque develops as an enlargement of the outer layer of the spindle pole body. Prospore wall formation initiates from this outer plaque (Moens and Rapport 1971; Davidow et al. 1980). A modification to the spindle pole bodies at the end of meiosis I, such as a hypothetical Sps1-dependent phosphorylation event, might contribute to the efficiency of the next round of spindle pole body duplication as well as to outer plaque development. In this scenario, the defect caused by the absence of Sps1 would ultimately prevent spore maturation and indirectly prevent activation of late *SPS* genes.

An alternative explanation for the phenotype of *sps1/sps1* cells is that Sps1-dependent phosphorylation leads to activation of a transcriptional regulator that is responsible for the expression of a set of mid-late sporulation-specific genes. This hypothetical set of *SPS1*-dependent genes not only would include structural genes required for efficient completion of meiosis II and spore wall maturation but also regulatory gene(s) required to activate expression of late *SPS* genes such as *SPS100*. Because *DIT1*, a mid-late gene required for spore wall maturation, is expressed in the asporogenous *sps1/sps1* cells, it is necessary to also invoke an *SPS1*-independent pathway for expression of some of the mid-late genes involved in spore wall formation. Additionally, the unusually high level of expression of *DIT1* in the *sps1/sps1* strain suggests that an *SPS1*-dependent gene might be responsible for turning down expression of *DIT1*.

The identification of Sps1 as a putative protein kinase adds to the growing list of kinases that have been found to play a role in sporulation in *S. cerevisiae* (for review, see Hoekstra et al. 1991). Entry into meiosis is regulated in part by a decrease in the activity of cAMP-dependent protein kinase in response to nutrient limitation (for review, see Broach 1991). The expression of *IME1*, a key regulatory gene required for almost all meiotic gene expression (for review, see Mitchell 1994), is stimulated by *MCK1*, which encodes a dual specificity kinase (Dailey

et al. 1990; Neugeborn and Mitchell 1991). This kinase also plays a role in ascus maturation (Neugeborn and Mitchell 1991) and centromere function (Shero and Heter 1991). The *RIM11*-encoded kinase is required for Ime1 to activate expression of *IME2* (Mitchell and Bowditch 1992; Smith et al. 1993; Mitchell 1994). Ime2/Sme1, which contributes to early meiotic gene expression, is itself a putative protein kinase (Yoshida et al. 1990). Mek1, another meiosis-specific protein kinase homolog, is required for chromosome synapsis and recombination (Rockmill and Roeder 1991). Additionally, the cell cycle kinases Cdc28 and Cdc7 and the DNA repair-associated kinase Hrr25 are required for progression through meiosis (for review, see Hoekstra et al. 1991).

A protein kinase cascade regulating spore wall development?

Four functionally distinct signal transduction pathways that operate via a protein kinase cascade to activate a MAP kinase have been identified in yeast (for review, see Errede and Levin 1993; Neiman 1993; Ammerer 1994; Marshall 1994). The observation that the catalytic domain of Sps1 has 44% identity with the catalytic domain of Ste20, a member of the MAP kinase pathway involved in response to mating pheromone, suggests the existence of a novel sporulation-specific kinase cascade. Indeed, a sporulation-specific gene, *SMK1*, encoding a protein with homology to MAP kinases (Krisak et al., this issue) has been identified. The phenotype of *smk1/smk1* cells is strikingly similar to that of *sps1/sps1* cells (Krisak et al., this issue). This raises the possibility that Sps1 and Smk1 are components of a kinase cascade required to coordinate events involved in spore wall formation.

Ste20 is stimulated by release of the $\beta\gamma$ subunit complex from the heterotrimeric G-protein associated with the membrane-bound pheromone receptor and acts in conjunction with Ste5 to activate Ste11 kinase (Leberer et al. 1992; Ramer and Davis 1993; for review, see Sprague and Thorner 1992). Ste11 then activates Ste7 kinase (Neiman and Herskowitz 1994), which leads to activation of the MAP kinase homologs Fus3 and Kss1, which in turn activate the transcription factor Ste12 (for review, see Sprague and Thorner 1992). In contrast to the role of a heterotrimeric G-protein in regulating Ste20 kinase, a Ras-related small GTP-binding protein recently has been shown to activate p65^{PAK}, a mammalian relative of Ste20 (Manser et al. 1994). Future experiments will reveal whether the activity of Sps1 is regulated solely by transcription, by a member of the G-protein family, or by some other mechanism. Further insight into the putative Sps1/Smk1 kinase pathway awaits the identification of other members of the cascade as well as the ultimate target of the pathway.

