

Identification of a Developmentally Regulated Septin and Involvement of the Septins in Spore Formation in *Saccharomyces cerevisiae*

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Abstract. The *Saccharomyces cerevisiae* *CDC3*, *CDC10*, *CDC11*, and *CDC12* genes encode a family of related proteins, the septins, which are involved in cell division and the organization of the cell surface during vegetative growth. A search for additional *S. cerevisiae* septin genes using the polymerase chain reaction identified *SPR3*, a gene that had been identified previously on the basis of its sporulation-specific expression. The predicted *SPR3* product shows 25–40% identity in amino acid sequence to the previously known septins from *S. cerevisiae* and other organisms. Immunoblots confirmed the sporulation-specific expression of Spr3p and showed that other septins are also present at substantial levels in sporulating cells. Consistent with the expression data, deletion of *SPR3* in either of two genetic backgrounds had no detectable effect on exponentially growing cells. In one genetic background, de-

letion of *SPR3* produced a threefold reduction in sporulation efficiency, although meiosis appeared to be completed normally. In this background, deletion of *CDC10* had no detectable effect on sporulation. In the other genetic background tested, the consequences of the two deletions were reversed. Immunofluorescence observations suggest that Spr3p, Cdc3p, and Cdc11p are localized to the leading edges of the membrane sacs that form near the spindle-pole bodies and gradually extend to engulf the nuclear lobes that contain the haploid chromosome sets, thus forming the spores. Deletion of *SPR3* does not prevent the localization of Cdc3p and Cdc11p, but these proteins appear to be less well organized, and the intensity of their staining is reduced. Taken together, the results suggest that the septins play important but partially redundant roles during the process of spore formation.

IN the yeast *Saccharomyces cerevisiae*, *MATa/MATa* cells undergo sporulation when subjected to nitrogen starvation in the presence of a nonfermentable carbon source (Esposito and Klapholz, 1981; Mitchell, 1994). During sporulation, a single round of DNA replication is followed by the two divisions of meiosis, which occur within a single, intact nuclear envelope. At the beginning of meiosis II, a flattened membrane sac, the “prospore wall”, forms in close apposition to the cytoplasmic face of each spindle-pole body (Moens, 1971; Moens and Rapport, 1971; Guth et al., 1972; Beckett et al., 1973; Byers, 1981). As meiosis proceeds, these membrane sacs extend along the outer surface of the nuclear envelope to form cup-shaped structures that surround the nuclear lobes containing the spindle poles and separating chromosomes. At the completion of meiosis II, the prospore walls separate from the spindle-pole bodies and nuclear envelope, and portions of the mother-cell cytoplasm and organelles enter the intervening space. Eventually, the lobes of the nucleus pinch off to form the daughter nuclei, and the prospore

walls complete the engulfment of these nuclei and associated cytoplasm to form the four spores. Several layers of spore-wall material are then deposited between the two membranes of the prospore wall (Lynn and Magee, 1970; Moens, 1971; Byers, 1981; Briza et al., 1988, 1990b, 1994).

Several approaches have been used to identify genes and proteins involved in spore formation. Some relevant genes have been identified by screening directly for mutants defective in sporulation (Esposito and Esposito, 1974; Esposito and Klapholz, 1981; Tsuboi, 1983; Briza et al., 1990a; Pammer et al., 1992); in other cases, mutants isolated in other ways were subsequently found to have defects in spore formation (Leibowitz and Wickner, 1976; Bulawa, 1993; Krisak et al., 1994). Other genes whose products might be involved in spore formation were identified on the basis of their specific expression during the later stages of sporulation (Clancy et al., 1983; Percival-Smith and Segall, 1984; Weir-Thompson and Dawes, 1984; Kurtz and Lindquist, 1984, 1986; Gottlin-Ninfa and Kaback, 1986; Law and Segall, 1988; Coe et al., 1992; San Segundo et al., 1993; Burns et al., 1994). Of the genes identified in this way, one (*SPS1*) was found to be essential for spore-wall maturation (Percival-Smith and Segall, 1986; Friesen et al., 1994), but mutations in the other genes ana-

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lyzed to date have had either modest or undetectable effects on the efficiency of spore formation (Percival-Smith and Segall, 1986; Gottlin-Ninfa and Kaback, 1986; Law and Segall, 1988; Kao et al., 1989; Kallal et al., 1990; Muthukumar et al., 1993; San Segundo et al., 1993).

Among the genes expressed differentially late in sporulation is *CDC10* (Kaback and Feldberg, 1985), which encodes one of the septin family of proteins. The septins were first identified in *S. cerevisiae* (see below) but have subsequently been found also in a variety of other organisms, where an involvement in cell division and/or septum formation appears to be a general aspect of their function (reviewed by Longtine et al., 1996). In *S. cerevisiae*, electron microscopy studies of vegetative cells revealed a ring of filaments lying in close apposition to the cytoplasmic face of the plasma membrane in the mother-bud neck (Byers and Goetsch, 1976; Byers, 1981). These filaments are absent in temperature-sensitive-lethal mutants defective in any of four genes, *CDC3*, *CDC10*, *CDC11*, and *CDC12* (Byers, B., and L. Goetsch. *J. Cell Biol.* 70:35a; Byers, 1981; Adams, 1984). In immunofluorescence and immunoelectron microscopy experiments, antibodies specific for the products of these four genes decorate the neck region of wild-type cells in the pattern expected if these proteins are constituents of the neck filaments; moreover, upon shift of any of the four mutants to restrictive temperature, immunofluorescence staining of the necks with each of the four specific antibodies disappears at the same rate as do the filaments as judged by electron microscopy (Haarer and Pringle, 1987; Kim et al., 1991; Ford and Pringle, 1991; Kim, H., B. Haarer, and J. R. Pringle, unpublished results; Mulholland, J., D. Preuss, and D. Botstein, personal communication). Sequencing revealed that Cdc3p, Cdc10p, Cdc11p, and Cdc12p constitute a family of related proteins (25–37% identical in amino acid sequence) (Steensma and van der Aart, 1991; Haarer, B. K., S. H. Lillie, S. K. Ford, S. R. Ketcham, and J. R. Pringle, unpublished results; Longtine et al., 1996). All four proteins contain sequences conserved among nucleotide-binding proteins, a feature shared with the related proteins from other organisms. In addition, three of the *S. cerevisiae* septins (Cdc3p, Cdc11p, and Cdc12p), like most of the septins from other organisms, contain predicted coiled-coil domains near their COOH-termini. The septins are not otherwise closely related to other known proteins.

The loss of the neck filaments in the *cdc3*, *cdc10*, *cdc11*, and *cdc12* mutants is accompanied by a failure to form a chitin ring in the cell wall at the base of the bud (suggesting a defect in the localization of chitin synthase or of an associated regulatory factor), the production of abnormally elongated buds (perhaps associated with a hyperpolarization of the actin cytoskeleton), and a failure of cytokinesis (presumably reflecting a defect in actin/myosin organization and/or in the localization of cell-wall deposition) (Hartwell, 1971; Adams, 1984; Adams and Pringle, 1984; Slater et al., 1985). Septin mutants also show defects in the axial budding pattern (Flescher et al., 1993; Chant et al., 1995), apparently reflecting an inability to localize Bud3p, a putative component of the axial positional signal (Chant et al., 1995). Despite the high level of expression of *CDC10* in sporulating cells, diploids homozygous for temperature-sensitive alleles of *CDC3*, *CDC10*, or *CDC11*

showed no defects in sporulation (Simchen, 1974). However, because the parent strain did not sporulate well at high temperatures, the experiments were done using a restrictive temperature several degrees lower than that used in the initial isolation of the mutants.

In every organism examined so far, the septins are present as a family with multiple members. To ask if *S. cerevisiae* contains septins other than Cdc3p, Cdc10p, Cdc11p, and Cdc12p, we used the polymerase chain reaction (PCR) with primers based on the known sequences. This identified a fifth *S. cerevisiae* septin gene, which proved identical to *SPR3*, a gene isolated previously on the basis of its high level of expression late in sporulation (Clancy et al., 1983). This led us to investigate septin localization and function during sporulation; as reported below, this analysis suggests that the septins are involved in the interesting and poorly understood process by which the haploid nuclei and associated cytoplasm become encapsulated in a newly formed plasma membrane and cell wall within the body of the mother cell.

Materials and Methods

Genetic and Recombinant-DNA Procedures

Standard procedures of yeast genetics (Guthrie and Fink, 1991) and DNA manipulation (Sambrook et al., 1989) were used except where noted. The *E. coli* strain DH5 α F' (Gibco BRL, Gaithersburg, MD) was used except where indicated. Clones for sequencing were generated by subcloning in the pBluescript vectors (Stratagene, La Jolla, CA). Primers were used to generate overlap in the sequence. Single-stranded sequencing was done for both strands using the Sequenase kit (version 2.0) as described in the U. S. Biochemicals (Cleveland, OH) manual. PCR was performed in two ways. For the initial isolation of an *SPR3* fragment (see Results), Taq DNA polymerase was used as described by Gould et al. (1989). In all other PCR reactions, Vent DNA polymerase was used according to the New England Biolabs (Beverly, MA) specifications. Filters containing λ clones covering the yeast genome (Riles et al., 1993) were obtained from the American Type Culture Collection (Rockville, MD).

Yeast Strains, Growth Conditions, and Sporulation

Most of the yeast strains used are described in Table I; others are described where appropriate below. All yeast media (YPD rich medium, synthetic complete [SC] medium, SC lacking specific nutrients, minimal medium) and *E. coli* media were as described previously (Guthrie and Fink, 1991; Sambrook et al., 1989). Sporulation experiments were performed by streaking cells from a frozen stock onto a YPD plate. A fresh colony from this plate was used to inoculate YPD liquid medium, and the culture was grown for 1 d to early stationary phase. 0.5 ml of this culture was centrifuged, and the cells were washed once in 1% YPA (1% potassium acetate, 1% yeast extract, 2% peptone) supplemented with all required nutrients, resuspended in 5 ml of the same medium, and grown to an OD₆₆₀ of 1.0–1.3. The cells were then centrifuged, washed once in 1% potassium acetate supplemented with all required nutrients, and resuspended in the same medium at 2×10^7 cells/ml. The cultures were then incubated at 22°C for assays of sporulation efficiency or at 30°C for protein analysis and immunolocalization experiments.

