The cation-transporting P-type ATPase Cta4 is required for assembly of the forespore membrane in fission yeast

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A novel sporulation-deficient mutant, *sev4-L5*, was isolated in a genetic screen of a collection of temperature-sensitive mutants of *Schizosaccharomyces pombe*. The wild-type *sev4* gene was identified as *cta4*⁺, which encodes a putative cation-transporting P-type ATPase. The *sev4-L5* allele harbored a single missense mutation that caused replacement of Gly615 with a glutamate at the putative ATP-binding site. Similar to *cta4*-null mutants, *sev4-L5* exhibited defects in growth at high and low temperatures, and sensitivity to high and extremely low concentrations of Ca²⁺. The *cta4*⁺ mRNA level was considerably enhanced during meiosis. When *sev4-L5* cells were incubated in sporulation medium at the permissive temperature, meiotic nuclear divisions proceeded with normal kinetics, but spores were not formed. Structural alteration of the spindle pole body, which is prerequisite to construction of the forespore membrane in wild type, was incomplete. Consequently, formation of the forespore membrane was severely impaired. These observations show that perturbation of Ca²⁺ homeostasis by mutation of *cta4/sev4* blocks sporulation mainly by interfering with forespore membrane assembly.

Key words: fission yeast, forespore membrane, meiosis, P-type ATPase, calcium homeostasis

INTRODUCTION

Sporulation in yeasts provides a model for gametogenesis in higher eukaryotes. An important step leading to ascospore formation is *de novo* assembly of the plasma membrane of newborn spores (Neiman, 1998; Shimoda, 2004). The spore plasma membrane is formed in the cytoplasm of zygotes as a double-layered intracellular membrane, termed the forespore membrane (FSM) in the fission yeast *Schizosaccharomyces pombe* (Yoo et al., 1973; Hirata and Tanaka, 1982; Tanaka and Hirata, 1982). The nascent FSM extends by fusing with vesicles derived from the ER/Golgi and encapsulates each of the meiotic nuclei (Hirata and Tanaka, 1982; Tanaka and Hirata, 1982; Nakamura et al., 2001). The inner membrane of the FSM becomes the plasma membrane of the spores. Formation of the FSM initiates during meiosis II near the spindle pole body (SPB), a microtubule-organizing center in yeast that is equivalent to the centrosome.

Before formation of the FSM, the SPB undergoes a morphological change from a dot to a crescent shape (Hagan and Yanagida, 1995), and this modification seems to be essential for FSM formation (Hirata and Shimoda, 1994; Ikemoto et al., 2000). These facts imply that the SPB plays an important role in membrane assembly, but the mechanistic details are still elusive.

Genetic and molecular analyses of many sporulation-specific mutants in *S. pombe* have dissected the molecular events that occur during meiosis and sporulation (Bresch et al., 1968; Kishida and Shimoda, 1986; Shimoda and Nakamura, 2004). In addition to specific genes termed *spo*, we have also screened for asporogenous mutants out of a collection of temperature-sensitive mutants (Yoshida et al., 2003). These mutants, referred to as “*sev*” (for sporulation genes essential for vegetative growth), have defined five genes. *sev1*⁺ is identical to *cdt2*⁺, which is responsible for mitotic and premeiotic DNA replication (Yoshida et al., 2003). In this article, we report the analysis of *sev4*⁺, which encodes Cta4, a putative Ca²⁺-ATPase.

Ca²⁺ is an essential component of cellular signal transduction. The intracellular concentration of Ca²⁺ is strictly regulated, and perturbation of Ca²⁺ homeostasis

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causes various disorders in cell growth and function. Analysis of the *cta4*-null mutant previously revealed that Ca^{2+} homeostasis is crucial for growth and cell polarity (Okorokova-Facanha et al., 2002). Influx of Ca^{2+} into cells is triggered during conjugation in budding yeast (Iida et al., 1994). The Ca^{2+} -binding protein calmodulin and its downstream protein phosphatase calcineurin are also involved in cell polarity and mating in yeast (Liu et al., 1990; Cyert et al., 1991; Ohya and Botstein, 1994; Yoshida et al., 1994). To date, however, little information is available on the requirement of intracellular Ca^{2+} homeostasis for meiosis and sporulation. Here we report that mutation of *sev4/cta4* causes severe disorders in sporulation.

