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SPR28, a sixth member of the septin gene family in *Saccharomyces cerevisiae* that is expressed specifically in sporulating cells

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The septins are a recently recognized family of proteins that are present in a wide variety of fungal and animal cells, where they are involved in cytokinesis and apparently in other processes involving the organization of the cell surface. Five previously described *Saccharomyces cerevisiae* septins are associated with the neck filaments of vegetative cells and/or with the developing prospore wall of sporulating cells. We report here the characterization of *SPR28*, a sixth member of the *S. cerevisiae* septin gene family whose existence was revealed by the yeast genome project. Analysis of mRNA levels showed that *SPR28* is a new member of the group of 'late genes' that are expressed at high levels during the meiotic divisions and ascospore formation. The septin it encodes, Spr28p, exhibited specific two-hybrid interactions with itself and with three other septins that are expressed in sporulating cells. Consistent with these results, an Spr28p–green fluorescent protein fusion was induced during meiosis I and appeared to be associated with the developing prospore walls. Deletion of *SPR28* in either a wild-type or an *spr3*Δ background produced no obvious abnormalities in vegetative cells and had little or no effect on sporulation, suggesting that the septins have redundant roles during spore formation.

Keywords: septins, *SPR28*, *Saccharomyces cerevisiae*, sporulation, cell wall synthesis

INTRODUCTION

Sporulation of the yeast *Saccharomyces cerevisiae* involves a regulated programme of cell development that includes pre-meiotic DNA replication, the two meiotic divisions, and encapsulation of the haploid nuclei within the spore walls (Esposito & Klapholz, 1981). Sporulation is initiated when *MAT***a**/*MAT*α cells are starved for nitrogen in the presence of a nonfermentable carbon source and is under the control of master regulatory genes that include *IME1*, *IME2* and *RME1* (reviewed by Mitchell, 1994). To help produce the proper sequence of genetic and morphological events during sporulation, three classes of sporulation-specific genes are activated at distinct times during the process (Mitchell, 1994): early genes are expressed at the beginning of meiotic prophase, middle genes are expressed later in prophase, and late genes are expressed at the time of the meiotic divisions and spore formation. Activation of the late genes is accompanied and followed by the morphological events of spore formation, which include (i) formation of a flattened membrane sac (the 'prospore wall') in close apposition to the cytoplasmic faces of the spindle-pole bodies early in meiosis II; (ii) extension of the prospore walls along the outer surface of the nuclear envelope; (iii) separation of the prospore walls from the spindle-pole bodies and the nuclear envelope, and movement of cytoplasm and organelles into the intervening space; (iv) final engulfment of the nuclear lobes (containing the haploid chromosome sets) and associated cytoplasm; and (v) deposition of spore-wall components between the two membranes of the prospore wall (Moens, 1971; Moens & Rapport, 1971; Guth *et al.*, 1972; Beckett *et al.*, 1973; Byers, 1981).

Among the genes expressed differentially late in sporulation are *CDC10* (Kaback & Feldberg, 1985) and *SPR3* (Holaway *et al.*, 1987; Kao *et al.*, 1989; Ozsarac *et al.*,

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Abbreviations: AD, activation domain; DBD, DNA-binding domain; GFP, green fluorescent protein.

1995; Fares *et al.*, 1996), which encode proteins of the septin family. Septins were originally identified in *S. cerevisiae* as a set of homologous proteins (encoded by *CDC3*, *CDC10*, *CDC11* and *CDC12*) associated with the ~ 10 nm filaments that lie immediately subjacent to the plasma membrane in the region of the mother-bud neck (Byers & Goetsch, 1976; Byers, 1981; Haarer & Pringle, 1987; Ford & Pringle, 1991; Kim *et al.*, 1991; Longtine *et al.*, 1996). In temperature-sensitive mutants defective in any of these four septins, a shift to restrictive temperature results in a pleiotropic phenotype that includes loss of the neck filaments, failure to form a chitin ring in the cell wall at the base of the bud, production of abnormally elongated buds, and a failure to complete cytokinesis, resulting in the formation of multibudded, multinucleate cells (Hartwell, 1971; Byers, 1981; Adams, 1984; Adams & Pringle, 1984; Slater *et al.*, 1985; D. DeMarini, A. E. M. Adams and J. R. Pringle, unpublished results). Recent work has shown that septins are also present in a variety of other organisms, including other fungi, insects, amphibia and mammals (reviewed by Longtine *et al.*, 1996). The septins appear to be generally involved in cytokinesis and/or septum formation, and protein-localization data suggest that they may also have a variety of other roles (Longtine *et al.*, 1996).

In *S. cerevisiae*, immunofluorescence analysis of the sporulation-specific septin Spr3p, and of Cdc3p and Cdc11p (which are expressed in both vegetatively growing and sporulating cells), suggested that these proteins are all localized to the prospore walls throughout the development of this structure (Fares *et al.*, 1996). These results, together with the finding that deletion of *SPR3* or of *CDC10* reduced the efficiency of sporulation, suggest that the septins might play an important role during the formation of the prospore wall and/or in the eventual formation of the spore wall within it.

In this study, we report the analysis of a sixth *S. cerevisiae* septin gene, *SPR28* (SPorulation-Regulated), whose existence was revealed during the yeast genome project. Like *SPR3*, *SPR28* is expressed specifically as one of the late group of sporulation genes, and its product appears to be localized to the prospore wall. Deletion of *SPR28* in a wild-type or an *spr3*Δ background did not produce a substantial decrease in sporulation efficiency, suggesting that the septins play partially redundant roles during spore formation.

