Introduction

As one of the major degradative mechanisms conserved among eukaryotic cells, autophagy mediates the turnover and recycling of long-lived cytosolic proteins and organelles in the lysosome/vacuole. In the yeast *Saccharomyces cerevisiae*, the integral membrane protein Atg9 (autophagy-related protein 9) cycles between mitochondria and the preautophagosomal structure (PAS), the nucleating site for formation of the sequestering vesicle, suggesting a role in supplying membrane for vesicle formation and/or expansion during autophagy. To better understand the mechanisms involved in Atg9 cycling, we performed a yeast two-hybrid–based screen and identified a peripheral membrane protein, Atg11, that interacts with Atg9. We show that Atg11 governs Atg9 cycling through the PAS during specific autophagy. We also demonstrate that the integrity of the actin cytoskeleton is essential for correct targeting of Atg11 to the PAS. We propose that a pool of Atg11 mediates the anterograde transport of Atg9 to the PAS that is dependent on the actin cytoskeleton during yeast vegetative growth.
(Kim et al., 2002; Noda et al., 2002). In most endomembrane trafficking systems, such as the early secretory pathway, vesicles form by budding from the surface of a preexisting organelle. However, in autophagy-related processes, the double-membrane system is used to create vesicles and may be transported to the assembly site. Among all Atg proteins, Atg9 is the best candidate to help us understand this pivotal issue. Atg9 is the only characterized integral membrane protein required for both autophagosome and Cvt vesicle formation (Noda et al., 2000). However, this protein is absent from the completed vesicles, suggesting that it is retrieved before the vesicle sealing/completion step. Atg9 localizes to multiple punctate sites, with one of them corresponding to the PAS and others to mitochondria in addition to unidentified membrane sites (Reggiori et al., 2005b). Recent studies reveal that Atg9 cycles between mitochondria and the PAS vesicle assembly site (Reggiori et al., 2004, 2005b). These characteristics make Atg9 a potential membrane carrier for vesicle formation.

We decided to investigate the molecular regulatory mechanisms underlying Atg9 cycling and, in particular, what factors regulate the anterograde transport of Atg9 to the site of vesicle formation. In this study, we discovered that a peripheral membrane protein, Atg11 (Kim et al., 2001), is an interaction partner of Atg9. The interaction requires the second coiled-coil (CC) domain of Atg11 and the Atg9 N-terminal cytosolic domain. A misense mutation (H192L) in the Atg9 N-terminal domain that disrupts its interaction with Atg11 results in the impaired cycling of Atg9 and a defect in selective autophagy. In addition, we found that in actin mutant cells, Atg11 colocalized with Atg9 and was retained on mitochondria, indicating that Atg11 is not able to direct Atg9 to the PAS in the absence of an intact cytoskeletal network. These data support a model in which a pool of Atg11 links Atg9 to the PAS along the actin cable under vegetative growth conditions.

**Results**

**Atg11 is an interaction partner of Atg9**

Atg11 interacts with Atg9 independently of Atg1 and Atg19. Wild-type (WT) strains expressing Atg11-GFP and Atg9–protein A fusions were used for affinity isolation. Total lysates and eluted polypeptides (Aff.Pur.) were separated by SDS-PAGE and detected with anti-YFP antibody. The same amounts of the total lysate (T) and immunoaffinity purified isolate (IP) were loaded per gel lane. (C) Atg11 is coprecipitated with Atg9 in the absence of Atg23 and Atg27. Wild-type (WT), atg23Δ (CCH004), and atg27Δ (CCH006) strains expressing integrated Atg11-GFP and Atg9-PA fusions were used for affinity isolation. Total lysates and eluted polypeptides (Aff.Pur.) were separated by SDS-PAGE and visualized by immunoblotting with antibody to YFP. An Atg11-GFP strain (PSY101) expressing CUP1-driven PA (pCuPA[414]) alone was used as a control.

Figure 1. **Atg9 interacts with Atg11.** (A) A yeast two-hybrid assay reveals that full-length Atg9 interacts with full-length Atg11. The two-hybrid strain PJ69-4A was cotransformed with plasmids containing the activation domain (AD)–fused Atg11 and the binding domain (BD)–fused Atg9 or with empty vectors (AD and BD). Interactions were monitored by the ability of cells to grow on plates without histidine for 3 d. [B] Atg11 is coprecipitated with Atg9 independently of Atg1 and Atg19. Wild-type (YY161), atg1Δ (YY162), and atg19Δ (CCH005) strains expressing integrated Atg11-GFP and Atg9–protein A (PA) fusions were used for affinity isolation. Wild-type (WT) strains expressing integrated Atg9-PA alone (FRY171) or integrated Atg11-GFP [PSY101] and CUP1-promoter-driven PA (pCuPA[414]) were used as controls. Eluted polypeptides were separated by SDS-PAGE and detected with anti-YFP antibody. The same amounts of the total lysate (T) and immunoaffinity purified isolate (IP) were loaded per gel lane. (C) Atg11 is coprecipitated with Atg9 in the absence of Atg23 and Atg27. Wild-type (WT), atg23Δ (CCH004), and atg27Δ (CCH006) strains expressing integrated Atg11-GFP and Atg9-PA fusions were used for affinity isolation. Total lysates and eluted polypeptides (Aff.Pur.) were separated by SDS-PAGE and visualized by immunoblotting with antibody to YFP. An Atg11-GFP strain (PSY101) expressing CUP1-driven PA (pCuPA[414]) alone was used as a control.

