CELL STRUCTURE AND FUNCTION 30: 15–24 (2005)

A Fission Yeast SNAP-25 Homologue, SpSec9, Is Essential for Cytokinesis and Sporulation

Taro Nakamura*, Jun Kashiwazaki, and Chikashi Shimoda

Department of Biology, Graduate School of Science, Osaka City University, Sumiyoshi-ku, Osaka 558-8585, Japan

ABSTRACT. **The soluble NSF attachment protein 25 (SNAP-25) is a component of the SNARE complex that is essential for regulated exocytosis in diverse cell types. Here, we identified a fission yeast SNAP-25 homologue, SpSec9. The *sec9*$^+$ gene was essential for vegetative growth. *sec9* mRNA was detected in vegetative cells and further increased during sporulation. This increase during sporulation was dependent on Mei4, a meiosis-specific transcription factor. A sporulation-deficient *sec9* mutant was isolated by random PCR mutagenesis (*sec9-10*). The *sec9-10* mutant also exhibited temperature sensitivity for growth and cell division was found to arrest before completion of cell separation at restrictive temperatures. In *sec9-10* cells, the forespore membrane was normally initiated near spindle pole bodies during meiosis II. However, subsequent extension of the membrane was severely impaired. These results indicate that SpSec9 plays an important role both in cytokinesis and in sporulation.**

Key words: fission yeast/sporulation/cytokinesis/SNARE/SNAP-25

Introduction

Sporulation is the process of gametogenesis in the fission yeast *Schizosaccharomyces pombe*, which involves a regulated program of cell development that includes two overlapping events, meiosis and spore formation. The latter event requires assembly of double-layered intracellular membranes, termed forespore membranes (FSMs), which is equivalent to the prospore membrane in *Saccharomyces cerevisiae* (Yoo *et al*., 1973). During meiosis II, FSM are assembled by the fusion of membrane vesicles. From metaphase II to anaphase II, the spindle pole body (SPB), which plays a crucial role in spindle microtubule formation, undergoes a morphological transformation from a single plaque into a multilayered structure. Membrane vesicles are then transported to the vicinity of the modified SPBs and subsequently fuse there to generate FSMs. As the nucleus divides in meiosis II, the FSM extends, and eventually encapsulates, each of the four nuclei. Assembly of FSMs provides a model system for studying the *de novo* biogenesis of membrane compartments within the cytoplasm (Yoo *et al*., 1973, Tanaka and Hirata, 1982; Hirata and Shimoda, 1992, 1994; Ikemoto *et al*., 2000; Nakase *et al*., 2001; Nakamura *et al.*, 2001; Nakamura-Kubo *et al.*, 2003; Takegawa *et al*., 2003b; Shimoda, 2004).

Recent studies suggest that a general protein secretion apparatus is involved in FSM assembly (Nakase *et al*., 2001, 2004; Nakamura *et al*., 2001; Nakamura-Kubo *et al*., 2003; Koga *et al*., 2004). The *S. pombe* Sec12 homologue, Spo14, is necessary for proper construction of the FSM (d'Enfert *et al*., 1992; Nakamura-Kubo *et al*., 2003). Sec12 is responsible for vesicle transport from the endoplasmic reticulum (ER) to the Golgi apparatus by activating the Sar1 GTPase in *S. cerevisiae* (Nakano *et al*., 1988). This protein is known as a GEF (GTP-GDP exchange factor) for a small GTPase, Sar1 (Nakano and Muramatsu, 1989; Barlowe and Schekman, 1993). *S. pombe* Spo20 protein (SpSpo20) is structurally and functionally related to the major *S. cerevisiae* phosphatidylinositol/phosphatidylcholine-transfer protein Sec14, which is required for vesicle formation from the Golgi apparatus (Bankaitis *et al*., 1989, 1990; Nakase *et al*., 2001). SpSpo20 regulates formation of the FSM, in addition to its known roles in post-Golgi vesicle trafficking (Nakase *et al*., 2001). The soluble NSF attachment protein receptor (SNARE) proteins play a central role both in providing specificity and in catalyzing the fusion of vesicles with the

*To whom correspondence should be addressed: Taro Nakamura, Department of Biology, Graduate School of Science, Osaka City University, Sumiyoshi-ku, Osaka 558-8585, Japan.
Tel: +81–66605–3156, Fax: +81–66605–3158
E-mail: taronaka@sci.osaka-cu.ac.jp

Abbreviations: FSM, forespore membrane; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SNARE, SNAP receptor; SPB, spindle pole body.

target membrane (Protopopov *et al*., 1993; Sollner *et al*., 1993; Rothman, 1994; Rothman and Warren, 1994; Pelham, 1999; Jahn *et al*., 2003). There are at least 20 different SNAREs in a higher eukaryote cell and 17 in an *S. pombe* cell, each associated with a particular membrane-enclosed organelle involved in the secretory pathway (Takegawa *et al*., 2003a). These transmembrane proteins exist as complementary sets of vesicle membrane SNAREs (v-SNAREs), and target membrane SNAREs (t-SNAREs). These SNARE proteins have characteristic helical domains, which are responsible for interactions between a specific pair of v-SNARE and t-SNARE. In the secretion of neurotransmitter, the SNAREs responsible for docking and fusion of synaptic vesicles with the plasma membrane consist of three proteins: synaptobrevin, syntaxin-1 and SNAP-25 (Rothman, 1994). The former two are transmembrame proteins each of which contributes one α-helix to the complex. SNAP-25 is a peripheral membrane protein contributing two α-helices to the four-helix bundle. We isolated the *S. pombe psy1*$^+$ gene encoding a syntaxin 1-like protein as a dose-dependent suppressor of the sporulation-deficient mutant, *spo3*. Psy1 localizes to the plasma membrane during vegetative growth. *psy1*$^+$ is essential for vegetative growth, and its transcription is further enhanced during sporulation. Interestingly, Psy1 disappears from the plasma membrane of the mother cell immediately after the first meiotic division and reappears at the nascent FSM. These results support the idea that the FSM is assembled by the fusion of membrane vesicles, mediated by the SNARE complex (Nakamura *et al*., 2001). In budding yeast, *SEC9* and *SPO20* encode SNAP-25 homologues (Brennwald *et al*., 1994; Neiman, 1998). *S. cerevisiae* Sec9 (ScSec9) and Spo20 (ScSpo20) interact with the syntaxin 1-like protein Sso1/Sso2 (Ssop) and the synaptobrevin-like protein Snc1/Snc2 (Sncp) (Gerst *et al*., 1992; Aalto *et al*., 1993; Protopopov *et al*., 1993). The ScSec9/Ssop/Sncp complex mediates the fusion of exocytic vesicles with the plasma membrane during vegetative growth, whereas ScSpo20/Ssop/Sncp mediates the fusion of the prospore membrane during sporulation (Neiman, 1998).

In this study, we identified a SNAP-25 homologue, SpSec9, whose existence was predicted from the *S. pombe* genome sequence. The *sec9*$^+$ gene is essential for vegetative growth, and its transcription is further enhanced during sporulation. We also isolated the sporulation-deficient mutant, *sec9-10*, in which assembly of the FSM is severely impaired. Our analysis suggests that SpSec9 plays an essential role both in cytokinesis and in sporulation.

