CELL STRUCTURE AND FUNCTION 30: 43–49 (2005)

A Role for Fission Yeast Rab GTPase Ypt7p in Sporulation

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ABSTRACT. **Ypt7p, a fission yeast (*Schizosaccharomyces pombe*) homologue of Rab7 GTPase, mediates fusion of endosomes to vacuoles and homotypic vacuole fusion. Here, we report that Ypt7p plays important roles in sporulation. Most *ypt7*Δ asci produced less than four spores, which were apparently immature and germinated at low frequency. Furthermore, *ypt7*Δ cells were defective in development of the forespore membranes. Vacuoles in sporulating cells were found to undergo extensive homotypic vacuole fusion to form a few large compartments occupying the entire cytoplasm of asci. This extensive vacuole fusion depended on Ypt7p.**

Key words: fission yeast/sporulation/Rab7 GTPase/vacuole fusion

Introduction

Sporulation in the fission yeast *Schizosaccharomyces pombe* is a unique biological process in that the plasma membrane of daughter cells is assembled *de novo* within the mother cell cytoplasm. A double unit membrane called the forespore membrane (FSM) is constructed during the meiotic second division; the inner membrane becomes the plasma membrane of newborn spores (Yoo *et al*., 1973; Tanaka and Hirata, 1982; Nakase *et al*., 2001; Nakamura *et al.*, 2001; Nakamura-Kubo *et al.*, 2003; Shimoda, 2004). FSM formation begins on the cytoplasmic face of the spindle pole body and extends by fusion with membranous vesicles derived from the endoplasmic reticulum via the Golgi apparatus, engulfing meiotic nuclei and cellular organelles (Nakamura *et al.*, 2001). The cell wall materials are synthesized and organized into spore walls within the gap between double layers of the FSM. After the spore wall construction is completed, mature spores are liberated from asci by autolysis of the ascal cell walls.

The yeast vacuole is a relatively large organelle functionally equivalent to the lysosome of animal cells (Takegawa *et al.*, 2003a). Vacuoles regulate cytosolic pH and osmolality, degrade macromolecules, and store various intermediary metabolites such as amino acids. Upon starvation, degradation of the bulk cytosol occurs in vacuoles by a process known as autophagy (Noda *et al.*, 2002). Remodeling of cellular structures may be necessary for sporulation, which proceeds under starvation conditions.

The budding yeast Ypt7p, a homologue of mammalian Rab7 GTPase, mediates docking and fusion of late endosomes to vacuoles, as well as mediating homotypic vacuole fusion (Wichmann *et al.*, 1992; Haas *et al.*, 1995; Wurmser *et al.*, 2000; Wickner, 2002). Ypt7p is activated by the guanine-nucleotide exchange factor (GEF), Vps39p. A GTP-bound form of Ypt7p associates with the HOPS tethering complex that is responsible for vacuole fusion. Following formation of the *trans*-SNARE complex, membranes are fused. *S. pombe* Ypt7p might play a similar role in vacuolar morphology and function (Bone *et al*., 1998; Murray and Johnson, 2001; Iwaki *et al*., 2003; Iwaki *et al*., 2004). In fact, a *ypt7*Δ mutant contains fragmented vacuoles (Bone *et al*., 1998; Iwaki *et al*., 2003; Iwaki *et al*., 2004). Earlier studies have suggested the importance of Ypt7p in sporulation. Transcript levels of *ypt7*$^{+}$ have been reported to be elevated about 3-fold during sporulation (Mata *et al*., 2002), and the *ypt7* null mutant forms asci less frequently (Iwaki *et al*., 2004). We report here that Ypt7p is implicated in extensive vacuole fusion in the late stage of sporulation, which might be important for spore maturation. The *ypt7*Δ mutant was found to form few and immature spores in asci. In addition, formation of the FSM was found to be partially dependent on Ypt7p function.

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Abbreviations: FSM, forespore membrane; MAP kinase, mitogen-activated protein kinase; PI, phosphatidylinositol; SNARE, soluble NSF attachment protein receptor; Vps, vacuolar protein sorting.

