Autophagy-deficient *Schizosaccharomyces pombe* mutants undergo partial sporulation during nitrogen starvation

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Autophagy is triggered when organisms sense radical environmental changes, including nutritional starvation. During autophagy, cytoplasmic components, including organelles, are enclosed within autophagosomes and are degraded upon lysosome–vacuole fusion. In this study, we show that processing of GFP-tagged Atg8 can serve as a marker for autophagy in the fission yeast *Schizosaccharomyces pombe*. Using this marker, 13 Atg homologues were also found to be required for autophagy in fission yeast. In budding yeast, autophagy-deficient mutants are known to be sterile, whereas in fission yeast we found that up to 30 % of autophagy-defective cells with amino acid auxotrophy were able to recover sporulation when an excess of required amino acids was supplied. Furthermore, we found that approximately 15 % of the autophagy-defective cells were also able to sporulate when a prototrophic strain was subjected to nitrogen starvation, which suggested that fission yeast may store sufficient intracellular nitrogen to allow partial sporulation under nitrogen-limiting conditions, although the majority of the nitrogen source is supplied by autophagy. Monitoring of the sporulation process revealed that the process was blocked non-specifically at various stages in the *atg1Δ* and *atg12Δ* mutants, possibly due to a shortage of amino acids. Taking advantage of this partial sporulation ability of fission yeast, we sought evidence for the existence of a recycling system for nitrogen sources during starvation.

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INTRODUCTION

Autophagy is a degradative pathway conserved among eukaryotic cells, and is responsible for turnover of damaged organelles and long-lived proteins. When living organisms are exposed to radical environmental changes such as nutrient starvation, differentiation or development, autophagy is rapidly induced and inner cell components are reorganized. In the budding yeast *Saccharomyces cerevisiae*, autophagy is triggered by starvation for nitrogen or carbon, after which endogenous proteins and organelles are enclosed within isolated membranes called autophago-

somes. The outer membrane of the autophagosome then fuses with the vacuole, allowing the contents of the autophagosome, designated autophagic bodies, to be released and degraded in the vacuole. The molecular mechanisms involved in the process of autophagy have been subjected to detailed genetic analysis and more than 20 Atg-related (*ATG*) genes essential for autophagy have been identified in *Sac. cerevisiae* (Ichimura *et al.*, 2000; Kamada *et al.*, 2000; Mizushima *et al.*, 1998).

One of the most striking findings with respect to function of the Atg proteins was the discovery of two ubiquitin-like conjugation systems involving Atg12p and Atg8p (Ohsumi, 2001). Atg12p, which has no apparent homology to ubiquitin, covalently attaches to Atg5p in a manner analogous to ubiquitination, and then forms a complex with Atg16p. Atg8p also lacks similarity to ubiquitin, but

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; FSM, forespore membrane.

Two supplementary tables are available with the online version of this paper.

mediates a ubiquitination-like modification. Interestingly, Atg8p forms a conjugate not with a protein, but with phosphatidylethanolamine (PE), an abundant membrane phospholipid (Ichimura *et al.*, 2000). For normal autophagosome formation, Atg8p-PE must be deconjugated by the processing enzyme, Atg4p. Therefore, the cycle of conjugation and deconjugation is critical for normal progression of autophagy (Ichimura *et al.*, 2000). In budding yeast, Atg8p is upregulated during autophagy and the Atg8-lipidation reaction is required for the membrane dynamics that are central to the process of autophagy. Thus, Atg8p could be a good marker for monitoring the autophagic process. In addition to these two autophagy-specific ubiquitin-like conjugation systems, two kinase complexes have also been described. One is the Atg1 protein kinase, which associates with Atg13p, Atg17p and Cvt9p (Matsuura *et al.*, 1997; Straub *et al.*, 1997). Atg13p is negatively regulated by Tor kinases (Chan *et al.*, 2001; Kirisako *et al.*, 1999). Once a starvation signal inactivates Tor, dephosphorylation of Atg13p occurs, activating Atg1p by conjugation with Atg1p-Atg13p, Atg17p and Atg11p, resulting in upregulation of autophagy.

In *Sac. cerevisiae*, all *atg* mutants that have been reported grow normally in rich medium, but lose viability during nitrogen starvation. A diploid homozygous for an *atg* mutation was found not to sporulate (Tsukada & Ohsumi, 1993). These mutants start to die after 2 days of starvation and lose almost 80 % viability after 5 days (Tsukada & Ohsumi, 1993). This loss of viability may be caused by deficient recycling of endogenous proteins.