Materials and Methods

Yeast strains, media and culture conditions

S. pombe strains used in this study are listed in Table I. Strains were grown in complete medium YEA supplemented with 75 μg/ml adenine sulfate and 50 μg/ml uracil. Malt extract medium MEA and synthetic sporulation medium SSL–N and MM–N were used for mating and sporulation. These media were described by Egel and Egel-Mitani, 1974, Gutz *et al*., 1974, and Moreno *et al*., 1990. *S. pombe* cells were grown at 30°C and sporulated at 28°C except for the *sec9-10* mutant, which was grown and sporulated at 25°C.

Plasmid construction

pREP1(ade6) (Tamai *et al*., unpublished) was digested with *Pst*I, filled in, and then ligated with an *Apa*I linker, yielding pL-A. Plasmid pL-A(syb1) was constructed as follows. The two oligonucleotides 5'-CCCCTCGAG(*Xho*I)TGAACCTTTCGCAAGGGATTC-3' and 5'-CCCGCGGCCGC(*Not*I)AGGGGAGCAAATATACTAC-3' were used to amplify the *syb1*$^+$ gene. The corresponding PCR product was digested with *Xho*I and *Not*I, and then subcloned into the corresponding sites in pAL-KS (Tanaka *et al*., 2000), yielding pAL(syb1). The *Apa*I-*Sac*I fragment of pAL(syb1) was inserted into the corresponding sites in pL-A, yielding pL-A(syb1). Plas-

Table I. STRAINS USED IN THIS STUDY

Strain	Genotype	Source
TN4	h$^-$ *leu1-32*	This study
TN52	h^{90} *ade6-M216*	Nakamura-Kubo *et al*., 2003
TN75	h^{90}/h^{90} *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*	This study
TN363	h^{90}/h^{90} *sec9::ura4/sec9*$^+$ *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*	This study
TN396	h^{90} *ade6<<GFP-Psy1 leu1<<sec9-10 sec9::ura4 ura4-D18*	This study
TN402	h^{90} *ade6<<GFP-Psy1*	This study
TN9SP10	h^{90} *leu1<<sec9-10 sec9::ura4 ade6-M210 ura4-D18*	This study
MKW5	h^{90}	Nakamura-Kubo *et al.*, 2003
MM59-4D	h^{90} *ade6-M210*	M. Morishita
JZ670	h$^-$/h$^-$ *pat1-114/pat1-114 ade6-M210/ade6-M216 leu1-32/leu1-32*	M. Yamamoto
AB4	h$^-$/h$^-$ *mei4::ura4*$^+$*/mei4::ura4*$^+$ *pat1-114/pat1-114 ade6-M210/ade6-M216 leu1-32/leu1-32*	Abe and Shimoda, 2000

mid pL-A(psy1) was constructed as follows. The two oligonucleotides 5'-CCCGTCGAC(*Sal*I)AATGAATAAAGCAAACGAT-3' and 5'-CCCGAGCTC(*Sac*I)ATCTAACCGGCCATATCACT-3' were used to amplify the *psy1*$^+$ gene. The corresponding PCR product was digested with *Sal*I and *Sac*I, and then subcloned into the corresponding sites in pAL-KS, yielding pAL(psy1). The *Apa*I-*Sac*I fragment of pAL(psy1) was inserted into the corresponding sites in pL-A, yielding pL-A(psy1). Plasmid pL-A(sec9) was constructed as follows. The two oligonucleotides 5'-CCCCTCGAG (*Xho*I)TAGTCCATCGGAAAGACAGAA-3' and 5'-CCCGAGCTC (*Sac*I)TAGCGGCATCAAAGCTGCTCA-3' were used to amplify the *sec9*$^+$ gene. The corresponding PCR product was digested with *Xho*I and *Sac*I, and then subcloned into the corresponding sites in pAL-KS, yielding pAL(sec9). The *Apa*I-*Sac*I fragment of pAL(sec9) was inserted into the corresponding sites in pL-A, yielding pL-A(sec9).

Gene disruption of sec9

The two oligonucleotides 5'-CCCCTCGAG(*Xho*I)TAGTCCATCGGAAAGACAGAA-3' and 5'-AGTGTGCGGCCGC(*Not*I)CTCGTAGTTTTGGTTAGCTAT-3' were used to amplify the *sec9*$^+$ gene by PCR. The PCR product was digested with *Xho*I and *Not*I, and then ligated into the same sites in pBluescript II (STRATAGENE, La Jolla, CA, USA), yielding pBS(sec9). A 1.8-kb *ura4*$^+$ fragment (Grimm *et al*., 1988) was then inserted into the internal *Pst*I site, yielding pBS(*sec9::ura4*$^+$). A 4.8-Kb *Nsp*V-*Cfr*10I fragment containing the interrupted *sec9* allele (*sec9::ura4*$^+$) was used to transform strain TN75. The disruption was confirmed by Southern hybridization (data not shown).

In vitro mutagenesis of sec9 by PCR

PCR was used to introduce a random point mutation into the *sec9*$^+$ gene (one cycle at 95°C for 3 min followed by 30 cycles at 95°C for 30 sec, 50°C for 30 sec, and 72°C for 3 min with *Taq* polymerase). The forward and reverse primers were 5'-ACACACACAGTCGAC(*Sal*I)TAGTCCATCGGAAAGACAGAAAAATG-3' and 5'-CCACCAACAGAGCTC(*Sac*I)CTCGTAGTTTTGGTTAGCTATGAG-3', respectively. The amplified DNA fragment contained the promoter and terminator regions, in addition to the *sec9*$^+$ ORF. The *S. pombe* genomic library pTN-L1 (Nakamura *et al*., 2001) was included in the reaction mixture as a template. The amplified fragment was digested with *Sal*I and *Sac*I and cloned into pBR(leu1) (Nakamura-Kubo *et al*., 2003). The resulting library was digested at the *Sna*BI site within the *leu1*$^+$ gene and then integrated at the *leu1* locus of a heterozygous diploid carrying a *sec9::ura4*$^+$ allele (TN363). Colonies of transformants on SSA plates were treated with ethanol to kill non-sporulating vegetative cells and were then spread again on SSA sporulation plates, which were then exposed to iodine vapor (Gutz *et al*., 1974). Iodine-negative (white) colonies were selected and inspected for zygotic ascus formation.

Immunofluorescence microscopy

Cells were fixed according to the procedure of Hagan and Hyams (1988) using glutaraldehyde and paraformaldehyde. For microtubule staining, the anti-α-tubulin antibody TAT-1 (Woods *et al.*, 1989) was used with Cy3-conjugated anti-mouse IgG (Sigma, St Louis, MO, USA) at a 1:1000 dilution. The nuclear chromatin region was stained with DAPI (4',6-diamidino-2-phenylindole) at 1 μg/ml. Stained cells were observed under a fluorescence microscope (model BX51; Olympus, Tokyo) and images were captured using a Cool SNAP CCD camera (Roper Scientific, San Diego, CA, USA).

