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Identification of a SNARE protein required for vacuolar protein transport in *Schizosaccharomyces pombe*

Kaoru Takegawa,* Akira Hosomi, Tomoko Iwaki, Yasuko Fujita, Tomotake Morita, and Naotaka Tanaka

Department of Life Sciences, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan

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Abstract

Intracellular vesicle trafficking is mediated by a set of SNARE proteins in eukaryotic cells. Several SNARE proteins are required for vacuolar protein transport and vacuolar biogenesis in *Saccharomyces cerevisiae*. A search of the *Schizosaccharomyces pombe* genome database revealed a total of 17 SNARE-related genes. Although no homologs of Vam3p, Nyv1p, and Vam7p have been found in *S. pombe*, we identified one SNARE-like protein that is homologous to *S. cerevisiae* Pep12p. However, the disruptants transport vacuolar hydrolase CPY (SpCPY) to the vacuole normally, suggesting that the Pep12 homolog is not required for vacuolar protein transport in *S. pombe* cells. To identify the SNARE protein(s) involved in Golgi-to-vacuole protein transport, we have deleted four SNARE homolog genes in *S. pombe*. SpCPY was significantly missorted to the cell surface on deletion of one of the SNARE proteins, Fsv1p (SPAC6F12.03c), with no apparent *S. cerevisiae* ortholog. In addition, sporulation, endocytosis, and in vivo vacuolar fusion appear to be normal in *fsv1*Δ cells. These results showed that Fsv1p is mainly involved in vesicle-mediated protein transport between the Golgi and vacuole in *S. pombe* cells.

Keywords: *Schizosaccharomyces pombe*; SNARE; Vacuolar protein sorting; Syntaxin; Membrane trafficking

Protein transport through the secretory pathway in eukaryotic cells requires an ordered series of vesicular budding and fusion events. Biochemical and genetic studies have identified a large number of protein families that perform analogous functions at different stages in the transport process. For example, members of the SNARE family contribute to transport specificity by regulating interactions between transport intermediates and their appropriate target membranes [1]. The first complete inventory of SNARE proteins in eukaryotic cells was carried out in *Saccharomyces cerevisiae* [2]. *S. cerevisiae* has been reported to contain a total of 24 SNARE-encoding genes including SNARE-like *SEC20* [3], and the recently identified YAL014c/*SYN8* [4] and YGL098W/*USE1* [5]. The localization and specific interactions of SNARE proteins are thought to provide much of the specificity for vesicle fusion in *S. cerevisiae* cells [6,7].

The yeast vacuole is an equivalent of the mammalian lysosome and the vacuole of plant cells. The vacuole of *S. cerevisiae* plays an important role in macromolecular degradation, metabolite storage, and ion homeostasis [8]. Protein transport to the vacuole is also a vesicle-mediated process and several SNARE proteins are required for vacuolar protein transport and vacuolar biogenesis. Pep12p is a mammalian syntaxin homolog and it is regulating the docking and fusion of Golgi-derived transport vesicles with the endosome [9]. Membrane transport from the endosome to the vacuole requires a second SNARE complex composed of Vam3p [10,11], Vam7p [12], and Vti1p [13]. Vam3p, Vam7p, and Nyv1p also mediate the homotypic fusion of vacuoles in *S. cerevisiae* cells [14].

The fission yeast *Schizosaccharomyces pombe* is widely used as a model system for eukaryotic cell biology. Although the complete genome sequence of *S. pombe* has been reported [15] and a group of GTP-binding proteins known as Ypt proteins which act 'upstream' of SNARE

* Corresponding author. Fax: +81-87-891-3021.
E-mail address: takegawa@ag.kagawa-u.ac.jp (K. Takegawa).

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proteins have been well studied [16], there are no reports on the SNARE proteins required for vacuolar protein transport and vacuolar biogenesis in *S. pombe*. So far only two SNARE proteins have been reported and characterized in *S. pombe*. Psy1p is a mammalian syntaxin homolog localized to the plasma membrane during vegetative growth and to the forespore membrane during sporulation [17]. Syb1p is a synaptobrevin homolog accumulated in the medial region of dividing cells depending on F-actin and myosin [18]. Both the *psy1*$^+$ and *syb1*$^+$ genes are essential for growth [17,18].