Materials and Methods

Yeast strains, media and culture conditions

S. pombe strains used in this study are listed in Table I. Complete medium YE was used for growth, and malt extract medium MEA and synthetic sporulation media (SSA, SSL-N and MM-N) were used for mating and sporulation (Egel and Egel-Mitani, 1974; Gutz *et al*., 1974; Moreno *et al*., 1991).

Construction of a strain expressing GFP-tagged Ypt7p

Plasmid pBS(GFP) was constructed by inserting DNA encoding a modified version of *Aequorea* green fluorescent protein (GFP^{S65T}) into the *Xho*I-*Bam*HI sites of pBluescript II (Stratagene, La Jolla, CA, USA). Plasmid pTN381 was constructed by inserting the *leu1*$^+$ gene into the *Pvu*II site of pBR322, and *Apa*I and *Sac*I linkers into the *Eco*RI and *Bam*HI sites, respectively. Plasmid pTN381 ($ypt7_{promoter}$-GFP-*ypt7*) was constructed as follows. The *ypt7* ORF was amplified by PCR using 5'-ACGTACTCGAG (*Xho*I)TATGGCCGGCAAAAAGAAG-3' and 5'-TCGATGAGCTC (*Sac*I)TTCAAGCCAAAGAACCATT-3' as forward and reverse primers, respectively. The PCR product was digested with *Xho*I and *Sac*I, and then inserted into *Xho*I- and *Sac*I-digested pBS (GFP), yielding pBS(GFP-*ypt7*). The *ypt7* promoter region was amplified by PCR using 5'-TCAGAGGGCCC(*Apa*I)GCAGCTA-CCTCAAGTTGTA-3' and 5'-GATCTCTCGAG(*Xho*I)ATTTACA-GCGTAAAAACGA-3' as forward and reverse primers, respectively. The PCR product was digested with *Apa*I and *Xho*I, and then ligated into pBS(GFP-*ypt7*), yielding pBS($ypt7_{promoter}$-GFP-*ypt7*). This plasmid was then digested with *Apa*I and *Sac*I, and the resulting DNA fragment carrying the *ypt7* promoter was subcloned into pTN381, generating pTN381($ypt7_{promoter}$-GFP-*ypt7*). This plasmid was linearized with *Sna*BI in the middle of the *leu1* sequence and was then introduced into the *ypt7* disruptant KJ100-7BY. The obtained GFP-*ypt7* integrant was designated ZK11 (Table I). An integration strain (ZK1) expressing GFP-Psy1 was constructed similarly.

Immunofluorescence microscopy

Cells were fixed according to Hagan and Hyams (1988) with glutaraldehyde and paraformaldehyde. Microtubules were stained with anti-α-tubulin antibody TAT-1 (Woods *et al*., 1989) and 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 obtained using a Cool SNAP CCD (charge-coupled device) camera (Roper Scientific, San Diego, CA, USA).

Staining of vacuolar membranes

Vacuolar membranes were stained with FM4-64 [N(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridiniumdibromide] (Molecular Probes, Eugene, OR, USA) according to Morishita *et al.* (2002) with a minor modification. Cells were harvested, resuspended in 0.5 ml of liquid YE medium containing 0.5 μl of 8 μM FM4-64 in dimethyl sulfoxide, and then were incubated with shaking at room temperature for 30 min. Stained cells were chased with YE medium for 60 min, and then cultured on SSA sporulation medium for 1 day.