Results

Identification of SNAP-25 homologue in fission yeast

The SNAP-25-like protein (SPBC26H8.02C), designated *sec9*$^+$, was found by means of a conventional BLAST search of an *S. pombe* genome database (The Sanger Institute, UK) for *S. cerevisiae* Sec9. *sec9*$^+$ encodes a 46.2-kDa protein containing 419 amino acids. Budding yeast has duplicated genes, *SEC9* and *SPO20*. ScSpo20 is dispensable for growth but essential for formation of prospore membranes. In contrast, ScSec9 is essential for vegetative growth (Brennwald *et al*., 1994; Neiman, 1998). SpSec9 is the sole SNAP-25-like protein in *S. pombe*. The N-terminal region of SpSec9 does not share similarity to homologues from other organisms. However, over the C-terminal 228 residues, the SpSec9 protein shares 41, 28, 26, and 23% identity and 61, 48, 41, and 41% similarity with the budding yeast ScSec9, ScSpo20, zebrafish SNAP-25A, and human SNAP-25 proteins, respectively (Fig. 1). In neurons, the SNARE complex is composed of a bundle of four helices with synaptobrevin and syntaxin 1 contributing one helix each and SNAP-25 contributing two. SpSec9 is predicted to have two α-helical regions, which are highly conserved among SNAP-25 proteins. These structural features strongly suggest that SpSec9 functions as a plasma membrane t-SNARE component.

SpSec9 is essential for cell viability

To explore the consequences of complete loss of *sec9*$^+$ function, a plasmid was constructed in which the *ura4*$^+$ cassette was inserted at the *Pst*I site within the *sec9*$^+$ ORF. After transformation of the *S. pombe* diploid TN75 with a linear DNA fragment containing the *sec9* disruption allele, Ura$^+$ transformants were obtained. Tetrad analysis indicated that every ascus consisted of two viable and two inviable spores, and that all viable spores were phenotypically Ura$^-$. Microscopic observation of nonviable meiotic progeny showed that these spores could not germinate (data not shown). Therefore, *sec9*$^+$ is essential for vegetative cell growth and spore germination.

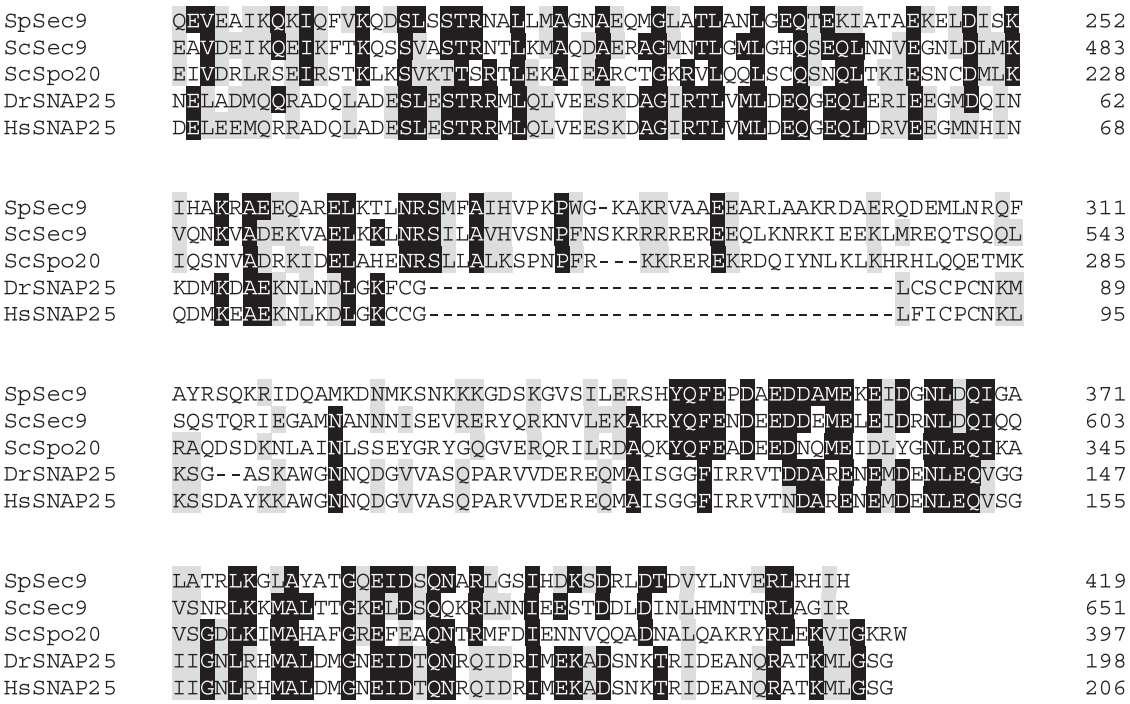

Fig. 1. Primary structure of the fission yeast SpSec9 protein. Comparison of the amino acid sequence of the conserved domains among SpSec9, SNAP-25 homologues from *S. cerevisiae* (ScSec9 and ScSpo20), *Danio rerio* (DrSNAP25), and *Homo sapiens* (HsSNAP25). Identical amino acids are shown in white against black. Similar amino acids are shaded.

Transcriptional regulation of sec9^{+}

Log-phase cells of a homothallic haploid strain (MKW5) were incubated in the sporulation medium MM–N, and the *sec9* mRNA abundance was monitored by Northern analysis. mRNA was detected in vegetative cells and was found to increase during sporulation (data not shown). To determine exactly when the rise in *sec9* mRNA level occurred, a similar Northern analysis was carried out using a *pat1-114* mutant. Meiosis was found to proceed in a synchronous fashion (Iino *et al*., 1995). The level of *sec9* mRNA began to increase about 6 hr after induction and peaked at about 9 hr, when cells were in early meiosis II (Fig. 2A, 2B).

The *mei4*$^{+}$ gene encodes a forkhead transcription factor that regulates an array of genes required for meiosis and sporulation (Horie *et al.*, 1998; Abe and Shimoda, 2000; Nakamura *et al*., 2001; Watanabe *et al*., 2001; Nakamura *et al*., 2002). To determine whether the elevation in *sec9* mRNA during sporulation is dependent on Mei4, the *sec9* mRNA level was measured in a *mei4*Δ mutant. As shown in Fig. 2A, accumulation of *sec9* mRNA was completely abolished in the *mei4*Δ mutant. Furthermore, ectopic overexpression of *mei4*$^{+}$ was found to induce *sec9*$^{+}$ mRNA in vegetative cells (Fig. 2C). *sec9*$^{+}$ has a consensus recognition sequence for Mei4, GTAAAYA (Horie *et al*., 1998) in the 5' upstream region. We conclude that transcription of *sec9*$^{+}$ during meiosis is strictly regulated by Mei4.

Isolation of sporulation-deficient sec9 mutants

To determine whether SpSec9 is involved in sporulation, an attempt was made to isolate sporulation-deficient mutant by random PCR mutagenesis (Materials and Methods). A single sporulation-deficient mutant, *sec9-10* was identified. *sec9-10* cells failed to develop spores but completed normal meiotic nuclear divisions (data not shown).

