Visualization of Atg3 during Autophagosome Formation in Saccharomyces cerevisiae*

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Background: Atg3 is an E2-like enzyme in the Atg8 system, an autophagy-related ubiquitin-like conjugation system.

Results: Atg3 is localized to the isolation membrane, which is an intermediate structure of an autophagosome.

Conclusion: Atg3 is likely to play an important role in autophagosome formation at the isolation membrane.

Significance: This result provides new insights into the role of the ubiquitin-like system in organelle biogenesis.

Macroautophagy (autophagy) is a highly conserved cellular recycling process involved in degradation of eukaryotic cellular components. During autophagy, macromolecules and organelles are sequestered into the double-membrane autophagosome and degraded in the vacuole/lysosome. Autophagy-related 8 (Atg8), a core Atg protein essential for autophagosome formation, is a marker of several autophagic structures: the pre-autophagosomal structure (PAS), isolation membrane (IM), and autophagosome. Atg8 is conjugated to phosphatidylethanolamine (PE) through a ubiquitin-like conjugation system to yield Atg8-PE; this reaction is called Atg8 lipidation. Although the mechanisms of Atg8 lipidation have been well studied in vitro, the cellular locale of Atg8 lipidation remains enigmatic. Atg3 is an E2-like enzyme that catalyzes the conjugation reaction between Atg8 and PE. Therefore, we hypothesized that the localization of Atg3 would provide insights about the site of the lipidation reaction. To explore this idea, we constructed functional GFP-tagged Atg3 (Atg3-GFP) by inserting the GFP portion immediately after the handle region of Atg3. During autophagy, Atg3-GFP transiently formed a single dot per cell on the vacuolar membrane. This Atg3-GFP dot colocalized with 2× mCherry-tagged Atg8, demonstrating that Atg3 is localized to autophagic structures. Furthermore, we found that Atg3-GFP is localized to the IM by fine-localization analysis. The localization of Atg3 suggests that Atg3 plays an important role in autophagosome formation at the IM.

Macroautophagy (herein referred to as autophagy) is a catabolic process, conserved throughout eukaryotes, in which cytoplasmic constituents are degraded in the vacuole/lysosome during severe environmental conditions. The degradative products are subsequently reused in biosynthetic reactions critical for survival. This self-eating process can be up-regulated under conditions such as nutrient deprivation or rapamycin treatment that inhibit the target of rapamycin complex 1 (1). Upon induction of autophagy, a perivacuolar structure termed the pre-autophagosomal structure (PAS) (2–6), to which most autophagy-related (Atg) proteins are localized, generates an isolation membrane (IM). Eventually, the IM elongates to form a characteristic double-membrane autophagosome enclosing cytoplasmic materials designated for degradation (7). The autophagosome fuses with the outer membrane of the vacuole to discharge a cargo-loaded single-membrane structure (termed the autophagic body) into the vacuolar lumen (8).

Among the 19 Atg proteins required for autophagosome formation in Saccharomyces cerevisiae, Atg8 has been used as a reliable marker for monitoring the progression of autophagy (9). Microscopic analysis has demonstrated that Atg8 is localized to the PAS, IM, complete autophagosome, and the autophagic body (2, 9, 10). Atg8 is conjugated to the phospholipid phosphatidylethanolamine (PE) by a ubiquitin-like conjugation system (termed the Atg8 system) to produce Atg8-PE (11, 12). Translated Atg8 is initially cleaved by the cysteine protease Atg4 (13) prior to its binding with the homodimeric E1-like activating enzyme Atg7 (14). Activated Atg8 is transferred from Atg7 to the E2-like enzyme Atg3 (15) and ultimately attached to the amino group of PE by interacting with the E3-like Atg16-Atg5-Atg12 complex (16, 17). This reaction is referred to as Atg8 lipidation. Atg8-PE plays a role in mediating membrane tethering and hemifusion activities in vitro, suggesting that Atg8 lipidation is important for IM expansion (18).

Although Atg8 lipidation is believed to be critical for autophagosome membrane expansion, the intracellular site of Atg8 lipidation remains unknown. With the goal of determining where Atg8 lipidation occurs, we focused on the E2-like enzyme Atg3, which is catalytically sufficient for Atg8-PE formation in vitro (19). However, to date no report has described the intracellular localization of Atg3.

For this study, we constructed N and C terminally GFP-tagged Atg3 constructs; however, neither of these fusion pro-

* This work was supported by grants from the International Kyowa Scholarship Foundation (to M. N.), the Hamaguchi Foundation for the Advancement of Biochemistry (to K. S.), the Mishima Kaiun Memorial Foundation (to K. S.), the NOVARTIS Foundation (Japan) for the Promotion of Science (to K. S.), the Takeda Science Foundation (to K. S.), the Yamada Science Foundation (to K. S.), and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (24121707, 24657083, and 25291040) (to K. S.).