Plasmids

The YEp24 library (Carlson and Botstein, 1982) contains Sau3A-digested yeast genomic DNA inserted at the BamHI site of vector YEp24. Plasmid pSP10 (Porter, S., and T. Petes, personal communication) contains *HIS4* on a derivative of YCplac33 (URA3, low copy; Gietz and Sugino, 1988). Plasmid pB5-1a was an *SPR3*-containing isolate from the YEp24 library (see Results). To amplify *SPR3* sequences by PCR, three primers were made that were complementary to sequences in the 2.0-kb fragment shown in Fig. 3 A. Primers, 1 (ACAGTCGACGAAATGTAGAGGT-TCAAG) and 2 (ACAGTCGACATGAAGTCAAAGGGAGT) have

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source or reference
C276-4A	<i>a</i> (prototrophic)	Wilkinson and Pringle, 1974
YEF473	<i>a/α his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3</i>	E. Bi*
YEF473-A	<i>a his3 leu2 lys2 trp1 ura3</i>	Segregant from YEF473
SY1263	<i>α his3 leu2 ura3 can1</i>	Bender and Sprague, 1989
NKY278	<i>a/α ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3</i>	Cao et al., 1990 [‡]
NKY1059	<i>a ho::hisG ade2::LK his4X leu2::hisG lys2 ura3</i>	N. Kleckner [‡]
52	<i>a/α his4/his4 trp1/trp1 ura3/ura3</i>	Chant and Herskowitz, 1991
HF17	<i>a leu2 met1 ura3 cdc3-6</i>	This study [§]
HF18	<i>a his3 leu2 ura3 cdc10-1</i>	This study
HF19	<i>α leu2 met1 ura3 cdc11-6</i>	This study [¶]
HF20	<i>α leu2 ura3 cdc12-6</i>	This study [¶]
HF1	<i>a/α ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 spr3::URA3/SPR3</i>	This study ^{**}
HF2	<i>a/α his4/his4 trp1/trp1 ura3/ura3 spr3::URA3/SPR3</i>	This study ^{**}
HF1-1A	<i>a ho::LYS2 lys2 ura3 spr3::URA3</i>	Segregant from HF1 ^{§§}
HF1-1B	<i>α ho::LYS2 lys2 ura3 spr3::URA3</i>	Segregant from HF1 ^{§§}
HF1-1C	<i>a ho::LYS2 lys2 ura3</i>	Segregant from HF1 ^{§§}
HF1-1D	<i>α ho::LYS2 lys2 ura3</i>	Segregant from HF1 ^{§§}
HF7	<i>a/α ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 spr3::URA3/spr3::URA3</i>	HF1-1A X HF1-1B
HF8	<i>a/α ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 spr3::URA3/SPR3</i>	HF1-1A X HF1-1D
HF9	<i>a/α ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3</i>	HF1-1C X HF1-1D
HF2-1A	<i>α his4 trp1 ura3</i>	Segregant from HF2
HF2-1B	<i>a his4 trp1 ura3 spr3::URA3</i>	Segregant from HF2
HF2-1C	<i>a his4 trp1 ura3</i>	Segregant from HF2
HF2-1D	<i>α his4 trp1 ura3 spr3::URA3</i>	Segregant from HF2
HF15	<i>a/α his4/his4 trp1/trp1 ura3/ura3 spr3::URA3/SPR3</i>	HF2-1A X HF2-1B
HF16	<i>a/α his4/his4 trp1/trp1 ura3/ura3 spr3::URA3/spr3::URA3</i>	HF2-1B X HF2-1D
HF31	<i>a/α ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 cdc10-Δ1/CDC10</i>	This study ^{**}
HF30	<i>a/α his4/his4 trp1/trp1 ura3/ura3 cdc10-Δ1/CDC10</i>	This study ^{**}
HF31-1A	<i>a ho::LYS2 lys2 ura3 cdc10-Δ1</i>	Segregant from HF31
HF31-1B	<i>α ho::LYS2 lys2 ura3 cdc10-Δ1</i>	Segregant from HF31
HF22	<i>a/α ho::hisG/ho::LYS2 his4X/HIS4 leu2::hisG/LEU2 lys2/lys2 ura3/ura3 cdc10-Δ1/CDC10 spr3::URA3/SPR3</i>	This study ^{¶¶}
HF22-1A	<i>a ho::LYS2 his4X lys2 ura3 cdc10-Δ1</i>	Segregant from HF22
HF22-1B	<i>α ho::hisG his4X leu2::hisG lys2 ura3 cdc10-Δ1</i>	Segregant from HF22
HF25	<i>a/α ho::hisG/ho::LYS2 his4X/his4X leu2::hisG/LEU2 lys2/lys2 ura3/ura3 cdc10-Δ1/cdc10-Δ1</i>	HF22-1A X HF22-1B ^{***}
HF30-1A	<i>a his4 trp1 ura3 cdc10-Δ1</i>	Segregant from HF30
HF30-1B	<i>α his4 trp1 ura3 cdc10-Δ1</i>	Segregant from HF30
HF28	<i>a/α his4/his4 trp1/trp1 ura3/ura3 cdc10-Δ1/cdc10-Δ1</i>	HF30-1A X HF30-1B ^{***}
HF29	<i>a/α his4/his4 trp1/trp1 ura3/ura3 cdc10-Δ1/CDC10 spr3::URA3/SPR3</i>	HF2-1B X HF30-1B

*Constructed by diploidizing (using *HO* on a plasmid; Herskowitz and Jensen, 1991) a segregant from C276-4A X YPH500 (Sikorski and Hieter, 1989).

[‡]Isogenic derivatives of strain SK-1 (Kane and Roth, 1974). Genetic markers were introduced by gene transplacement.

[§]Derived from the original *cdc3-6* mutant (isolated in the screen described by Adams and Pringle, 1984) by several crosses with other strains.

^{||}Derived from strain 17012 (Hartwell et al., 1973) by several crosses with other strains.

[¶]Derived from strains JPT194 and JPTA1493 (Adams and Pringle, 1984) by several crosses with other strains.

**Transformant of NKY278 (see text).

††Transformant of 52 (see text).

§§HF1-1A, HF1-1B, HF1-1C, and HF1-1D were from the same tetrad.

|||HF2-1A, HF2-1B, HF2-1C, and HF2-1D were from the same tetrad.

¶¶HF31-1A was mated to a segregant from NKY1059 X HF1-1B.

***See text for details.

the *SalI* recognition sequence (underlined) included near their 5' ends, and primer 3 (ACAGAATTCGTTGTTGGTAGTGGAAAG) has the *EcoRI* recognition sequence (underlined) included near its 5' end. A 1.8-kb fragment including the *SPR3* open reading frame and 350 bp of 3'-untranslated sequence was amplified by PCR using primers 1 and 2 (see Fig. 3 A) and plasmid pB5-1a as template; this fragment was inserted at the *SalI* site of the pBluescript vector SK(+) (Stratagene) in the orientation that would generate a 1.6-kb fragment upon digestion with *HindIII*, creating plasmid pFUS5. The 1.6-kb *HindIII* fragment from pFUS5 was inserted in frame at the *HindIII* site downstream of the *ADHI* promoter of vector pADNS (Colicelli et al., 1989), creating plasmid pADSPR3. In a second PCR reaction, primers 1 and 3 were used with plasmid pB5-1a as template, and the resulting 1.9-kb fragment was digested with *SalI* and *EcoRI* and inserted at the corresponding sites in vector SK(+), creating plasmid pSKSPR3. Plasmid pSKSPR3 was digested with *HindIII* and *HpaI*, removing 1.4 kb of sequence (including most of the *SPR3* open reading frame; see Fig. 3 A), which was then replaced with the 1.1-kb *HindIII*-*SmaI* fragment containing *URA3* from plasmid YIpURA3 (Lillie, S., personal

communication), creating the *spr3::URA3* allele in plasmid pSPR3ΔURA3. The 1.6-kb fragment from pSPR3ΔURA3 was then used to disrupt the chromosomal *SPR3* gene (see Results).

The 2.5-kb *KpnI*-*XbaI* fragment carrying *CDC10* (Clarke and Carbon, 1980) was subcloned at the corresponding sites in vector pAlter-1 (Promega, Madison, WI), creating plasmid pAlter(CDC10). The 2.5-kb *SacI*-*XbaI* fragment carrying *CDC10* from pAlter(CDC10) was subcloned at the corresponding sites in vector YCplac111 (*LEU2*, low copy; Gietz and Sugino, 1988), creating plasmid YCp111(CDC10). The 2.5-kb *KpnI*-*SalI* fragment carrying *CDC10* from pAlter(CDC10) was subcloned at the corresponding sites in vector pRS314 (*TRP1*, low copy; Sikorski and Hieter, 1989), creating plasmid pRS(CDC10). Plasmid p239 contains the *cdc10-Δ1* allele (*CDC10* disrupted with *URA3*) on a *KpnI*-*XbaI* fragment (Flescher et al., 1993).