MATERIALS AND METHODS

Yeast strains, media and genetic techniques. The *S. pombe* strains used in this study are listed in Table 1. Complete medium YE and minimal medium EMM2+N were used for growth. Sporulation media MEA, SSA and EMM2–N were used for mating and sporulation (Egel and Egel-Mitani, 1974; Gutz et al., 1974; Moreno et al., 1991). The original *sev4-L5* mutant was derived from C982-16C. The method for synchronous meiosis has been described in our previous report (Yoshida et al., 2003).

To construct a *cta4*-null allele, a major part of the ORF (amino acids 64 to 993) was replaced by a *ura4⁺* cassette (Grimm et al., 1988). The DNA fragment containing the *cta4::ura4⁺* allele was introduced into the diploid strain SYD1 and uracil prototrophs were isolated. Correct integration of the disrupted allele at the *sev4⁺* locus was

verified by Southern blot analysis, as described previously (Yoshida et al., 2003).

Construction of *cta4*-HA. Triple hemagglutinin (HA) epitope-tagged *cta4⁺* was constructed as follows. The *cta4⁺* coding region was amplified by PCR using primers CCCGTCGAC(*Sal*I)GGGGAGTAAGGCTTTA and GGG-GCGGCCGC(*Not*I)AATTACGAAGAACAAT. The amplified DNA fragment was digested with *Sal*I and *Not*I and then inserted into pTN218 containing the HA (3x) and the *nmt1* terminator (Nakamura et al., 2002) to yield a *cta4*-HA integration plasmid pSY05004. pSY05004 was linearized at a single *Stu*I site in the *Cta4*-coding region and transformed into TN29 to create SY70, in which a single copy of the *Cta4*-HA allele with a *cta4* native promoter was integrated at the chromosomal *cta4* locus.

Northern blot analysis. Total RNA was prepared from the *pat1-114* mutant strain (JZ670) at hourly intervals after induction of meiosis (Jensen et al. 1983). RNA was fractionated on a 1.0% agarose gel containing 3.7% formaldehyde (Thomas, 1980), blotted and then probed with the *cta4⁺*-specific probe. The probe DNA was labeled with [32 P]dCTP by the random primer method (Feinberg et al., 1983).

Nucleotide sequence analysis of the *sev4-L5* allele. The complete *sev4-L5* ORF was amplified by PCR by using genomic DNA prepared from SY13 as a template. Amplified DNA fragments were directly sequenced with an ABI-PRISM 310 autosequencer (Applied Biosystems, CA, USA). The nucleotide sequence of the *sev4-L5* allele was compared with the published

Table 1. Strain list

Strains	Genotype	Reference/Source
C982-16C	<i>h</i> ⁹⁰ <i>ura4-D18</i>	Yoshida et al. (2003) / YGRC*
C996-11D	<i>h</i> ⁹⁰ <i>leu1-32</i>	Yoshida et al. (2003) / YGRC*
FS26	<i>h</i> ⁹⁰	YGRC*
SY13	<i>h</i> ⁹⁰ <i>sev4-L5</i>	This study
SY15	<i>h</i> ⁹⁰ <i>sev4-L5 leu1-32</i>	This study
SY34	<i>h</i> ⁹⁰ <i>cta4::ura4⁺ ura4-D18</i>	This study
SY60	<i>h</i> ⁹⁰ <i>sev4-L5 leu1 << GFP-psy1 leu1-32</i>	This study
SY70	<i>h</i> ⁹⁰ <i>cta4-HA<<LEU2 ura4-D18 leu1-32</i>	This study
TN29	<i>h</i> ⁹⁰ <i>ura4-D18 leu1-32</i>	Ikemoto et al. (2000) / YGRC*
YN68	<i>h</i> ⁹⁰ <i>leu1 << GFP-psy1 leu1-32</i>	Nakase et al. (2004) / YGRC*
JZ670	<i>h⁻/h⁻</i> <i>pat1-114 / pat1-114 ade6-M210 / ade6-M216 leu1-32 / leu1-32</i>	Iino et al. (1995) / YGRC*
SYD1	<i>h</i> ⁹⁰ / <i>h</i> ⁹⁰ <i>ura4-D18 / ura4-D18 leu1-32 / leu1-32</i>	This study
SYD2	<i>h⁺/h⁻</i> <i>ade6-M210 / ade6-M216</i>	This study
SYD3	<i>h⁺/h⁻</i> <i>sev4-L5 / sev4-L5 ade6-M210 / ade6-M216</i>	This study

*YGRC, Yeast Genetic Resource Center Japan (<http://bio3.tokyo.jst.go.jp/jst/>). The strains constructed in this study will be deposited in the YGRC.

sequence of *cta4*⁺ (Okorokova-Facanha et al., 2002).