Here, we found by a BLAST search of protein databases that *S. pombe* contains 17 genes that are homologous to SNARE proteins of *S. cerevisiae*. To identify the SNARE protein(s) involved in Golgi-to-vacuole protein transport, five SNARE-encoding genes have been deleted in *S. pombe* in this study. This paper describes the first identification of a SNARE protein essential for vacuolar protein transport in *S. pombe*.

Materials and methods

Strains, media, and genetic methods. The wild-type *S. pombe* strains ARC039 (*h- leu1 ura4*) and KJ100-7B (*h*^{90} *leu1 ura4*) were obtained from Yuko Giga-Hama (Asahi Glass, Japan), and Koichi Tanaka (Tokyo Univ., Japan). The mutants *cpy1*Δ (*h*$^+$ *leu1-32 his2 ura4-D18 ade6-M216 cpy1::ura4*$^+$) and *vps34/pik3*Δ (*h*$^+$ *leu1-32 ura4-D18 ade6-M216 vps34::ura4*$^+$) were constructed as described previously [19–21]. FM4-64 and Lucifer yellow CH were purchased from Molecular Probes and Sigma Chemicals, respectively. The genetic methods have been reported previously [22].

Cloning and disruption of S. pombe SNARE-related genes. The *fsv1*$^+$ locus was disrupted in the wild-type *S. pombe* strain ARC039 by replacing an internal *fsv1*$^+$ gene fragment with the *S. pombe ura4*$^+$ gene. To amplify the DNA fragment of the *fsv1*$^+$ gene from chromosomal DNA of *S. pombe* by PCR, the following oligonucleotides were synthesized: sense, 5′-GATCCCTGGTTCGAATCCTGGTTAGGCC CC-3′ and antisense, 5′-TAGCTTTGTGAACAAGGGGGACGGC CC-3′. A fragment of 1.6 kb was recovered and ligated into Promega pGEM-T EASY vector. A 0.4 kb *Hpa*I–*Cla*I fragment was eliminated from the cloned *fsv1*$^+$ open reading frame and a 1.6 kb *ura4*$^+$ cassette was inserted. A linearized DNA fragment carrying this disrupted *fsv1*$^+$ gene was used to transform wild-type haploid ARC039 and KJ100-7B strains, and *ura*$^+$ transformants were selected. To confirm that the *fsv1*$^+$ gene had been disrupted, *ura*$^+$ transformants were analyzed by Southern blotting and PCR to verify correct integration of the deletion constructs.

Schizosaccharomyces pombe homologs of *S. cerevisiae PEP12* (SPBC31E1.04), *TLG1* (SPBC36B7.07), *TLG2* (SPA823.05c), and *GOS1* (SPAC4G8.10) were amplified from chromosomal DNA by PCR with appropriate primers and cloned into pGEM-T EASY vector. These genes are named according to the *S. cerevisiae* nomenclature [2]. The genes were disrupted by inserting the *ura4* gene for parts of the coding sequence using *Hin*dIII restriction sites. The *ura4*$^+$-containing DNA fragments were used to transform strain ARC039 and gene disruptions were analyzed by PCR.

Plasmid constructions. pREP41-GFP-Fsv1 was constructed as follows. The *fsv1* cDNA clone was amplified from an *S. pombe* cDNA library (a gift from Dr. T. Nakamura, Osaka City Univ., Japan). *Xho*I and *Not*I sites were introduced at the 5′ and 3′ ends, and two oligonucleotides were used to amplify *fsv1* by PCR. The corresponding PCR product was digested with *Xho*I and *Not*I, and cloned into the corresponding site of pTN54 derived from pREP41 [17]. The HA-tagged Gms1 fusion plasmid was constructed as described previously [23]

Fluorescence microscopy. For localization of Fsv1p, cells were grown to early log phase and fixed with glutaraldehyde and paraformaldehyde as described previously [17]. The HA-tagged Gms1p was visualized by indirect immunofluorescence microscopy with the use of rat anti-HA antibody (Boehringer Mannheim, Germany) and Alexa 568-conjugated goat anti-rat IgG (Molecular Probes).

The vacuoles of fission yeast wild-type and mutant cells were labeled with FM4-64 [24] and Lucifer yellow CH [25]. The cells were observed with an Olympus BX-60 fluorescence microscope (Olympus, Tokyo, Japan), and images were captured with a Sensys Cooled CCD camera using a MetaMorph (Roper Scientific, San Diego, CA).