Disruption of *CDC10*

Strains NKY278 and 52 were transformed with the 1.8-kb *KpnI*-*XbaI*

fragment of plasmid p239 (see above). Transformants that had one copy of *CDC10* replaced by *cdc10-Δ1* (strains HF31 and HF30, respectively) were confirmed by Southern blot analysis (see Fig. 3 D) and by the rescue of the defects in the haploid *cdc10-Δ1* segregants by *CDC10* on a plasmid (data not shown). Tetrad analysis of HF31 and HF30 yielded four viable segregants per tetrad, and the *URA3* marker segregated 2:2. Haploid *cdc10-Δ1* cells had a severe mating defect when mated to other *cdc10-Δ1* cells, although not when mated to *CDC10* cells (data not shown). Thus, to construct diploids homozygous for *cdc10-Δ1* in the strain NKY278 background, strain HF22-1B was transformed with plasmids pSP10 and YCp111(CDC10), and a transformant was then mated to strain HF22-1A. Diploids were selected on minimal medium and eight diploid clones were streaked repeatedly on YPD plates to promote plasmid loss. We were able to cure plasmid YCp111(CDC10) from only one strain, HF25, as judged by its inviability and abnormal morphology at 37°C. All eight diploids remained His⁺, presumably due to gene conversion of the chromosomal *his4* allele during the initial transformation of strain HF22-1B with plasmid pSP10. To construct diploids homozygous for *cdc10-Δ1* in the strain 52 background, strain HF30-1A was transformed with plasmid pRS(CDC10) and mated to strain HF30-1B that had been transformed with plasmid pSP10, and diploids were selected on minimal medium. Eight diploid clones were streaked repeatedly on YPD to promote plasmid loss. All eight diploids lost plasmid pSP10 (i.e., became His⁻), but only one, HF28, lost plasmid pRS(CDC10), as judged by its failure to grow on SC-Trp medium and by the inviability and abnormal morphology of the cells at 37°C.

Protein Manipulations and Preparation of Antibodies

Total yeast proteins were extracted by adding 0.15 ml of a solution containing 1.85 M NaOH and 7.4% β-mercaptoethanol to 1 ml of culture and incubating the mix on ice for 10 min. Proteins were precipitated with TCA (Sambrook et al., 1989), resuspended in 2× Laemmli buffer (Laemmli, 1970), solubilized by boiling for 10 min, electrophoresed on SDS-PAGE, and transferred electrophoretically to nitrocellulose membranes as described previously (Ford and Pringle, 1991). For the immunodetection of proteins, longitudinal strips of these membranes were incubated with primary antibodies and then with alkaline phosphatase-conjugated secondary antibodies as described previously (Haarer and Pringle, 1987).

To prepare antibodies to Spr3p, the 1.8-kb *Sall* fragment from plasmid pFUS5 (see above) was inserted at the *Sall* site of plasmid pATH3 (Koerner et al., 1991), resulting in a *trpE-SPR3* fusion, and at the *Sall* site of pMal-2c (Maina et al., 1988), resulting in a *malE-SPR3* fusion. *E. coli* strain BS100 (Lutkenhaus et al., 1986) was transformed separately with the resulting plasmids, expression of the fusion proteins was induced (Koerner et al., 1991; Maina et al., 1988), and the insoluble fractions were isolated from both strains (Koerner et al., 1991). In both cases, these fractions were highly enriched (>95% of the total protein) for proteins of the sizes predicted for the desired fusion proteins (96.8 kD for TrpE-Spr3p and 99.4 kD for MalE-Spr3p; data not shown) as judged by SDS-PAGE. Each insoluble fraction was injected into rabbits using standard protocols (Cocalico Biologicals, Reamstown, PA). 800 μg of fusion protein were used for each primary injection and 400 μg were used for the subsequent boosts. Sera were tested for antibody titer by immunoblotting (as described above) of proteins from the appropriate *E. coli* strains. When strong signals were seen (after two boosts of the rabbits injected with each fusion protein), antibodies were affinity purified for further use. Affinity purification was performed as described previously (Pringle et al., 1989) using transverse strips of nitrocellulose blots (prepared as described above) of the fusion proteins; each such strip had ~400 μg of immobilized fusion protein, and eight such strips were used for every 0.6 ml of serum. Two steps of purification were used. Antibodies raised against TrpE-Spr3p were purified first on MalE-Spr3p and then on TrpE-Spr3p; antibodies raised against MalE-Spr3p were purified first on TrpE-Spr3p and then on MalE-Spr3p. All experiments were done in parallel using the twice-affinity-purified antibodies raised against each fusion protein at dilutions of 1:100 (for immunoblotting) and 1:5 (for immunofluorescence). Identical results were obtained with the two antibody preparations; the results shown below were all obtained with the antibodies raised against MalE-Spr3p.

Other Reagents

The antibodies to Cdc3p (Kim et al., 1991) and Cdc11p (Ford and Pringle, 1991) were described previously. YOL1/34 anti-tubulin antibody (Kilmar-

tin et al., 1982) was obtained from Accurate Chemical and Scientific (Westbury, NY). Goat anti-rabbit-IgG and anti-rat-IgG secondary antibodies conjugated to rhodamine, FITC, or alkaline phosphatase were purchased from either Jackson ImmunoResearch Labs (West Grove, PA) or Boehringer Mannheim (Indianapolis, IN).

Morphological Observations

The overall morphology of cells and asci was determined after growth in liquid medium except where indicated. Cultures were sonicated briefly and observed using either an oil-immersion 60× objective with differential interference-contrast (DIC) optics or a 40× objective with phase-contrast optics. In some cases, cells were fixed by adding formaldehyde to the culture medium to 3.7% after sonication. Cells were prepared for Calcofluor staining and immunofluorescence and observed by fluorescence microscopy as described previously (Pringle et al., 1989). Nuclei were stained by including 0.05 μg/ml bisBenzimide (Sigma, St. Louis, MO) in the mounting medium. All microscopy was performed using a Nikon Microphot SA microscope.

Results

Isolation of *SPR3*

Four *S. cerevisiae* septin genes (*CDC3*, *CDC10*, *CDC11*, and *CDC12*) had been identified on the basis of temperature-sensitive-lethal mutants (see Introduction). To screen for additional *S. cerevisiae* septins, two highly degenerate primers were designed based on regions conserved among the four known proteins and related proteins from other fungi (Fig. 1, A and B). PCR was then performed on genomic DNA from strain C276-4A (see Materials and Methods). The major amplified product was 257 bp, close in size to the ~275 bp expected from the other sequences (data not shown). This product was isolated, cut with *EcoRI* and *PstI* at sites included in the primers (Fig. 1 B), and cloned into the pBluescript SK(+) vector. DNA sequencing revealed an open reading frame whose predicted product showed homology to the known septins.

A radioactive probe prepared from the cloned PCR product was used to screen the *S. cerevisiae* YEp24 genomic-DNA library by colony hybridization, yielding a plasmid (pB5-1a) with a 7.5-kb insert. Subclones from the region of pB5-1a that hybridized to the PCR product were sequenced, revealing an open reading frame of 512 codons; the sequenced region included 111 bp of apparent 5'-noncoding sequence and 317 bp of apparent 3'-noncoding sequence (Fig. 1 C). The DNA sequence of the open reading frame was identical to that of the PCR product within the region of overlap (underlined in Fig. 1 C). The sequence also matched that of *SPR3* (Ozsarac et al., 1995), except for a few single-base discrepancies that could represent polymorphisms or sequencing errors. *SPR3* had originally been identified as a gene that is preferentially expressed during sporulation (Clancy et al., 1983; Holaway et al., 1987; Kao et al., 1989). The 1.8-kb *Sall* fragment containing *SPR3* from plasmid pFUS5 (see Materials and Methods) was used to probe filters containing an ordered set of λ clones (Riles et al., 1993) covering the yeast genome. The fragment hybridized uniquely to clones from the right arm of chromosome VII (λ' clones 6247 [ATCC No. 70703] and 6581 [ATCC No. 70745]). This places *SPR3* between *TFC4* and *SPR2* on the current map of this chromosome arm in the *Saccharomyces* Genome Database (Stanford University).

A

	1	2
Cdc3p	IDTEGFG.....(72aa).....	IPVIAKS
Cdc10p	IDTPGFG.....(72aa).....	IPVIGKS
Cdc11p	IDTPGFG.....(72aa).....	IPVIGKS
Cdc12p	IDTPGFG.....(71aa).....	IPVIAKA
Spn1p	IDTPGFG.....(71aa).....	IPVIAKA
CaCdc3p	ITAPGFG.....(71aa).....	IPVIAKS
CaCdc10p	IDTPGFG.....(72aa).....	VPIIAKS