METHODS

Strains, media, and microbiological and recombinant DNA methods. The *S. cerevisiae* strains used in this study are listed in Table 1. Strains CDV46-1 to CDV46-6 and CDV47-1 to CDV47-6 were constructed by crossing haploid segregants from five different tetrads from CDV45 as follows: CDV45-3C × CDV45-3D (CDV46-1), CDV45-2A × CDV45-4A (CDV46-2), CDV45-1B × CDV45-3D (CDV46-3), CDV45-1D × CDV45-4A (CDV46-4), CDV45-2A × CDV45-3D (CDV46-5), CDV45-4C × CDV45-5C (CDV46-6), CDV45-1A × CDV45-4B (CDV47-1), CDV45-1A × CDV45-3B (CDV47-2), CDV45-1C × CDV45-3B (CDV47-3), CDV45-2B × CDV45-4B (CDV47-4), CDV45-3A × CDV45-3B (CDV47-5) and CDV45-3B × CDV45-4D (CDV47-6).

Escherichia coli strain JMB9 [(r^- m^+) *ΔtrpF*] (Sterner *et al.*, 1995) was used to rescue pJG4-5-based plasmids from strain EGY48. The transformed cells were plated directly onto Vogel–Bonner minimal plates (Davis *et al.*, 1980) that were supplemented with 0·2 % glucose, 0·5 % Casamino acid hydrolysate, 0·01 mM $FeCl_3$ and 100 mg ampicillin l^{-1}. Other plasmid manipulations were performed in *E. coli* strain DH5α (Gibco BRL) using standard procedures (Sambrook *et al.*, 1989) except where noted. Standard procedures of yeast genetics and molecular biology (Guthrie & Fink, 1991; Sambrook *et al.*, 1989) were used except where noted. Yeast transformations were performed using a modification of the Li^+-ion method (Gietz *et al.*, 1992).

Yeast and *E. coli* media, including the rich, glucose-containing media YM-P and YPD, the acetate-containing medium YPAc, defined media (SD with appropriate supplements) and sporulation medium, were prepared by standard recipes (Lillie & Pringle, 1980; Sambrook *et al.*, 1989; Rose *et al.*, 1990). Sporulation experiments were performed essentially as described by Fares *et al.* (1996) except that all experiments were carried out at 22 °C.

Deletion of *SPR28* and construction of an *SPR28–GFP* fusion. The complete *SPR28* coding region was deleted by the PCR method (Baudin *et al.*, 1993) using plasmid pRS304 (*TRP1*; Sikorski & Hieter, 1989) as template and *Taq* DNA polymerase (Boehringer). Oligonucleotides that contained 40 nucleotides immediately upstream or downstream of the *SPR28* coding region and 20 nucleotides either upstream or downstream of *TRP1* were used to create a PCR product that contained flanking sequences of *SPR28* separated by *TRP1* and *TRP1*-flanking sequences. This DNA was extracted with phenol/chloroform (1:1, v/v), precipitated and transformed into strain YEF473 (Table 1). A transformant (CDV45) that had one copy of *SPR28* replaced with *TRP1* was confirmed by PCR and Southern blot analysis and used to construct other strains as described above.

To construct an *SPR28–GFP* fusion gene and a *GFP* gene under control of the *SPR28* promotor, the 446 nucleotides upstream of and including the *SPR28* start codon, with or without the full-length *SPR28* coding region, were amplified by PCR using Vent DNA polymerase (New England Biolabs) and genomic DNA as template. *Eco*RI and *Not*I restriction sites were introduced at the ends of each fragment. The two resulting fragments were purified, digested with *Eco*RI and *Not*I, and co-ligated with a *Not*I–*Bam*HI fragment encoding the green fluorescent protein (GFP; see below) to *Eco*RI/*Bam*HI-digested YEplac181 (Gietz & Sugino, 1988), thus creating YEplac181–SPR28–GFP and YEplac181–GFP, respectively. The *GFP* fragment had been amplified previously by PCR using Vent DNA polymerase and pS65T-C1 (Clontech) as template. *Not*I and *Bam*HI restriction sites that allowed in-frame fusions were introduced with the primers.

Two-hybrid analyses. The two-hybrid assay (Fields & Sternglanz, 1994) was performed as described by Gyuris *et al.* (1993). To fuse the various full-length septin genes to the LexA DNA-binding domain (DBD) coding sequences in plasmid pEG202 (Zervos *et al.*, 1993) and to the activation domain (AD) coding sequences in a modified version of plasmid pJG4-5 (Gyuris *et al.*, 1993) that contains the polylinker region from pEG202 (C. De Virgilio and D. DeMarini, unpublished results), *S. cerevisiae* *CDC3*, *CDC10*, *CDC11*, *CDC12*, *SPR3* and *SPR28* (accession numbers P32457, P25342, P32458, P32468, P41901 and Z48612, respectively) full-length coding sequences were amplified by PCR using Vent DNA polymerase and either cloned genes