Pulse-chase and immunoblot analyses of the S. pombe CPY. Pulse-chase analysis and immunoprecipitation of the vacuolar carboxypeptidase Y from *S. pombe* (SpCPY) were carried out as previously described [19]. Antibody incubations were carried out using rabbit polyclonal antibody against SpCPY [19]. The CPY colony blot assay was performed by replica-plating freshly grown spots onto nitrocellulose for 3-days growth as reported previously [26].

Results

Identification of SNARE-related proteins in S. pombe

BLAST searches of the *S. pombe* genome database with the SNARE motif identified a total of 17 open reading frames encoding SNARE-related proteins (Table 1). Two fission yeast SNARE proteins (Psy1p and

Table 1
Summary of fission yeast homologs of the budding yeast SNARE proteins

S. cerevisiae	*S. pombe*	Similarity (*E* value)
UFE1 (346 aa)	SPCC895.04c (319 aa)	0.047
SEC20 (383 aa)	—	
SEC22 (214 aa)	SPBC2A9.08c (209 aa)	2.5e − 51
USE1 (245 aa)	SPAC17G6.07c (222 aa)	0.0008
SED5 (340 aa)	SPBC8D2.14c (309 aa)	6.1e − 32
BET1 (142 aa)	SPAC23C4.13 (117 aa)	1.1e − 11
BOS1 (244 aa)	SPAP14E8.03 (235 aa)	5.6e − 22
YKT6 (200 aa)	SPBC13G1.11 (197 aa)	3.3e − 63
SFT1 (97 aa)	SPAC31A2.13c (91 aa)	3.4e − 06
VTI1 (217 aa)	SPBC3B9.10 (214 aa)	1.2e − 26
GOS1 (223 aa)	SPAC4G8.10 (182 aa)	3.1e − 09
TLG1 (224 aa)	SPBC36B7.07 (225 aa)	1.8e − 16
—	SPAC6F12.03c (247 aa)	
TLG2 (397 aa)	SPAC823.05c (301 aa)	3.7e − 26
SYN8 (255 aa)	—	
PEP12 (288 aa)	SPBC31E1.04 (317 aa)	3.8e − 23
VAM3 (283 aa)	—	
VAM7 (316 aa)	—	
NYV1 (253 aa)	—	
SSO2 (SSO1) (295 aa)	SPCC825.03c/*psy1*$^+$ (284 aa)	3.7e − 32 (4.8e − 32)
SNC2 (SNC1) (115 aa)	SPAC6G9.11/*syb1*$^+$ (121 aa)	1.6e − 31 (1.2e − 26)
SEC9 (651 aa)	SPBC26H8.02c (419 aa)	3.9e − 42
SPO20 (397 aa)	—	

On the basis of sequence homology, potential homologs of *S. cerevisiae* SNARE proteins have been identified in *S. pombe*. Five *S. pombe* genes deleted and characterized in this study are underlined.

Syb1p) have been characterized and found homologous to Sso1/2p and Snc1/2p of *S. cerevisiae*, respectively [27,28] (Table 1). Several SNARE proteins required for vacuolar biogenesis, such as Vam3p, Vam7p, and Nyv1p, have been reported in *S. cerevisiae* [10–14]. Interestingly, no fission yeast equivalent of Vam3p, Vam7p, and Nyv1p has been found in the *S. pombe* genome (Table 1). To gain insight into the role of SNARE protein(s) in vacuolar protein transport in *S. pombe*, we first tried to characterize the SPBC31E1.04 gene, which we will hereafter refer to as *pep12*$^+$, because the gene product is highly homologous to Pep12p/Vps6p (30% identity in 223 aa) which is essential for vacuolar protein transport in *S. cerevisiae* cells [9].