B

Primer 1 5' ACA GAA TTC ATT GAC ACC CCC GGC TTT GG 3'
 EcoRI A A A A

Primer 2 5' ACA CTG CAG GA TTT CGC GAT CAC CGG CA 3'
 PstI C ATT A A A

C

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-111 TTTTCCCCCCTTTTGTGTTGGTAGTGGAAAGAAAAGGATCAATTACAGAAATAAA
-51 TAAATAAATAAATAAACCTAAAATTCCTTTGGCTCATTGAATTTTATTAGAGTCA
1 K H K S
10 AAAGGGAGTCGGTGTGCACAGACTGCTGTCGAATTTCCCAAGATAGTCTCTGGATTC
4 K G S R L S T D C P V E F P K I V S G F
70 GCTGAGGAAGTGAANAATACGTAGACAAGTTCCTCCAGGACAGTACGCCGTGATTCACAT
24 A E E V K I R S Q S Q G Q Y A V D S H
130 CCTCCGAAAAGCCCTGAAGTGAACAGACAGAGGTCATCTCTTTTGTTAACGGT
44 P P K S P E L K H R R Q R S S S F V N G
190 AAATGCAGGAACAGGACCTTCCATTTGLTATATAACAAGAAGCAAGAANAATACACC
64 K C R N R D L P T L D N K K A Q E I N T
250 AATTCACAGGCCAAGACATCGGTATCAAAAATACCACGTCAACGTGAGTTGTAAT
84 N S H G Q D I G I K N L P R Q R E L L N
310 GCCAAAATGGAATGATTTTACCGTGTATGGTGGCGGGTCAAGTGGATTGGGTAAAACC
104 A K N G I D F T L M V G A G Q S G L G K T
370 ACGTTTATCAATTCCTTATTCTTACTTCTTTAATTGATGATGACATCAAGAAAACAAA
124 T F I N S L F S T S L I D D D I K E N K
430 CCTATTATCGTTATAAAGCATTGTAGAAGGAGATGGAACACCTTAAATTCACGTC
144 P I I R Y K S I V E G D G T H L N F N V
490 ATCGATACACCTGGTTTGGCTAACAATATGGATAATGCATTAGCTGGAGGACAATGGTT
164 I D T P G P G N N M D N A F T W R T M V
550 AACTATATGATGAAGAATAAGATCTGTACTTTCCAGAGAACAACCTGATAGAGCA
184 N Y I D E E I R S Y I F Q E E Q P D R T
610 AAAATGGTGTATAGAGTCCATTGTTTGTACTTTCTGAGACCTTCAATAAGGGA
204 K M V D N R V H C C L Y F L R P S N K G
670 ATTGATACCTTTAGACCTGTAACAATGAAAATAATAGCGAAGAGAGTGAATTTAATCCCG
224 I D T L D V V T M K K L A K R V N L I P
730 GTTATTGCTAAATCAGATTGCTAACGAAAGAGGAATGAAAATCTCAAAACACAAAGTT
244 V I A K S D L L T K E E L K N F K T Q V
790 AGAGAAATAAAGAGTACAGATATCCCTGATGTTTCTTTTCGGCGATGAAGTTTGG
264 R E I I R V Q D I P V C F F F G D E V L
850 AATGCAACACAAGATATTTTCAAAAATATCCATTCAGTATAATGCACTAATGAGTAC
284 N A T Q D I F Q K P F S I I A S N E
910 ATTTTAAATGAAAAGCGGAAAAGTTAAAGGAAGACAATACAATGGGCGCTGTGTGAC
304 I F N E K G E K V K G R Q Y K W G A V D
970 ATTGAAAATGAAAAGTCTGACTTCAAAAATCTGCAAAAAGACGATTTTGTGTTGAAAT
324 I E N E K Y C D F K C L Q K T I F D V
1030 TTAATTGATCTTGTAGAAGTACCGAGGATTATTGAAAATGCAAGATCTGAAATGCTA
344 L I D L V E S T E D Y Y E K C R S E M L
1090 AGAATAGGCTATTAAAGCCAGAGATGCTTAAACAACGAAAAGTGTGACATAACGGAA
364 R T R L L K A R D L T T K S V D I T E
1150 GAACAAAGGAAAATTTTGGAGGAAGAAATGAACCTCGATGAATCGAGGAAAACAAACTC
384 E Q R K F L E E E M N F D E I E E N K L
1210 AAAAATFACAAGTCTTGAATAATTAATAAACGGTCAATGGATAAGGTGGCTACAGAA
404 K N Y K C Y E I I N K P F S I V M D K V A T E
1270 TGGATCTCGAATTTATAACTAGACAATAGAACCTAAGAAAATTCACAGGCTGTGCC
424 W D P E F I T R Q L E A K K K F N E L S
1330 AACAGAGAAATTTCAAAATTCGAGACTGGAAAAAGAGCCCTATTCTGGAACAAGAGAAAT
444 N R E I S K R F D W K S L F M E Q A N
1390 TTTAACCAAGAGATTGAACAATGAATCAAGATGGAAAACCTTACAACCTGGAATGTGAC
464 F N Q E I E Q L N H K L E N L Q L E C Q
1450 GACTTGAATACAAGCTGTAATCGGAAAAGTTCCNAACGCCATCCACAGATAGTCTD
484 D L E Y K L L I G K S L S C S H S T D S A
1510 ACTTTAGTAAACCTTCCATCAAAAAGGTAGTATTATAAAAAAAGAAAGCCAAATAA
504 T L V N V H I K R *
#IndIII
1570 TTTGCGCAGCTTTGCTCTACATAATAAATAAATAAATAAATAGGATAGAAATGAATTTTT
1630 TTACGGCAGTTTACCAATAAGTAGCAAAACCTTAAACAATATGATGAGACTAAGATAA
1690 ATCCGGGTAATACAAAATAAATTTTTTTTATTCTTCCACAGCGTTTTTCGACGGAGGA
1750 GGGAAAAAATAAATGATTTAAGGCATCAATATGCGCGACATCATATACCTTTGTCCGC
1810 TGTTATCATACCTTGAACCTTACATTTTCAGCTAGCGCGCCGCAAAA
    
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Characterization of the SPR3 Product

The four previously known *S. cerevisiae* septins share 25–37% amino acid-sequence identity with each other (Longtine et al., 1996; Haarer, B. K., S. H. Lillie, S. K. Ford, S. R. Ketcham, and J. R. Pringle, unpublished results). The predicted Spr3p sequence (59.8 kD) shows clearly that it is a fifth member of this protein family (Fig. 2 A); sequence identity values range from 28–36% with the other *S. cerevisiae* septins. Spr3p shares with Cdc3p an NH₂ terminus that is extended relative to those of the other *S. cerevisiae* septins; some sequence similarity between Spr3p and Cdc3p is detectable within this region (Fig. 2 A). Spr3p is also related to septins from other organisms but does not have a particularly close homologue among the known proteins; sequence identity values range from 30–32% with the *Candida albicans* septins (DiDomenico et al., 1994), from 25–40% with the *Schizosaccharomyces pombe* septins (Longtine et al., 1996; Pugh, T. A., H. B. Kim, O. Al-Awar, and J. R. Pringle, unpublished results), from 31–35% with the *Drosophila melanogaster* septins (Neufeld and Rubin, 1994; Fares et al., 1995), and from 31–37% with the mouse septins DIFF6, H5, and NEDD-5 (Nottenburg et al., 1990; Kato, 1990; Kumar et al., 1992). Like all other known members of the septin family, Spr3p has amino acid motifs that are conserved among nucleotide-binding proteins (Fig. 2 A), suggesting that it may be able to bind a nucleotide (Dever et al., 1987; Saraste et al., 1990). In addition, Spr3p, like most (but not all) of the other known septins, has a region at its carboxyl terminus that is strongly predicted to form a coiled-coil by the empirical formula of Lupas et al. (1991) (Fig. 2, A and B).

Effect of SPR3 Mutation or Overexpression on Vegetative Cells

Previous studies had not detected *SPR3* expression in vegetative cells (Clancy et al., 1983; Holaway et al., 1987). In addition, although two deletion alleles of *SPR3* were constructed (Kao et al., 1989), their effects on vegetative growth were not examined in detail. To characterize further the function(s) of Spr3p, we replaced one copy of *SPR3* in strains NKY278 and 52 with the *spr3::URA3* allele (which removes all of the *SPR3* coding region except for the first 60 codons; see Materials and Methods and Fig. 3 A), creating strains HF1 and HF2, respectively. Tetrads

Figure 1. (A) Alignment of the relevant parts of the proteins used to design the PCR primers shown in B. Cdc3p, Cdc10p, Cdc11p, and Cdc12p (GenEmbl Accession Numbers L16548, L16549, L16550, and L16551) are from *S. cerevisiae* (Haarer, B. K., S. H. Lillie, S. K. Ford, S. R. Ketcham, and J. R. Pringle, unpublished results); Spn1p (GenEmbl Accession No. U31742) is from *S. pombe* (Pugh, T. A., H. B. Kim, and J. R. Pringle, unpublished results); and CaCdc3p and CaCdc10p are from *C. albicans* (DiDomenico et al., 1994). Primer 1 is 8,192-fold degenerate and primer 2 is 110,592-fold degenerate. (C) Nucleotide sequence of 1,966 bp of genomic DNA containing *SPR3* (GenEmbl Accession No. L31767) and predicted amino acid sequence of Spr3p. The nucleotide sequence included in the PCR-amplified product (excluding the sequences of the primers) is underlined. The restriction sites shown in Fig. 3 A are indicated except for the EcoRV sites at the ends of the clone, which were not sequenced.

A

Spr3p	1	MKSKGSRSLTDCPVEFPKIVSGFAEEVKIRROSSQGYAVDSSHPPKS...	
Cdc3p	1	MSLKEEQVSIKQDFEQEERQHDQFNDVQI.KQESQDHDGVDSQYTNQTQ	
Spr3p	48PELKRRRQSSSSVFNKCRNRDLPLLDNKAQEINTNSHGQD	
Cdc3p	50	DDSERFEAAESDVKVEPGLMGITSSSQSEKQVLPDQPEIKFIRRQINGY	
Spr3p	90	IGIKNLPQRELLNAKNGIDFTLMVAGOSGLGKTFINSLFSTSLIDDDI	
Cdc3p	100	VGFANLPKQWHRRSIKNGFSFNLLCVGPDGIGKTTLMKTLFNNDIEANL	
Spr3p	140KENKPIIRYKSIIVEGDGTHLN	
Cdc3p	150	VKDYEEELANDQEEEGQEGEGHENQSQEQRHVKIKSYESVIEENGVKLN	
Spr3p	161	FNVIDTFEGGNMNA.FTWRTMVNYIDEEIRSYIFQEEQPDRTKMVDNR	
Cdc3p	200	LNVIDTEGEGDFLNDQKSWDPIIKEIDSRFDQYLDENKINRHSINDKR	
Spr3p	210	VHCCLYFLRPSNKGIDTLDVVTMKKLAKRVNLIPVIAKSDLLTKEELKNF	
Cdc3p	250	IHACLIFYIEPTGHYKPLDLKFMQSVYKCNLIPVIAKSDILTDEEILSF	
Spr3p	260	KTQVREIIRVQDIPV...CFFFGDEVLNA..TQDIFQKYPFSIIASNEYI	
Cdc3p	300	KKTIMNQLIQSNIELFKPPIYSNDDAENSHLSERLFSLLPYAVIGNSDIV	
Spr3p	305	FNEKGEKVKGRQYKQWGAVIDENKYCDFKILQKTFIDWNLIDLVESTED.	
Cdc3p	350	ENYSGNQVRGRSYPWGVIEVDNDNHSDFNLLKNLIRKQFMEELKERTSKI	
Spr3p	354	YYEKCRSEMLRTRLLKARDCLTTSKVDITFEORKELEEMNFDEIENKLI	
Cdc3p	400	LYENYRSSLAKLGIKQDNSVFKFDPISKQO....EKKTLHEAKLAKI	
Spr3p	404	KNYKCYEIIKNTVMDKVATEWDFEFITRQLEAKKFNELSNREISKFRDW	
Cdc3p	445ELEMKTTFVFOOKYSEKPKKLOKSETELFARHKEM.....	
Spr3p	454	<u>KKSLFMEQENFQEIQLNHHKLENLOLECODLEYKLLIGKSSNSHSTDSA</u>	
Cdc3p	478 <u>KEKLTKOLKALEDKKQKOLELSIN</u> <u>SASPNVNHSPVP</u>	
Spr3p	504	TLVNVHIKR	512
Cdc3p	513	TKKKGFRLR	520