Results

Ypt7p is required for proper ascospore formation

*ypt7*Δ has been reported to sporulate poorly (Iwaki *et al.*, 2004). We explored sporulation in the *ypt7* null mutant in greater detail. The frequency of four-spored asci was remarkably lower than that of the wild-type strain (Fig. 1A). A significant fraction of asci contained one, two or three spores. Furthermore, spores of *ypt7*Δ were apparently smaller than wild-type spores. The mean volume of wild type and *ypt7*Δ spores was 10.7 μm^3 and 6.6 μm^3, respectively (Fig. 1B). After spores fully mature, they are liberated from asci by autolysis of the ascal walls. However, *ypt7*Δ asci required more time before autolysis occurred (data not shown) (Iwaki *et al.*, 2004). These observations suggested that spores of *ypt7*Δ were unable to mature fully. To confirm this, the germination ability of *ypt7*Δ spores was examined. As spores were scarcely liberated from an ascus in *ypt7*Δ, single spores could hardly be manipulated. Instead, single asci were separated by micromanipulation and incubated on

Table I. STRAINS USED IN THIS STUDY

Strains	Genotype		Reference or Source
MKW5	h^{90}		Nakamura-Kubo *et al.*, 2003/YGRC*
KJ100-7BY	h^{90}	*ypt7*::*ura4*$^+$ *ura4-D18 leu1-32*	Iwaki *et al.*, 2003/YGRC*
YN68	h^{90}	*leu1*<<*GFP-psy1*$^+$	Nakase *et al.*, 2004/YGRC*
ZK1	h^{90}	*ypt7*::*ura4*$^+$ *ura4-D18 leu1*<<*GFP-psy1*$^+$	This study
ZK3	h^{90}	*ypt7*::*ura4*$^+$ *ura4-D18*	This study
ZK11	h^{90}	*ypt7*::*ura4*$^+$ *ura4-D18 leu1*<<*GFP-ypt7*$^+$	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.

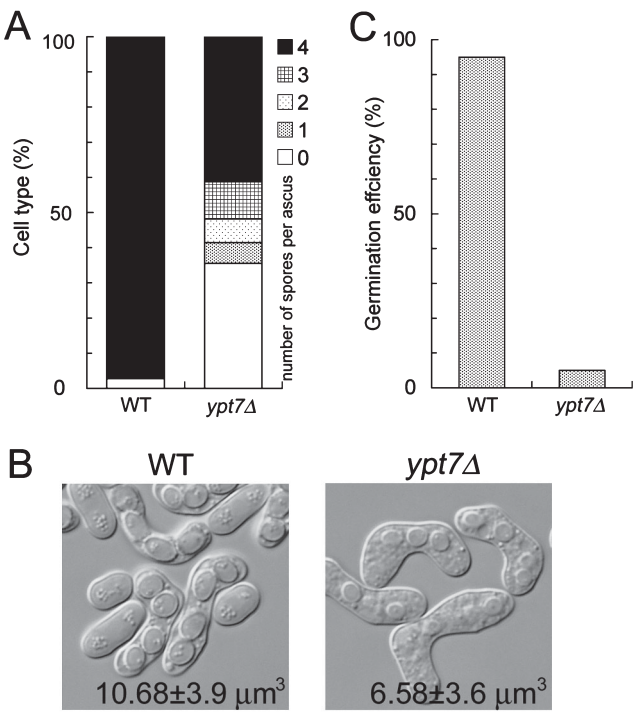

Fig. 1. Ypt7p is required for proper sporulation. MKW5 (wild type) and ZK3 (*ypt7*Δ) were sporulated on SSA medium for 2 days at 28°C, and observed by differential interference contrast (DIC) microscopy. (A) Percentage of tetra-nucleated asci containing different number of spores. At least 500 cells were counted for each sample. (B) DIC images of asci. The mean volume of spores and standard deviations are indicated. Long and short axes were measured by AquaCosmos (version 2.5) image analysis software (Hamamatsu Photonics, Hamamatsu). Volumes were calculated assuming that spores are spheroids. Bar, 10 μm. (C) Germination of wild-type and *ypt7*Δ spores. Asci formed on SSA for 3 days were randomly chosen and placed on YE solid medium by micromanipulation. Colonies formed after 3 days of incubation were counted.

YE complete medium for 3 days. Only 5% of asci formed colonies, while the frequency was ~95% in a wild-type strain (Fig. 1C). These results indicated that Ypt7p is required for spore morphogenesis, autolysis of ascal walls and spore germination.