In the fission yeast *Schizosaccharomyces pombe*, nitrogen starvation stimulates large-scale protein degradation involving the starvation-specific serine protease Isp6p following initiation of autophagy (Nakashima *et al.*, 2006). Kohda *et al.* (2007) recently reported that null mutants with deletions in *ATG1*, *ATG8* and *ATG13*, which are orthologues of *ATG1*, *ATG8* and *ATG13*, respectively, in budding yeast, also failed to undergo autophagy. These *atg* mutants were also reported not to undergo sexual differentiation and to lose viability during nitrogen starvation. Interestingly, *atg1*, *atg8* and *atg13* mutants that also harboured auxotrophic mutations exhibited a more drastic loss of viability during nitrogen starvation (Kohda *et al.*, 2007). These data suggest that autophagy is also a critical system to ensure survival in fission yeast. However, because other *ATG* homologues have not been characterized in fission yeast, we undertook the present study. Here we show that several fission yeast Atg proteins, whose roles in autophagy had until now only been predicted based on homology, are indeed essential for the process. Moreover, we found that approximately 15 % of prototrophic autophagy-defective cells were able to sporulate, and an autophagy-defective strain with amino acid auxotrophy also recovered sporulation in about 30 % of cells when an excess of required amino acids was supplied. This observation suggested that the majority of the nitrogen source for *de novo* protein synthesis needed for sporulation

is dependent on the autophagic protein degradation system. However, fission yeast may store sufficient intracellular nitrogen to allow partial sporulation to occur under nitrogen-limiting conditions. In this study, we sought evidence for the existence of a recycling system for nitrogen sources by taking advantage of this partial sporulation ability of fission yeast.

METHODS

Database searches of ATG genes. Fission yeast *ATG* genes were identified by screening the *Sch. pombe* genome database for new homologues of known *Sac. cerevisiae* *ATG* genes using a BLAST search of protein sequences. BLAST scores lower than 10^{-8} were considered significant. The fission yeast Atg proteins were classified based on the most similar *Sac. cerevisiae* Atg proteins.

Strains, media and materials. *Sch. pombe* strains used in this study are listed in Supplementary Table S1, available with the online version of this paper. *Escherichia coli* strain XL1-Blue (Stratagene) was used for all cloning procedures. *Sch. pombe* strains were grown in yeast extract medium with supplements [YES: 0.5 % Bacto-yeast extract (Becton Dickinson), 3 % glucose and SP supplements (Qbiogene)] or in minimal medium (MM), of which 1000 ml contains 3 g potassium hydrogen phthalate, 2.2 g Na_2HPO_4 , 5 g NH_4Cl , 20 g glucose, 1.05 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 11 mg CaCl_2 , 1 g KCl, 40 mg Na_2SO_4 , 1 mg pantothenic acid, 10 mg nicotinic acid, 10 mg inositol, 10 μg biotin, 0.5 mg boric acid, 0.4 mg MnSO_4 , 0.4 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 mg $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$, 0.04 mg $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$, 0.1 mg KI, 0.04 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 mg citric acid, and 75 mg each of adenine, histidine and lysine. A total of 37.5 mg uracil or 75 mg leucine was added per 1000 ml to cover auxotrophies. Sporulation was performed in sporulation medium (MEA or MM-N) (Moreno *et al.*, 1991). *Sch. pombe* cells were transformed by the lithium acetate method or by electroporation as described previously (Morita & Takegawa, 2004; Okazaki *et al.*, 1990; Suga & Hatakeyama, 2001). Standard genetic methods were as described by Alfa *et al.* (1993). Restriction enzymes and other DNA-modifying enzymes were purchased from New England Biolabs. All other chemicals were from Sigma-Aldrich or Wako Pure Chemicals Co.

Construction of GFP-Atg8p. The *Sch. pombe atg8* ORF was amplified from a cDNA library by PCR, using the oligonucleotides 5'-GTTTTCATATGCGTTCTCAATTCAAGGACGAC-3' and 5'-GTTTGGATCCCTAAAAAGGAAACACTGTTCC-3'. The PCR products were digested with *NdeI* or *BamHI*, and cloned into the corresponding sites of pTN54, derived from pREP41 (Nakamura *et al.*, 2001), which is a thiamine-repressible expression vector.

Cloning and disruption of *Sch. pombe ATG* and *AVT* homologues. Genes encoding Atg proteins were cloned into pGEM-T EASY or pGEM-T vectors (Promega) (Nakamura *et al.*, 2001) digested with the restriction enzymes listed in Supplementary Table S2, followed by insertion of a *ura4⁺* gene cassette to generate gene disruption constructs. Disruption of each *atg* or *avt* gene was performed by direct chromosomal integration as described by Bahler *et al.* (1998). Gene disruptions were confirmed by colony-PCR, using appropriate primers (Supplementary Table S2).

Immunofluorescence and fluorescence microscopy. Cells were fixed with glutaraldehyde and paraformaldehyde according to Hagan & Hyams (1988). Microtubules were stained with anti- α -tubulin antibody TAT-1 (Woods *et al.*, 1989) and Cy3-conjugated anti-mouse IgG (Sigma) at 1:1000 dilution. The nuclear chromatin region was stained with DAPI (4',6-diamidino-2-phenylindole) at 1 $\mu\text{g ml}^{-1}$.

Stained cells were observed under a fluorescence microscope (model BX-60; Olympus) and images were obtained using a Cool SNAP CCD camera (Roper Scientific).