In addition to causing a defect in ascospore formation, the *sec9-10* mutation compromised vegetative growth. As shown in Fig. 3B, the *sec9-10* mutant grew well at 25°C but was unable to form colonies at 37°C. Thus, the *sec9-10* mutation confers temperature sensitivity for growth. It is known that mutations in several genes involved in membrane trafficking cause defects in cytokinesis (Nakase *et al*., 2001; Poloni and Simanis, 2002; Wang *et al*., 2002; Cheng *et al*., 2002; Edamatsu and Toyoshima, 2003). For example, the *sec8*$^{+}$ gene, which encodes a component of the exocyst complex, is essential for cell separation (Wang *et al*., 2002). Therefore, the cell morphology of *sec9-10* mutants incubated at permissive and restrictive temperatures was examined. *sec9-10* mutants were indistinguishable from wild type cells with respect to septum formation when incubated at 25°C (Fig. 3C). In marked contrast, *sec9-10* cells exhibited a rather uniform arrest morphology at the restrictive temperature (Fig. 3C). At 12 hr after the shift to 34°C, approximately 43% of the *sec9-10* cells had a single sep-

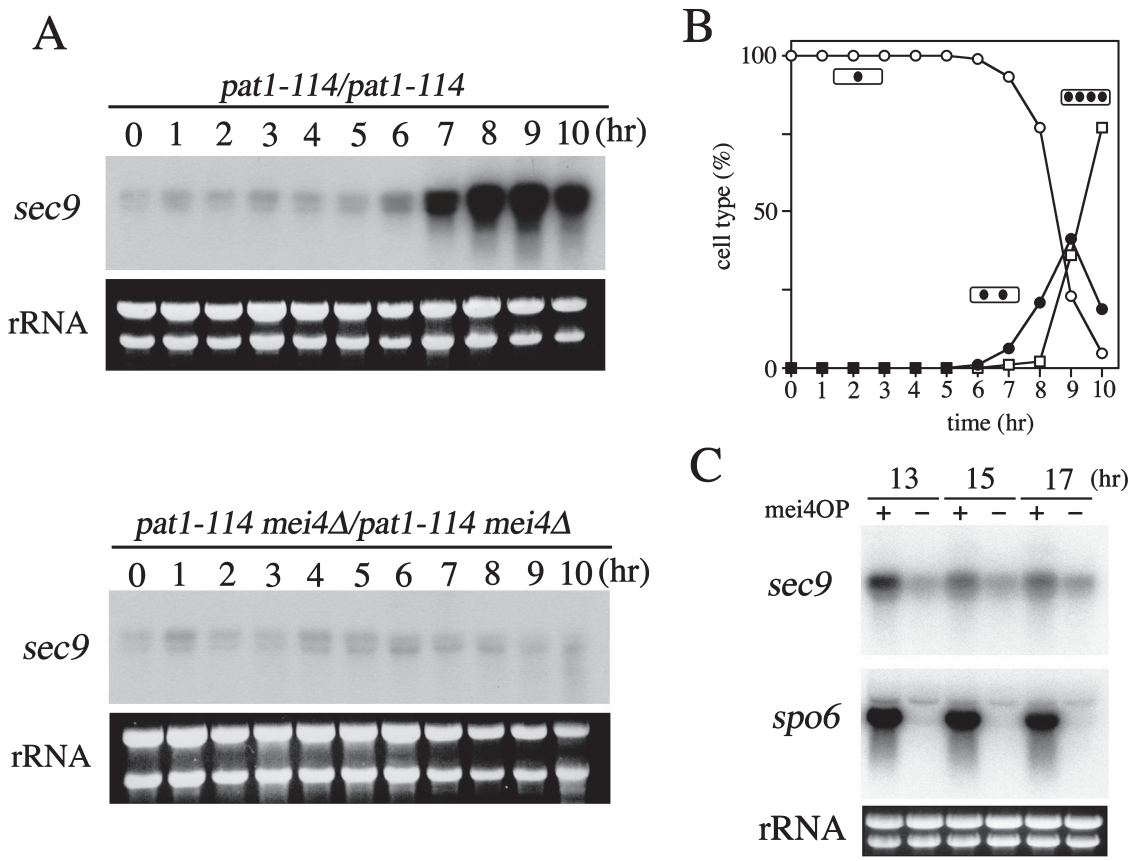

Fig. 2. Expression of *sec9*$^+$ during meiosis. (A) Transcription of *sec9*$^+$ in *pat1*-driven meiosis. Meiosis in the diploid strains JZ670 (*mei4*$^+$) and AB4 (*mei4*Δ), homozygous for *pat1-114*, was synchronously induced by a temperature shift-up (Iino *et al.*, 1995). At intervals, total RNA was prepared from *S. pombe* cultures (Jensen *et al*., 1983) and fractionated on a 1.0% gel containing 3.7% formaldehyde as previously described (Thomas, 1980). The approximate quantity of RNA was assessed by staining with ethidium bromide. (B) Meiotic nuclear division of JZ670 was monitored by DAPI staining. Open circles, mononucleate; closed circles, binucleate; squares, tetranucleate cells. (C) Transcription of *sec9*$^+$ is induced by ectopic overproduction of Mei4 in vegetative cells. TN4 cells harboring plasmid pREP1(*mei4*$^+$) were grown in MM medium without thiamine (Mei4 OP, +) or MM with thiamine (Mei4OP, –) (Maundrell., 1993). After an incubation of 13, 15 and 17 hr, total RNA was subjected to Northern analysis. A Mei4-dependent gene *spo6*$^+$ is included as a positive control.

tum, and 4% exhibited multiple septa (Table II). In wild-type cells, only 13% of the cells had a single septum (Table II). These results indicate that SpSec9 plays an important role in cytokinesis, especially in cell separation.

We next determined the mutation point of the *sec9-10* allele. The mutant gene was isolated from genomic DNA by PCR. Nucleotide sequencing demonstrated that *sec9-10* is the result of a single nucleotide change (T to C) that results in replacement of leucine 228 with proline in the conserved N-terminal α-helical region.

Table II. CELL MORPHOLOGY OF WILD TYPE AND *sec9-10* STRAINS

Number of septa per cell	% Frequency (mean±SD)			
	wild type		*sec9-10*	
	25°C	34°C	25°C	34°C
0	81.1±2.4	87.3±1.7	87.5±0.45	52.8±2.1
1	18.8±2.5	12.7±1.7	12.5±0.45	42.7±2.1
2	0.1±0.1	0	0	4.3±0.22
3	0	0	0	0.19±0.09

Values are mean±SD for three independent experiments as for Figure 3C.