Ypt7p is also required for forespore membrane development

The size of spores is basically determined by development of the FSM (Nakamura *et al.,* 2001). We then monitored growth of the FSM in *ypt7*Δ by visualization with GFP-Psy1. Psy1 is a fission yeast homologue of mammalian syntaxin 1A, which is a t-SNARE (soluble NSF attachment protein receptor) on the plasma membrane (Nakamura *et al.*, 2001). We have previously reported that Psy1 is translocated from the plasma membrane to the nascent FSM at meiosis II (Nakamura *et al.*, 2001). Formation of the FSM in *ypt7*Δ was normally initiated near the spindle poles at meiosis II. However, the membrane did not grow sufficiently and often failed to encapsulate a nucleus (Fig. 2A). We classified these aberrant zygotes into three types with respect to their terminal phenotypes (Fig. 2B). The majority of the population (type I; 72%) formed four nucleated prespores. These prespores were considerably smaller than wild-type prespores. A fraction of the zygotes formed four prespores but some of them were anucleated (type II; 22%), and the rest of the population formed only 1 to 3 prespores (type III; 6%). These results indicate that Ypt7p is also required for normal development of the FSM.

Extensive vacuole fusion at the late stage of sporulation

Bulk degradation of cellular macromolecules and remodeling of organelles occur during sporulation (Klar and Halvorson, 1975; Betz and Weiser, 1976). We noted the morphological changes of vacuoles, because they are responsible for degradation of macromolecules. Vacuolar membranes were stained with a fluorescent styryl dye, FM4-64 (Vida and Emr, 1995), and the FSM was marked with GFP-Psy1 to monitor the process of sporulation. Cells were exposed to FM4-64 for 30 min, during which the plasma membrane was preferentially stained, and were then chased in the dye-free medium. The signals quickly moved to the endosome-like compartments and eventually to the vacuolar membrane. Thereafter cells were transferred to sporulation medium and incubated. Vacuoles in early stage of sporulation (before completion of the FSM) were a little larger than those in vegetative cells. Surprisingly, in the late stage of sporulation, when spore wall materials accumulate between two layers of the FSM, vacuoles fused extensively to form a few large membranous compartments that occupied the entire cytoplasm (Fig. 3A, asterisk). The vacuolar membrane was found to be in close contact with the surface of the nascent spores and with the plasma membrane of mother cells. We also noted that signals of FM4-64 were not detected on the FSM (Fig. 3A), indicating that there is practically no membrane flow from the vacuolar membrane to the FSM.

Ypt7p is required for vacuole fusion during sporulation

Hypotonic conditions induce fusion of vacuoles in vegetative cells, and this fusion process is inhibited by the *ypt7* null mutation (Bone *et al.*, 1998). As *ypt7* mutation affects spore formation, we explored morphological changes in vacuoles in *ypt7*Δ during sporulation. As shown in Fig. 3B, enlargement of vacuoles was not remarkable, indicating that extensive vacuole fusion in the late stage of sporulation is highly dependent on Ypt7p.

We next explored intracellular localization of GFP-Ypt7 in sporulating cells. The GFP-tagged *ypt7* gene was chromosomally integrated and driven by its own promoter in a