Western blot analysis. Cells expressing GFP-Atg8 were suspended in $1 \times$ PBS with appropriate protease inhibitors and disrupted using a Multi-Beads Shocker (YASUI KIKAI) with 0.5 mm glass beads. Following centrifugation at 15 000 *g* for 15 min, cell-free extract was dissolved in an equal volume of $2 \times$ SDS-PAGE sample buffer and incubated at 80 °C for 5 min. A total of 20 μ l of sample was applied to an SDS-PAGE gel containing 10% polyacrylamide and electro-blotted onto a PVDF membrane (Bio-Rad). Immunodetection analysis was performed on the blotted membrane with SNAP i.d. (Millipore) using mouse polyclonal anti-GFP antibody (Molecular Probes) and horseradish-peroxidase-conjugated anti-rabbit IgG serum (Millipore). GFP-specific signals were visualized by enhanced chemiluminescence (ECL-plus, GE Healthcare) and were detected with an LAS4000 imaging system (Fuji Film Co.).

Vacuole staining. Vacuolar membranes were stained with FM4-64 [N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium-dibromide] (Molecular ProbesA) as described by Morishita *et al.* (2002) with a minor modification. Cells were harvested, resuspended in 0.5 ml liquid YE medium containing 0.5 μ l 8 μ M FM4-64 in DMSO, and then incubated with shaking at room temperature for 30 min. Stained cells were chased with YE medium for 60 min, and then cultured on MM-N starvation medium for the appropriate time. Stained cells were observed with a fluorescence microscope (model BX-60; Olympus) equipped with a U-MGFPHQ filter set (Olympus) for GFP, and a U-MWIG filter set (Olympus) for FM4-64. Images were captured with a Sensys Cooled CCD camera using MetaMorph (Roper Scientific).

RESULTS

Western blot analysis of GFP-Atg8 in fission yeast *ATG* homologue mutants

More than 20 autophagy-related (*ATG*) genes that are essential for autophagy have been identified in the budding yeast *Sac. cerevisiae* (Klionsky *et al.*, 2007; Suzuki *et al.*, 2001). In fission yeast, 15 *atg* orthologues of the budding yeast genes were identified in a BLAST search using *Sch. pombe* GeneDB (<http://www.genedb.org/genedb/pombe/>) (Supplementary Table S2). Database analysis showed that SPAC589.07c, SPAC823.16c and SPAC458.06 possess two WD repeat domains that are putative orthologues of *ATG18*, named *atg18a*⁺, *atg18b*⁺ and *atg18c*⁺, respectively. Orthologues of *ATG10*, 11, 14, 16, 19, 23, 27, 29 and 31 were not found in the *Sch. pombe* GeneDB because of low levels of similarity. From among these orthologues, Kohda *et al.* (2007) previously reported that *atg1*⁺, *atg8*⁺ and *atg13*⁺, homologous with budding yeast *ATG1*, *ATG8* and *ATG13*, respectively, were essential for autophagy induced by nitrogen starvation in fission yeast. We then constructed null mutants of the *atg* orthologues (Supplementary Table S1). In order to analyse the autophagy-specific requirements of the encoded Atg proteins, we introduced GFP-Atg8 into each *atg* mutant strain. In *Sac. cerevisiae*, Atg8p is a ubiquitin-like protein that is conjugated to phosphatidylethanolamine at the C-terminal glycine and which is essential for organization of

the autophagosome (Kirisako *et al.*, 2000). Once GFP-Atg8p on the autophagosome is delivered to the vacuole by the normal autophagic process, the GFP moiety is proteolytically removed and free GFP, which is relatively stable, is observed by immunochemical assay (Cheong *et al.*, 2005; Hosokawa *et al.*, 2006; Klionsky *et al.*, 2007; Shintani & Klionsky, 2004). Accordingly, the appearance of free GFP can be used to monitor autophagy in *Sch. pombe*. The processing of GFP-Atg8p was examined in *atg* mutants expressing GFP-Atg8p under the control of the attenuated thiamine-repressible *nmt* promoter. Vegetatively grown cells were transferred to the nitrogen-free medium MM-N to induce autophagy. Cells were harvested at appropriate times after starvation and GFP was detected by immunoblot analysis using anti-GFP antibody (Klionsky *et al.*, 2007). In wild-type cells expressing GFP-Atg8p, only full-length GFP-Atg8p was detected under nutrient-replete conditions, but free GFP increased during the course of starvation (Fig. 1a), indicating that processing of GFP-Atg8p was promoted during induction of autophagy. This phenomenon was also observed in *atg8Δ* cells expressing GFP-Atg8p, indicating that this fusion plasmid is functional.