B

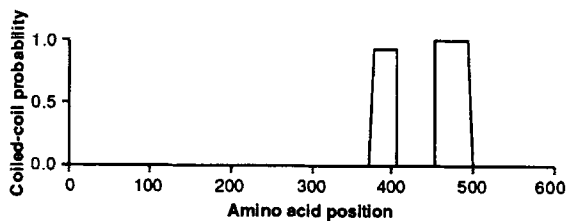


Figure 2. Analysis of the Spr3p sequence. (A) Comparison of the Spr3p and Cdc3p sequences. Vertical bars indicate identical amino acids and asterisks indicate similar ones (I/L/V; S/T; R/K; N/Q; D/E). Dots indicate gaps introduced to maximize sequence alignment. Single underlining indicates amino acid motifs conserved in nucleotide-binding proteins (Dever et al., 1987; Saraste et al., 1990); double underlining indicates the regions predicted (probability $\geq 90\%$) to form coiled-coils. (B) Probability of coiled-coil formation for Spr3p calculated using the program of Lupas et al. (1991) with a window size of 28.

from strains HF1 and HF2 yielded four viable spores with *URA3* segregating 2:2, indicating that the deletion is not lethal. The expected bands were seen in Southern blots of genomic DNA from the diploid transformants and their haploid segregants (Fig. 3, B and C). No obvious defects were observed in the growth rate, morphology, budding pattern, or Cdc11p localization of exponentially growing haploid *spr3::URA3*, diploid *spr3::URA3/SPR3*, or diploid *spr3::URA3/spr3::URA3* cells of either background, and haploid *spr3::URA3* cells showed no obvious mating defect (data not shown).

To determine the effects of ectopic expression of *SPR3* in vegetative cells, we transformed strain SY1263 with plasmid pADSPR3 (carrying *SPR3* under the control of the strong, constitutive *ADHI* promoter). The transformants grew as well as transformants carrying the control plasmid pADNS in medium selective for the plasmids and appeared normal in morphology (data not shown). Immunoblotting of proteins extracted from these strains using antibodies against Spr3p did not detect any protein in cells harboring pADNS, but did detect a strongly staining polypeptide of ~ 59 kD in cells harboring pADSPR3 (Fig. 3 E). The same plasmids were transformed into strains YEF473 and YEF473-A and the localization of Spr3p in the transformed cells was examined by immunofluorescence. No staining was seen in cells carrying plasmid pADNS (Fig. 4, A and B), whereas cells carrying plasmid pADSPR3 displayed a general cytoplasmic staining (Fig. 4, C and D). Despite the homology of Spr3p to the other septins, Spr3p was not detectable at the bud neck in the majority of cells. Some cells did appear to have staining at the neck that was slightly more intense than the background cytoplasmic staining, but the neck staining did not seem to represent the usual tight band and was difficult to characterize because of the brightness of the cytoplasmic staining.

To test for possible genetic interactions between *SPR3* and the other septin genes, we transformed strains HF17 (*cdc3-6*), HF18 (*cdc10-1*), HF19 (*cdc11-6*), and HF20 (*cdc12-6*) with plasmids pADNS and pADSPR3. pADSPR3 did not rescue the growth defect of any strain at 37°C. At 22°, cells of all four strains carrying plasmid pADNS were morphologically normal (Fig. 4, E and G; and data not shown), as were cells of strains HF17 and HF18 carrying plasmid pADSPR3 (Fig. 4 F; and data not shown). In contrast, $\sim 15\%$ of the cells of strains HF19 and HF20 carrying pADSPR3 had elongated buds and failed to complete cytokinesis at 22°C (Fig. 4 H; and data not shown), suggesting that ectopic expression of Spr3p could interfere with normal septin function in these strains.

Effects of *SPR3* and *CDC10* Mutations on Sporulation

SPR3 transcripts are very abundant during the late stages of sporulation (Clancy et al., 1983; Holaway et al., 1987). The same is true for at least one other septin gene, *CDC10* (Kaback and Feldberg, 1985). Consistent with these data, Spr3p and two other septins, Cdc3p and Cdc11p, were detected at substantial levels in sporulating cells (Fig. 3 F, lanes 1–3). These expression data suggest that the septins might have a role in spore-wall formation. Previous attempts to identify such a role genetically had yielded ambiguous results. Strains homozygous for temperature-sensitive *cdc3*, *cdc10*, or *cdc11* mutations showed no defect in sporulation at 33.5°C (Simchen, 1974), but interpretation of the results was complicated by the possibility that the temperature used was not fully restrictive and by the fact that the strains used were not isogenic (which might have obscured subtle effects on sporulation). In addition, diploids homozygous for the previously constructed *spr3* deletions displayed only small (although apparently reproducible) decreases in sporulation efficiency in comparison to an isogenic control strain (Kao et al., 1989).

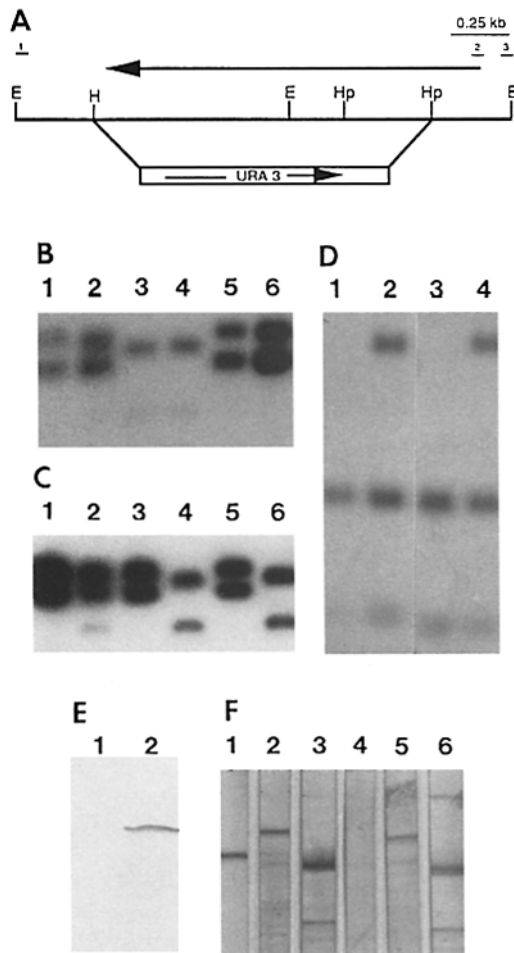


Figure 3. (A) Restriction map of the 2.0-kb fragment containing *SPR3* and structure of the *spr3::URA3* allele. The arrow above the map indicates the *SPR3* coding sequence; the arrow inside the box shows the direction of *URA3* transcription. The small numbered segments above the map (not drawn to scale) represent the approximate positions of the primers used for PCR (see Materials and Methods). Restriction sites shown are *EcoRV* (*E*), *HindIII* (*H*), and *HpaI* (*Hp*). There were no *EcoRI* or *SalI* sites in the fragment. (B) Southern blot analysis of parent strain NKY278 (lane 1), its *spr3::URA3/SPR3* transformant HF1 (lane 2), and the four segregants from a single tetrad (HF1-1A, lane 3; HF1-1B, lane 4; HF1-1C, lane 5; and HF1-1D, lane 6). Genomic DNAs were digested with *EcoRV* and probed with the 2.0-kb *SalI*-*EcoRI* fragment from pSKSPR3 (i.e., essentially the segment shown in A; see Materials and Methods). *SPR3* yields fragments of 1.1 and 0.9 kb (see A); the fragments from *spr3::URA3* are 1.0 and 0.7 kb because of an *EcoRV* site in *URA3*. The disruption bands are weaker because of smaller regions of overlap with the probe and apparently also because of poor transfer in this experiment (cf. C). (C) Southern blot analysis of parent strain 52 (lane 1), its *spr3::URA3/SPR3* transformant HF2 (lane 2), and the four segregants from a single tetrad (HF2-1A, lane 3; HF2-1B, lane 4; HF2-1C, lane 5; and HF2-1D, lane 6), digested and probed as described for B. (D) Southern blot analysis of parent strains NKY278 (lane 1) and 52 (lane 3) and of their *cdc10-Δ1/CDC10* transformants HF31 (lane 2) and HF30 (lane 4). Genomic DNAs were digested with *EcoRI* and probed with the 1.2-kb *PvuI*-*XhoI* fragment carrying *CDC10*. *CDC10* yields fragments of 1.0 kb and 0.45 kb; *cdc10-Δ1* yields a fragment of 1.75 kb. (E) Immunoblot of proteins from exponentially growing cells of strain SY1263 harboring (lane 1) pADNS (control plasmid) or (lane 2) pADSPR3 (expressing *SPR3* from the *ADHI* promoter; see Ma-

To examine the possible effects of septin mutations in more detail, we examined the sporulation of strains homozygous for the *spr3::URA3* allele in two different genetic backgrounds. The effects of deleting *CDC3*, *CDC11*, or *CDC12* could not be tested in this way, because such deletions are lethal (*CDC3*, *CDC12*) or severely affect cell growth (*CDC11*) (Haarer, B. K., S. H. Lillie, M. S. Longtine, and J. R. Pringle, unpublished results). However, the viability of a *cdc10Δ* strain (Flescher et al., 1993) suggested that the effects of this mutation on sporulation could be tested. Accordingly, we deleted *CDC10* in the same two genetic backgrounds as used for the deletion of *SPR3* (see Materials and Methods). In agreement with the results of Flescher et al. (1993), the *cdc10-Δ1* (*Ura*⁺) segregants from strains HF31 and HF30 were viable but grew more slowly than the *CDC10* (*Ura*⁻) segregants at 22°C and did not grow at 37°C, at which temperature they exhibited the typical defects seen in temperature-sensitive *cdc3*, *cdc10*, *cdc11*, or *cdc12* mutants (see Introduction). When the *cdc10-Δ1* segregants from either strain were grown in YPD at 22°C, some cells had aberrant morphologies, including large cells, enlarged mother-bud necks, a failure to complete cytokinesis, and the presence of more than one nucleus in some compartments (Fig. 4, I-L). A partial explanation for the viability of the *cdc10-Δ1* strains is provided by the observation that other septins can localize to the neck in these strains (Fig. 4, I and K); in contrast, strains carrying temperature-sensitive-lethal *cdc10* mutations lose localization of the other septins at restrictive temperatures (Haarer and Pringle, 1987; Kim et al., 1991; Ford and Pringle, 1991), perhaps because the septin complex at the mother-bud neck is unstable at the higher temperatures in the absence of Cdc10p.