**Atg11 interacts with Atg9.** Atg11 is the only characterized integral membrane protein required for both autophagosome and Cvt vesicle formation (Reggiori and Klionsky, 2005). One of the major current challenges is to unveil where the membrane materials for autophagosome or Cvt vesicles come from and how the lipids are transported to the assembly site. Among all Atg proteins, Atg9 is the best candidate that can help us understand this pivotal issue. Atg9 is the only characterized integral membrane protein required for both autophagosome and Cvt vesicle formation (Noda et al., 2000). However, this protein is absent from the completed vesicles, suggesting that it is retrieved before the vesicle sealing/completion step. Atg9 localizes to multiple punctate sites, with one of them corresponding to the PAS and others to mitochondria in addition to unidentified membrane sites (Reggiori et al., 2005b). Recent studies reveal that Atg9 cycles between mitochondria and the PAS vesicle assembly site (Reggiori et al., 2004, 2005b). These characteristics make Atg9 a potential membrane carrier for vesicle formation.

We decided to investigate the molecular regulatory mechanisms underlying Atg9 cycling and, in particular, what factors regulate the anterograde transport of Atg9 to the site of vesicle formation. In this study, we discovered that a peripheral membrane protein, Atg11 (Kim et al., 2001), is an interaction partner of Atg9. The interaction requires the second coiled-coil (CC) domain of Atg11 and the Atg9 N-terminal cytosolic domain. A misense mutation (H192L) in the Atg9 N-terminal domain that disrupts its interaction with Atg11 results in the impaired cycling of Atg9 and a defect in selective autophagy. In addition, we found that in actin mutant cells, Atg11 colocalized with Atg9 and was retained on mitochondria, indicating that Atg11 is not able to direct Atg9 to the PAS in the absence of an intact cytoskeletal network. These data support a model in which a pool of Atg11 links Atg9 to the PAS along the actin cable under vegetative growth conditions.

Results

Atg11 is an interaction partner of Atg9

Atg9 is the only known transmembrane protein required for both bulk autophagy and selective autophagic processes (e.g., the Cvt pathway; Noda et al., 2000). Unlike most other Atg proteins, which are restricted to the perivacuolar PAS, Atg9 localizes to several punctate structures; one of them is at the PAS, whereas the others are primarily confined to mitochondria (Reggiori et al., 2005b). Atg9 cycles between the two compartments, suggesting that it plays a role in providing lipids to the forming autophagosomes or Cvt vesicles (Reggiori et al., 2004). However, other than actin (Reggiori et al., 2005a), the factors that regulate the anterograde transport of Atg9 to the PAS have not been identified. Therefore, we performed a yeast two-hybrid–based screen of Atg proteins to identify potential Atg9-interacting proteins. Yeast two-hybrid cells harboring Atg9 and a peripheral membrane protein, Atg11, showed robust growth on plates lacking histidine, indicating that Atg11 could interact with Atg9 (Fig. 1 A). The same result was obtained on plates lacking adenine (unpublished data).

Atg11 functions in selective types of autophagy (i.e., the Cvt pathway and pexophagy) but is not essential for bulk autophagy (Kim et al., 2001). Atg11 plays a role in organizing the PAS and linking cargo to the vesicle-forming machinery at the PAS (Shintani et al., 2002; Yorimitsu and Klionsky, 2005). This protein is a component of at least two complexes in yeast. One is the Atg1–Atg11 complex, which is involved in the induction of bulk and selective autophagy (Kamada et al., 2000). The other is the Atg19–Atg11 complex, which recognizes and delivers preApe1 and α-mannosidase to the PAS (Shintani et al., 2002; Yorimitsu and Klionsky, 2005). We decided to determine whether these complexes are involved in Atg11 and Atg9 interaction. To address this issue, we used a biochemical approach to examine whether Atg11 was able to form a complex with Atg9. We tagged Atg11 with GFP and tagged Atg9 with protein A (PA) at the chromosomal locus. Wild-type, atg1Δ, and atg19Δ
cells expressing the integrated Atg9-PA and Atg11-GFP fusions were lysed, and the PA-tagged protein was isolated with IgG–Sepharose beads. Atg11 was coprecipitated with Atg9-PA in all three strains (Fig. 1 B), which verifies that these two proteins are present in a complex, although we do not know whether they interact directly. Thus, the absence of either Atg1 or Atg19 did not affect the formation of a complex between Atg9 and Atg11. This finding suggests that there might be multiple populations of Atg11 within the cell that interact with different sets of Atg proteins.