Materials and methods

Plasmids

p18 (Percival-Smith and Segall 1984) contains a 3.7-kb fragment of yeast DNA encompassing the entire *SPS1* gene inserted into

the *Bam*HI site of pBR322. pSPS1-URA3, a replicating yeast plasmid containing the *SPS1* gene, was obtained by cloning a 2.3-kb *Bam*HI-*Xba*I fragment purified from p18 (Percival-Smith and Segall 1986) that had been propagated in a *dam*⁻ strain of *Escherichia coli* between the corresponding sites of pRS316 (Sikorski and Hieter 1989). pCAN^S1-LEU2, used to integrate the CAN^S1 allele into the yeast genome, contained a 1.8-kb *Bam*HI-*Sal*I fragment encompassing the CAN^S1 gene (Hoffman 1985) cloned between the corresponding sites of pRS306 (Sikorski and Hieter 1989). p18-2 (Percival-Smith and Segall 1986) was used to disrupt the chromosomal *SPS1* gene by insertion of URA3 12 codons upstream of the predicted stop codon of *SPS1*. This plasmid contains a DNA fragment encompassing the URA3 gene inserted into the *Bgl*III site at the 3' end of *SPS1* (see Fig. 1). YlpΔST, the plasmid used to delete the 5' end of the chromosomal *SPS1* gene, was a derivative of pRL1. pRL1 contained a 2.5-kb *Bam*HI-*Bgl*III fragment from p18, encompassing most of the *SPS1* gene, inserted into the *Bam*HI site of pUC18. YlpΔST was constructed by replacement of the *Clal*-*Eco*RV fragment of pRL1, which spans the 5'-end of the *SPS1* gene (see Fig. 1), with an 0.85-kb *Bgl*III-*Bgl*III fragment containing the *TRP1* gene. In the construction of YlpΔST, the *Clal* and *Eco*RV site of pRL1 and the *Bgl*III ends of the *TRP1*-containing fragment were filled in with the Klenow form of DNA polymerase I. *E. coli* strains used for cloning were TG1 [K12 Δ(*lac-pro*) *supE thi hsdD5/F' traD36 proA⁺B⁺ lacI^q lacZΔM15*], DH5α (*supE44*

Δ*lacU169* [φ80 *lacZΔM15*] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*], and W3110dam3 [*HsdR⁺ sup⁺ dam⁻*]. CaCl₂ transformations and recombinant DNA manipulations were carried out as described by Sambrook et al. (1989).

Yeast strains

All yeast strains used in this study were derived from the isogenic strains W3031A and W3031B (constructed by R. Rothstein) and their derivatives W3031A-H and W3031B-T (Law and Segall 1988) (see Table 1). LP112, provided by S. Lindquist, represents the diploid obtained by mating W3031A and W3031B; LP-HT represents the diploid obtained by mating W3031A-H and W3031B-T. With the exception of LP112, all diploids were obtained by prototrophic selection taking advantage of either chromosomal markers or markers introduced on plasmids for this purpose. The diploid strain YRL1 (*MATa/MATα sps1::URA3/sps1::URA3*) was constructed in three steps. First, we transformed W3031A-H and W3031B-T with p18-2 DNA that had been digested with *Bam*HI and *Xba*I, as described in Percival-Smith and Segall (1986). Second, insertion of the URA3-containing DNA fragment at the 3' end of the *SPS1* gene was confirmed by Southern blot analysis of genomic DNA from Ura⁺ transformants. Finally, a *MATa sps1::URA3* strain and a *MATα sps1::URA3* strain were mated to give the