When the effects of the *spr3* and *cdc10* deletions were examined in the SK-1 (NKY278) genetic background, strain HF7 (*spr3::URA3/spr3::URA3*) showed no detectable difference in sporulation efficiency from the control strains (Fig. 5, A and B; Table II), but strain HF25 (*cdc10-Δ1/cdc10-Δ1*) had threefold fewer four-spored asci than did the other strains tested (Fig. 5 C; Table II). In contrast, when the effects of the deletions were tested in the strain 52 background, strain HF16 (*spr3::URA3/spr3::URA3*) had three to fourfold fewer four-spored asci than did the control strains (Fig. 5, D and E; Table II), but strain HF28 (*cdc10-Δ1/cdc10-Δ1*) showed no detectable deficit in sporulation efficiency (Fig. 5 F; Table II). Strain HF16 did not differ detectably from the control strain in the percentage of cells that had four well-separated masses of DNA or in the percentages of one-, two-, and three-spored asci (data not shown). Moreover, tetrads dissected from the com-

materials and Methods) probed with antibodies against Spr3p. The polypeptide detected in lane 2 is ~59 kD. (F) Immunoblots of proteins from sporulating cultures of parent strain NKY278 (lanes 1-3) or *spr3::URA3/spr3::URA3* strain HF7 (lanes 4-6) probed with antibodies against Spr3p (lanes 1 and 4), Cdc3p (lanes 2 and 5), or Cdc11p (lanes 3 and 6). The major bands detected correspond to polypeptides of ~59 kD (Spr3p), ~62 kD (Cdc3p), and ~55 kD (Cdc11p). For both strains, the protein preparations were made after 6 h in sporulation medium at 30°C, at which time ~50% of the cells were in meiosis I and the rest were in (or had completed) meiosis II, as judged by DNA staining.

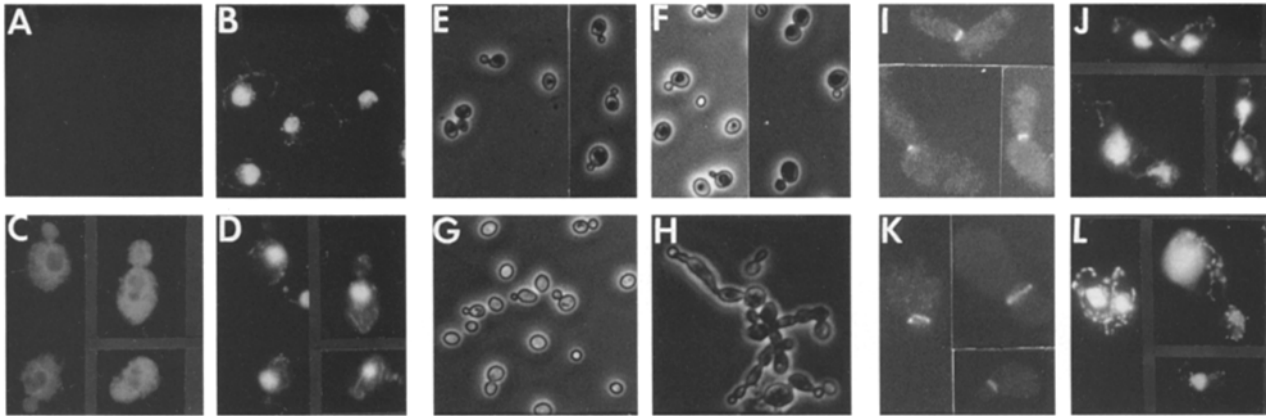


Figure 4. (A–D) Wild-type strain YEF473 harboring either pADNS (A and B) or pADSPR3 (C and D) double stained for Spr3p (A and C) and for DNA (B and D). A and C were photographed and printed at the same exposures. (E–H) Cells of strain (E and F) HF18 (*cdc10-1*) or (G and H) HF19 (*cdc11-6*) carrying plasmid pADNS (E and G) or pADSPR3 (F and H) were removed from SC-Leu plates at 22°C, resuspended in drops of water on a slide, and viewed using phase-contrast microscopy. (I–L) Cells of the *cdc10-Δ1* strains HF31-1A (I and J) and HF30-1A (K and L) were double stained to visualize Cdc11p (I and K) and DNA (J and L).

plete asci of strain HF16 yielded four viable segregants. These data suggest that the loss of Spr3p did not cause defects in meiosis. Such quantitative comparisons were not feasible for the *cdc10Δ* strain HF25 because many asci had more than four spores (cf. Fig. 5 F), presumably because of the defect in nuclear segregation during vegetative growth (see above), and because a significant proportion of the cells undergoing sporulation already had an aberrant morphology or had lysed (Fig. 5 C), again presumably because of the abnormalities during vegetative growth.

These results suggest that Spr3p and Cdc10p (and perhaps other septins) might have overlapping functions during spore formation and that the relative importance of individual septins might vary from strain to strain. In this regard, it is relevant that the absence of Spr3p had little or no effect on the expression of Cdc3p and Cdc11p during sporulation (Fig. 3 F, lanes 5 and 6).

Localization of Septins in Sporulating Cells

The timing of septin gene expression in sporulating cells suggests that these proteins might play a role in spore-wall formation. To ask if the localization of the proteins were consistent with such a role, we performed immunofluorescence on sporulating cells using antibodies to Spr3p, Cdc3p, and Cdc11p. As expected, Spr3⁺ cells stained with the antibodies to Spr3p (Fig. 6, A, E, F–I), whereas Spr3[−] cells did not (Fig. 6 B). In the Spr3⁺ cells, Spr3p staining was first detected during meiosis I (Fig. 6 A, cells 1 and 2), at which point the protein appeared to be dispersed in the cytoplasm. This timing of Spr3p appearance is consistent with the timing of appearance of *SPR3* mRNA (Holaway et al., 1987). At the onset of meiosis II, Spr3p, Cdc3p, and Cdc11p all appeared to be concentrated in distinct ringlike structures around each of the four spindle-pole bodies (Fig. 6, C, E, F, J, and N). This staining pattern suggests that the septins are concentrated at the leading edges of the membrane sacs that first appear near the spindle-pole bodies and within which the spore wall will later form (Moens, 1971; Moens and Rapport, 1971; Byers, 1981; Fig. 7, A and B). The staining patterns at successively later

stages of meiosis II (Fig. 6, G, K, O, H, L, and P) suggest that the septins remain concentrated at the leading edges (and perhaps in the lateral regions) of these membrane sacs as they gradually extend to surround the chromosome-containing lobes of the nucleus and eventually enclose the spores (Fig. 7 C). At the end of meiosis II, it ap-

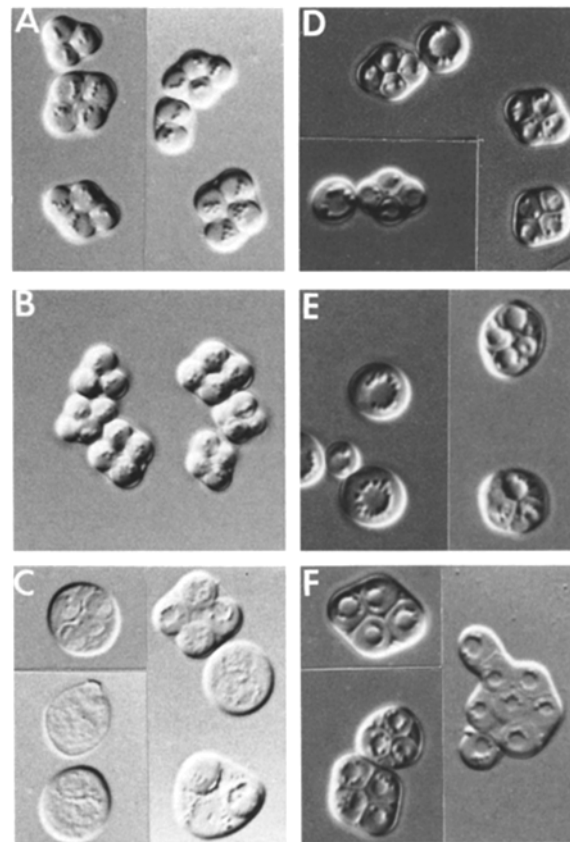


Figure 5. DIC images of sporulated cultures of strains (A) NKY278 (wild type); (B) HF7 (*spr3::URA3/spr3::URA3*); (C) HF25 (*cdc10-Δ1/cdc10-Δ1*); (D) 52 (wild type); (E) HF16 (*spr3::URA3/spr3::URA3*); and (F) HF28 (*cdc10-Δ1/cdc10-Δ1*).

Table II. Efficiencies of Sporulation of Strains Deleted for *SPR3* or *CDC10*

Strain	Relevant genotype	Four-spored Asci %
SK-1 background		
NKY278	<i>CDC10/CDC10 SPR3/SPR3</i>	82
HF8	<i>CDC10/CDC10 spr3/SPR3</i>	79
HF7	<i>CDC10/CDC10 spr3/spr3</i>	71
HF31	<i>cdc10/CDC10 SPR3/SPR3</i>	79
HF25*	<i>cdc10/cdc10 SPR3/SPR3</i>	26 [†]
HF22	<i>cdc10/CDC10 spr3/SPR3</i>	82
52 background		
52	<i>CDC10/CDC10 SPR3/SPR3</i>	39
HF15	<i>CDC10/CDC10 spr3/SPR3</i>	38
HF16	<i>CDC10/CDC10 spr3/spr3</i>	8 [§]
HF30	<i>cdc10/CDC10 SPR3/SPR3</i>	37
HF28*	<i>cdc10/cdc10 SPR3/SPR3</i>	55 [†]
HF29	<i>cdc10/CDC10 spr3/SPR3</i>	38

Data represent the mean values from two independent experiments. Cultures were examined using DIC optics (see Fig. 5). 200 cells were counted from each culture after 3 d in sporulation medium at 22°C. No differences in the counts were seen after 7 d in sporulation medium.