Autophagy deficiency in *atg* mutants in fission yeast

In budding yeast, as cells are induced to undergo autophagy in the presence of the serine proteinase inhibitor PMSF, autophagy can be followed by monitoring accumulation of autophagic bodies inside the vacuoles. Under such conditions, the autophagic process is blocked prematurely and does not terminate normally (Kohda *et al.*, 2007; Takeshige *et al.*, 1992). Similarly, in fission yeast, addition of PMSF during nitrogen starvation resulted in exclusive accumulation of full-length GFP-Atg8p (Fig. 2, lane 5). An *isp6Δ* mutant, disrupted in a gene encoding a putative vacuolar serine protease, was previously reported to grow normally under nutrient-replete conditions, but was defective in large-scale protein degradation during nitrogen starvation (Nakashima *et al.*, 2006). The addition of PMSF could inactivate Isp6p, resulting in arrest of the autophagic process. The above results suggest that processing of GFP-Atg8p is also dependent on nitrogen-induced autophagy in fission yeast. Among the other 13 *atg* null mutants, except for *atg18b* and *atg18c*, only full-length GFP-Atg8p was observed before and after nitrogen starvation (Fig. 1b). *atg8Δ* cells transformed with GFP-Atg8p accumulated both full-length GFP-Atg8p and the isolated GFP moiety under starvation conditions, indicating that GFP-Atg8p complements the autophagic deficiency of the *atg8Δ* mutant (Fig. 1b). Interestingly, *atg18bΔ* and *atg18cΔ* showed a diluted GFP signal. In contrast, *atg18aΔ* or the *atg18bΔatg18cΔ* double mutant showed only a full-length GFP-Atg8p signal during nitrogen starvation (Fig. 1b). These phenomena suggest that a combination of either Atg18a-Atg18b or Atg18a-Atg18c was necessary for

Fig. 1. Cleavage of GFP-Atg8p for monitoring autophagy. (a) Time-course processing of GFP-Atg8p. Cells were grown in MM containing required amino acids and were then shifted to MM-N medium. At the indicated times, aliquots were removed, and proteins were prepared as described in Methods. Full-length GFP-Atg8p and the GFP moiety were detected by immunoblotting using anti-GFP antibodies. The position of the GFP moiety alone indicates processed GFP-Atg8p. (b) Cleavage of GFP-Atg8p in *atg* mutant cells. The multicopy plasmid encoding GFP-Atg8p was introduced into various *atg* mutants. Cells were collected after 6 h of starvation, lysates were prepared and immunoblotting was performed as described above. The *atg* mutants analysed are indicated below the respective lanes. All mutants, except for *atg8Δ*, also expressed endogenous ATG8.

autophagy, although all three Atg18 orthologues are essential for complete autophagic degradation. In contrast, the *atg22Δ* mutant accumulated both the full-length fusion protein and the isolated GFP moiety, consistent with the results of *atg22Δ* in budding yeast (Fig. 1a) (Yang *et al.*, 2006). In budding yeast, Atg22p was reported to be a vacuolar membrane amino acid transporter, which could mediate amino acid efflux during autophagy. Therefore, it is unlikely that *atg22Δ* is involved in the GFP-Atg8p processing reaction caused by autophagic degradation in the vacuole.

Fig. 2. Cleavage of GFP-Atg8p under various conditions. WT (ARC039) and *atg1Δ* (SK16) cells were grown to mid-exponential phase in MM medium containing required amino acids and were then shifted to the conditions indicated below. Lysates were prepared from WT cells grown in nutrient-replete medium (lane 1), 6 h after shift to MM-N (lanes 2 and 7), 6 h after shift to MM-N containing 1 mM PMSF (lane 5), before shift to MM-N containing 0.2 μ g rapamycin ml^{-1} (lane 3) and 6 h after shift to MM-N containing 0.2 μ g rapamycin l^{-1} (lane 4), and 6 h after shift to glucose-depleted MM (lane 6). Lysates from *atg1Δ* cells were prepared 6 h after shift to MM-N (lane 7).

Processing of GFP-Atg8 under various autophagy-inducing conditions reported in budding yeast

The conditions that induce autophagy in fission yeast are not the same as those in budding yeast. In budding yeast, glucose depletion also induces autophagy (Takeshige *et al.*, 1992). Environmental glucose is monitored by a cAMP-PKA cascade in both budding and fission yeast (Isshiki *et al.*, 1992; Maeda *et al.*, 1990, 1994; Welton & Hoffman, 2000). Noda & Ohsumi (1998) reported that addition of a high concentration of cAMP repressed autophagy in budding yeast. In contrast, the addition of excess cAMP did not influence the induction of autophagy in fission yeast (Kohda *et al.*, 2007). Our results are consistent with the results obtained upon cAMP addition, as GFP-Atg8p was not processed following the shift from nutrient-replete conditions to glucose starvation (Fig. 2) or to use of the non-fermentable glycerol as carbon source (data not shown). In budding yeast, vegetative cells undergo a nitrogen-starvation response induction of autophagy upon addition of rapamycin (Helliwell *et al.*, 1994; Weisman, 2004; Weisman *et al.*, 1997, 2001). In contrast, rapamycin treatment does not induce autophagy in fission yeast (Uritani *et al.*, 2006). We tested whether processing of GFP-Atg8p occurred under such conditions. In nutrient-replete conditions, only full-length GFP-Atg8p accumulated in spite of the addition of rapamycin (Fig. 2). These data also indicate that the GFP-Atg8p processing assay strictly reflects the existence of an autophagic process.