Table 1. *S. cerevisiae* strains used in this study

Strain		Genotype	Source or reference
YEF473	**a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3*	Bi & Pringle (1996)
CDV45	**a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 spr28Δ::TRP1/SPR28*	See text
CDV45-3A	α	*his3 leu2 lys2 trp1 ura3*	Segregant from CDV45*
CDV45-3B	**a**	*his3 leu2 lys2 trp1 ura3*	Segregant from CDV45*
CDV45-3C	**a**	*his3 leu2 lys2 trp1 ura3 spr28Δ::TRP1*	Segregant from CDV45*
CDV45-3D	α	*his3 leu2 lys2 trp1 ura3 spr28Δ::TRP1*	Segregant from CDV45*
CDV46-1 to CDV46-6	**a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 spr28Δ::TRP1/spr28Δ::TRP1*	This study†
CDV47-1 to CDV47-6	**a**/α	*his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3*	This study†
HF1-1A	**a**	*lys2 ura3 ho::LYS2 spr3::URA3*	Fares *et al.* (1996)
CDV48	**a**/α	*his3/HIS3 leu2/LEU2 lys2/lys2 trp1/TRP1 ura3/ura3 ho::LYS2/HO spr28Δ::TRP1/SPR28 spr3::URA3/SPR3*	CDV45-3D × HF1-1A
EGY48	**a**	*his3 trp1 ura3 LEU2::pLexAop6-LEU2*	Zervos *et al.* (1993)
AMP109	**a**/α	*leu2::hisG/leu2::hisG lys2/lys2 trp1::hisG/trp1::hisG ura3/ura3 ho::LYS2/ho::LYS2*	Bowdish *et al.* (1995)

* CDV45-3A, CDV45-3B, CDV45-3C and CDV45-3D are segregants from the same tetrad.

† Constructed by mating segregants from CDV45 as specified in the text.

(*CDC3*, *CDC10*, *CDC11* and *CDC12*) or genomic DNA (*SPR3* and *SPR28*) as templates. Appropriate restriction sites were introduced with the primers. Sequences that encode either the amino-terminal portions (-*N*) of Cdc3p (amino acids 1–422), Cdc11p (amino acids 1–346), Cdc12p (amino acids 1–336), Spr3p (amino acids 1–370) or Spr28p (amino acids 1–383), or the carboxy-terminal portions (-*C*) of Cdc3p (amino acids 421–520), Cdc11p (amino acids 342–415), Cdc12p (amino acids 325–407), Spr3p (amino acids 363–512) or Spr28p (amino acids 378–423) were also amplified by PCR. The PCR products were cloned at the *Eco*RI site (*CDC3*), the *Xho*I site (*CDC10*, *CDC11*, *CDC11-N* and *CDC12*), or at the *Eco*RI–*Xho*I sites (all other constructs) of pEG202 and pJG4-5. The constructs cloned at the *Eco*RI or *Eco*RI–*Xho*I sites of pEG202 contain two additional amino acids (EF), and the ones cloned at the *Xho*I site contain 13 additional amino acids (EFPGIRRPWRPLE) between the LexA DBD and the first amino acid of the fused protein. All constructs but those encoding the amino-terminal sequences have the original stop codons of the fused genes. The constructs with the amino-terminal sequences have a stop codon immediately downstream of the polylinker.

Strain EGY48 (Table 1) containing the *LexAop–lacZ* reporter plasmid pSH18-34 (Gyuris *et al.*, 1993) was cotransformed with a pEG202 derivative expressing a LexA-DBD fusion protein and with pJG4-5 or a pJG4-5 derivative expressing an AD-fusion protein. β-Galactosidase activities were measured in cultures of several independent transformants grown for 16 h at 30 °C in minimal medium containing 0·1 mg leucine ml^{-1}, 2% (w/v) galactose and 1% (w/v) raffinose.

RNA and protein analyses. The preparation of RNA and of the RNA blot (generously provided by A. Mitchell, Columbia University, New York, USA), and a control verifying that the lanes contain equivalent amounts of RNA, have been described previously (Bowdish *et al.*, 1995). ^{32}P-labelled probes were prepared using the Random Primed DNA Labeling kit (Boehringer Mannheim) according to the manufacturer's instructions. For *SPR28*, the fragment encoding the amino-terminal 383 amino acids (as used in the two-hybrid constructs; see above) was labelled. For *CDC12*, a PCR product corresponding to the complete ORF was labelled. Hybridizations were performed at 68 °C in ExpressHyb Hybridization Solution (Clontech) according to the manufacturer's instructions.

Proteins were extracted from *S. cerevisiae* by pelleting the cells from 10 ml of a sporulating culture (2×10^7 cells ml^{-1}) and resuspending the cells in 240 µl 2·5 × Laemmli buffer (Laemmli, 1970). The cells were sonicated for 5 s and boiled for 10 min. Samples (30 µl) were run on an SDS-polyacrylamide gel (8%, w/v, acrylamide) and transferred electrophoretically to nitrocellulose membranes as described previously (Ford & Pringle, 1991). For immunodetection of the Spr28p–GFP fusion protein, the membrane was incubated with anti-GFP antibodies (Clontech), which were then visualized using the ECL Western blotting method (Amersham) according to the manufacturer's instructions.

Amino acid sequence comparisons. The University of Wisconsin Genetics Computer Group (GCG) programs were used to compile and analyse the sequence data. Alignments were performed using the GAP, PILEUP and PRETTY comparison programs.

Morphological observations. The overall morphologies of cells and asci were determined after growth for 4 d in liquid sporulation medium. 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. For visualization of the GFP-fusion protein, cells were fixed in ice-cold 70% (v/v) ethanol, washed once with water, resuspended in mounting medium (Pringle *et al.*, 1991) containing 0·05 µg bisBenzimide (Sigma) ml^{-1} and observed using a Nikon Microphot SA microscope with an IF380-490 FITC filter set.