Immunofluorescence microscopy. For cell fixation, we followed the method of Hagan and Hyams (1988) using glutaraldehyde and paraformaldehyde. Cta4-HA was visualized by indirect immunofluorescence microscopy using rat anti-HA antibody 3F10 (Boehringer Mannheim, Mannheim, Germany) and Alexa 546-conjugated goat anti-rat IgG (Molecular Probes, Eugene, USA). The SPB was visualized by indirect immunofluorescence microscopy using rabbit anti-Sad1 antibody and Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probes). For microtubule staining, TAT-1 anti- α -tubulin antibody (Woods et al., 1989) and Cy3-conjugated secondary antibody (Sigma Chemical Co., St. Luis, USA) were used. To visualize the nuclear chromatin region, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) at 1 μ g/ml. Stained cells were observed under a fluorescence microscope (model BX51; Olympus, Tokyo) equipped with a charge-coupled device (CCD) camera (Cool-SNAP; Roper Scientific, San Diego, USA).

RESULTS AND DISCUSSION

Isolation of a sporulation-deficient *sev4-L5* mutant.

In a genetic screen for sporulation-deficient mutants present in a collection of temperature-sensitive mutants of *S. pombe*, we previously isolated *sev* mutants and identified five *sev* loci (Yoshida et al. 2003). Genetic analysis of the *sev4-L5* mutant indicated that its mutant phenotype was due to a single recessive mutation. *sev4-L5* cells were unable to form colonies either at 37°C or at 19°C on YEA plates (Fig. 1A). The mutant cells grew very poorly in EMM2+N supplemented with 0.5 M CaCl₂ or in Ca²⁺-free EMM2+N containing 10 mM EGTA (data not shown). The growth in the latter medium was recovered by adding 20 mM CaCl₂ (data not shown). A homothallic haploid strain carrying *sev4-L5* was incubated in nitrogen-limited medium MEA at the permissive temperature (28°C) for two days. Cells conjugated to form zygotes at a lower efficiency than did wild-type control cells, and notably produced no asci (Table 2 and Fig. 1B).

Identification of the *sev4*⁺ gene. An *S. pombe* genomic library (pTN-L1) (Nakamura et al., 2001) was introduced into the *sev4-L5* mutant. Plasmid DNA was prepared from a few Spo⁺ transformants, and both termini of the inserted fragment were sequenced to identify the cloned gene. Comparison with the *S. pombe* genome sequence database (http://www.sanger.ac.uk/Projects/S_pombe/) showed that the insert contained two ORFs. Subcloning experiments determined the ORF (SPACUNK4.07c, also known as *cta4*⁺) responsible for the complementation activity, and integration mapping

Fig. 1. Phenotype of the *sev4* mutant. (A) Colony formation at different incubation temperatures. Wild-type (C996-11D) and *sev4-L5* (SY15) cells were incubated on YE plates at 28°C for 2 days, at 37°C for 2 days, or at 19°C for 3 days. (B) Mating and sporulation ability of the *sev4-L5* mutant. Wild-type (C996-11D) and *sev4-L5* (SY15) cells were incubated on MEA plates at 28°C for 2 days. After fixation with 70% ethanol, cells were stained with DAPI. Bar, 10 μ m.

Table 2. Efficiency of conjugation and sporulation in *sev4* mutant

	Conjugation (%)	Sporulation (%)
WT	64.1	86.1
<i>sev4-L5</i>	24.3	0
Δ <i>cta4</i>	30.8	0

Wild-type (FS26), *sev4-L5* (SY13), and *cta4* Δ (SY34) cells were cultured on MEA plates at 28°C for 2 days. Over 300 cells were counted for each strain. Efficiencies of conjugation and sporulation were calculated by the following equations:

Conjugation (%) = $(2 \times [\text{Zygotes}] + 2 \times [\text{Asci}]) \times 100 / (2 \times [\text{Zygotes}] + 2 \times [\text{Asci}] + [\text{Vegetative cells}])$