We examined the localization of GFP-Atg8p in several *atg* mutants before and after starvation. Initial observations were made in *atg8Δ* cells expressing GFP-Atg8p under the control of the attenuated *nmt* promoter. Fluorescence microscopy revealed that almost all GFP-Atg8p was localized in the cytosol, although some fluorescence was also detected in the nucleus, presumably due to over-expression by the *nmt* promoter. After 3 h of starvation, a few bright dots were localized near the vacuole. These bright dots may represent the preautophagosomal structure (PAS), which is the putative site where Atg components localize to compose the vesicle-forming machinery for autophagy and the Cvt pathway, or an autophagosomal structure (Fig. 3). After 6 h of starvation, GFP fluorescence was observed within the vacuole (Fig. 3). This translocation of GFP fluorescence suggests a similar autophagic process to that in budding yeast, as autophagosomes containing GFP-Atg8p fused with the vacuole and their contents, the 'autophagic bodies' were released within the vacuole. When localization of GFP-Atg8p was investigated in other *atg* mutants, including *atg1Δ*, *atg3Δ*, *atg4Δ*, *atg5Δ*, *atg7Δ*, *atg9Δ*, and *atg12Δ* (data not shown), GFP fluorescence was not observed within the vacuole after 6 h of starvation. These data also suggested that *atg* null mutants of *Sch. pombe* were defective in the autophagic process, for GFP-Atg8p could not be transported into the vacuole lumen.

Fig. 3. Localization of GFP-Atg8p expressed in an *atg* null mutant before and after starvation. *atg8Δ* (SK23) cells were grown to mid-exponential phase in MM medium and were then shifted to MM–N for 3 or 6 h. Staining of the vacuole with FM4-64 and observation by fluorescence microscopy were performed as described in Methods. Arrows indicate the vacuole.

Rescue of sporulation deficiency by amino acid supplementation in autophagy-defective mutants

Because defects in autophagy may interfere with sporulation signal transduction or with depletion of materials necessary for induction of sporulation during starvation, almost all *Sac. cerevisiae atg* mutants exhibit sporulation defects (Deutschbauer *et al.*, 2002). We therefore tested whether a similar phenomenon occurred in *atg* mutants in fission yeast. In MEA sporulation-inducing medium, microscopic observations revealed that sporulation occurred in over 90 % of TN8 cells, which are auxotrophic for leucine (Fig. 4c), whereas sporulation was rarely observed in a leucine-auxotrophic *atg1Δ* (SK29) strain derived from KJ100-7B or an arginine auxotrophic *atg1Δ* (SK49) strain (Fig. 4b). Mating was observed in only some cells (Fig. 5a). Further investigation revealed that a defect in sporulation was also observed in other leucine auxotrophic *atg* null mutants, including *atg2Δ* to *atg9Δ*, *atg13Δ*, *atg15Δ* and *atg17Δ* (data not shown). These results have previously been reported by Kohda *et al.* (2007), but we observed a novel aspect of the sporulation in *atg* null mutants during nitrogen starvation: sporulation was observed in some cells of *atg* null mutants, including *atg1Δ*, *atg2Δ*, *atg12Δ* and *atg13Δ* derived from the TN9 strain, which is not auxotrophic for amino acids. Each of the TN9-derived *atg* mutants exhibited sporulation in 8–15 % of cells during nitrogen starvation (Fig. 4a), whereas over 90 % of wild-type TN7 (non-auxotrophic) cells sporulated as well as TN8 cells (Fig. 4a, c). This result suggests that fission yeast is able to sporulate partially with a pooled nitrogen source. Moreover, it suggests that the majority of nitrogen essential for sporulation might be dependent on a nitrogen source supplied by the autophagic degradation process.

Next, we examined whether addition of amino acids to MEA sporulation medium could rescue the sexual differentiation defects of the KJ100-7B-derived *atg* mutants. In the *atg1Δ* mutant (SK29, leucine auxotroph), autophagy and sporulation were induced in MEA medium containing several different concentrations of leucine. As the leucine concentration in the medium increased, the rate of spore formation increased and became indistinguishable from that of the non-auxotrophic strain *atg1Δ* (Fig. 4a) by addition of 120 μg leucine ml^{-1} . About 30 % of the cells sporulated in ME medium containing 240 μg leucine ml^{-1} (Fig. 4b), corresponding to a sporulation efficiency that was higher than that of the non-auxotrophic *atg1* null mutant.

Random spore analysis revealed that each spore derived from the *atg* mutants germinated and grew normally in nutrient medium (data not shown). These results were also observed in the other *atg* mutants investigated (Fig. 4c). In budding yeast, a defect in sporulation has been reported in *atg* null mutants; in fission yeast, however, mating ability was rescued by addition of leucine (Figs 4b and 5a) when cells were auxotrophic for leucine. This phenomenon was