*Some peculiarities in strain construction (see Materials and Methods) make it possible that these strains may not be fully isogenic with the other strains tested.

[†]Asci with more than four spores are included in these counts.

[§]Several additional *spr3/spr3* strains constructed independently using the same protocol showed similar reductions in sporulation efficiency.

pears that the septins may be more uniformly associated with the entire membrane system surrounding the developing spores (Fig. 6, *I*, *M*, and *Q*). At this point, spore-wall formation was still not detectable by DIC microscopy. (At a later stage, when spore walls were detectable by DIC, no staining of septins was observed, presumably because of impermeability of the asci and/or spores to the antibodies under the conditions used.)

In the *Spr3*⁻ cells, Cdc3p and Cdc11p showed localization similar to that observed in *Spr3*⁺ cells, but the staining was consistently less intense (and perhaps less tightly localized, particularly later in development) in the *Spr3*⁻ cells (Fig. 6, *R*-*Y*).

Discussion

In every organism examined so far, the septins are present as a family with multiple members (Longtine et al., 1996). In this paper, we report that *SPR3*, a gene identified previously on the basis of its differential expression in sporulating cells, encodes a fifth septin in *S. cerevisiae*. It is possible that *Spr3p*, *Cdc3p*, *Cdc10p*, *Cdc11p*, and *Cdc12p* do not represent the full set of *S. cerevisiae* septins; *S. pombe*, for example, contains at least six septins (Longtine et al., 1996; Pugh, T. A., H. B. Kim, O. Al-Awar, and J. R. Pringle, unpublished results). Like all of the known septins, *Spr3p* contains a putative nucleotide-binding domain; however, the function of this domain, and whether it is indeed involved in nucleotide binding, remain to be determined. Like all of the known septins except *Cdc10p* (Steensma and van der Aart, 1991; Haarer, B. K., S. R. Ketcham, and J. R. Pringle, unpublished results), *C. albicans* *Cdc10p* (DiDomenico et al., 1994), and *S. pombe* *Spn2p* (Al-Awar, O., T. Pugh, and J. R. Pringle, unpublished results), *Spr3p* contains a region near its COOH terminus that is strongly predicted to form a coiled coil. This region in *Spr3p* is un-

usual in consisting of two predicted coiled-coil domains separated by ~50 amino acids, rather than the single, continuous domain found in the other known septins. The coiled-coil domains are likely to be involved in interactions between the septins, or between them and other proteins, but the details await elucidation.

Unlike the other known *S. cerevisiae* septins, *Spr3p* does not seem to play a role during vegetative growth. Expression of *SPR3* has not been detected in exponentially growing cells (Clancy et al., 1983; Holaway et al., 1987; Fig. 3 *E*), and its deletion produces no obvious defect in such cells. Interestingly, when *Spr3p* is expressed from a heterologous promoter in vegetatively growing cells, it coassembles poorly, if at all, with the other septins at the mother-bud neck. Nonetheless, *Spr3p* apparently has at least some capacity to interact with other septins in vegetative cells, as overexpression of *Spr3p* at permissive temperature in cells carrying temperature-sensitive *cdc11* or *cdc12* mutations caused morphological abnormalities like those normally seen in these mutants at restrictive temperature; presumably, interaction with *Spr3p* reduced the level of functional *Cdc11p* or *Cdc12p* below that needed for normal morphogenesis. In addition, *Spr3p* does colocalize with *Cdc3p* and *Cdc11p* during sporulation and indeed appears to be necessary for fully normal localization of these other septins under these conditions. Taken together, the data suggest that *Spr3p* lacks some sequence(s) present in the other septins that are necessary for regulation of their assembly during the budding cycle.

SPR3 mRNA and protein first appear in sporulating cells during meiosis I (Holaway et al., 1987; Fig. 6 *A*), at which time *Spr3p* is diffusely distributed in the cytoplasm. Subsequently, during meiosis II, both *Spr3p* and the other septins examined become localized in patterns that suggest an association with the developing prospore wall and thus an involvement in the formation of the spore plasma membrane and/or cell wall. This interpretation is consistent with the analysis of *spr3* and *cdc10* deletion mutants: in an appropriate genetic background, each mutation produces a partial defect in spore formation. At least in the case of *spr3* (*cdc10* was difficult to analyze), this defect did not appear to result from a defect in meiosis. The observations that the deletions produce only partial blocks of spore formation and that deletion of *SPR3* causes only partial delocalization of *Cdc3p* and *Cdc11p* probably reflect some functional redundancy among the septins. Evidence for partial redundancy has also been obtained in studies of vegetative cells. For example, overexpression of *CDC10* and *CDC12* can partially rescue temperature-sensitive *cdc3* and *cdc11* mutants, respectively (Haarer, B. K., S. H. Lillie, L. Bloom, and J. R. Pringle, unpublished results). In addition, deletion of *CDC10* is not lethal and does not prevent the localization of at least one other septin to the bud-neck at 22–24.5°C, although it is lethal at 37°C (Flescher et al., 1993; Fig. 4, *I*-*L*). In sporulating cells, the reciprocal severities of deleting *SPR3* and *CDC10* in the two genetic backgrounds tested might reflect simply the relative expression levels of different septins in these strains. It would be desirable to test the sporulation competence of a strain homozygous for deletions of both *SPR3* and *CDC10*; however, we have been unable to construct such a strain, despite repeated efforts using methods like those

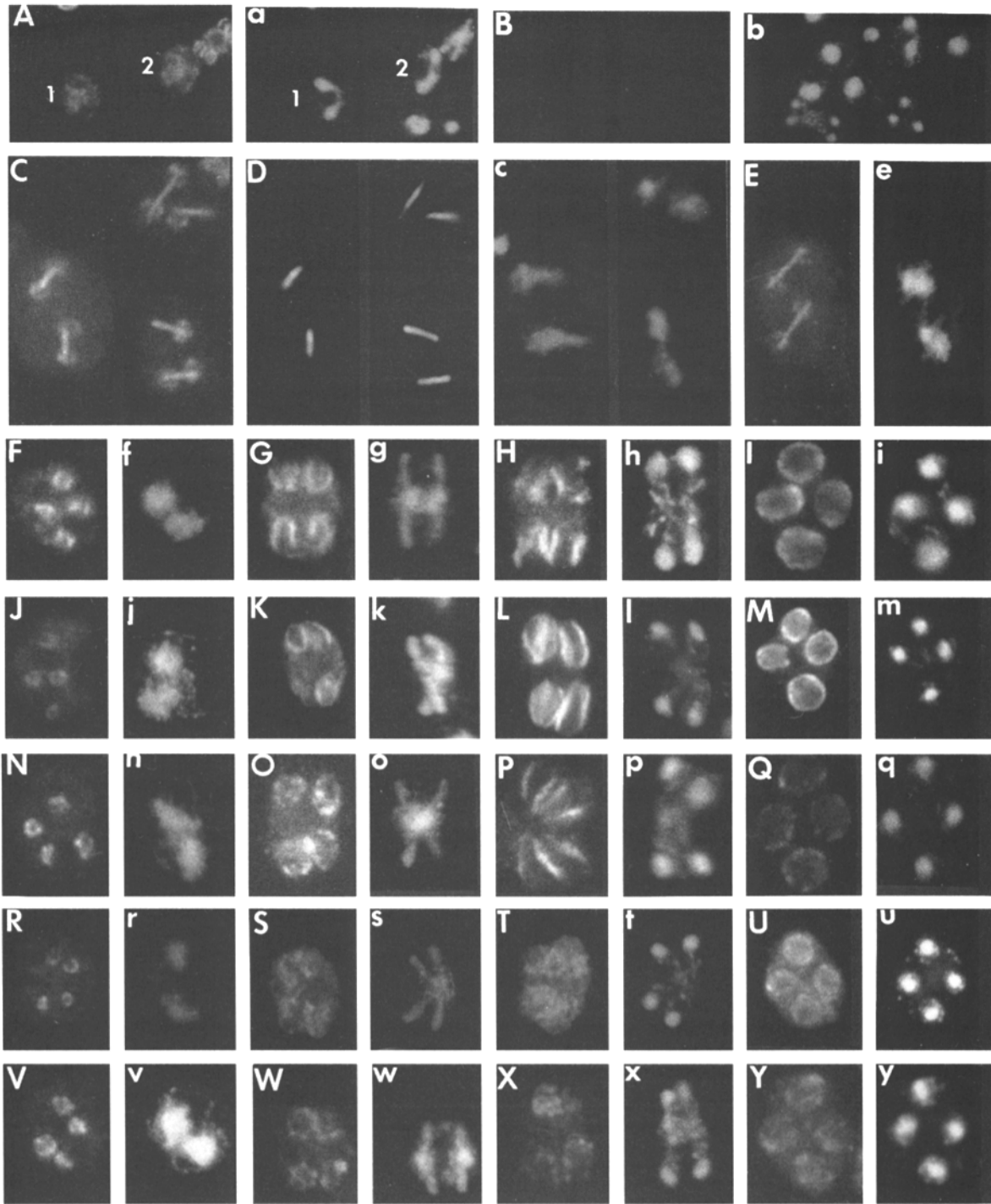


Figure 6. Localization of septins in sporulating cells. Cultures of the *SPR3/SPR3* strains HF9 (A) and NKY278 (C–Q) and the *spr3::URA3/spr3::URA3* strain HF7 (B, R–Y) were sporulated at 30°C, and cells taken at various times were examined by immunofluorescence using antibodies to Spr3p (A, B, E–I), tubulin (D), Cdc3p (J–M, R–U), or Cdc11p (C, N–Q, V–Y). Accompanying panels (lower case letters) show DNA staining of the same cells. Cells in C–E were double stained for the septin and for tubulin. The bright rhodamine staining of the antitubulin antibodies (shown separately in D for the cells of C) was visible also when using the filters for visualization of the FITC label on the antiseptin antibodies; thus, C and E show superimposed septin and tubulin staining. Some cells are numbered for reference in the text. (A and B) Cells at various stages of sporulation. A and B were photographed and printed at the same exposure settings. (C–Y) Cells at the beginning (C–F, J, N, R, and V), at a later stage (G, K, O, S, and W), at a still later stage (H, L, P, T, and X), and after the end (I, M, Q, U, and Y) of meiosis II. Cells in I, M, Q, U, and Y had not yet produced spore walls visible by DIC microscopy.