Our recent data show that two other Atg proteins, Atg23 and Atg27, interact with Atg9 and are required for Atg9 cycling. The interaction between Atg9 and either Atg23 or Atg27 is not mediated through Atg11 (Tucker et al., 2003; and unpublished data). Thus, we extended the analysis by determining whether the Atg9–Atg11 interaction was dependent on these other Atg9-interacting proteins. We found that Atg11 was coprecipitated with Atg9 in atg23Δ and atg23Δ atg27Δ cells despite a lower overall efficiency of recovery (Fig. 1 C). This suggests that Atg9 and Atg11 were able to form a complex in the absence of Atg23 and Atg27, although these two proteins may facilitate the interaction. The interaction between Atg9 and Atg11 in the atg23Δ or atg27Δ strains was confirmed by yeast two-hybrid analyses; the atg23Δ or atg27Δ two-hybrid cells expressing Atg9 and Atg11 were able to grow on -histidine (-His) selective plates, which is comparable with the wild-type cells (unpublished data).

Atg11 is predicted to contain four CC domains (Fig. 2 A; Yorimitsu and Klionsky, 2005). Each CC domain mediates interactions of Atg11 with different Atg proteins. To test whether these CC domains are responsible for the interaction between Atg11 and Atg9, we used CC domain deletion mutants in a series of yeast two-hybrid assays. As shown in Fig. 2 B, the two-hybrid mutant activation domain (AD)–Atg11 C-terminal truncation (11N; Δ627–1,178 and lacking CC3-4) allowed the cells to grow in the presence of binding domain (BD)–Atg9 as well as the full-length AD-Atg11. In contrast, cells expressing an AD-Atg11 N-terminal truncation (11C; Δ1–817 and lacking CC1-3) could not grow on selective -His plates (Fig. 2 B). Thus, the Atg11 N terminus is sufficient for Atg9–Atg11 interaction. In addition, Atg11ΔCC2 (Δ536–576) abolished the interaction with Atg9, whereas cells containing mutants Atg11ΔCC1 (Δ272–321), Atg11ΔCC3 (Δ627–858), or Atg11ΔCC4 (Δ859–1,178) grew well on selective plates (Fig. 2 C). These data indicated that Atg11 CC domain 2 is required for the Atg9–Atg11 interaction.

From a hydrophathy plot analysis, Atg9 contains hydrophilic N and C termini flanking six to eight transmembrane domains (Noda et al., 2000). To define the topology of Atg9, we investigated the protease sensitivity of the Atg9 N and C termini. Spheroplasts derived from pep4Δ cells expressing either the N-terminal–tagged PA-Atg9 fusion or the C-terminal–tagged Atg9-PA fusion were osmotically lysed and centrifuged at 13,000 g. In agreement with previous studies, approximately two thirds of the total Atg9 was present in the S13 supernatant fraction that contained the PAS, and one third was present in the P13 pellet fraction (Fig. 2 D; Noda et al., 2000; Reggiori et al., 2005b).

Figure 2. The Atg9–Atg11 interaction is mediated by the Atg11 CC2 domain and the Atg9 N-terminal region. (A) Schematic representation of Atg11 highlighting the position of the four CC domains and indicating the extent of the N- and C-terminal domains used in the two-hybrid analysis. (B) The Atg11 N terminus is sufficient to interact with Atg9 as demonstrated by the yeast two-hybrid assay. Cells expressing an Atg11 N-terminal fragment and Atg9 (11N-9), full-length Atg11 and Atg9 (11-9), the Atg11 C terminus and Atg9 (11C-9), Atg11 (11), or Atg9 (9) with the corresponding empty vector were grown for 3 d on plates lacking histidine. (C) Atg11 CC domain 2 is essential for Atg11–Atg9 interaction. Cells expressing Atg9 and Atg11 lacking the indicated coiled-coil (CC) domains were grown for 3 d on -His plates. (D) Both the N and C termini of Atg9 are protease sensitive. A schematic representation of Atg9 topology is shown on the left. pep4Δ cells expressing either integrated C-terminally tagged Atg9-PA (FY172) or CUP1-driven N-terminally tagged PA-Atg9 (TVY1; pCuPAAtg9[9.1]) were converted to spheroplasts and osmotically lysed, and the pellet fraction was subjected to protease K [PK] treatment in the presence or absence of Triton X-100. Fractions were analyzed by immunoblotting with antibodies to PA and Pho8. T, total. (E) The Atg9 N terminus interacts with Atg11. A schematic diagram of Atg9 and domains used for two-hybrid analysis are depicted. Cells expressing Atg11 and the Atg9 N-terminal fragment [N], transmembrane domain [TM], C terminus [C], or full-length Atg9 [FL] were grown for 3 d on -His plates. (F) Atg9 N-terminal amino acids 159–255 are the minimal sufficient region needed to interact with Atg11. A truncation series of the Atg9 N-terminal region in the binding domain [BD] two-hybrid plasmid is depicted. The strength of the corresponding proteins to interact with activation domain [AD]–Atg11 is indicated on the right by the robustness of cell growth on plates without histidine [-His] or adenine [-Ade] for 5 d.
When the P13 fraction was treated with exogenous proteinase K, both the N- and C-terminal PA tags were cleaved in the absence or presence of detergent, and no bands of smaller molecular mass were detected (Fig. 2 D), indicating that both the N and C termini of Atg9 were accessible to protease on the cytosolic side of the membrane. To verify that intracellular membranous structures were intact after osmotic lysis, we simultaneously monitored the protease sensitivity of an endogenous vacuole membrane protein, Pho8. The precursor form of Pho8 that accumulated in the pep4Δ background contains a small cytosolic tail and a luminally oriented propeptide (Fig. 2 D). In the absence of detergent, only the Pho8 cytosolic tail but not the luminal propeptide was accessible to proteinase K. Upon the addition of both detergent and proteinase K, the Pho8 luminal propeptide was removed as a result of the disruption of all membranous compartments, which is shown as a further shift of the molecular mass (Fig. 2 D). Thus, these data verified the integrity of the relatively fragile vacuole and presumably other intracellular membranous compartments after osmotic lysis, suggesting that both the N and C termini of Atg9 are exposed to the cytosol (Fig. 2 D). Accordingly, the topology of Atg9 appears to be conserved between yeast and mammalian cells (Young et al., 2006).