RESULTS

Characterization of *SPR28* and its product

A search of the Genbank and SwissProt databases revealed a previously unidentified member of the septin gene family in *S. cerevisiae*. Its nucleotide sequence, located on chromosome IV, was deposited by B. Barrell & M. A. Rajandream under the accession number Z48612 (ORF number YD9934·03c) as part of the yeast genome project.

(a)

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Spr3p     MKSKGSRLSTDCPVEFPKIVSGFAEEVKIRRQSSQGQYAVDSHFPKSPEL   50

Cdc11p                                      MSGIIDASSALRKRKH   16
Spr28p                                MFPMKDHSALEQHTLSRDELRRRKG   25
Spr3p     KHRRQRSSSFVNGKCRNRDLPLLDNKKAQEINTNSHGQDIGIKNLPRQRE  100

Cdc11p    L...KRGITFTVMIVGQSGSGKSTFINTLCGQQVV.....DTSTTI   54
Spr28p    Y...KKGLQLSILLLGEKGSGKSTFLNNLCGQDISLSDGDYDDDDEKVTN   72
Spr3p     LLNAKNGIDFTLMVAGQSGLGKTTFINSLFSTSLI.....DDDIKENK  143

Cdc11p    LLPTDTSTEID.....LQLREETVELEDDEGVKIQLNIIDT   90
Spr28p    NVTPENGNAIEDIDPGYKTAHLSPGLKLVTRRVYLNDELGVPITLDIILF  122
Spr3p     PIIRYKS.....IVEGDGTHLNFNVIDT  166

Cdc11p    PGFGDSLDNSPSFEIISDYIRHQYDEILLEESRVRRNPRFKDGRVHCCLY  140
Spr28p    PGCGDNVDNSQSSVVIKNYLDQQFANVLKEEVRIKRNTKETDGRPHVCLY  172
Spr3p     PGFGNNMDNAFTWRTMVNYIDEEIRSYIFQSEQPDR.TKMVDNRVHCCLY  215

Cdc11p    LINPTGHGLKEIDVEFIRQLGSLVNIIPVISKSDSLTRDELKLNKKLIME  190
Spr28p    FLKSTPRGVKKFDIELMKTICDKVNLIPIIPKADGLTETELNLHKDIVRQ  222
Spr3p     FLRPSNKGIDTLDVVTMKKLAKRVNLIPVIAKSDLLTKEELKNFKTQVRE  265

Cdc11p    DIDRWNLPIYNFPFD.....EDEISDEDVETNMYLRTLLPFA  227
Spr28p    EISQNNIRVFDFKSDTLGETLALYDMDIDSSSAKSKYDNDTKIKEISPFA  272
Spr3p     IIRVQDIPVCFF.....FGDEVLNATQDIFQKYPFS  296

Cdc11p    IIGSNEVYEMGGDVGTIRGRKYPWGILDVEDSSISDFVILRNALLISHLH  277
Spr28p    IVCSK.TFNKNSENRVEHIRTYEWGSLVVEDQNTSDFIYLKAILLGSHLQ  321
Spr3p     IIASNE.YIFNEKGEKVKGRQYKWGAVDIENEKYCDFKILQKTIFDWNLI  345

Cdc11p    DLKNYTHEILYERYRTEALSGE.....SVAAESIRPNLTKLNGSSS  318
Spr28p    ELKDVTNNVLYENYRAKVLTEK.....KNNYDI..PNYSYIDETSR  360
Spr3p     DLVESTED.YYEKCRSEMLRTRLLKARDCLTTKSVDITEEQRKFLEEEMN  394

Cdc11p    SS...TTTRRNTNPFKQSNNINNDVLNPASD..MHGQSTGENNETYMTRE  363
Spr28p    GSVSNVSTRRNSASRTLGNPDTND..ENAYQ..IH.KEIDEKNRIIEDYQ  405
Spr3p     FDEIEENKLKNYKCYEIINKTVMDKVATEWDPEFITRQLEAKKKFNELSN  444

Cdc11p    EQIRLEEERLKA.F..EERVQQELLLKRQELLQREKELREIEARLEKEAK  410
Spr28p    RKIDLLEKMLAAPH..QNKV*  423
Spr3p     REISKFRDWKKSLFMEQENFNQEIEQLNHKLENLQLECQDLEYKLLIGKS  494

Cdc11p    IKQEE*  415
Spr3p     SNSHSTDSATLVNVHIKR*  512
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(b)

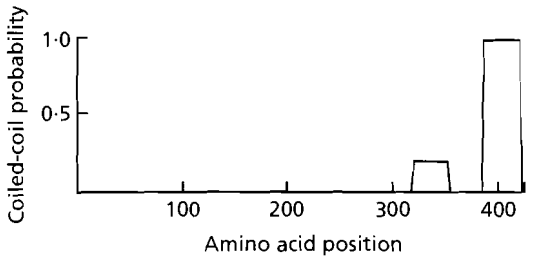
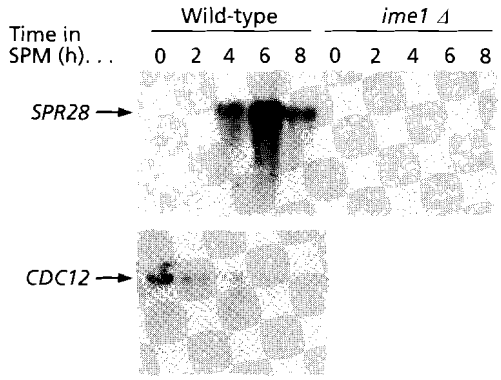


Fig. 1. (a) Sequence alignment of *S. cerevisiae* Spr28p, Cdc11p and Spr3p (see Methods). Dots indicate gaps introduced to maximize sequence alignment; numbers indicate amino acid positions. Shading indicates amino acids that are the same in Spr28p and one or both of the other proteins. Motifs of the predicted nucleotide-binding site are underlined. (b) Probability of coiled-coil formation for Spr28p as predicted by the empirical program of Lupas *et al.* (1991) using a window size of 28 residues.