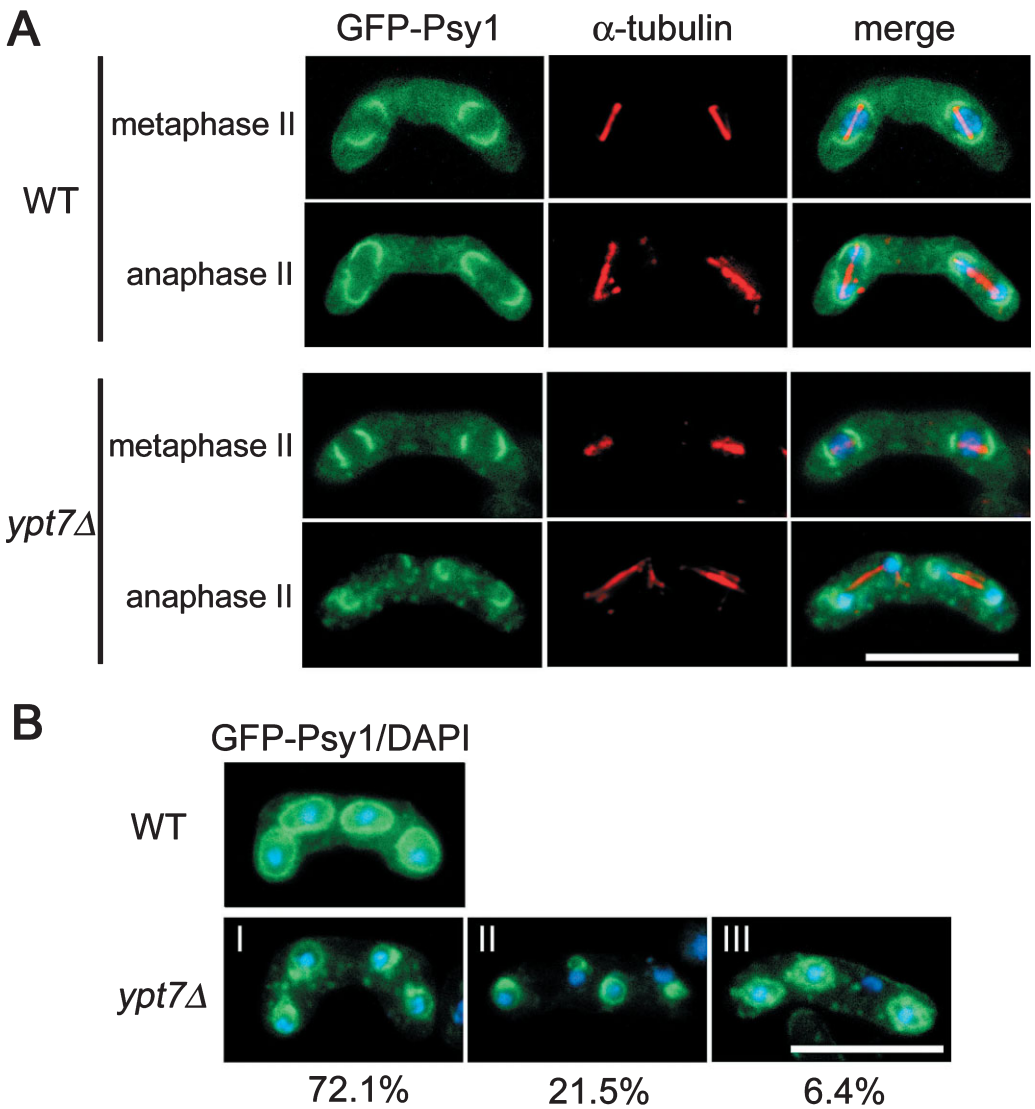

Fig. 2. Development of the FSM during meiosis. (A) YN68 (wild type) and ZK1 (*ypt7*Δ) were sporulated for 16 hr as described in the legend to Fig. 1. The FSM was visualized by GFP-Psy1. α-tubulin was immunostained by TAT-1 antibody. DNA was stained by DAPI. Bar, 10 μm. (B) Morphology of the FSM in *ypt7*Δ. Aberrant zygotes were classified into three types (Type I, II and III) and their relative frequency is shown. Bar, 10 μm.

*ypt7*Δ strain. This strain carrying a single-copy of the GFP-*ypt7* gene was able to sporulate and undergo normal and extensive vacuole fusion, suggesting that the GFP-*ypt7* fusion construct is functional. Fig. 4 shows that GFP-Ypt7 localized to vacuolar membranes in both vegetative and sporulating cells. Large vacuoles generated by extensive fusion were also observed by using GFP-Ypt7. These results support the notion that Ypt7p plays an important role in fusion of vacuoles during sporulation. Interestingly, strong GFP signals appeared within the cytoplasm of newly formed spores. We thus speculate that Ypt7p produced during sporulation is incorporated into the vacuolar membrane of spores (Fig. 4).