The fission yeast homolog of Pep12p is not required for vacuolar protein transport

Saccharomyces cerevisiae Pep12p is a mammalian syntaxin homolog thought to reside on the membrane of the prevacuolar compartments. Pep12p controls the trafficking of newly synthesized proteins to the vacuole [9]. If *S. pombe pep12*$^+$ is an ortholog of *S. cerevisiae PEP12*, the *pep12* mutant should show a defect in vacuolar protein transport in *S. pombe* cells. We have previously reported the isolation and characterization of a vacuolar marker protein, carboxypeptidase from *S. pombe* (SpCPY) [19]. We examined the processing of SpCPY in the *pep12*Δ strain. During its synthesis, SpCPY undergoes characteristic modifications and changes in apparent molecular mass; after a 15-min pulse period, the ER- and Golgi-specific precursor form (proCPY) and a small amount of the vacuole-specific mature form (mCPY) were labeled in the wild-type cells, and after a 30-min chase, all SpCPY had been transported to the vacuole and matured (Fig. 1). The *pep12*Δ mutant cells did not show a sorting defect for SpCPY, and SpCPY had matured and not been missorted to the cell surface like in wild-type cells (Fig. 1), suggesting that the Pep12p homolog is not required for the delivery of SpCPY to the vacuole in *S. pombe*. Moreover, no obvious differences were observed between the *pep12*Δ and wild-type strains with respect to growth rates, sporulation, cell and vacuolar morphologies, or ion sensitivities.

Identification of a SNARE protein required for vacuolar protein transport

Then we inspected SNARE-related genes from the *S. pombe* genome that may act on vesicle trafficking from the late-Golgi to plasma membrane. We succeeded in disrupting four additional genes and examined the localization of vacuolar CPY in the mutant strains (Table 1). To confirm the missort of SpCPY to the cell surface in the mutant cells, we employed the CPY colony blot assay that directly tests cells for secretion of SpCPY. In wild-type,

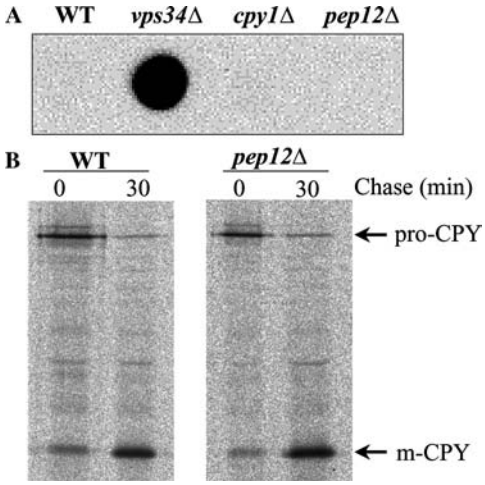

Fig. 1. The fission yeast *PEP12* homolog is not required for the transport of SpCPY to the vacuole. (A) Immunoblot analysis of SpCPY. Cells were grown on a nitrocellulose filter for 2 days at 30 °C and the filter was processed for immunoblotting using rabbit polyclonal antibody against SpCPY. *cpy1*Δ was used as a negative control and *vps34*Δ as a positive control for SpCPY missorting. (B) Processing of SpCPY in vitro. Wild-type (WT) and *pep12*Δ cells were pulse-labeled with Express-^{35}S-label for 15 min at 28 °C and chased for 30 min. The immunoprecipitates were separated on an SDS–10% polyacrylamide gel. Autoradiograms of the fixed, dried gels are shown. The positions of proCPY (110 kDa) and mature CPY (mCPY; 32 kDa) are indicated.

*tlg1*Δ, *tlg2*Δ, and *gos1*Δ cells, SpCPY is efficiently sorted to the vacuole and therefore is not secreted. Only the strain deleted of SPAC6F12.03c showed strong secretion of SpCPY (Fig. 2A). These results indicate that the SPAC6F12.03c gene is required for delivery of SpCPY to the vacuole in *S. pombe*. Therefore we designated this gene *fsv1*$^+$ (fission yeast syntaxin homolog required for vacuolar protein transport). The *fsv1*$^+$ gene encodes a typical SNARE protein of 247 amino acids with one transmembrane domain at the C-terminus. A BLAST search revealed that Fsv1p shows weak similarity to *S. cerevisiae* Syn8p (22% identity in 238 aa) and Tlg1p (22% identity in 212 aa) (Fig. 2B).

We further examined the processing of SpCPY in *fsv1*Δ strain in pulse-chase experiments. The *fsv1*Δ null mutant showed a severe sorting defect for SpCPY. After a 30-min chase, ~60% of proCPY was still detected in *fsv1*Δ cells (Fig. 2C). The strong block in SpCPY maturation indicated that Fsv1p is required for vacuolar transport to the vacuole in *S. pombe*.