Fig. 4. Sporulation recovery in autophagy-deficient mutants during nitrogen starvation. (a) Sporulation rate of non-auxotrophic WT (TN7), *atg1Δ* (SK43), *atg2Δ* (SK44), *atg5Δ* (SK45), *atg12Δ* (SK46) and *atg13Δ* (SK47) cells under sporulation conditions (MEA medium). The *atg* mutant designations are indicated below the respective columns. Cells were incubated at 28 °C in MEA medium for 2 days and each culture was then examined under a light microscope. A minimum of 600 cells were counted per culture to determine the proportion of asci relative to vegetative cells. (b) Sporulation rate of *atg1Δ leu* (SK29), *atg1Δ arg* (SK49), and *atg1Δ leu arg* (SK50) cells under sporulation conditions (MEA medium containing required amino acids). Sporulation rate was determined as described above. (c) Sporulation rate of WT (TN8), *atg1Δ* (SK29), *atg2Δ* (SK30), *atg3Δ* (SK31), *atg4Δ* (SK32), *atg5Δ* (SK33), *atg6Δ* (SK34), *atg7Δ* (SK35), *atg8Δ* (SK36), *atg9Δ* (SK37), *atg12Δ* (SK38), *atg13Δ* (SK39), *atg15Δ* (SK40), *atg17Δ* (SK41) and *atg22Δ* (SK42) cells under sporulation conditions with leucine (MEA medium containing 240 μg leucine ml⁻¹). SK42 was also induced to sporulate under starvation conditions without leucine. Sporulation rate was determined as described above. The *atg* mutant designations are indicated below the respective columns. (d) Sporulation rate of WT (KJ100-7B), *avt3Δ* (SK57), *avt5Δ* (SK58) and *atg22Δ avt3Δ avt5Δ* (SK59) under sporulation conditions (MEA medium) and under sporulation conditions with leucine. Sporulation rate was determined as described above.

not restricted to leucine auxotrophy. We constructed *atg1Δ* strains auxotrophic for both leucine and arginine (SK50) and for arginine alone (SK49). The sporulation defect of SK49 was partially rescued by addition of arginine alone, whereas addition of 240 μg ml⁻¹ of both leucine and arginine was required to rescue the sporulation defect of SK50 (Fig. 4b). Sporulation efficiency was not increased by the addition of more than 240 μg ml⁻¹ of either amino acid. These results suggest that the defect in sporulation of the *atg* mutants during nitrogen starvation was not caused by a genetic defect of proteins involved in the spore formation process, but rather by insufficient expression of proteins owing to a lack of amino acids in the cytosol. Kohda *et al.* (2007) showed that autophagy-defective mutants produced spores when 30 mM NH₄Cl was added after the induction of autophagy. This observation suggests that the sporulation defect of autophagy mutants was caused by a depletion of nitrogen source, but does not show whether the amino acids generated proteolytically in

the vacuole through the autophagic process were indeed recycled for use in *de novo* protein synthesis in the cytosol.

In budding yeast, seven genes have been predicted to be vacuolar amino acid transporters (AVT); Avt3p, Avt4p and Avt6p are reported to be effluxers of acidic or branched amino acids from the vacuole (Russnak *et al.*, 2001), but substrates for Avt2p, Avt5p and Avt7p have not been identified as yet. Yang *et al.* (2006) showed that *atg22p* is also a vacuolar effluxer of leucine and other amino acids, functioning redundantly with Avt3p and Avt4p, and these proteins are partially required to supply amino acids for *de novo* protein synthesis after the autophagic process. The fission yeast genome database revealed the existence of two AVT homologues, SPAC3H1.09C and SPBC1685.07C, the encoded proteins of which share 63 % and 67 % similarity with budding yeast Avt3p and Avt5p, respectively. But the function of these two homologues has not yet been determined. We generated null mutants of SPAC3H1.09C

Fig. 5. Development of the FSM during meiosis. (a) Mating and sporulation rate of GFP-Psy1p-expressing WT (YN68), *atg1Δ* (TN400) and *atg12Δ* (TN401). Cells were cultured on MEA plates with or without leucine for 2 days at 28 °C, and then observed by DIC microscopy. (b) WT (YN68), *atg1Δ* (TN400) and *atg12Δ* (TN401) mutants were sporulated on MEA medium for 2 days at 28 °C. The FSM was visualized by GFP-Psy1p. α -Tubulin was immunostained by TAT-1 antibody. DNA was stained by DAPI. Arrows indicated the visualized FSM by GFP-Psy1p. (c) Morphology of the cells upon forespore formation. Sporulation was performed as described above. DNA was stained by DAPI. Arrows indicate the FSM visualized by GFP-Psy1p.

and SPBC1685.07C (*avt3⁺* and *avt5⁺*, respectively), and observed the sporulation efficiency of these mutants. As shown in Fig. 4(d), *avt3Δ*, *avt5Δ* and an *atg22Δavt3Δavt5Δ* triple mutant that is also leucine auxotrophic showed a sporulation defect similar to that observed in *atg22Δ* (Fig. 4c), although addition of leucine restored the rate of spore formation such that it was indistinguishable from that of the wild-type parent strain KJ100-7B. These data suggest that *Sch. pombe* Avt3p and Avt5p are also responsible for spore formation, but the *avt*-defective mutants are able to process GFP-Atg8p during starvation as observed in *atg22Δ* (data not shown). From the similarity in proteins, fission yeast Atg22p, Avt3p and

Avt5p could be vacuole effluxers of amino acids. Our data suggest that amino acids that are proteolytically produced through the autophagic process might be recycled for maintenance of cellular function during nitrogen-starvation conditions in *Sch. pombe* cells.