Discussion

In this study, we demonstrated that *S. pombe* Rab family GTPase Ypt7p plays important roles in sporulation. In the *ypt7*Δ strain, each ascus contained less than four spores. Furthermore, the spores were significantly smaller than wild-type spores, and their germination efficiency was greatly reduced. We investigated development of the FSM by means of the GFP-fused FSM marker protein. The FSM develops through a few steps that culminate in nucleated prespores. The FSM failed to encapsulate the nucleus in a portion of *ypt7*Δ cells. Even nucleated prespores were considerably smaller than wild-type prespores. Additionally, the number of nucleated and anucleated prespores per

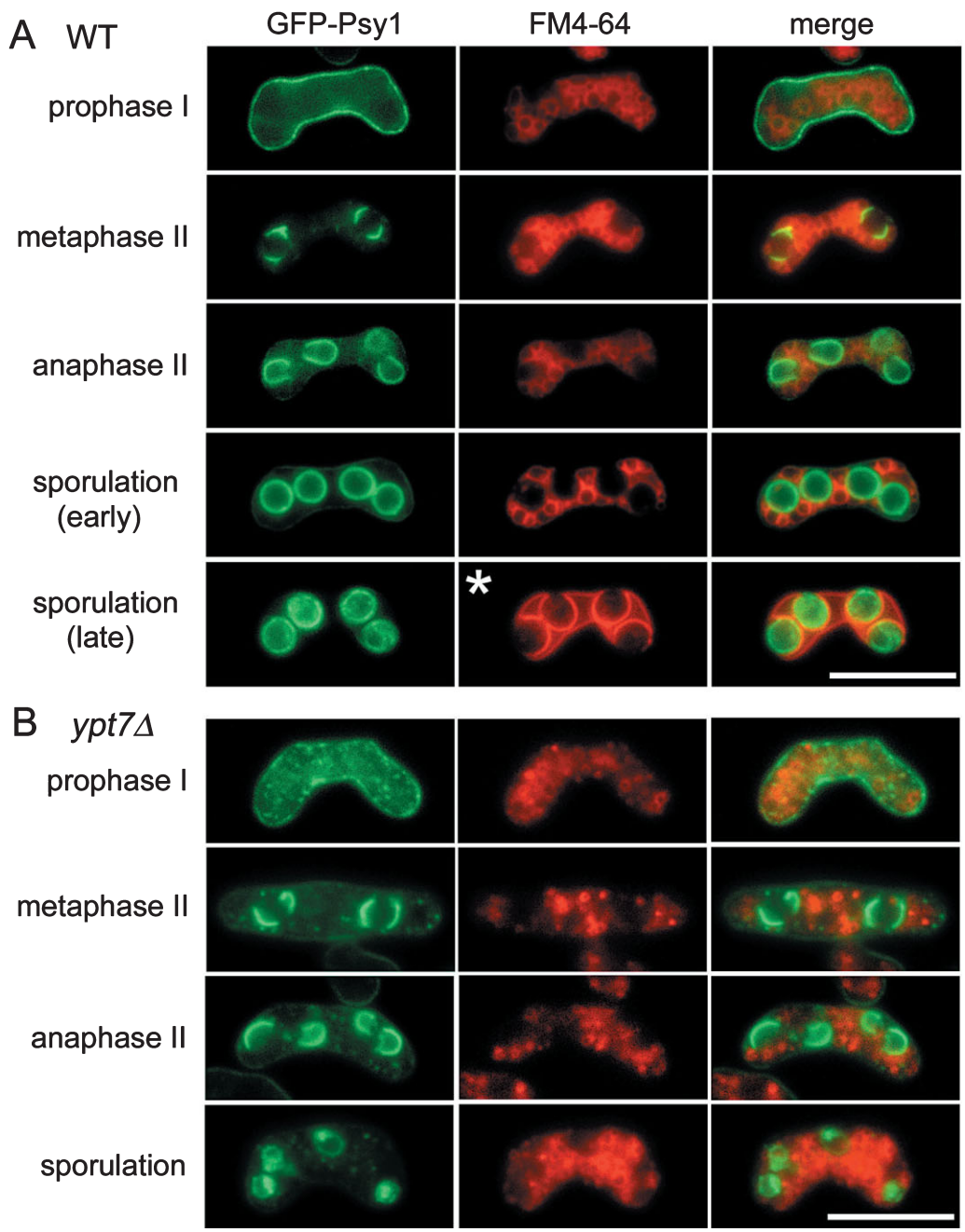

Fig. 3. Vacuolar morphology during sporulation. To monitor progress of sporulation, YN68 (wild type) and ZK1 (*ypt7*Δ) expressing GFP-Psy1 were used. Cells were first labeled with FM4-64 for 30 min in nutrient medium, and then sporulated on SSA medium for 1 day. The FSM visualized by GFP-Psy1 and the vacuolar membranes were observed under a fluorescence microscope. Note that markedly enlarged vacuoles are formed at late stage of sporulation (asterisk). Bar, 10 μm.