So far, no known functional domains have been identified in the Atg9 N- or C-terminal regions. To further analyze the Atg9–Atg11 interaction, we generated truncated Atg9 mutants containing the N terminus, C terminus, or transmembrane region. As shown in Fig. 2 E, in the presence of Atg11, the Atg9 N-terminal domain supported the growth of two-hybrid cells as well as the full-length Atg9, whereas neither the C-terminal nor the transmembrane domains of Atg9 were able to do so (Fig. 2 E). This result showed that Atg11 interacts with the N-terminal region of Atg9. We further constructed a series of Atg9 N-terminal truncation mutants and analyzed them for interaction with Atg11 by yeast two-hybrid analysis. Atg9 N-terminal amino acids 159–255 appeared to be the minimal region that mediates the interaction with Atg11 (Fig. 2 F). Collectively, we concluded that Atg11 and Atg9 interact through the Atg11 CC domain 2 and the Atg9 N terminus.

**Atg11 recruits Atg9 to the PAS**

In wild-type cells, Atg11 localizes at the PAS (Kim et al., 2001), and Atg9 cycles between the PAS and mitochondria (Reggiori et al., 2004, 2005b). To examine the role of the interaction between Atg9 and Atg11, we analyzed the cycling of Atg9 in the presence of overexpressed Atg11 or in the absence of this protein. The chromosomally tagged Atg9-YFP chimera displayed a multiple punctate distribution in wild-type cells, with one of the puncta colocalizing with the PAS marker blue fluorescent protein (BFP)–Ape1 (Fig. 3 A). In contrast, in the atg1Δ background, Atg9 was restricted to the PAS, which is in agreement with previous observations that indicated a role for Atg1 in the retrograde transport of Atg9 from the PAS to mitochondria (Reggiori et al., 2004, 2005a,b). In 91% (109/120) of the cells overexpressing Atg11, Atg9-YFP localized solely to the perivacuolar PAS (represented by CFP-Atg11), which is similar to the situation observed in atg1Δ cells (Fig. 3 A; Kim et al., 2002), and did not localize to mitochondria (not depicted). Thus, excess Atg11 was able to restrict Atg9 to the PAS. Overexpressed Atg11 displays a dominant-negative phenotype, interfering with...
the vacuolar import of prApe1 through the Cvt pathway (unpublished data). The dominant-negative phenotype presumably reflects the defect in Atg9 cycling.

To further examine the role of Atg11 in recruiting Atg9 to the PAS, we used the TAKA (transport of Atg9 after knocking out ATG1) assay (Cheong et al., 2005). This assay examines the epistasis of a second mutation relative to atg1Δ with regard to Atg9 localization at the PAS. We visualized chromosomally tagged Atg9-YFP in atg1Δ single and atg1Δ atg11Δ double deletion cells and simultaneously labeled the vacuolar membrane with FM 4-64. As shown previously, in atg1Δ cells, Atg9-YFP localized to a single perivacuolar punctum, which corresponds to the PAS (Fig. 3 B). In contrast, in nearly 90% of the atg11Δ or atg1Δ atg11Δ mutants, Atg9-YFP fluorescence showed multiple puncta and did not localize at a single perivacuolar structure, suggesting that its anterograde transport was blocked because of the ATG11 deletion (Fig. 3 B). These results suggest that Atg11 is involved in the anterograde transport of Atg9.