(a)



(b)

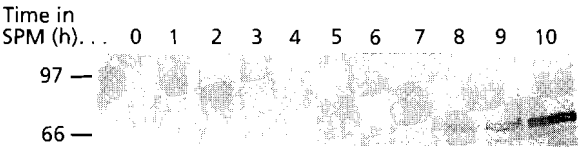


Fig. 2. (a) Accumulation of *SPR28* mRNA and loss of *CDC12* mRNA in sporulating cells. RNA was prepared from cells growing exponentially in YPAc (0 h) and from cells harvested 2, 4, 6 or 8 h after a shift to sporulation medium (SPM) (Bowdish *et al.*, 1995). The Northern blot was probed with ^{32}P-labelled *SPR28* or *CDC12* DNA as described in Methods. The strains used were AMP1179 (*MATa/α*, wild-type) and AMP258×1184 (*MATa/α ime1Δ/ime1Δ*) (see Bowdish *et al.*, 1995). (b) Immunoblot analysis of Spr28p-GFP fusion protein expression. Cultures were grown to mid-exponential phase in YPAc and transferred to sporulation medium. Proteins were extracted from the YPAc-grown cells (0 h) and from cells removed at hourly intervals after transfer to sporulation medium. The proteins were separated by SDS-PAGE, blotted and probed with anti-GFP antibodies as described in Methods. The predicted molecular mass of the Spr28p–GFP fusion protein (76·9 kDa) is in good agreement with the estimated sizes (~ 74 and ~ 77 kDa) of the two polypeptides induced between 7 and 10 h after the transfer to sporulation medium. The smaller of these two polypeptides may be a degradation product of the larger. The mobilities of the flanking molecular size markers (97 and 66 kDa) are indicated.

Analysis of the sequence showed an ORF of 1269 nucleotides predicting a protein of 423 amino acids (48·2 kDa). Based on (i) its SPorulation-Regulated transcription (see below) and (ii) the previous identification of 27 other *SPR* genes (Clancy *et al.*, 1983; Holaway *et al.*, 1987), we designated this gene *SPR28*. The *SPR28* ORF is flanked by several in-frame stop codons.

The predicted Spr28p sequence shows clearly that it is a sixth member of the *S. cerevisiae* septin family (Fig. 1a); sequence identities range from 27% (with Cdc3p) to 35% (with Cdc11p) over the full length of the shorter protein in each case. Spr28p is also related to septins from other organisms (sequence identities ranging from 27% to 34%) but does not have a particularly close homologue among the known proteins. Like all other known members of the septin family, Spr28p has amino acid motifs that are conserved among nucleotide-binding

Table 2. Timing of meiosis in strain AMP109

The percentages of cells with one, two or four distinct nuclear DNA masses are shown at various time points after transfer to sporulation medium (SPM). At least 400 individual cells were analysed for each time point.

Time in SPM (h)	Percentage of cells with:		
	1 DNA mass	2 DNA masses	4 DNA masses
1	100	0	0
2	100	0	0
3	100	0	0
4	100	0	0
5	100	0	0
6	99	1	0
7	91	9	0
8	75	15	10
9	51	19	22
10	44	12	44

proteins (Fig. 1a) (Saraste *et al.*, 1990; Bourne *et al.*, 1991). In addition, Spr28p, like most (but not all) of the other septins, contains a region near its carboxy-terminus (amino acids 385–417) that is predicted (> 98% probability) to form a coiled-coil (Lupas *et al.*, 1991) (Fig. 1b).

Sporulation-specific expression of *SPR28*

To determine if *SPR28* is expressed in vegetative cells, sporulating cells, or both, a filter containing total RNA isolated from cells at various times after a shift to sporulation medium was hybridized with labelled *SPR28* DNA. No *SPR28* transcripts were detected in vegetatively growing wild-type cells (Fig. 2a, 0 h). However, the *SPR28* transcript levels increased dramatically after 4 h in sporulation medium and peaked after 6 h (Fig. 2a). Thus, given the time course of sporulation in this and related strains (Table 2; Bowdish *et al.*, 1995), the *SPR28* transcript was first detectable at the beginning of meiosis I and was maximal just prior to meiosis II. In contrast, transcript levels for the vegetatively expressed septin gene *CDC12* declined progressively as sporulation proceeded (Fig. 2a).

Because deletion of *IME1* eliminates expression of almost all sporulation-specific genes and blocks all tested events in meiosis and sporulation (Mitchell, 1994), we also examined the expression pattern of *SPR28* in an *ime1Δ/ime1Δ* strain. No *SPR28* transcripts were detected in either vegetatively growing or sporulating cells (Fig. 2a), indicating that *SPR28* expression requires *IME1*, as expected.