zygote was less than four in a small portion of the mutant cells. These observations suggest that extension of the FSM was insufficient in *ypt7*Δ. As to how Ypt7p is involved in the FSM formation, several previous studies have indicated that the FSM elongates by fusion with vesicles derived from the endoplasmic reticulum via the Golgi apparatus (Nakase *et al.*, 2001; Nakamura *et al.*, 2001; Nakamura-Kubo *et al.*, 2003; Shimoda, 2004; Nakamura *et al*., 2005). Ypt7p plays essential roles in membrane fusion between endosomes and vacuoles as well as homotypic vacuole fusion. As membrane flux from the vacuoles to the FSM was not found, it seems unlikely that Ypt7p is directly implicated in fusion of

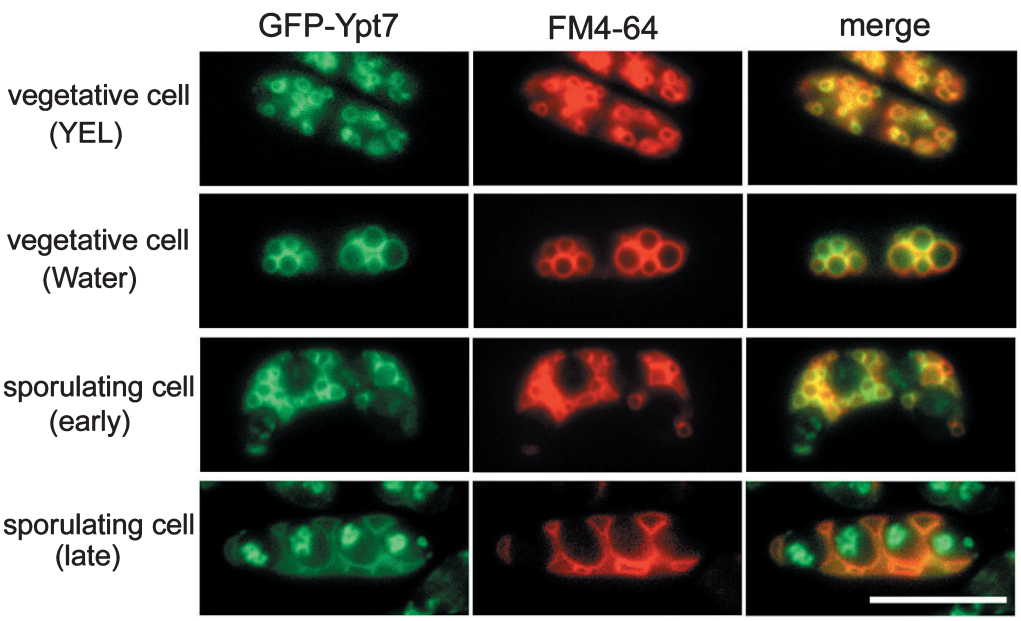

Fig. 4. Localization of GFP-Ypt7 during sporulation. ZK11 expressing GFP-Ypt7 was stained with FM4-64 in YE liquid medium. An aliquot of the culture was transferred to water for 60 min. Cells stained with FM4-64 were sporulated on SSA. Zygotes displaying immature spores (early) or mature spores (late) were observed. Bar, 10 μm.

vesicles to the FSM. To address whether vacuole fusion is directly involved in the FSM formation, it appears to be important to examine the phenotypes of vacuole-specific v-SNARE mutants. In *S. cerevisiae*, three proteins (Vam3p, Vam7p and Nyv1p) have been reported. However, the corresponding proteins have not yet been identified in *S. pombe*, and a search of the *S. pombe* genome sequence database for the ORFs with a high sequence homology was not successful (Takegawa *et al.*, 2003b).