Our mapping of the Atg9 and Atg11 interaction domains indicated that Atg11ΔCC2 but not Atg11 lacking its other CC domains was defective in forming a complex with Atg9 (Fig. 2). Accordingly, we examined the effect of Atg11 CC deletions on Atg9 subcellular distribution. As shown in Fig. 3 C, in cells expressing Atg11ΔCC1 in the atg1Δ background, Atg9 localized to the PAS, which is similar to the result seen in atg1Δ cells expressing wild-type Atg11. In contrast, in cells expressing Atg11ΔCC2, which lacks the Atg9-interacting domain, Atg9 displayed a multiple punctate distribution (Fig. 3 C) resembling to the PAS, which is similar to the result seen in atg1Δ cells expressing wild-type Atg11, Atg9-YFP localized to a single perivacuolar punctum, which corresponds to the PAS (Fig. 3 B). In contrast, in nearly 90% of the atg11Δ or atg1Δ atg11Δ mutants, Atg9-YFP fluorescence showed multiple puncta and did not localize at a single perivacuolar structure, suggesting that its anterograde transport was blocked because of the ATG11 deletion (Fig. 3 B). These results suggest that Atg11 is involved in the anterograde transport of Atg9.

The Atg9H192L mutant is defective for the Cvt pathway

To further clarify the physiological functions of the Atg9–Atg11 interaction, we decided to test whether autophagic processes were affected by its disruption. It has been reported that Atg11 CC domain 2, the Atg9-interacting domain, interacts with multiple Atg proteins, including at least one (Atg1) that is involved in Atg9 retrograde transport (Yorimitsu and Klionsky, 2005). Thus, we concluded that the interaction of Atg9 with Atg11 CC domain 2 is required to direct Atg9 to the PAS.

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This indicates that the interaction between Atg9 and Atg11 was abolished by the H192L mutation. The loss of Atg9–Atg11 interaction was confirmed by a communoprecipitation assay. As shown in Fig. 4 B, endogenous Atg11 was recovered only with wild-type Atg9 but not with Atg9H192L. In contrast, yeast two-hybrid data indicated that the interaction between Atg9 and Atg11 was abolished in growing conditions.

Anterograde movement of Atg9H192L is blocked in growing conditions

We were interested in determining whether the impairment of the Cvt pathway seen with Atg9H192L (Fig. 4 B) was caused by defects in Atg9 cycling, particularly anterograde movement to the PAS. Accordingly, we used the TAKA assay to visualize the cycling of Atg9H192L–GFP in growing conditions and concurrently stained mitochondria with the dye MitoFluor red. In the atg1Δ background, wild-type Atg9-GFP was restricted to the...
Atg9-GFP (pAPG9GFP(416)) or Atg9H192L-GFP (pAPG9H192L-GFP(416)), and a plasmid expressing wild-type Atg1 (pAPG9GFP(416)) or Atg9H192L-GFP (pAPG9H192L-GFP(416)). Cells were cultured to mid-log phase and stained with MitoFluor red 589 as described in Materials and methods before imaging by fluorescence microscopy. Arrows mark the locations of BFP-Ape1. DIC, differential interference contrast. Bar, 2 μm.

Atg9-GFP BFP-Ape1 Mitochondria Merge DIC

Atg9H192L-GFP BFP-Ape1 Mitochondria Merge DIC

Figure 5. Anterograde transport of Atg9 to the PAS is impaired in the Atg9H192L mutant under growing conditions. The atg1Δ atg9Δ strain (CCH001) was cotransformed with a BFP-Ape1 plasmid (pBF-Pape1(414)) and a plasmid expressing wild-type Atg9-GFP (pAPG9GFP(416)) or Atg9H192L-GFP (pAPG9H192L-GFP(416)). Cells were cultured to mid-log phase and stained with MitoFluor red 589 as described in Materials and methods before imaging by fluorescence microscopy. Arrows mark the locations of BFP-Ape1. DIC, differential interference contrast. Bar, 2 μm.

Bulk autophagy is not affected by disruption of the Atg9–Atg11 interaction

Atg9 is essential for both bulk autophagy and the Cvt pathway, whereas Atg11 is required solely in the Cvt pathway (Noda et al., 2000; Kim et al., 2001). To clarify whether Atg9 anterograde transport via Atg11 is involved in bulk autophagy, we used the Atg9H192L mutant to analyze the progression of bulk autophagy by several established assays.

Atg8 conjugated to phosphatidylethanolamine remains associated with the completed autophagosome and is a marker for autophagic delivery to the vacuole (Kirisako et al., 1999, 2000; Huang et al., 2000). After delivery of the GFP-tagged Atg8 chimera, the GFP moiety is cleaved and remains relatively stable in the vacuole, whereas Atg8 is rapidly degraded. Thus, the accumulation of free GFP reflects the progression of bulk autophagy, which can be readily detected by Western blotting (Shintani and Klionsky, 2004b; Cheong et al., 2005). In atg9Δ cells, essentially no free GFP was detected, indicating that bulk autophagy was blocked by ATG9 deletion (Fig. 6 A). In cells expressing wild-type Atg9 or Atg9H192L, free GFP was detectable starting 2 h after cells were shifted to starvation conditions (synthetic medium lacking nitrogen [SD-N]) to induce bulk autophagy, although GFP-Atg8 processing showed a delay in Atg9H192L cells compared with wild-type cells. This result demonstrated that bulk autophagy retained similar activity even when Atg9 failed to interact with the Cvt-specific component Atg11.