The sec9-10 mutant is defective in forespore membrane formation

To examine in detail how the *sec9-10* mutation impairs sporulation, the assembly of FSMs in the *sec9-10* mutant was analyzed, using GFP-tagged Psy1, a syntaxin 1-like protein (Nakamura *et al*., 2001). Overexpression of GFP-Psy1 did not overcome the sporulation defect in *sec9-10* cells. Progression of meiosis was monitored by observing the construction and elongation of spindle microtubules. In wild type cells, most haploid nuclei produced by meiotic second divisions were encapsulated by the FSM (Fig. 4A). In *sec9-10* mutant cells, FSMs initiated normally at both

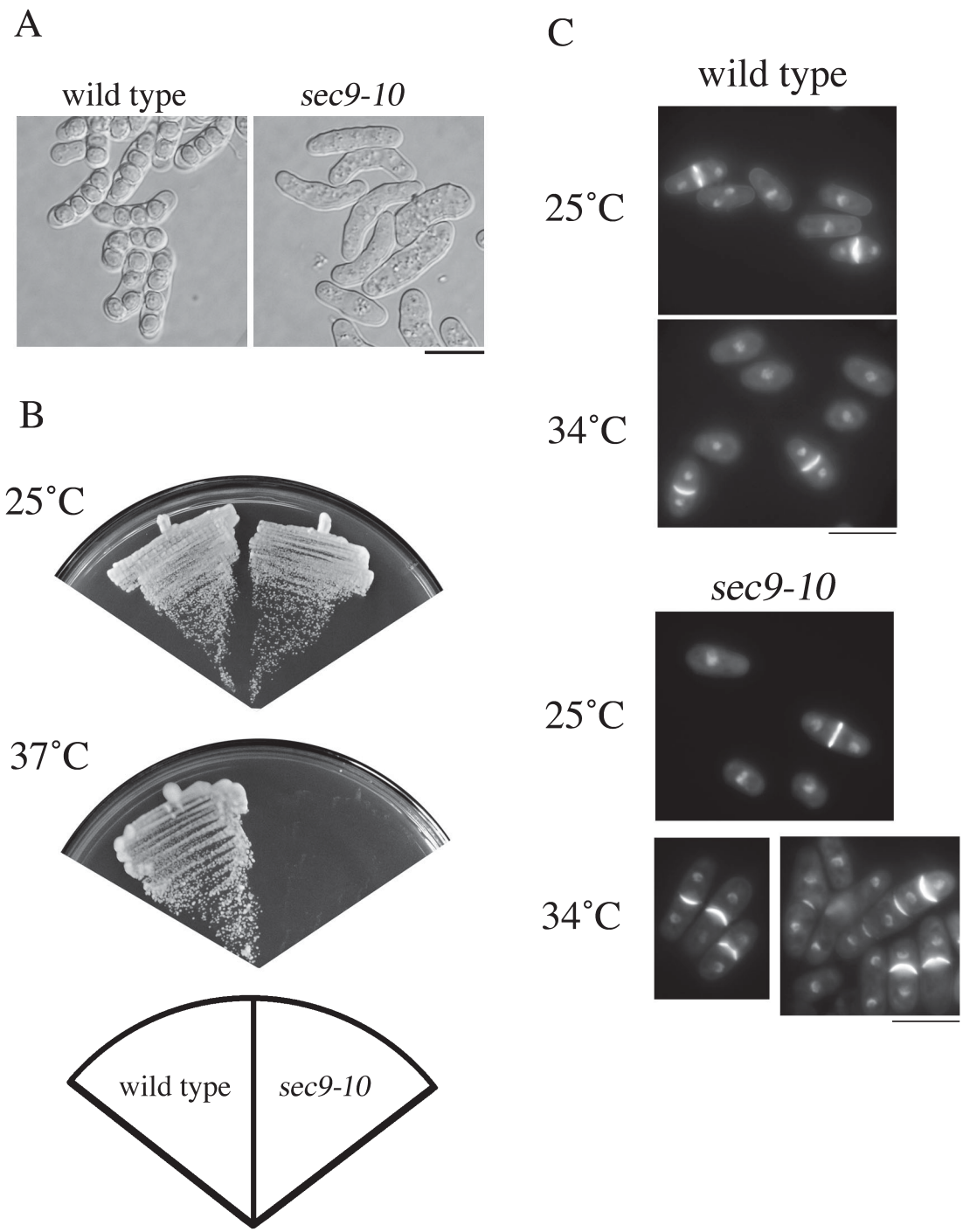

Fig. 3. Phenotypes of the *sec9-10* mutant. (A) MM59-4D (wild type) and TN9SP10 (*sec9-10*) were sporulated on SSA medium for 2 days at 25°C. Bar, 10 μm. (B) Strains MM59-4D (wild type) and TN9SP10 (*sec9-10*) were streaked on complete medium (YEA) and incubated at 25°C or 37°C for 3 days. (C) MM59-4D (wild type) and TN9SP10 (*sec9-10*) were incubated in liquid complete medium (YEL) for 12 hr at 25°C or 34°C. Cells were fixed and stained with calcofluor and DAPI. Bars, 10 μm.

poles of the meiosis II spindles (Fig. 4A), but extension of the FSMs was soon blocked, resulting in anucleated small prespores (Fig. 4B). These results indicated that the FSM initiated normally, but its subsequent development was abnormal. In conclusion, SpSec9 appears to be required for the normal construction of the FSM.