Expression and localization of Spr28p in sporulating cells

To examine the timing of Spr28p expression and its intracellular localization, we examined wild-type cells (AMP109; Table 1) that expressed an Spr28p–GFP fusion protein under the control of the *SPR28* promoter. Consistent with the results of the RNA analyses, no Spr28p–GFP was detected by immunoblotting of extracts from vegetatively growing cells (Fig. 2b, 0 h), but Spr28p–GFP was present in increasing amounts in cells incubated for 7–10 h in sporulation medium (Fig. 2b). Initial detection of the Spr28p–GFP fusion protein coincided with the first appearance of cells that had completed meiosis I (cells with two DNA masses; Table 2). This observed timing of Spr28p–GFP expression in sporulating cells is very similar to that of Spr3p under the same conditions (Fares *et al.*, 1996).

To determine the intracellular localization of Spr28p–GFP, we examined cells that had been in sporulation medium for 10 h. Consistent with the previously reported localizations of Spr3p, Cdc3p and Cdc11p (Fares *et al.*, 1996), the Spr28p–GFP fusion protein localized to ring-like structures around each of the four nuclear lobes at the onset of and during meiosis II (Fig. 3a, d). At later stages, Spr28p–GFP appeared to be found over the entire

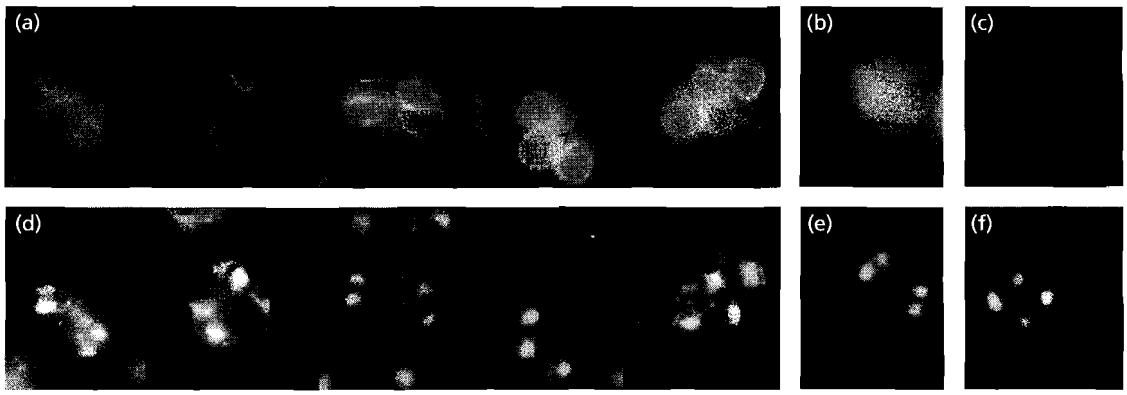


Fig. 3. Localization of Spr28p–GFP in sporulating cells. Strain AMP109 carrying YEplac181–SPR28–GFP (a, d), YEplac181–GFP (b, e) or YEplac181 (c, f) was pregrown on SD-Leu medium, grown to mid-exponential phase in YPAc and incubated for 10 h in sporulation medium. The fluorescence of cells expressing Spr28p–GFP (a), GFP (b) or no additional protein (c) was visualized as described in Methods. (d, e, f) DNA staining of the cells shown in a, b and c, respectively.

Table 3. Two-hybrid interactions of Spr28p with itself and other septins

Possible interactions between Spr28p and other *S. cerevisiae* septins were evaluated using the two-hybrid system as described in Methods. Numbers represent mean β-galactosidase activities (in Miller units) from three different transformants for each pair of plasmids. The characters '*N*' and '*C*' designate sequences encoding the amino- or carboxy-terminal portions, respectively, of the various septins as specified in Methods. pJG4-5 indicates the AD vector with no septin insert. Values that were above 100 units and at least sixfold higher than the corresponding control (pJG4-5 without insert) are shown in bold.

AD fusion	DBD fusion					
	SPR28	*SPR28-N*	*SPR28-C*	*SPR3*	*SPR3-N*	*SPR3-C*
SPR28	6	14	39	51	**914**	7
SPR28-N	**1278**	**130**	20	39	**374**	6
SPR28-C	90	37	44	21	16	4
SPR3	**207**	**171**	6	14	8	5
SPR3-N	**1841**	**1840**	25	6	14	11
SPR3-C	2	7	16	2	9	7
CDC3	**150**	9	13	12	8	15
CDC3-N	9	12	19	6	11	8
CDC3-C	6	12	35	5	5	18
CDC10	10	8	24	25	11	38
CDC11	**3402**	**3927**	39	3	16	3
CDC11-N	**3957**	**3776**	13	3	13	7
CDC11-C	53	69	48	3	6	7
CDC12	31	45	32	4	16	10
CDC12-N	43	45	30	5	6	2
CDC12-C	8	17	14	6	11	38
pJG4-5	18	10	31	21	10	4

prospore wall (Fig. 3a, d and data not shown). Cells expressing GFP alone under control of the *SPR28* promoter showed only dispersed fluorescence over the entire cell at comparable stages of sporulation (Fig. 3b, e). The fluorescence signal was completely absent in cells carrying only an empty control vector (Fig. 3c, f). Thus, Spr28p, like the other septins examined to date (Fares *et al.*, 1996), appears to be concentrated initially at the leading edges of the developing prospore wall and later is found over this entire structure.

Two-hybrid analysis of Spr28p interactions

A variety of data suggests that the septins interact intimately with each other (Longtine *et al.*, 1996). To investigate the possible interactions of Spr28p with the other septins, directed two-hybrid analysis (Fields & Sternglanz, 1994) was performed. Interactions were detected between Spr28p and itself, Spr3p, Cdc3p and Cdc11p (Table 3). Thus, Spr28p appears to interact directly with itself and with some, but not all, of the other septins that are expressed in sporulating cells. [Based on its high mRNA levels (Kaback & Feldberg, 1985), Cdc10p is also expected to be abundant in sporulating cells. The levels of Cdc12p have not been examined].