To confirm the aforementioned result, we quantitatively measured bulk autophagy activity using another marker protein, Pho8Δ60, which encodes an altered form of alkaline phosphatase that is only delivered to the vacuole via autophagy (Noda et al., 1995). The Pho8Δ60 enzymatic activity was measured in wild-type and atg1Δ cells and in atg9Δ cells transformed with a plasmid expressing wild-type Atg9 or Atg9H192L or an empty vector in growing (synthetic minimal medium [SMD]) and starvation (SD-N) conditions (Fig. 6 B). The atg1Δ or atg9Δ cells transformed with an empty vector showed only the basal level of Pho8Δ60 activity after autophagy induction (SD-N), indicating that bulk autophagy was defective after deleting either gene. In contrast, there was an increase of Pho8Δ60 activity in atg9Δ cells expressing either wild-type Atg9 or Atg9H192L, indicating that Atg9H192L rescued the autophagy defect in atg9Δ cells, which is comparable with the wild-type Atg9 protein. Collectively, these data suggested that bulk autophagy does not depend on the interaction between Atg9 and an Atg protein that is needed for anterograde movement during specific autophagy (Atg11).

Figure 6. Bulk autophagy is normal with the Atg9H192L mutant. (A) GFP-Atg8 processing is not affected by Atg9H192L. The atg9Δ strain (JKY007) was cotransformed with a GFP-Atg8 plasmid (pGFPAtg8(414)) and a plasmid expressing wild-type Atg9 (pAPG9GFP(416)), Atg9H192L (pAPG9H192L-GFP(416)), or an empty vector (pRS416; vec). Cells were grown in SMD to mid-log phase and shifted to starvation conditions (SD-N). At the indicated time points, aliquots were taken, and protein extracts were analyzed by Western blotting using anti-GFP antibody. The positions of GFP-Atg8 and free GFP are indicated. The asterisks mark nonspecific bands. (B) Pho8Δ60 activity is normal in Atg9H192L-expressing cells. The wild-type (WT; YTS158) and atg1Δ strain (TY1158) and atg9Δ strain (CCH002) transformed with a plasmid expressing wild-type Atg9 (pAPG9GFP(416)), Atg9H192L (pAPG9H192L-GFP(416)), or an empty vector (pRS416; vec). Cells were grown in SMD to mid-log phase and shifted to SD-N for 4 h. The Pho8Δ60 activity was measured as described in Materials and methods. Error bars indicate the SEM of three independent experiments.
**Atg9H192L cycling is normal during bulk autophagy**

Because bulk autophagy activity was not affected by the Atg9 H192L mutation, it was tempting to speculate that Atg9H192L cycled normally under starvation conditions even though it was not capable of forming a complex with Atg11. To test this hypothesis, we visualized the movement of Atg9H192L by the TAKA assay after autophagy induction. Cells were treated with the drug rapamycin, which mimics starvation conditions and induces bulk autophagy. As shown in Fig. 7, in the atg1Δ background without rapamycin treatment (−rap), wild-type Atg9-GFP was restricted to the PAS, whereas Atg9H192L-GFP could not move to the PAS and displayed a multiple punctate localization. After treatment with rapamycin (Fig. 7, +rap), in 85% (41/48) of the cells, Atg9H192L-GFP colocalized with the PAS marker BFP-Ape1 similarly to wild-type Atg9, indicating that Atg9 recruitment to the PAS was normal. Therefore, this result demonstrated that during bulk autophagy, the Atg9H192L mutation did not interfere with the cycling of Atg9.

**Atg11 localization to the PAS is dependent on the actin cytoskeleton**

Recently, we have shown that Atg9 anterograde traffic to the PAS is blocked when the actin cytoskeleton is disrupted by either treatment with the drug latrunculin A or point mutations in ACT1, the gene encoding actin (Reggiori et al., 2005a). In particular, the impairment of actin function leads to a defect in the Cvt pathway, whereas bulk autophagy is normal; a similar phenotype was also observed with the Atg9H192L mutant. Because Atg9 anterograde transport was also dependent on its interaction with Atg11 (Fig. 3), we wondered whether there was a functional connection between actin and Atg11 in Atg9 cycling and autophagic processes. Previous data show that Atg11 is needed to recruit prApe1 to the PAS (Yorimitsu and Klionsky, 2005). The atg9-null mutant does not affect the recruitment of prApe1 to the PAS or the localization of Atg11 (unpublished data), suggesting that Atg11 localization at the PAS is not dependent on the Atg9–Atg11 interaction. Thus, we propose that Atg11 mediates actin-dependent Atg9 cycling in the Cvt pathway.