Sporulation (%) = $[\text{Asci}] \times 100 / ([\text{Asci}] + [\text{Zygotes}])$

verified that the cloned gene was indeed the defined *sev4*⁺/*cta4*⁺ gene.

cta4⁺ encodes a cation-transporting P-type ATPase that specifically localizes to the endoplasmic reticulum (ER) (Okorokova-Facanha et al., 2002). The *cta4*-null mutation markedly increases the cellular Ca²⁺ concentration, indicating that Cta4 is responsible for Ca²⁺ homeostasis in *S. pombe* (Okorokova-Facanha et al., 2002). Sequencing of the *sev4-L5* allele revealed that it contained a single mutation of G to A at nucleotide 1844. This mutation

predicts the substitution of Gly615, which is located in the conserved ATP-binding site, with a glutamate residue. As the phenotype of the *sev4-L5* mutant closely resembled that of the *cta4*-null mutant, the ATP-binding activity must be essential for the function of Cta4.

Expression and localization of Cta4. To examine the expression of *cta4*⁺, we estimated the mRNA abundance by Northern blot analysis. Synchronous meiosis was conducted in the *pat1-114* mutant, JZ670 (Iino and Yamamoto, 1995). The *cta4* hybridization signal was detected in the cell sample at 0 hr (vegetative cells) and intensified at around 6–7 hr after the induction of meiosis, corresponding to meiosis I (Fig. 2). *cta4* mRNA was also present in exponentially growing cultures (data not shown). This result is consistent with DNA microarray data showing that transcription of *cta4*⁺ is transiently upregulated during meiosis (Mata et al., 2002). We thus conclude that *cta4*⁺ transcription is constitutive in vegetative cells and is upregulated significantly during meiosis.

Fig. 2. Northern blot analysis of *cta4*⁺ mRNA. The *pat1-114* homozygous diploid strain (JZ670) was incubated in EMM2-N for 15 hr at 25°C and then shifted to 34°C to induce synchronous meiosis. At hourly intervals, aliquots were removed for RNA preparation. (A) Autoradiogram of RNA blots with a *cta4*-specific probe. Ethidium bromide staining of ribosomal RNAs is presented as a loading control (bottom). (B) To monitor the meiotic nuclear divisions, the number of nuclei per cell was determined after DAPI staining.

Fig. 3. Localization of Cta4-HA to the ER. Vegetative cells of an integrant strain SY70 was fixed, treated with anti-HA antibody (3F10), and stained with Alexa 594-conjugated anti-rat IgG antibody and DAPI. A phase-contrast image is also presented. Bar 10 μ m.

A GFP-Cta4 fusion protein has been reported to localize to the ER (Okorokova-Facanha et al., 2002). To verify this result, we constructed a strain carrying a single copy of the *cta4-HA* allele that was expressed under the control of a *cta4* native promoter. The integrant strain was then transformed with pREP82(13g6-GFP), which was a *ura4*⁺-carrying version of pREP81(13g6-GFP) (Nakamura-Kubo et al. 2003), to visualize an ER marker protein 13g6 (Brazer et al., 2000). Both signals were colocalized to the nuclear periphery and beneath the plasma membrane; this localization is typical of proteins associated with the ER (Fig. 3). These findings support the idea that Cta4 is a cation-transporting ATPase localized to the ER.

***sev4-L5* was defective in development of the FSM.** The *cta4*-null mutant showed elevated levels of intracellular Ca^{2+} concentration (Okorokova-Facanha et al., 2002). We thus examined whether externally added CaCl_2 influenced conjugation and sporulation in wild-type

cells. Homothallic wild-type strain (FS26) was cultured in nitrogen-free liquid medium, EMM²-N, containing different concentrations of CaCl_2 . Cells were cultured with shaking at 28°C for 2 days. CaCl_2 at concentrations higher than 0.5 M markedly inhibited conjugation. At lower concentrations, 0.1–0.4 M, CaCl_2 moderately inhibited conjugation, but did not significantly affect sporulation (data not shown).

As external Ca^{2+} addition preferentially blocked the zygote formation, we studied the requirement of Ca^{2+} homeostasis for the later stages of sexual development, meiosis and sporulation, with the *sev4-L5* mutant. First, the kinetics of meiosis in diploid strains was monitored by DAPI staining of the nuclear chromatin region. As shown in Fig. 4, timing of the meiotic nuclear divisions in the *sev4-L5* homozygous diploid was similar to that in the wild-type strain with a slight increase in the remaining mononucleate cells. Thus, it seems unlikely that the sporulation deficiency in *sev4-L5* is due to a failure in the progression of meiosis.