Fsv1p is not required for sporulation, endocytosis, and in vivo vacuolar fusion

In *S. cerevisiae*, several SNARE proteins play essential roles not only in vacuolar protein transport but also in sporulation [29], endocytosis [30], and vacuolar fusion [31]. In an effort to define the function of Fsv1p, the *fsv1*Δ cells were analyzed further. No obvious differences

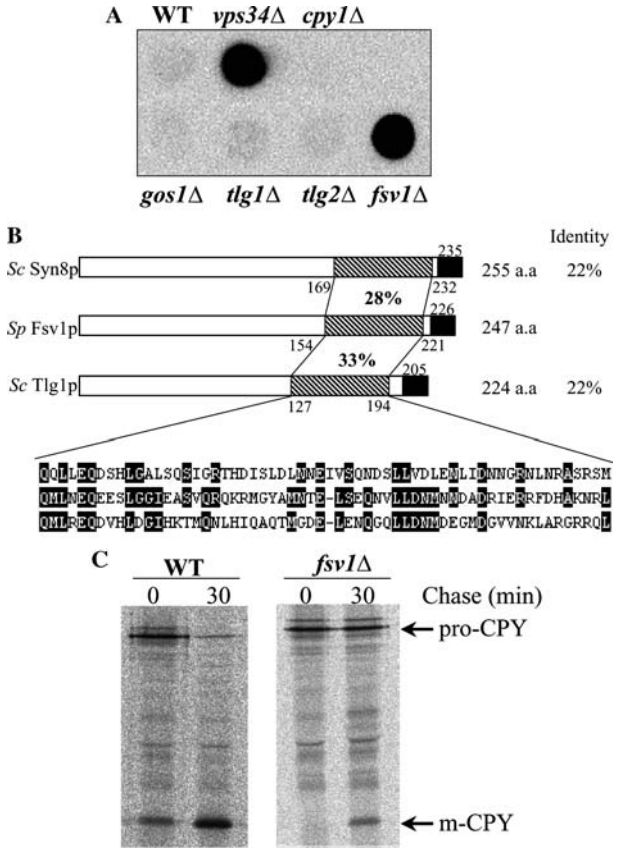

Fig. 2. The fission yeast SPAC6F12.03c (*fsv1*$^{+}$) gene is required for vacuolar localization of SpCPY. (A) Wild-type (WT) and mutant cells were grown on a nitrocellulose filter for 2 days at 30 °C and the filter was processed for immunoblotting as described in Fig. 1. (B) Schematic view of homology between Fsp1 and *S. cerevisiae* Syn8p or Tlg1p. The numbers between the proteins show percent identity between Fsv1p and the other two proteins. The black boxes indicate a transmembrane domain and the hatched boxes, a SNARE motif. (C) Wild-type and *fsv1*Δ cells were pulse-labeled for 15 min at 28 °C and chased for 30 min as described in Fig. 1.