**Discussion**

Atg9 is the only characterized transmembrane protein involved in the formation of the sequestering vesicles that form during the Cvt pathway, pexophagy, and autophagy. Accordingly, it is the best candidate to mark the source of the vesicle membrane. Recently, we have shown that Atg9 localizes to mitochondria in addition to the PAS, implicating this organelle in supplying membrane during autophagy-related processes (Reggiori et al., 2005b). Only Atg19, which is a receptor for biosynthetic cargos, and Atg8 remain associated with the completed vesicles; most of the soluble Atg proteins involved in vesicle formation presumably dissociate from the membrane before or upon vesicle completion. In contrast, a specific retrieval mechanism operates in the cycling of Atg9 and the associated protein Atg23 (Reggiori et al., 2004). The retrograde movement of Atg9 from the PAS to mitochondria requires Atg1–Atg13, Atg2, Atg18, and the PtdIns3–kinase complex. The transit of Atg9 to the PAS involves Atg23, Atg27, and actin (Reggiori et al., 2005a;
Yen et al., 2006; and unpublished data); however, the mechanism by which actin mediates Atg9 movement is not known.

To identify other Atg components involved in the anterograde movement of Atg9, we performed a yeast two-hybrid screen for proteins that interact with Atg9 and identified Atg11 (Fig. 1). This result is intriguing because Atg11 has previously been shown to interact with Atg19 and Atg1, which are components involved in distinct steps of a specific autophagic process. In conjunction with the present result, we propose that Atg11 acts as a scaffold to coordinate the delivery of multiple components, including the cargo–receptor complex, components involved in vesicle formation, and proteins involved in supplying membrane, to the site of vesicle formation, the PAS (Fig. 9). The Atg11 CC domain interacts with the N terminus of Atg9 (Fig. 2), and the interaction occurs in the absence of Atg1 or Atg19 (Fig. 1), suggesting that there are distinct and multiple populations of Atg11 within the cell. Atg11 self-interaction (Yorimitsu and Klionsky, 2005) may then allow these various populations of Atg proteins to be delivered to the PAS in a coordinated manner. Consistent with this model, the overexpression of Atg11 restricted Atg9 to the PAS, presumably as a result of enhanced delivery (Fig. 3). In contrast, a mutation of H192L that disrupts interaction between Atg9 and the specific autophagy component Atg11 resulted in a defect in transporting Atg9 to the PAS (Fig. 5). Furthermore, the absence of Atg9 at the PAS caused by this point mutation led to a block in the Cvt pathway (Fig. 4).

It is known that Atg11 is needed for specific types of autophagy such as the Cvt pathway but is not essential for nonspecific autophagy (Kim et al., 2001). We found that the Atg9 point mutant that disrupts the interaction with Atg11 imposed little effect on the bulk autophagy induced during starvation (Fig. 6). The essentially normal autophagy function was correlated with the normal localization/transport of the binding-defective Atg9 mutant in the presence of the autophagy inducer rapamycin (Fig. 7). This finding suggests that the anterograde transport of Atg9 during bulk autophagy may be mediated by a different mechanism that is at least relatively independent of Atg11. Thus, other proteins that may interact with Atg9 deserve further investigation to reveal the Atg9 cycling machinery that operates during bulk autophagy; however, we cannot rule out the possibility that a low level of interaction between Atg9H192L and Atg11 allows the anterograde transport of Atg9H192L after autophagy induction.

Finally, we found that actin is required for the localization of Atg11 to the PAS (Fig. 8). This observation, coupled with the role of Atg11 in Atg9 anterograde movement but not vice versa, suggests that Atg11 mediates the connection between actin and Atg9 delivery from the mitochondria to the PAS. Actin is not needed for bulk autophagy in yeast (Reggiori et al., 2005a), which is in agreement with our findings in the present paper that the Atg9H192L mutant is not defective for nonspecific autophagy. It is not known how Atg9 might move along actin cables. The third Atg11 CC domain displays some similarity with that of Yen et al., 2006; and unpublished data; however, the mechanism by which actin mediates Atg9 movement is not known.

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Myo2; however, Atg11 lacks the N-terminal motor domain that functions in Myo2 movement (Monastyrsk et al., 2006). Thus, it is not clear how Atg11 might actually mediate the anterograde movement of Atg9. Continued analysis of Atg9 cycling and its interactions with Atg11 and other Atg proteins may provide insight into the underlying mechanisms of membrane delivery during Cvt vesicle and autophagosome formation.

Materials and methods

Strains, plasmids, and media
The S. cerevisiae strains used in this study are listed in Table I. For gene disruption, the entire coding region was replaced by the S. cerevisiae TRP1, the Kluyveromyces lactis LEU2 or URA3, the Saccharomyces klyveri HIS3, or the Escherichia coli kan gene using PCR primers containing ~50 bases of identity to the regions flanking the open reading frame. For PCR-based integrations of the PA and GFP tags at the 3’ end of the ATG9 and ATG11 genes, pHAB102 and pFA6a-CFP-HIS3 were used as templates to generate strains expressing fusion proteins under the control of their native promoters (Longtine et al., 1998; Abellovich et al., 2003).