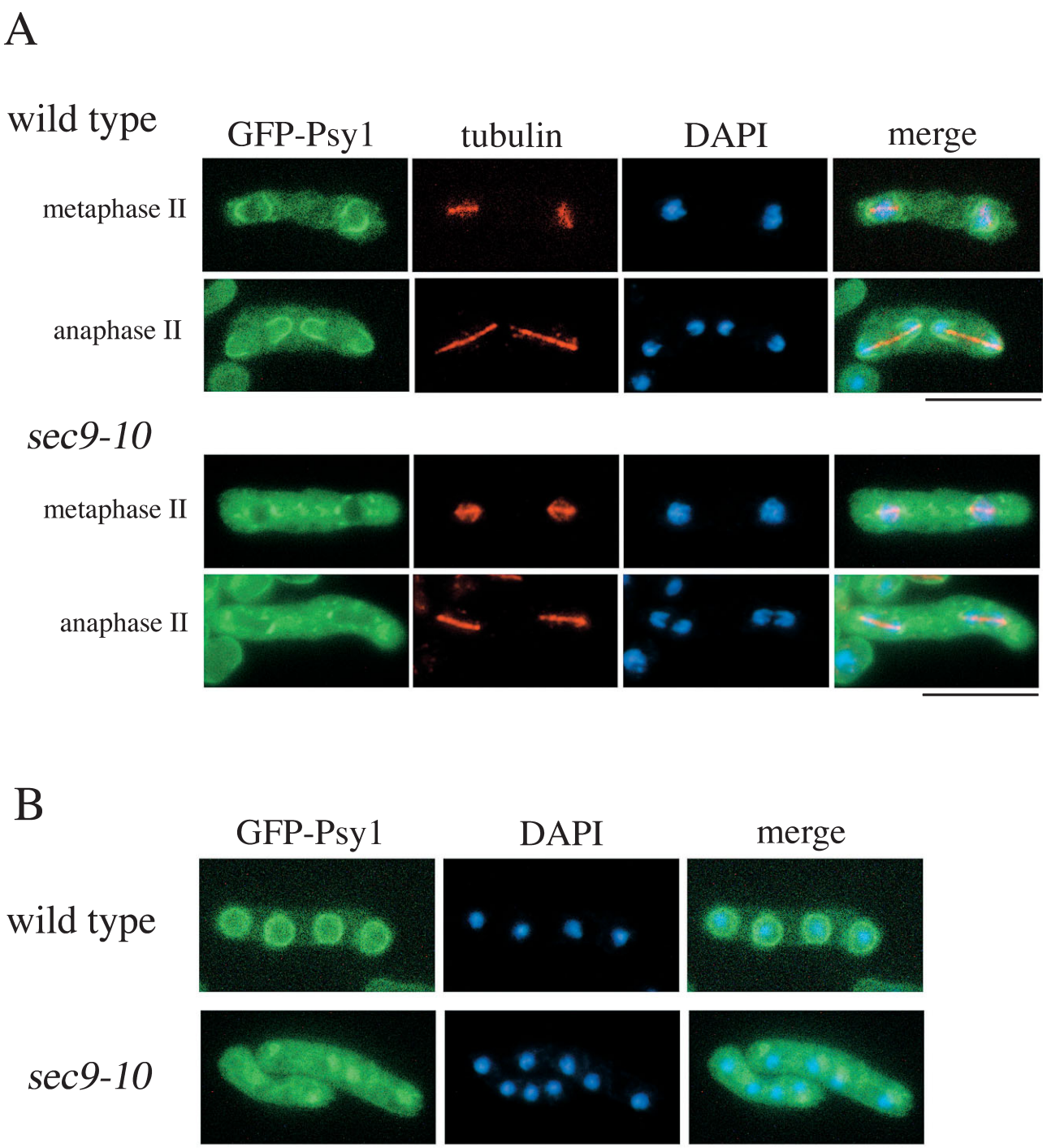

Fig. 4. Aberrant assembly of FSMs in *sec9-10*. Assembly of the FSM during metaphase II and anaphase II (A), and in post-meiosis (B). TN402 (wild type) and TN396 (*sec9-10*) were cultured on SSA medium for 1 day. Fixed cells were doubly stained with the anti-α-tubulin and DAPI. Bars, 10 μm.

sec9^{+} *interacts with syntaxin1 homologue, psy1*$^{+}$

In higher eukaryotes, SNAP-25 forms a complex with syntaxin-1 and synaptobrevin, and this interaction is important for specific membrane fusion. *S. pombe* has a synaptobrevin homologue, Syb1, which is involved in cytokinesis and cell elongation (Edamatsu and Toyoshima, 2003). To examine whether overproduction of Syb1 and Psy1 suppresses the

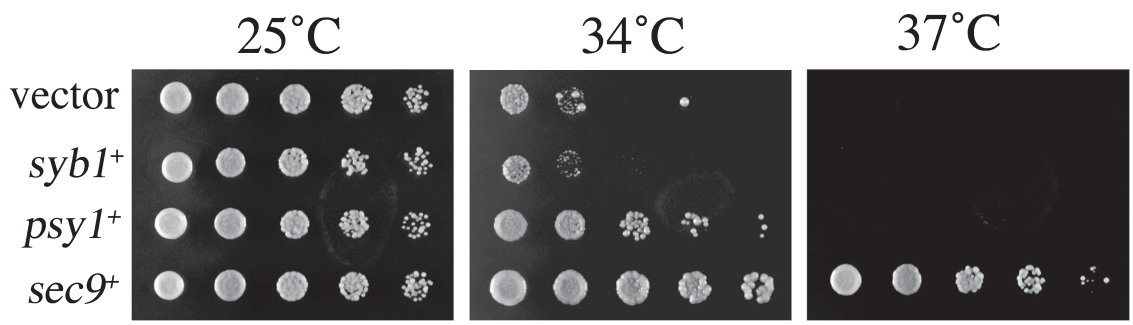

Fig. 5. Suppression of temperature sensitivity of *sec9-10* by *psy1*$^+$. TN9SP10 (*sec9-10*) cells transformed with empty vector (pL-A), pL-A(syb1), pL-A(psy1), or pL-A(sec9) were serially diluted and plated onto YEA medium. Photographs were taken after 2 days of incubation at 25, 34, and 37°C.

sec9-10 mutation in *S. pombe*, a multicopy plasmid harboring either *syb1*$^+$ or *psy1*$^+$ was introduced into *sec9-10* strains. Psy1 was found to suppress *sec9-10* well at 34°C but poorly at 37°C. In contrast, Syb1 could not suppress *sec9-10* even at 34°C (Fig. 5). These data indicate that *sec9*$^+$ interacts genetically with the syntaxin1 homologue, *psy1*$^+$. With respect to sporulation, overexpression of both genes was tested and found not to suppress the sporulation defect of the *sec9-10* mutant.

Discussion

This is the first report indicating that the *S. pombe* SNAP-25 homologue, SpSec9, plays essential roles in cell separation, a final step of cytokinesis, and in assembly of the FSM, an important step in sporulation. While SpSec9 is the sole SNAP-25 homologue in *S. pombe*, two SNAP-25 homologues exist in *S. cerevisiae*, ScSec9 and ScSpo20. Interestingly, formation of the prospore membrane in budding yeast does not require one of the SNAP-25 paralogs, ScSec9, while its sporulation-specific counterpart, ScSpo20, is indispensable. These findings imply that precursor vesicles for spore membranes are provided through a general secretory pathway and that sporulation-specific components are substituted in the case of the SNAP-25 homologue (Neiman, 1998). Recent studies have shown that ScSpo20 is subject to both positive and negative regulation by its amino terminal domain. A short, amphipathic helix in the N-terminal region, which binds to acidic phospholipids such as phosphatidic acids, is essential for proper localization of ScSpo20 to the prospore membrane. Thus, the N-terminal region appears to be an important determinant for meiosis-specific function of Spo20 (Neiman *et al*., 2000; Nakanishi *et al*., 2004). However, an analogous amphipathic helix was not found in the N-terminal region of *S. pombe* Sec9. The mechanism by which the SNAP-25 homologue regulates FSM formation might be quite different between the two yeasts.

The *sec9-10* mutant is temperature-sensitive for growth yet exhibits a sporulation-defective phenotype at temperatures permissive for proliferation. Perhaps SpSec9 plays a functional role in sporulating cells but not in vegetative cells at these permissive temperatures. Alternatively, the threshold activity level of SpSec9 might be required for sporulation is higher than that required for vegetative growth. We presently favor the latter model because the *sec9* mRNA level increased dramatically during sporulation. Other sporulation mutants such as *spo20-KC104* and *spo20-H6* also show a sporulation-defective phenotype at temperatures permissive for proliferation, supporting this notion. *sec9-10* was also defective in cell separation at a restrictive temperature. In cell poles and the medial septation sites, membranes are actively remodeled by both biosynthetic and degradative mechanisms. These regions therefore may require the robust recruitment of membrane vesicles carrying secretory and membrane-associated proteins for cell surface growth and septation. Indeed, proteins required for septum formation and cell separation localize in the medial region of the cell as ring-like structures. Additionally, recent studies have revealed that a mutation in several genes involved in the membrane trafficking (Sec8, Sec10, Sec13, SpSpo20, Syb1, and Ypt3) causes a cytokinesis defect (Nakase *et al*., 2001; Poloni and Simanis, 2002; Wang *et al*., 2002; Cheng *et al*., 2002; Edamatsu and Toyoshima. 2003). Therefore, it is possible that SpSec9 might play a role in the transport of secretory proteins to these growing sites.