Interestingly, the interaction with Spr28p was the only interaction detected for Spr3p (see Discussion). Also of interest was the finding that the interactions of Spr28p with itself, Spr3p and Cdc11p all appeared to be independent of the carboxy-terminal coiled-coil regions, as the interactions were observed when one or both of the interacting proteins were truncated upstream of this region (Table 3).

Effects of *SPR28* deletion

To determine the consequences of a loss of Spr28p, we replaced the complete *SPR28* coding region with *TRP1* (Fig. 4a; see Methods). A transformant carrying the deletion (CDV45) was identified by PCR and confirmed by Southern blotting (Fig. 4b). Homozygous *spr28Δ/spr28Δ* diploids (CDV46-1 to CDV46-6; Table 1) and related homozygous wild-type diploids (CDV47-1 to CDV47-6) were constructed by inducing sporulation in CDV45 and mating appropriate pairs of segregants. Consistent with the apparent lack of *SPR28* expression in vegetative cells, *spr28Δ* haploids and homozygous mutant diploids showed no obvious change in growth rate at 20, 30 or 37 °C, and no morphological abnormalities or defects in budding pattern, when compared to wild-type sister segregants or homozygous wild-type diploids. More surprisingly, when the efficiencies of sporulation of the homozygous *spr28Δ/spr28Δ* and related wild-type diploids were compared, there was little or no difference.

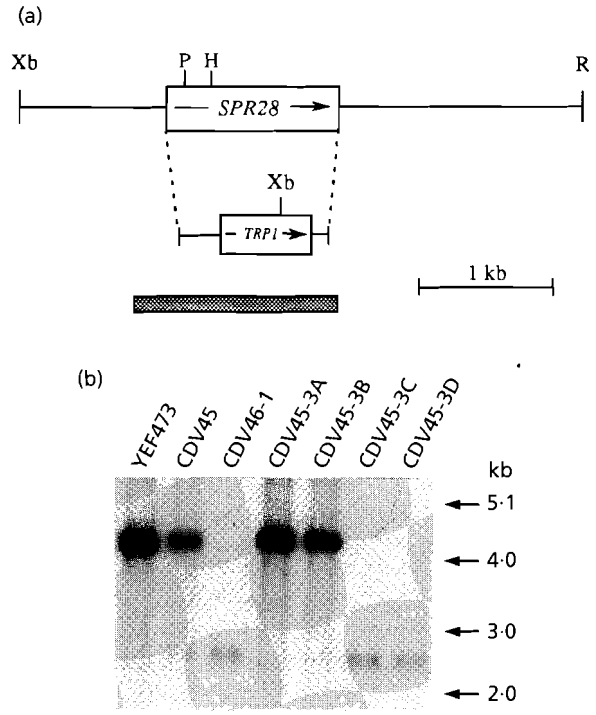


Fig. 4. (a) Physical map of the *SPR28* region and structure of the *spr28Δ::TRP1* deletion. All cleavage sites for *Eco*RI (R), *Hin*dIII (H), *Pst*I (P) and *Xba*I (Xb) are shown. The location and direction of transcription of the *SPR28* coding region are shown along with the structure of the deletion allele containing *TRP1* (direction of transcription indicated by the arrow) and *TRP1*-flanking regions (see Methods). The shaded bar represents the DNA fragment used as a probe for Southern blot analysis. (b) Southern blot analysis of strains with and without the *spr28Δ::TRP1* deletion. YEF473 is the parental diploid strain; CDV45 is the heterozygous diploid obtained by transformation of YEF473 with the *spr28Δ::TRP1* construct; CDV45-3A to CDV45-3D are the four segregants from one tetrad of CDV45; and CDV46-1 is the homozygous *spr28Δ::TRP1/spr28Δ::TRP1* diploid formed by mating CDV45-3C with CDV45-3D. Genomic DNAs were digested with *Xba*I and *Eco*RI, and DNA blots were hybridized with a PCR-generated DNA fragment containing the *SPR28* coding region and upstream sequences (see a). The mobilities of molecular size markers are indicated. Bands of the predicted sizes (4·2 kb for wild-type and 2·5 kb for *spr28Δ::TRP1* strains) were observed in the expected lanes.

After 4 d in sporulation medium, the six CDV46 series strains displayed 12–17% (mean 15%) 4-spored asci (counts of 400 cells per strain), compared to 14–22% (mean 17%) for the six CDV47 series strains.

Previous work had shown that deletion of either *SPR3* or *CDC10* reduced sporulation efficiency in some genetic backgrounds (Fares *et al.*, 1996). The possibility of redundancy in function between Spr3p and Cdc10p proved difficult to test because an *spr3/spr3 cdc10/cdc10* double mutant diploid could not be constructed. We thought that an *spr3/spr3 spr28/spr28* double mutant might be easier to construct (because neither gene appears to play a role in vegetative growth) and might display a more severe sporulation block than either of the single mutants. Thus, we constructed six homozygous *spr3Δ/spr3Δ spr28Δ/spr28Δ* diploids and six related homozygous wild-type diploids by inducing sporulation in CDV48 (a double heterozygote; Table 1) and mating appropriate pairs of segregants. As expected, vegetatively growing *spr3Δ spr28Δ* haploids as well as homozygous mutant diploids showed no obvious change in growth rate at 20 or 30 °C or morphological abnormalities when compared to wild-type sister segregants or homozygous wild-type diploids. Surprisingly, however, we also found little or no difference in sporulation efficiency when the *spr3Δ/spr3Δ spr28Δ/spr28Δ* homozygous diploids were compared to related wild-type diploids. After 4 d in sporulation medium, the six *spr3Δ/spr3Δ spr28Δ/spr28Δ* strains displayed 24–62% (mean 42%) 4-spored asci (counts of 200 cells per strain), compared to 52–76% (mean 63%) for the six homozygous wild-type strains.