Alternatively, Ypt7p may affect sporulation ability by mediating membrane fusion events between vacuoles, and between endosomes and vacuoles. Such vacuole fusion events may regulate the cellular function of vacuoles, which is supposed to be required for normal sporulation. In fact, various mutations affecting vacuolar protein sorting (vps mutations) are also defective in sporulation. For example, a Sec1 family protein Vps33p (Iwaki *et al.*, 2003) and phosphatidylinositol 3-kinase (Vps34p/Pik3p) play a role in assembly of the FSM (Onishi *et al.*, 2003). Additionally, the retromer components (Vps5p, Vps17p and Vps29p) that are involved in the retrograde transport from the endosomes to the Golgi apparatus are also required for normal development of the FSM (Koga *et al.*, 2004). Sporulation is a dynamic cell remodeling process, thus it requires bulk degradation of preexisting proteins in vacuoles. In fact, null mutations of the *isp6* gene encoding vacuolar proteinase B in fission yeast drastically block spore formation (Sato *et al.*, 1994). As the *ypt7*Δ mutation does not completely block spore formation, but rather specifically impairs the FSM assembly, it seems less likely that reduced protease activity is a major cause for sporulation defects observed in *ypt7*Δ cells.

Vacuoles of fission yeast rapidly fuse in water in response to hypotonic stress (Bone *et al.*, 1998). This hypotonic stress-induced vacuole fusion is regulated by the Sty1 MAP kinase cascade as well as the Pmk1 kinase (Bone *et al.*, 1998). We are interested in investigating the dynamic features of vacuoles in the sexual cycle. Unlike budding yeast, the nitrogen starvation signal itself does not affect the vacuole size (Bone *et al.*, 1998). However, vacuoles have been found to become enlarged in response to the mating pheromone signal (M. Morishita and C. Shimoda, unpublished results), which is transmitted via the Spk1 MAP kinase cascade. The present study demonstrates that vacuoles undergo extensive homotypic fusion at the late stage of sporulation, depending on Ypt7p. As a result, only a few greatly enlarged vacuoles were found to occupy the entire cytoplasm of asci at the final stage of sporulation. Defects in such vacuolar dynamics in *ypt7*Δ resulted in inefficient lysis of ascal walls. In this context, it is intriguing that endo-(1,3)-α-glucanase (Agn2) has been reported to be necessary for ascal wall autolysis and thus for release of spores from asci (Dekker *et al.*, 2004). Agn2p is expressed during sporulation (Mata *et al.*, 2002) and is present in the ascus cytoplasm, most probably within the vacuoles. Enlarged vacuoles may physically contact the ascal walls, and thus may facilitate access of α-glucanase to cell wall α-glucan. We speculate that disintegration of the ascal plasma membrane and the vacuolar membrane abruptly triggers autolysis of cell walls.

Our observations indicate that vacuolar fusion events

during the sexual process proceed through two distinct steps: a mating pheromone-induced fusion and a sporulation-associated fusion. At a minimum, the second step is strongly inhibited by disruption of *ypt7*. We presume that the first step is also under the control of Ypt7p, because *ypt7* disruption influences mating ability (Iwaki *et al.*, 2004). Understanding the molecular mechanisms and biological significance of extensive homotypic vacuolar fusion during sporulation will require further detailed study.

Acknowledgments. We thank Dr. K. Gull of the University of Oxford for the anti-α-tubulin antibody, TAT-1. 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/).

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(Received for publication, September 20, 2005 and accepted, October 14, 2005)