In *S. cerevisiae*, ScSec9 localizes to the plasma membrane during vegetative growth and both ScSec9 and ScSpo20 localize to the prospore membrane during sporulation (Brennwald *et al*., 1994; Neiman *et al*., 2000). We tried to observe the localization of SpSec9 using GFP-tagged SpSec9. However, GFP-SpSec9 was not fully functional and showed no characteristic localization.

The *sec9-10* mutation site is positioned in one of the two conserved α-helices that are essential for formation of the SNARE complex. Therefore this mutation may reduce the affinity for the other t-SNARE, Psy1. Overexpression of Psy1 suppressed the temperature sensitivity, but not sporulation defect of *sec9-10*. These data suggest that the phenotype of *sec9-10* could be due to *in vivo* instability of the

SpSec9-Psy1 complex. The process of sporulation might require a more stable SpSec9-Psy1 complex than vegetative growth. Alternatively, the Sec9-10 mutant protein might be more labile than wild type Sec9. Indeed, *cam1-F116* mutation, which reduces the abundance of the Cam1 protein level both in vegetative growth and in sporulation, exhibits sporulation-specific phenotype (Takeda *et al*., 1989).

The fluorescence microscopic analysis showed that, in *sec9-10* mutants, FSM formation initiated normally near the SPB during meiosis II, but subsequent development into closed membrane compartments containing a nucleus, called prespores, was severely impaired. The sporulation-specific mutants, *spo3* and *spo14*, exhibit similar phenotypes. Compared to these mutants, the FSM in the *sec9-10* mutant arrested at an earlier stage of membrane formation. These data suggest that SpSec9 functions at a very early stage of FSM extension. This is consistent with the fact that the SNARE complex plays a central role in membrane vesicle fusion.

In summary, we have shown that a fission yeast SNAP-25 homologue, SpSec9, functions in completion of cell septation in vegetative cells and also plays a crucial role in sporulation, especially in biogenesis of the FSM. The present study together with our previous work demonstrate that spore formation accompanies a dynamic membrane fusion process which occurs under the strict control of SNARE proteins. Further work will be needed to understand how these SNARE proteins function at the molecular level during sporulation.

Acknowledgments. We thank Dr. K. Gull of the University of Manchester for the anti-α-tubulin antibody, TAT-1, Dr. M. Yamamoto of the University of Tokyo for strains, and Ms. Y. Maeda of Osaka City University for plasmids. The present study was supported in part by Grants-in-Aid for Scientific Research "B" (14380338) and Priority Areas "Genome Biology" (15013249) from the Ministry of Education, Science, Sports and Culture of Japan to C. S. and Grants-in-Aid for Scientific Research on Priority Area 'Cell Cycle Control' (16026240) and 'Life of Proteins' (14037263) from the Ministry of Education, Science, Sports and Culture of Japan to T. N. A few strains used in this study were provided by the Yeast Genetic Resource Center Japan (http://bio3.tokyo.jst.go.jp/jst/).

References

Aalto, M.K., Ronne, H., and Keranen, S. 1993. Yeast syntaxins Sso1p and Sso2p belong to a family of related membrane proteins that function in vesicular transport. *EMBO J.*, **12**: 4095–4104.

Abe, H. and Shimoda, C. 2000. Autoregulated expression of *Schizosaccharomyces pombe* meiosis-specific transcription factor Mei4 and a genome-wide search for its target genes. *Genetics*, **154**: 1497–1508.

Bankaitis, V.A., Malehorn, D.E., Emr, S.D., and Greene, R. 1989. The *S. cerevisiae SEC14* gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex. *J. Cell Biol.*, **108**: 1271–1281.

Bankaitis, V.A., Aitken, J.R., Cleves, A.E., and Dowhan, W. 1990. An essential role for a phospholipid transfer protein in yeast Golgi function. *Nature*, **347**: 561–562.

Barlowe, C. and Schekman, R. 1993. *SEC12* encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. *Nature*, **365**: 347–349.

Brennwald, P., Kearns, B., Champion, K., Keranen, S., Bankaitis, V., and Novick, P. 1994. Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. *Cell*, **79**: 245–258.

Cheng, H., Sugiura, R., Wu, W., Fujita, M., Lu, Y., Sio, S.O., Kawai, R., Takegawa, K., Shuntoh, H., and Kuno, T. 2002. Role of the Rab GTP-binding protein Ypt3 in the fission yeast exocytic pathway and its connection to calcineurin function. *Mol. Biol.*, **13**: 2963–2976.

d'Enfert, C., Gensse, M., and Gaillardin, C. 1992. Fission yeast and a plant have functional homologues of the Sar1 and Sec12 proteins involved in ER to Golgi traffic in budding yeast. *EMBO J.*, **11**: 4205–4211.

Edamatsu, M. and Toyoshima, Y.Y. 2003. Fission yeast synaptobrevin is involved in cytokinesis and cell elongation. *Biochem. Biophys. Res. Commun.*, **301**: 641–645.

Egel, R. and Egel-Mitani, M. 1974. Premeiotic DNA synthesis in fission yeast. *Exp. Cell Res.*, **88**: 127–134.

Gerst, J.E., Rodgers, L., Riggs, M., and Wigler, M. 1992. *SNC1*, a yeast homolog of the synaptic vesicle-associated membrane protein/synaptobrevin gene family: genetic interactions with the RAS and CAP genes. *Proc. Natl. Acad. Sci. USA.*, **89**: 4338–4342.

Grimm, C., Kohli, J., Murray, J., and Maundrell, K. 1988. Genetic engineering of *Schizosaccharomyces pombe*: a system for gene disruption and replacement using the *ura4* gene as a selectable marker. *Mol. Gen. Genet.*, **215**: 81–86.

Gutz, H., Heslot, H., Leupold, U., and Loprieno, N. 1974. *Schizosaccharomyces pombe*. In *Handbook of Genetics,* vol. 1, ed. R. C. King, New York, Plenum Press, 395–446.

Hagan, I.M. and Hyams, J.S. 1988. The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.*, **89**: 343–357.

Hirata, A. and Shimoda, C. 1992. Electron microscopic examination of sporulation-deficient mutants of the fission yeast *Schizosaccharomyces pombe*. *Arch. Microbiol.*, **158**: 249–255.

Hirata, A. and Shimoda, C. 1994. Structural modification of spindle pole bodies during meiosis II is essential for the normal formation of ascospores in *Schizosaccharomyces pombe*: Ultrastructural analysis of *spo* mutants. *Yeast*, **10**: 173–183.

Horie, S., Watanabe, Y., Tanaka, K., Nishiwaki, S., Fujioka, H., Abe, H., Yamamoto, M., and Shimoda, C. 1998. The *Schizosaccharomyces pombe mei4*$^{+}$ gene encodes a meiosis-specific transcription factor containing a forkhead DNA-binding domain. *Mol. Cell Biol.*, **18**: 2118–2129.

Iino, Y., Hiramine, Y., and Yamamoto, M. 1995. The role of *cdc2* and other genes in meiosis in *Schizosaccharomyces pombe*. *Genetics*, **140**: 1235–1245.

Ikemoto, S., Nakamura, T., Kubo, M., and Shimoda, C. 2000. *S. pombe* sporulation-specific coiled-coil protein Spo15 is localized to the spindle pole body and essential for its modification. *J. Cell Sci.*, **113**: 545–554.