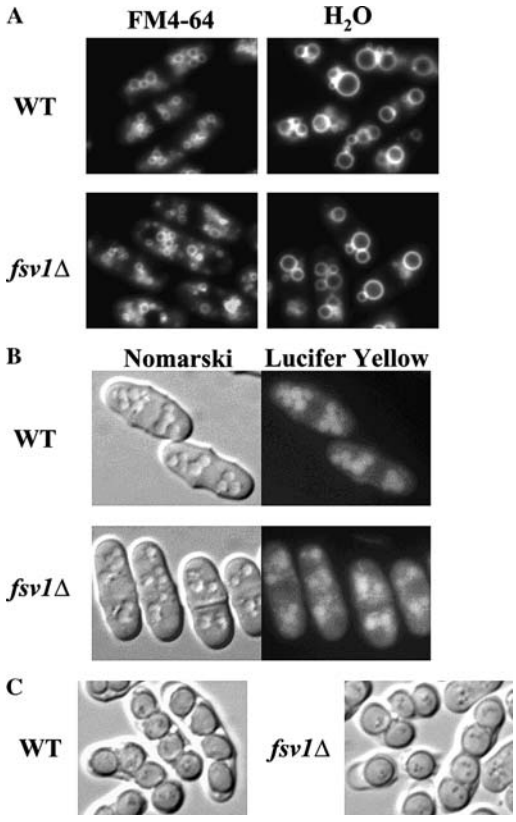

Fig. 3. Fsv1p is not required for vacuolar fusion *in vivo*, endocytosis, and sporulation (A) Wild-type (WT) and *fsv1*Δ cells were grown in YES at 27 °C and stained with FM4-64. Cells were shifted to water for 60 min and then visualized using Nomarski optics and fluorescence microscopy. (B) Wild-type and *fsv1*Δ cells were grown in YES at 27 °C and stained with Lucifer yellow for 60 min. The cells were then visualized using Nomarski optics and fluorescence microscopy. (C) Wild-type KJ100-7B (WT; h^{90}) and *fsv1*Δ (h^{90}) cells were cultured on ME plates at 28 °C for 3 days and then observed using Nomarski optics.

were observed between the *fsv1*Δ and wild-type strains with respect to growth rates, cell-shape, and septum formation. The *fsv1* gene was disrupted in a homothallic (h^{90}) strain, which was then plated onto MEA medium and observed microscopically. After incubation for 3 days, >90% of the wild-type (h^{90}) and *fsv1*Δ cells showed the formation of zygotes and sporulation, indicating that the *fsv1* gene is not required for spore formation (Fig. 3C).

Wild-type and *fsv1*Δ strains were examined for their ability to internalize Lucifer yellow, a fluorescent dye that is an established marker for fluid phase endocytosis [25]. Wild-type and *fsv1*Δ cells incubated with Lucifer yellow at 25 °C for 1 h showed distinct vacuolar staining (Fig. 3B). This result suggests that Fsv1p is not necessary for fluid phase endocytosis.

Isolated vacuoles from *S. cerevisiae* can undergo fusion in vitro [31] and vacuolar SNARE proteins including Vam3p, Vam7p, and Nyv1p are required for this process [10–14]. Under normal conditions, *S. pombe* has a large number of small vacuoles and hypotonic stress caused the transitory fusion of vacuoles [24,32]. Wild-type and *fsv1*Δ cells were grown in YES medium, stained with FM4-64, and moved into water to observe the vacuolar morphology (Fig. 3A). Both wild-type and *fsv1*Δ cells had a smaller number of larger vacuoles that resulted from vacuolar fusion, indicating that Fsv1p is not required for vacuolar fusion in vivo.

Intracellular localization of Fsv1p

We have constructed the fusion protein GFP-Fsv1 and observed its localization in the *fsv1*Δ cells. The expression of GFP-Fsv1 complemented the missorting of SpCPY in the *fsv1*Δ mutant, showing that GFP-Fsv1 is functional (Fig. 4A). The GFP fusion protein shows a punctate pattern of cytoplasmic staining in *fsv1*Δ cells. The fluorescent signals of GFP-Fsv1 partially overlapped those of the Golgi-localized Gms1p/UDP-galactose transporter [23] (Fig. 4B). FM4-64 has been

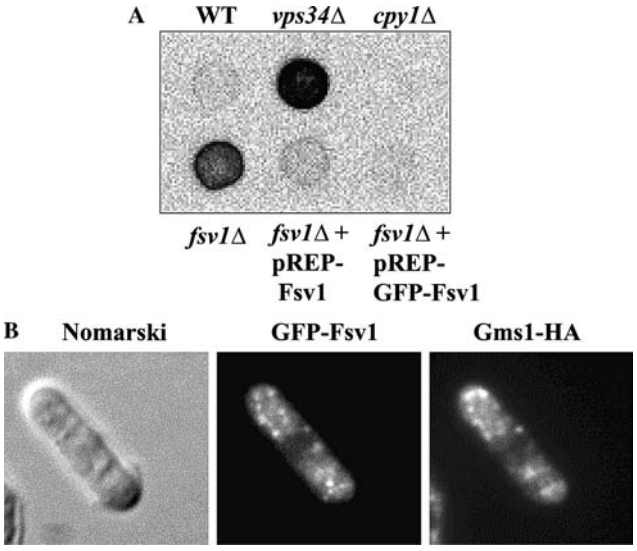

Fig. 4. Intracellular localization of Fsv1p. (A) The pREP-GFP-Fsv1 plasmid can complement the vacuolar protein sorting defects of *fsv1*Δ cells. *fsv1*Δ cells containing plasmid pREP41-Fsv1 or pREP41-GFP-Fsv1 were streaked on minimal medium minus leucine and thiamine at 28 °C for 2 days, and colonies were grown on nitrocellulose filter. The filter was processed for immunoblotting as described in Fig. 1. (B) Cells of strain *fsv1*Δ transformed with plasmids pREP-GFP-Fsv1 and pAU-Gms1-HA were cultured in minimal medium. Fixed cells were examined by GFP as well as with anti-HA antibody.