Yeast cells were grown in rich medium (YPD; 1% yeast extract, 2% peptone, and 2% glucose) or SMD (0.67% yeast nitrogen base, 2% glucose, amino acids, and vitamins as needed). Starvation experiments were conducted in SD-N (0.17% yeast nitrogen base without amino acids and 2% glucose).

Plasmids expressing Atg11 truncations (Yarimitsu and Klionsky, 2005), GFP-Atg8 (pGFP-AUT7(414); Abellovich et al., 2003), Atg9 (pAPG9(416); essentially constructed the same as pAPG9(414); Noda et al., 2000), Atg9-GFP (pAPG9GFP(416); Noda et al., 2000), and CFP-Atg11 (pCuHACFPCVTP(416); Kim et al., 2002) have been described previously.

Table I. Yeast strains used in this study

| Strain   | Genotype                        | Reference          |
|----------|---------------------------------|--------------------|
| BY4742   | MATα ural3a leu2Δ his3Δ lys2Δ   | Invitrogen         |
| CCH001   | SEY6210 atg9α::HIS5 atg1Δ::LEU2 | This study         |
| CCH002   | YTS158 atg9α::HIS5             | This study         |
| CCH004   | TYY161 atg23α::URA3            | This study         |
| CCH005   | TYY161 atg19α::URA3            | This study         |
| CCH006   | TYY161 atg23α::URA3 atg27α::LEU2| This study         |
| CCH007   | PSY101 atg9α::LEU2             | This study         |
| DDY1493  | MATα ura3-52 leu2-3,112 his3ΔΔ200 tub2-201 act1-159::HIS3 | Drubin et al., 1993 |
| FRY136   | SEY6210 ATG9-YFP::HIS3         | Cheong et al., 2005|
| FRY138   | SEY6210 ATG9-YFP::HIS3 atg1Δ::URA3 | Reggiori et al., 2004|
| FRY171   | SEY6210 ATG9-PA::TRP1          | This study         |
| FRY172   | SEY6210 ATG9-PA::TRP1 pep4Δ::LEU2| Reggiori et al., 2004|
| FRY245   | SEY6210 ATG9-RFP::HIS3         | Reggiori et al., 2005b|
| IRA004   | DDY1493 ATG9-RFP::KAN          | Reggiori et al., 2005b|
| JKY007   | SEY6210 atg9α::HIS3            | Unpublished data   |
| JLY8     | P69-4A atg23α::KAN-J.          | Noda et al., 2000  |
| JLY88    | P69-4A atg27α::KAN             | Unpublished data   |
| KTY53    | SEY6210 ATG9-YFP::HIS3 atg11Δ::URA3 | Reggiori et al., 2004|
| P69-4A   | MATα leu2-3,112 trp1-Δ901 ura3-52 his3ΔΔ200 gal4Δ gal80α δys2::GAL1-HIS3 GAL2ADE2 met2::GAL7-locZ | James et al., 1996|
| P6101    | SEY6210 ATG11-GFP::HIS3        | This study         |
| SEY6210  | MATα ura3-52 leu2-3,112 his3ΔΔ200 trp1-Δ901 lys2-801 suc2Δ9 mep GAL | Robinson et al., 1988|
| TY11     | SEY6210 pep4Δ::LEU2            | Gerhardt et al., 1998|
| TYY117   | YTS158 atg1Δ::HIS5             | This study         |
| TYY1161  | SEY6210 ATG11-GFP::HIS3 ATG9-PA::TRP1 | This study         |
| TYY1162  | TYY161 atg1Δ::URA3             | This study         |
| YTS150   | SEY6210 ATG9-YFP::HIS3 atg1Δ::URA3 atg11Δ::LEU2 | Shintani and Klionsky, 2004b|
| YTS158   | BY4742 pho8Δ pho8ΔΔ60 pho13Δ::KAN | This study         |
was introduced into the pAPG9GFP[416] plasmid by site-directed mutagenesis. The Atg9 N-terminal region containing the AvrII site was amplified and introduced into pGBDU-C1 with EcoRI and BamHI to generate pBD-Atg9N[AvrII]. A gapped pGBDU-C1 plasmid was generated by digestion with EcoRI and BamHI. The PCR reaction was performed using Taq polymerase (New England Biolabs, Inc.) with pBD-Atg9N[AvrII] as the template. The dATP concentration was lowered to three fifths that of the other three deoxynucleoside triphosphates. The resulting mutagenized PCR product shares overlapping sequences of ~100 bp at both S′ and 3′ ends with the gapped pGBDU-C1 plasmid. The PCR product and the gapped plasmid were cotransformed into the two-hybrid strain PJ69-4A. The transformants that were not able to grow on either plate were selected − His and gapped plasmid were cotransformed into the two-hybrid strain PJ69-4A.

Additional assays

The GFP-Atg8 processing assay, the Pho8Δ60 activity assay, and the protease protection assay were performed as previously described (Noda et al., 1995; Wang et al., 2001; Abelliovich et al., 2003; Shintani and Klionsky, 2004b; Cheong et al., 2005).

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