Table 1. *S. cerevisiae* strains

Strain ^a	Genotype
W3031A	<i>MATa ade2-1 his3-11,15 leu2-3,115 trp1-1 ura3-1 can^r1-100</i>
W3031B	<i>MATα ade2-1 his3-11,15 leu2-3,115 trp1-1 ura3-1 can^r1-100</i>
W3031A-H	<i>MATa ade2-1 HIS3 leu2-3,115 trp1-1 ura3-1 can^r1-100</i>
W3031B-T	<i>MATα ade2-1 his3-11,15 leu2-3,115 TRP1 ura3-1 can^r1-100</i>
LP112	<i>MATa ade2-1 his3-11,15 leu2-3,115 trp1-1 ura3-1 can^r1-100</i> <i>MATα ade2-1 his3-11,15 leu2-3,115 trp1-1 ura3-1 can^r1-100</i>
LP-HT	<i>MATa ade2-1 HIS3 leu2-3,115 trp1-1 ura3-1 can^r1-100</i> <i>MATα ade2-1 his3-11,15 leu2-3,115 TRP1 ura3-1 can^r1-100</i>
Y6	<i>MATa ade2-1 his3-11,15 leu2-3,115 trp1-1 ura3-1 can^r1-100 cyh^r2</i>
Y7	<i>MATa ade2-1 his3-11,15 LEU2-CAN^S1-leu2-3,115 trp1-1 ura3-1 can^r1-100 cyh^r2</i>
Y8	<i>MATa ade2-1 his3-11,15 LEU2-CAN^S1-leu2-3,115 trp1-1 ura3-1 can^r1-100 cyh^r2 Δ<i>sps1::TRP1</i></i>
Y31	<i>MATα ade2-1 his3-11,15 leu2-3,115 trp1-1 ura3-1 can^r1-100 Δ<i>sps1::TRP1</i></i>
Y26	<i>MATa ade2-1 his3-11,15 LEU2-CAN^S1-leu2-3,115 trp1-1 ura3-1 cyh^r2 can^r1-100 Δ<i>sps1::TRP1</i></i> <i>MATα ade2-1 his3-11,15 leu2-3,115 trp1-1 ura3-1 CYH^S2 can^r1-100 Δ<i>sps1::TRP1</i></i>
Y30	<i>MATa ade2-1 his3-11,15 LEU2-CAN^S1-leu2-3,115 trp1-1 ura3-1 cyh^r2 can^r1-100</i> <i>MATα ade2-1 his3-11,15 leu2-3,115 trp1-1 ura3-1 CYH^S2 can^r1-100</i>
YRL1	<i>MATa ade2-1 HIS3 leu2-3,115 trp1-1 ura3-1 can^r1-100 sps1::URA3</i> <i>MATα ade2-1 his3-11,15 leu2-3,115 TRP1 ura3-1 can^r1-100 sps1::URA3</i>

^a These strains were generated by the authors, with the exception of W3031A and W3031B (from R. Rothstein) and LP112 (from S. Lindquist).

diploid strain YRL1. Y8 (*MATa Δsps1::TRP1*) and Y31 (*MATα Δsps1::TRP1*) were obtained by transforming W3031B and Y7 (see below), respectively, with YIpΔST that had been digested with *Bam*HI and *Hind*III. Deletion–substitution of the 5' end of the chromosomal *SPS1* gene with *TRP1*-containing DNA, to give the allele referred to as *Δsps1*, was confirmed by Southern blot analysis. We have found that *MATa/MATα sps1::URA3/sps1::URA3* and *MATa/MATα Δsps1::TRP1/Δsps1::TRP1* strains behave identically in all our phenotypic and genetic analyses.

Diploid strains heterozygous for *can1* and *cyh2* were obtained as follows. First, a spontaneous cycloheximide-resistant mutant of W3031A, Y6, was isolated as described by Sikorski and Boeke (1991). Strain Y7, a canavanine-sensitive derivative of Y6, was generated by transforming Y6 with pCAN^s1–LEU2, which had been partially digested with *Eco*RI. This resulted in the targeting of the plasmid to the *leu2* locus giving a *CAN^s1* gene flanked by *leu2* and *LEU2*, generating the allele *LEU2–CAN^s1–leu2*. Replacement of the *SPS1* gene of Y7 with the *Δsps1::TRP1* allele by integrative transformation generated strain Y8 (see above). After pSPS1–URA3 had been introduced into strain W3031B and into strain Y31, the plasmid-containing W3031B strain was mated with Y7 and the plasmid-containing Y31 strain was mated with Y8. The plasmid provided a *URA3* marker for prototrophic selection of diploids and allowed us to confirm that the diploid obtained in the latter cross was able to sporulate efficiently. Diploid strains heterozygous for *cyh2* and *can1* and lacking pSPS1–URA3, referred to as Y30 (*SPS1/SPS1*) and Y26 (*Δsps1::TRP1/Δsps1::TRP1*), were then isolated on 5-fluoro-orotic acid (Boeke et al. 1984).

Yeast transformations were done as recommended by Schiestl and Gietz (1989). Growth and sporulation were as described by Percival-Smith and Segall (1984). In brief, cells were grown in YPD supplemented with 40 μg/ml of adenine or in SD supplemented with required amino acids and bases (Sherman 1991). To induce sporulation, cells were pregrown in YEPA (0.5% yeast extract, 1% Bacto-peptone, and 1% potassium acetate) supplemented with 40 μg/ml of adenine for at least three generations. When the cells had reached a density of 1×10^7 to 2×10^7 cells/ml, they were washed and transferred to sporulation medium (1% potassium acetate supplemented with required amino acids and bases) at 1×10^7 to 2×10^7 cells/ml.

Sequencing strategy

A 2.5-kb *Bam*HI–*Bgl*II fragment of p18 was subcloned in both orientations in the *Bam*HI site of pUC18. Nucleotide sequence was obtained from restriction endonuclease-generated derivatives of these plasmids by the dideoxy-chain termination method of Sanger et al. (1977) with commercially available primers using double-stranded DNA as template. Both strands of the inserts were sequenced. The last 291 nucleotides of *SPS1* have been sequenced previously (Percival-Smith and Segall 1986). The two sequences differ at codon 469; GGA is present in the current sequence and AGA was present in the previous sequence. A protein homology search was performed using the BLAST network service of the National Center for Biotechnology Information. The GenBank accession number for the *SPS1* sequence reported in this paper is U13018.