shown to be endocytosed via a route involving the prevacuolar compartment [33]. FM4-64 fluorescence was localized to punctate structures in cells that were incubated at 20 °C for 15 min, and the punctate structures stained by FM4-64 partially co-localized with GFP-Fsv1p fluorescence (data not shown). These results strongly suggest that Fsv1p is localized to Golgi and prevacuolar membranes in *S. pombe* cells.

Discussion

Here, we have demonstrated that one SNARE protein (Fsv1p) with no apparent *S. cerevisiae* ortholog is essential for Golgi-to-vacuole protein transport in *S. pombe* cells. Unexpectedly, the disruption of the *PEP12* homolog gene did not result in any defects in vacuolar protein transport or vacuolar biogenesis in *S. pombe*. Although no obvious differences were observed between the *pep12*Δ and wild-type strains, we found that the GFP-Pep12p fusion protein was specifically localized to the vacuolar membrane (data not shown). The most likely explanation for the lack of a *pep12* phenotype is that the function of Pep12p can be performed by another SNARE protein including Fsv1p. Biochemical and genetic analyses including co-precipitation experiments of Pep12p with other SNARE proteins will be required to elucidate the precise role of Pep12p in *S. pombe* cells.

We inspected and identified 17 SNARE-related genes from the *S. pombe* genome. This total is less than the 24 genes reported for *S. cerevisiae* [2]. Curiously, *S. pombe* lacks Vam3p, Nyv1p, and Vam7p homologs and these *S. cerevisiae* proteins are required for vacuolar fusion in vitro. Recently, we reported the characterization of *S. pombe vps33*$^{+}$, a gene encoding a homolog of *VPS33*, which is required for vacuolar fusion in *S. cerevisiae* [24]. The vacuolar structures of *vps33*Δ cells were not detected after the shift to water, indicating that the vacuolar fusion induced by hypotonic stress is dependent on *S. pombe* Vps33p [24]. The class C Vps protein complex including Vps33p plays an essential role in the process of docking and fusion to the vacuolar membrane in *S. cerevisiae* [31]. The class C Vps complex genetically and biochemically interacts with Ypt7p (mammalian Rab7 GTPase homolog) and Vam3p [31]. Vacuoles of *S. pombe ypt7*Δ cells are smaller than those of the wild-type, and vacuolar fusion was not induced by osmotic stress, indicating that Ypt7p is required for the vacuolar fusion process as in *S. cerevisiae* [24,32]. Therefore the components of the *S. cerevisiae* vacuolar fusion machinery appear to be conserved in *S. pombe* and a SNARE complex must exist to regulate the vacuolar membrane fusion in *S. pombe*. One possibility is that Fsv1p has expanded its role to include the vacuolar fusion process. However, our deletion analyses showed that vacuolar fusion was normal not only in *fsv1*Δ but also in *pep12*Δ, *tlg1*Δ, *tlg2*Δ, and *gos1*Δ cells, suggesting that these SNARE proteins are not required for the vacuolar fusion process in *S. pombe* cells (Fig. 3 and unpublished results). Wickner and co-workers reported that two additional SNARE proteins, Vti1p and Ykt6p, originally isolated as essential for retrograde trafficking to the Golgi membrane, also participate in the vacuolar fusion process in *S. cerevisiae* cells [34]. At present, we have not succeeded in isolating the strains deleted of the *VTI1* or *YKT6* homolog gene (Table 1), and these genes may be essential for vegetative growth like those of *S. cerevisiae*.

Analyses of whole genome sequences for many eukaryotic organisms have revealed that *S. cerevisiae* Vam3p is a specialized SNARE with no clear homologs in other fungi and multicellular eukaryotes [35]. Therefore, it will be of interest to identify the SNARE protein that interacts with class C Vps proteins in *S. pombe*. Characterization of the proteins involved in the vacuolar fusion process in *S. pombe* will reveal new insights into the molecular mechanisms of membrane fusion conserved in all eukaryotic cells.

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