Incomplete sporulation observed in *atg1Δ* or *atg12Δ* mutants

We observed that amino acid depletion led to incomplete sexual differentiation in *atg* mutants, but sporulation could be partially restored by amino acid supplementation. These data suggested that the sporulation process might be

influenced by amino acid depletion caused by a defect in the autophagic process. We then examined which process in sporulation of the *atg* mutant was affected. To this end, we monitored formation of the forespore membrane (FSM) by visualization with GFP-Psy1 in both *atg1Δ* (TN400) and *atg12Δ* (TN401) mutants. 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 previously reported that Psy1 is translocated from the plasma membrane to the nascent FSM at meiosis II (Nakamura *et al.*, 2001). GFP-Psy1 under the control of its native promoter was integrated into the *Sch. pombe* genome to facilitate monitoring of GFP fluorescence during sporulation. After induction of sexual differentiation in the absence of leucine, nuclear division and spore morphogenesis terminated normally in almost all wild-type cells. Formation of the FSM initiated normally near the spindle poles at meiosis II and proceeded normally to anaphase II (Fig. 5b). However, in *atg1Δ* and *atg12Δ* mutants, more than half of the cells appeared to exhibit a related deficiency in organization of the FSM and nuclear division (Fig. 5b). The *atg1Δ* and *atg12Δ* mutants exhibited abnormal elongation of the FSM. Chromosome segregations were suspended incompletely whereas the cells proceeded to anaphase II and the spindle microtubules were correctly organized (Fig. 5b). Moreover, some cells finished closure of the FSM without encapsulation of the nucleus (Fig. 5b, iv, vii and viii). In wild-type cells, closure of the FSM is typically observed more than 20 min after completion of anaphase II. We have never observed cells completing closure of the FSM without encapsulating the nucleus (Nakamura *et al.*, 2008). Failure of the spore formation process was also observed in forespore formation. WT cells were completely enclosed by forespore membrane, whereas in *atg1Δ* and *atg12Δ* mutants, only a few cells sporulated normally (Fig. 5c, i of TN400, and iv of TN401), and the majority of cells failed to complete sporulation as evidenced by incomplete tetrad formation or by abnormal forespore organization (Fig. 5c, ii and iii of TN400, v and vi of TN401). This sporulation deficiency was unlikely to be caused by repression of sporulation-specific genes because the sporulation process was not blocked at a particular stage in the same strain. Whole-genome microarray analyses have revealed that gene expression profiles are altered extensively during sporulation (Chu *et al.*, 1998; Mata *et al.*, 2002; Primig *et al.*, 2000) and this suggested that extensive *de novo* protein synthesis may be needed to execute the sporulation process. Depletion of nitrogen may decrease or halt *de novo* protein synthesis, resulting in a shortage of proteins critical for sporulation.

DISCUSSION

Autophagy is a crucial pathway for adaptation to changing environments, and thus mechanisms of autophagy are well

conserved in eukaryotes. We report five major findings from this study, using fission yeast. (1) Processing of GFP-Atg8 during nitrogen starvation could also serve as a useful marker for monitoring autophagy in *Sch. pombe*. (2) Through use of this marker, almost all Atgs predicted by sequence analysis were experimentally demonstrated to be involved in the autophagic process. (3) Autophagy in fission yeast was induced only by nitrogen starvation, and not by glucose starvation or addition of rapamycin. (4) The fission yeast AVT homologue genes (*avt3⁺* and *avt5⁺*) may be required for recycling of amino acids from the vacuolar compartment to the cytosol for maintenance of cellular function during nitrogen-starvation conditions in *Sch. pombe*. (5) Autophagy is responsible for supplying a large proportion of the amino acids required for *de novo* protein synthesis to adapt to starvation conditions.

Recent studies have also reported the existence of nitrogen-starvation-induced autophagy in fission yeast, and several budding yeast *ATG* orthologues (*ATG1*, *ATG13* and *ATG8*) have been shown to be involved in the autophagic pathway (Kohda *et al.*, 2007). Here, we identified 15 presumptive *atg* orthologues of budding yeast by sequence analysis of proteins in *Sch. pombe* GeneDB, and we suggest that they indeed function in autophagy on the basis of monitoring a GFP-Atg8p marker. However, several orthologues of budding yeast were not identified in the *Sch. pombe* genome database because of a low degree of similarity. Atg10p and Atg16p are small proteins that are critical for ubiquitin-like conjugation systems involving Atg12p. In *Pichia pastoris*, which was previously reported to undergo autophagy and pexophagy, an orthologue of *ATG10* has not yet been identified (Mukaiyama *et al.*, 2002). A homology search for *ATG14*, *ATG21*, *ATG27*, *ATG29* and *ATG31* resulted in no significant similarity. Microscopic observation of an *atg8* mutant expressing GFP-Atg8p revealed that GFP-Atg8p was translocated from the cytosol to the vacuolar lumen via an autophagosome. However, we could not observe GFP-Atg8p in the vacuolar lumen in other *atg* null mutant cells (data not shown). Western blotting showed that GFP-Atg8p expressed in an *atg8* mutant or in wild-type cells is present in two forms during nitrogen starvation: a full-length fusion protein or free GFP. In contrast, only full-length GFP-Atg8p was detected in the other *atg* null mutants expressing GFP-Atg8p, with the sole exception of *atg22Δ*. Moreover, addition of rapamycin or glucose starvation was not able to facilitate normal processing of GFP-Atg8p, consistent with studies described by Kohda *et al.* (2007). These observations also suggested that the GFP-Atg8p processing assay could provide a criterion for demonstrating autophagy in fission yeast.