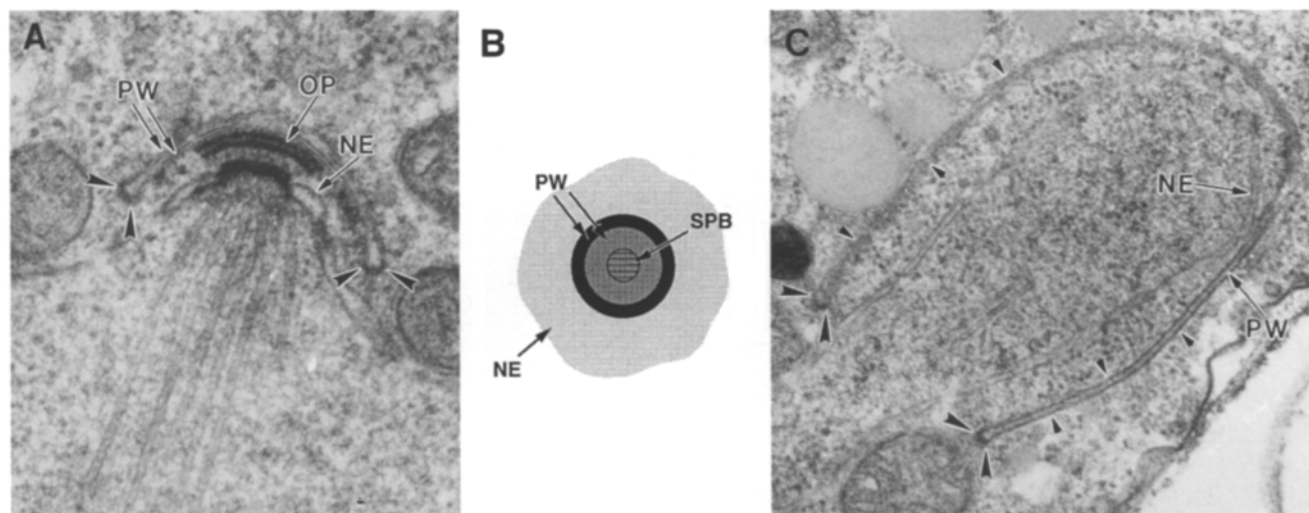


Figure 7. Electron microscopic visualization of the prospore wall and interpretation of septin localization as judged from the immunofluorescence images of Fig. 6. *PW*, prospore wall; *NE*, nuclear envelope; *SPB*, spindle-pole body; *OP*, outer plaque of the SPB. (*A* and *B*) Early stage of prospore wall formation (corresponding to Fig. 6, *C*, *E*, *F*, *J*, *N*, *R*, and *V*). (*A*) Cross-section electron micrograph showing the prospore wall as a cup-shaped, flattened membrane sac just external to the nuclear envelope and symmetrically arranged over the spindle-pole body. Arrowheads indicate the proposed localization of the septins at the leading edge of the extending prospore wall, although it is possible that septin localization also extends some distance back from the leading edge. The electron-dense material at the leading edge might be a concentration of the septins or of other materials associated with them. Modified, with permission, from Byers (1981). (*B*) Presumed appearance en face of the prospore wall at the same stage as visualized in *A*. *Lightest shading*, nuclear envelope; *darker shading*, prospore wall; *darkest shading*, proposed localization of the septins; *cross-hatching*, position of the underlying spindle-pole body. (*C*) Cross-section electron micrograph showing a later stage of prospore wall formation (corresponding to Fig. 6, *G*, *K*, *O*, *S*, and *W* or Fig. 6, *H*, *L*, *P*, *T*, and *X*). It appears that the septins are not uniformly distributed over the prospore wall at this stage (and, in particular, that the region over the spindle-pole body is devoid of septins), because cup-shaped structures with their open ends facing the center of the cell were rarely, if ever, observed in the immunofluorescence studies (see Fig. 6). However, it is not clear at present whether the septins are distributed only in a narrow band near the leading edge of the prospore wall (*large arrowheads*; note the continued presence of a zone of electron-dense material at this site) or more widely over the lateral surfaces (*small arrowheads*). (Note that the three-dimensional structures of the nuclear lobes and overlying prospore wall at this stage may be more complex than would be suggested by a simple rotation of a two-dimensional image such as this; see Moens, 1971; Moens and Rapport, 1971.)

used to construct the *SPR3/SPR3 cdc10Δ/cdc10Δ* diploids (see Materials and Methods).

Although a general description of the process by which the prospore wall appears and then gradually extends to envelop the spore contents and form the spore plasma membrane and cell wall has been available for many years (see Introduction), the mechanisms of this process remain largely unknown. The open questions include the following: (*a*) Where does the membrane of the prospore wall originate, and how does this flattened membrane sac extend along the outer surface of the nuclear envelope? The failure of spore formation in *kex2* mutants (Leibowitz and Wickner, 1976) suggests (not surprisingly) that vesicles derived from a late-Golgi compartment are involved (Redding et al., 1991), but no other information is available. (*b*) Does the curved (cup-like) shape of the prospore wall result from an adherence to the nuclear envelope or reflect an aspect of its internal architecture or a role of cytoskeletal elements? (*c*) How (and in response to what signal) does the prospore wall eventually separate from the spindle-pole body and nuclear envelope, and then accumulate cytoplasm and organelles within the intervening space? (*d*) How are the nuclear lobes pinched off to form the haploid nuclei, and how does the prospore wall complete the enclosure of the nuclei and associated cytoplasm? (*e*) How do the several layers of the spore wall then form between the two membranes that enclose the spore? Among the in-

triguing mysteries here is that formation of the outermost, dityrosine-containing wall layer is spore-autonomous (i.e., dependent on functions encoded within the spore), whereas formation of the inner, chitosan-containing layer is dependent on functions in the mother cell cytoplasm (Briza et al., 1990a). (*f*) Does the inner of the two membranes derived from the prospore wall evolve into the plasma membrane of the spore, and, if so, how? What becomes of the outer of the two membranes?

Although the septins may be involved in any of these aspects of spore formation, two possible roles seem particularly likely in light of the available information on septin localization during sporulation and septin function in other contexts. First, although more precise localization of the septins by immunoelectron microscopy will be necessary to provide an unequivocal picture, the immunofluorescence images suggest that the septins are associated with the leading edge (i.e., the lip of the cup-shaped structure) of the prospore wall after it first forms, and with the leading edge and perhaps the lateral regions of this membrane sac as it extends around the nuclear lobes during meiosis II (Figs. 6 and 7). This suggests that the septins might be involved in the extension of the prospore wall, perhaps by directing the fusion of membrane vesicles to the appropriate region. Such a role would be analogous to one suggested for the septins during the cellularization of the *Drosophila* embryo (Fares et al., 1995), where mem-

brane extension appears to arise from the fusion of pre-aligned vesicles (Loncar and Singer, 1995), and where the septins are concentrated at the very tip of the invaginating membrane (Neufeld and Rubin, 1994; Fares et al., 1995).

Second, the septins might be involved in the formation of one or more layers of the spore wall. Although immunoelectron microscopy will again be necessary to provide a detailed picture (e.g., Are the septins associated with the inner membrane, the outer membrane, or both?), the immunofluorescence images suggest that the septins are uniformly distributed around the nascent spore by the time that spore-wall formation begins, consistent with a role in this process. Moreover, synthesis of the chitosan layer of the spore wall depends on the activity of chitin synthase III (Briza et al., 1990a; Pammer et al., 1992; Bulawa, 1993), the same enzyme that appears to form the chitin ring at the beginning of bud emergence in vegetative cells (Bulawa, 1993). As the chitin ring does not form normally in *cdc3*, *cdc10*, *cdc11*, or *cdc12* mutants (Adams, 1984), the septins appear to play a role in the localization of chitin synthase III activity in vegetative cells, suggesting that they might have a similar role during spore-wall formation. In vegetative cells, the localization of chitin synthase III activity may be mediated by an interaction between the septins and Csd4p, a polypeptide necessary for enzyme activity but not the catalytic subunit itself (Bulawa, 1992, 1993), as synthetic lethality has been observed between *csd4* and *cdc12* mutations, and the product of a novel gene, *BNI4*, shows two-hybrid interactions both with Csd4p and with Cdc10p (DeMarini, D., H. Fares, and J. R. Pringle, unpublished results). It is intriguing that *CSD4* has a homologue, *SHC1*, that is expressed only in sporulating cells (Bulawa, 1993). The septins might also be involved in organizing other aspects of spore-wall formation; detailed biochemical and electron microscopic analyses (as in Briza et al., 1990a) of spore structure in *spr3* and *cdc10* mutants should clarify these possibilities.

In summary, the studies reported here have identified an involvement of the yeast septins in the interesting and poorly understood process of spore formation. Further study of the septin role (or roles) in this process should clarify both the mechanisms by which the spore plasma membrane and layers of cell wall are formed and the molecular mechanisms of septin action. As in vegetative cells, this action is likely to involve coassembly of the several septins into a functional complex in which the individual septins then interact (perhaps in a partially redundant way) with various other proteins to recruit them to, and/or organize them at, their proper site of action.

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