RNA isolation and analysis

RNA was prepared from yeast and analyzed as described by Percival-Smith and Segall (1984). The probes used in Northern blot analysis to detect various transcripts were as follows: *SPS1*, a 550-bp *Clal*–*Eco*RV fragment from pSPS1–URA3; *HOP1*, a

3.0-kb *Eco*RI–*Eco*RV fragment from pNH59-2 (Hollingsworth et al. 1990); *DIT1*, a 1.8-kb *Hind*III–*Hind*III fragment from pPB-13 (Briza et al. 1990); *PYK1*, a 1.0-kb *Hpa*I–*Hpa*I (Burke et al. 1983) fragment from pPYK101 (provided by Bryan McNeil); *SPS2*, a 0.8-kb *Pst*I–*Hind*III fragment from p18 (Percival-Smith and Segall 1986); *SPS4*, p275 digested with *Hind*III (Garber and Segall 1986); *SPS100*, a 750-bp *Bam*HI–*Nco*I fragment from pE18-B8a (Law and Segall 1988); and *SPS101*, pC2 digested with *Eco*RI (Law and Segall 1988). The probes were radioactively labeled using a random priming method (Feinberg and Vogelstein 1983).

DAPI staining

Approximately 1 ml of cells was harvested and fixed by resuspension in 0.5 ml of a solution containing 50% ethanol, 1 M sorbitol, and 10 mM NaPO₄ (pH 7.2) (Bishop et al. 1992). Slides were pretreated with 1% polyethyleneimine for 5 min and rinsed in water. Twenty microliters of fixed cells was placed on the slide and allowed to settle for 30 min. The cells were then rinsed in phosphate-buffered saline (PBS) (Sambrook et al. 1989) prior to being stained for 5 min with 10 μg/ml of DAPI in PBS. After rinsing the slides in PBS, 10 μl of mounting solution (0.8% propylgallate, 90% glycerol) was added, coverslips were put on, and the slides were sandwiched between paper towels and weighted for 30 min prior to being viewed with fluorescent optics.

Assays for resumption of mitotic growth and for haploid progeny derived from sps1-arrested cells

Resumption of growth was assessed using cells that had been pregrown in YEPA to a concentration of 1×10^7 to 2×10^7 cells/ml and then transferred to sporulation medium. At various times, samples of cells were diluted and plated on YPD. To determine the efficiency of haploidization, we examined the uncovering of recessive drug-resistant alleles. In these experiments *SPS1/SPS1 CAN^s1/can1 CYH^s2/cyh2* and *sps1/sps1 CAN^s1/can1 CYH^s2/cyh2* cells were pregrown in YEPA, transferred to sporulation medium at a concentration of 1×10^7 to 2×10^7 cells/ml, incubated for 21 hr in sporulation medium, diluted, and plated on YPD. After a 24 hr incubation, the resultant colonies were patched on YPD, incubated for 24 hr, and replica plated to SD containing the appropriate supplements and either 10 μg/ml of cycloheximide or 60 μg/ml of canavanine.

For FACS analyses, wild-type and mutant cells were incubated in sporulation medium for 21 hr, diluted in YPD, and grown for 21 hr. Approximately 10^7 cells were washed and fixed in 70% ethanol. Fixed cells were resuspended in 50 mM sodium citrate at pH 7.4, sonicated briefly, and treated with 0.25 mg/ml of RNase A for 1 hr at 50°C. Cells were then stained with 8 μg/ml of propidium iodide in citrate buffer for 30 min at 24°C in the dark, treated with 0.2 mg/ml of proteinase K for 1 hr at 50°C, and sonicated to disperse clumps. Fluorescence was measured using a Becton Dickinson FACScan and analyzed with LYSYS II software. No gating was used, and 20,000 cells were examined in each experiment.

Electron microscopy

Cells from strains Y30 and Y26 that had been in sporulation medium for 21 hr were centrifuged, washed in H₂O, and prepared for electron microscopy using procedures modified from Hayat (1970) and Briza et al. (1988). In brief, cells were fixed overnight in a solution containing 3% glutaraldehyde, 3% acrolein, and 0.1 M sodium cacodylate. Samples were washed and

postfixed in 1.5% potassium permanganate for 1 hr at 4°C, washed again, and fixed with 1% osmium tetroxide in veronyl acetate buffer for 30 min at 24°C. Samples were embedded in warm agar and dehydrated through a graded series of ethanol, and were then embedded in Spurr resin. Thin sections were stained with saturated uranyl acetate and Reynold's lead citrate and examined on a Philips 300 electron microscope.

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