Formation of the FSM, as visualized by GFP-Psy1, commences during meiosis II (Nakamura et al., 2001). Wild-type and *sev4-L5* cells expressing GFP-Psy1 were cultured at 28°C on SSA sporulation medium. Fixed cells were immunostained with an antibody against α -tubulin (TAT-1) (Woods et al., 1989) or an anti-Sad1 antibody (Hagan and Yanagida, 1995) to monitor microtubules or the SPB, respectively. Progression of meiosis was also traced by staining cells with DAPI. Assembly of the FSM began at metaphase II near the spindle poles in both

strains (Fig. 5). Cup-shaped structures typical of an early stage of FSM were observed in the *sev4-L5* mutant (Fig. 5, metaphase II), but extension of the FSM appeared to be aberrant (Fig. 5, anaphase II). As a result, malformed and anucleated prespores were frequently produced. Finally, we analyzed quantitatively the terminal morphology of the FSM in post-meiotic, tetranucleate cells. In most wild-type cells, the FSM developed into closed membrane compartments termed prespores, each of which contained one haploid nucleus (Fig. 6A, Class I). By contrast, normal nucleated prespores were rarely found in *sev4-L5*; instead, this mutant exhibited abnormal morphology that could be classified into three main groups: zygotes that formed four prespores, but some of which were anucleated (Fig. 6A, Class II); zygotes that formed malformed and/or anucleated prespores (Fig. 6A, Class III); and zygotes that formed only amorphous aggregates of the FSM (Fig. 6A, Class IV). The distribution of these classes in wild-type and *sev4-L5* zygotes is summarized in Fig. 6B. Normal zygotes containing four nucleated prespores were rarely found in *sev4-L5*.

Similar aberrant FSM morphology has been observed in several asporogenous mutants such as *spo3*, *spo14* and *spo20* (Nakase et al., 2001; Nakamura et al., 2001; Nakamura-Kubo et al., 2003), in which the fusion of membrane vesicles is impaired. Cta4 might be required for the vesicle fusion process to extend the FSM. We also noted that the structural modification of the compact SPBs to a crescent shape occurred only incompletely (Fig. 5B). The *S. pombe* calmodulin homologue encoded by

Fig. 4. Kinetics of meiotic nuclear divisions in the *sev4-L5* mutant. Diploid strains homozygous for *sev4⁺* (SYD2) and *sev4-L5* (SYD3) were incubated in EMM+N for 16 hr at 28°C and then shifted to EMM²-N to induce meiosis. To monitor the meiotic nuclear divisions, the number of nuclei per cell was determined after DAPI staining.

Fig. 5. Formation of the FSM in the *sev4-L5* mutant. Homothallic strains of *sev4*⁺ (YN68) and *sev4-L5* (SY60) were cultured on SSA plates for 24 hr and then fixed. GFP-Psy1 signals were observed under a fluorescence microscope. Immunostaining of microtubules (A) and SPBs (B) was performed with anti- α -tubulin and anti-Sad1 antibodies, respectively. Nuclear chromatin regions were stained with DAPI. Bar, 10 μ m.

Fig. 6. Aberrant assembly of the FSM in the *sev4-L5* mutant. Homothallic strains of *sev4*⁺ (YN68) and *sev4-L5* (SY60) were cultured on SSA plates for 2 days at 28°C. (A) Typical morphologies of the FSM and prespores visualized by GFP-Psy1. Cells were also stained with DAPI. Classification of zygotes: Class I, zygotes containing four normal nucleated prespores; Class II, zygotes containing anucleated prespores; Class III, zygotes containing malformed prespores; and Class IV, zygotes containing no prespores (only aggregates). (B) Relative frequency of cell types. Approximately 200 cells were scored for each sample.

cam1⁺ localizes to the SPBs (Moser et. al., 1997). A mis-sense *cam1-F116* mutant is also defective in sporulation (Takeda and Yamamoto, 1987) and shows marked defects in FSM formation (our unpublished data). This finding that the structural alteration of the SPB that normally takes place prior to formation of the FSM occurs only partially in the *sev4-L5* mutant suggests the possibility that disturbances in Ca²⁺ homeostasis may inhibit the function of the SPB in constructing the FSM.

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