The physiological role of autophagy is to maximize survival when cells are subjected to conditions of nutrient insufficiency. It is thought that cells can either degrade endogenous macromolecular constituents to provide nutrients until environmental conditions improve, or decrease dispensable functions, structures or organelles

during survival. In budding yeast, it is assumed that the nutrients recycled by the autophagic process may be used for sexual differentiation, because wild-type cells are able to initiate sporulation during starvation (Takeshige *et al.*, 1992). This amino acid recycling was not directly exhibited in budding yeast, however, because *atg* null mutants exposed to nitrogen starvation showed only cell death, and it was unclear whether nutrient shortage alone or also other factors such as apoptosis were triggers of this cell death.

Sporulation by *atg* null mutants (*atg1Δ* and *atg12Δ*) was assessed in the absence of auxotrophic requirements by monitoring formation of the forespore membrane (FSM) through visualization of GFP-Psy1. In these mutants, approximately 30 % of cells completed spore formation, but others formed incomplete tetrads caused by abnormal forespore organization or abnormal chromosome segregation (Fig. 5b, c). Interestingly, the sporulation process in these *atg* mutants was blocked at various stages. In budding yeast, autophagy-defective *atg7Δ* cells contain markedly smaller intracellular amino acid pools, and levels of *de novo* bulk protein synthesis are suppressed during nitrogen starvation (Onodera & Ohsumi, 2005). This amino acid depletion may cause rapid loss of viability and sporulation deficiency in *atg* mutants of budding yeast during nitrogen starvation. In budding yeast, *atg13Δ* or *atg1Δ* mutants are reported to lose 80 % viability after 5 days of nitrogen starvation (Funakoshi *et al.*, 1997; Tsukada & Ohsumi, 1993). In contrast, fission yeast *atg* mutant cells retain 100 % viability after 7 days of nitrogen starvation (Kohda *et al.*, 2007). This observation implies that fission yeast may possess larger pools of endogenous amino acids required for sporulation than budding yeast, and indeed we were able to observe partial complementation of the sporulation process during nitrogen starvation in fission yeast. In *Sch. pombe* or the filamentous fungus *Aspergillus fumigatus*, addition of ammonium chloride as a nitrogen source is able to initiate sporulation during nitrogen starvation (Kohda *et al.*, 2007; Richie *et al.*, 2007). This observation suggests that a nitrogen source is necessary for sporulation, but it does not reveal the pathway of amino acid recycling that follows autophagy.

Here, in fission yeast, we found that 8–15 % of non-auxotrophic cells were able to sporulate despite the absence of autophagy during starvation. In contrast, sporulation was not observed in an *atg1Δ* mutant with amino acid auxotrophy, whereas *atg1Δ* in a non-auxotrophic genetic background showed up to 15 % sporulation. These findings suggest that fission yeast is able to sporulate partially with a pooled nitrogen source despite an autophagy deficiency. On the other hand, these findings suggest that the majority of nitrogen essential for sporulation may be dependent on a source that is supplied by the autophagic degradation process.

The means by which yeast cells recycle degradation products generated by autophagy is of interest. In budding yeast, vacuolar amino acid permeases such as Atg22p,

Avt3p and Avt4p are partially redundant vacuolar amino acid efflux pumps that mediate the efflux of leucine and other amino acids resulting from autophagy (Yang *et al.*, 2006). Fission yeast also possesses *ATG22*, and an *atg22Δ* mutant was also starvation-sensitive, although *atg22Δ* cells have a much less pronounced sporulation defect than other *atg* null mutants (Fig. 4c). The fission yeast genome database revealed two AVT homologues, AVT3 and AVT5. Our investigation revealed that these two Avt proteins and Atg22p may function as vacuole effluxers of amino acids generated during the autophagic process (Fig. 4d), because null mutants of Avt3p and Avt5p showed almost the same sporulation defect as observed for *atg22Δ*. These data suggested that the amino acids generated by autophagy were effluxed redundantly by Avt3p, Avt5p and Atg22p. We then constructed an *avt3Δavt5Δatg22Δ* triple mutant; the sporulation rate of the triple mutant was almost same as that of the *atg22Δ* single null mutant (Fig. 4d). These results might suggest the existence of other redundant vacuole effluxers of amino acids. We recently reported that the fission yeast *fnx1⁺* and *fnx2⁺* genes, which are homologous to the budding yeast *VBA* (vacuolar transporter for basic amino acids) genes, are involved in vacuolar amino acid uptake in *Sch. pombe* (Chardwiriyaapreecha *et al.*, 2008). We tested sporulation of *fnx1Δ* and *fnx2Δ* cells, and found that they sporulate normally during nitrogen starvation (unpublished results). These results suggest that Fnx1p and Fnx2p function only in vacuolar amino acid influx, and may not be involved in vacuolar amino acid efflux in *Sch. pombe*. We are currently attempting to identify other proteins involved in the amino acid recycling that follows nitrogen starvation-induced autophagy.

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