Jahn, R., Lang, T., and Sudhof, T.C. 2003. Membrane fusion. *Cell*, **112**: 519–533.

Jensen, R., Sprague, G.F. Jr., and Herskowitz, I. 1983. Regulation of yeast mating-type interconversion: feedback control of HO gene expression by the mating-type locus. *Proc. Natl. Acad. Sci. USA.*, **80**: 3035–3039.

Koga, T., Onishi, M., Nakamura, Y., Hirata, A., Nakamura, T., Shimoda, C., Iwaki, T., Takegawa, K., and Fukui, Y. 2004. Sorting nexin homologues are targets of phosphatidylinositol 3-phosphate in sporulation of *Schizosaccharomyces pombe*. *Genes Cells*, **9**: 561–574.

Maundrell, K. 1993. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene.*, **123**: 127–130.

Moreno, S., Klar, A., and Nurse, P. 1990. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.*, **194**: 793–823.

Nakamura, T., Nakamura-Kubo, M., Hirata, A., and Shimoda, C. 2001. The *Schizosaccharomyces pombe spo3*$^{+}$ gene is required for assembly of the forespore membrane and genetically interacts with *psy1*$^{+}$ encoding syntaxin-like protein. *Mol. Biol. Cell*, **12**: 3955–3972.

Nakamura, T., Nakamura-Kubo, M., Nakamura, T., and Shimoda, C. 2002. A novel fission yeast Cdc7-Dbf4-like kinase complex required for the initiation and progression of meiotic second division. *Mol. Cell Biol.*, **22**: 309–320.

Nakamura-Kubo, M., Nakamura, T., Hirata, A., and Shimoda C. 2003. The fission yeast *spo14*$^{+}$ gene encoding a functional homologue of budding yeast Sec12 is required for the development of forespore membranes. *Mol. Biol. Cell*, **14**: 1109–1124.

Nakanishi, H., de los Santos, P., and Neiman, A.M. 2004. Positive and negative regulation of a SNARE protein by control of intracellular localization. *Mol. Biol. Cell*, **15**: 1802–1815.

Nakano, A., Brada, D., and Schekman, R. 1988. A membrane glycoprotein, Sec12p, required for protein transport from the endoplasmic reticulum to the Golgi apparatus in yeast. *J. Cell Biol.*, **107**: 851–863.

Nakano, A. and Muramatsu, M. 1989. A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Biol.*, **109**: 2677–2691.

Nakase, Y., Nakamura, T., Hirata, A., Routt, S.M., Skinner, H.B., Bankaitis, V.A., and Shimoda, C. 2001. *Schizosaccharomyces pombe spo20*$^{+}$ gene encoding a homologue of *Saccharomyces cerevisiae* Sec14 plays an important role in forespore membrane formation. *Mol. Biol. Cell*, **12**: 901–917.

Nakase, Y., Nakamura, T., Okazaki, K., Hirata, A., and Shimoda, C. 2004. The Sec14 family glycerophospholipid-transfer protein is required for structural integrity of the spindle pole body during meiosis in fission yeast. *Genes Cells*, **9**: 1275–1286.

Neiman, A.M. 1998. Prospore membrane formation defines a developmentally regulated branch of the secretory pathway in yeast. *J. Cell Biol.*, **140**: 29–37.

Neiman, A.M., Katz, L., and Brennwald, P.J. 2000. Identification of domains required for developmentally regulated SNARE function in *Saccharomyces cerevisiae*. *Genetics*, **155**: 1643–1655.

Pelham, H.R. 1999. SNAREs and the secretory pathway-lessons from yeast. *Exp. Cell Res.*, **247**: 1–8.

Poloni, D. and Simanis, V. 2002. A DMSO-sensitive conditional mutant of the fission yeast orthologue of the *Saccharomyces cerevisiae SEC13* gene is defective in septation. *FEBS Lett.*, **511**: 85–89.

Protopopov, V., Govindan, B., Novick, P., and Gerst, J.E. 1993. Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in *S. cerevisiae*. *Cell*, **74**: 855–861.

Rothman, J.E. 1994. Mechanisms of intracellular protein transport. *Nature*, **372**: 55–63.

Rothman, J.E. and Warren, G. 1994. Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Curr. Biol.*, **4**: 220–233.

Shimoda, C. 2004. Forespore membrane assembly in yeast: coordinating SPBs and membrane trafficking. *J. Cell Sci.*, **117**: 389–396.

Sollner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., and Tempst, P., and Rothman, J.E. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature*, **362**: 318–324.

Takeda, T., Imai, Y., and Yamamoto, M. 1989. Substitution at position 116 of *Schizosaccharomyces pombe* calmodulin decreases its stability under nitrogen starvation and results in a sporulation-deficient phenotype. *Proc. Natl. Acad. Sci. USA.*, **86**: 9737–9741.

Takegawa, K., Hosomi, A., Iwaki, T., Fujita, Y., Morita, T., and Tanaka, N. 2003a. Identification of a SNARE protein required for vacuolar protein transport in *Schizosaccharomyces pombe*. *Biochem. Biophys. Res. Commun.*, **311**: 77–82.

Takegawa, K., Iwaki, T., Fujita, Y., Morita, T., Hosomi, A., and Tanaka, N. 2003b. Vesicle-mediated protein transport pathways to the vacuole in *Schizosaccharomyces pombe*. *Cell Struct. Funct.*, **28**: 399–417.

Tanaka, K. and Hirata, A. 1982. Ascospore development in the fission yeasts. *Schizosaccharomyces pombe* and *S. japonicus*. *J. Cell Sci.*, **56**: 263–279.

Tanaka, K., Yonekawa, T., Kawasaki, Y., Kai, M., Furuya, K., Iwasaki, M., Murakami, H., Yanagida, M., and Okayama, H. 2000. Fission yeast Eso1p is required for establishing sister chromatid cohesion during S phase. *Mol. Cell Biol.*, **20**: 3459–3469.

Thomas, P.S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.*, **77**: 5201–5205.

Wang, H., Tang, X., Liu, J., Trautmann, S., Balasundaram, D., McCollum, D., and Balasubramanian, M.K. 2002. The multiprotein exocyst complex is essential for cell separation in *Schizosaccharomyces pombe. Mol. Biol. Cell*, **13**: 515–529.

Watanabe, T., Miyashita, K., Saito, T.T., Yoneki, T., Kakihara, Y., Nabeshima, K., Kishi, Y.A., Shimoda, C., and Nojima, H. 2001. Comprehensive isolation of meiosis-specific genes identifies novel proteins and unusual non-coding transcripts in *Schizosaccharomyces pombe*. *Nucleic Acids Res.*, **29**: 2327–2337.

Woods, A., Sherwin, T., Sasse, R., MacRae, T.H., Baines, A.J., and Gull, K. 1989. Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. *J. Cell Sci.*, **93**: 491–500.

Yoo, B.Y., Calleja, G.B., and Johnson, B.F. 1973. Ultrastructural changes of the fission yeast (*Schizosaccharomyces pombe*) during ascospore formation. *Arch. Microbiol.*, **91**: 1–10.

(Received for publication, August 15, 2005 and accepted, September 4, 2005)