DISCUSSION

SPR28, a gene identified during the yeast genome project (B. Barrell & M. A. Rajandream, unpublished results), encodes a sixth member of the *S. cerevisiae* septin family. Recently, the existence of a seventh (and final) *S. cerevisiae* septin gene has been revealed by the completion of the genome project. The expression and function of this gene and its product are currently under investigation.

Spr28p is ~ 30% identical in amino acid sequence to the other known septins and does not have a particularly close homologue among them; in particular, there is no obvious special similarity between Spr28p and Spr3p or the *Schizosaccharomyces pombe* septin(s) that appear to be expressed specifically in sporulating cells (Fig. 1; O. Al-Awar, H. B. Kim & J. R. Pringle, unpublished results). Like all of the other known septins, Spr28p contains a putative nucleotide-binding site that might be involved in the control of septin assembly and/or function. However, the role of this site has yet to be elucidated in any septin. In addition, like all but a few of the other known septins (Longtine *et al.*, 1996), Spr28p contains a predicted coiled-coil domain near its carboxy terminus. This domain is likely to be involved in interactions among the septins or between the septins and other proteins. Interestingly, the observed two-hybrid interactions between Spr28p and itself, Spr3p and Cdc11p were not mediated by the coiled-coil domains but rather by the amino-terminal portions of all three proteins. This finding does not necessarily exclude a role for the coiled-coil domains in inter-septin interactions. However, if the inter-septin interactions are indeed mediated solely by their amino termini, the coiled-coil domains might serve as anchors for other proteins being recruited to the site of septin assembly.

The localization of the Spr28p–GFP fusion protein during meiosis II and spore formation was very similar to that observed previously for Spr3p, Cdc3p and Cdc11p (Fares *et al.*, 1996), suggesting a close association of these (and perhaps other) septins with each other and with the developing prospore wall. Indeed, two-hybrid analyses suggested that Spr28p interacts directly with itself, Spr3p,

Cdc3p and Cdc11p. Interestingly, the strong interaction with Spr28p was the only interaction detected for Spr3p, suggesting that Spr28p may play a special role in recruiting Spr3p into the putative septin complex at the prospore wall. In previous studies, the localization of Cdc3p and Cdc11p to the prospore wall appeared to be partly, but not completely, dependent on the presence of Spr3p. Further work will be necessary to elucidate the full set of interactions necessary for proper localization and assembly of the septins in sporulating cells.

The apparent localization of the septins to the leading edge of the early prospore wall (Fig. 4; Fares *et al.*, 1996) suggests that these proteins may be involved in the extension of this structure, but we have as yet no strong evidence for such a role. Alternatively (or in addition), the septins may be involved in the formation of the spore wall, a process that appears to begin only after encapsulation of the spore nucleus and cytoplasm are complete or nearly so (Moens, 1971; Beckett *et al.*, 1973; Byers, 1981; Fares *et al.*, 1996). In this context, it should be noted that the formation of the spore wall chitosan layer depends on chitin synthase III (Briza *et al.*, 1988; Pammer *et al.*, 1992; Bulawa, 1993), the same enzyme that produces the chitin ring at the base of the bud in vegetative cells (Bulawa, 1993). The vegetatively expressed septins appear to be involved in the localization of chitin synthase III activity through interactions that involve the Chs4p (Csd4p) and Bni4p proteins (D. DeMarini, H. Fares & J. R. Pringle, unpublished results). Chs4p has a homologue, Shc1p, that is expressed specifically in sporulating cells (Bulawa, 1993). Thus, the septins may be involved in the organization of spore wall chitosan formation through interaction with Shc1p and Bni4p or a sporulation-specific homologue.

If the septins play an important role in extension of the prospore wall and/or in spore wall formation, why have the septin mutations examined had such modest (or even undetectable) effects on spore formation (Simchen, 1974; Kao *et al.*, 1989; Fares *et al.*, 1996; this study)? The failure to see effects with temperature-sensitive alleles of *CDC3*, *CDC10* and *CDC11* (Simchen, 1974) might have resulted from the need to do the tests of sporulation at a temperature that may not have been fully restrictive for the alleles used. However, deletions of *CDC10*, *SPR3* and *SPR28* also produce only modest effects on sporulation efficiency. The answer seems likely to lie, at least in part, in functional redundancy among the septins, a phenomenon for which evidence also exists in vegetative cells (Longtine *et al.*, 1996). However, we have as yet no strong evidence for this hypothesis. We were unable to construct stable *cdc10Δ/cdc10Δ spr3Δ/spr3Δ* diploids (Fares *et al.*, 1996), and results with *spr3Δ/spr3Δ spr28Δ/spr28Δ* diploids, although variable, showed no consistent, strong block of spore formation (this study). Thus, remarkably, neither of the known sporulation-specific septins is strictly required for sporulation. Further studies will be required to unravel the possible redundancies in function among the septins and thus (perhaps) to allow decisive studies of the septin role(s) in a strain with a strong defect in sporulation.

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