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‡ The abbreviations used are: PAS, pre-autophagosomal structure; IM, isolation membrane; PE, phosphatidylethanolamine; Atg, autophagy-related.
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Atg3 is involved in autophagosome formation at the IM. This construct could complement the autophagic defect in atg3Δ cells. This GFP-tagged Atg3 (Atg3-GFP) formed perivacuolar dots, which were also labeled with mCherry-tagged Atg8 (Ch-Atg8) during autophagy. Next, we visualized the IM under a fluorescence microscope and found that Atg3-GFP is localized to the IM, supporting the idea that Atg3 is involved in autophagosome formation at the IM.

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth Conditions—The yeast strains used in this study were SEY6210 (MATa lys2 suc2 his3 leu2 trp1 ura3) (21), KPY113 (SEY6210; atg3Δ::LEU2) (2), GYS638 (SEY6210; leu2Δ::mRFP::APE1::LEU2) (22), GYS973 (SEY6210; leu2Δ::mCherry-ATG8::IphNT1)) (10), and GYS1156 (GYS638; ypt7Δ::kanMX). To generate strain GYS1156, the ypt7Δ::kanMX fragment was amplified from the disruptant in laboratory stocks and used for transformation. All strains were grown in a synthetic dextrose casamino acid medium (SDCA; 0.67% Difco yeast nitrogen base without amino acids, 0.5% Bacto-casamino acids, and 2% glucose) generated the GFP-tagged Atg3 construct, we cloned the open reading frame of prApe1 (prApe1) is delivered into the vacuole by autophagic pathways: the Cvt pathway under nutrient-rich conditions, and autophagy under starvation conditions (29). In both pathways, prApe1 is processed into its mature form (mApe1) by vacuolar hydrolases. The conversion from prApe1 to mApe1 can be monitored by Western blotting with anti-Ape1 antiserum; two protein bands with molecular masses of ~60 and 50 kDa markers, as in wild-type cells (Fig. 1A, upper panels). Next, we examined the functionality of Atg3 by monitoring recovery of Ape1 maturation in atg3Δ cells. The premature form of Ape1 (prApe1) is delivered into the vacuole by autophagic pathways: the Cvt pathway under nutrient-rich conditions, and autophagy under starvation conditions (29). In both pathways, prApe1 is processed into its mature form (mApe1) by vacuolar hydrolases. The conversion from prApe1 to mApe1 can be monitored by Western blotting with anti-Ape1 antiserum; two protein bands with molecular masses of ~60 and 50 kDa correspond to prApe1 and mApe1, respectively (30). Ape1 maturation was restored in atg3Δ cells carrying prApe1 (Fig. 1A, lower panels), indicating that Atg3 expressed from prApe1 can complement the autophagic defect of atg3Δ cells.

Construction of Functional GFP-tagged Atg3 Protein—We examined N and C terminally GFP-tagged Atg3 constructs, but both failed to rescue defects in autophagy (data not shown). We concluded that a GFP tag at either terminus of Atg3 yielded a non-functional protein that would be inappropriate for use in this study. Therefore, we screened for permissive sites for GFP insertion, referring to structural information obtained previously for S. cerevisiae Atg3 (20). In particular, we evaluated three GFP insertion sites C-terminal to the handle region of Atg3: (I) Asp265/Gly266, (II) Asp269/Trp270, and (III) Asp276/Ile277 (Fig. 1B). Western blot analysis with anti-Atg3 antisera confirmed that all three internally GFP-tagged Atg3 proteins were expressed in atg3Δ cells (Fig. 1C, upper panels), and monitoring of Ape1 maturation revealed that all three were functional. atg3Δ cells carrying plasmids (I) or (II) exhibited Ape1 maturation at levels comparable with the wild-type control (Fig. 1C, lower panels). We chose plasmid (I) for microscopic

articles. We sought to visualize the subcellular localization of Atg3 using GFP-tagged Atg3. Before we generated the GFP-tagged Atg3 construct, we cloned the open reading frame of ATG3 with upstream and downstream flanking sequences into expression vector pRS316. With Western blot analysis with anti-Atg3 antiserum, we detected Atg3 expressed from the plasmid as a band between the 37- and 50-kDa markers, as in wild-type cells (Fig. 1A, upper panels). Next, we examined the functionality of Atg3 by monitoring recovery of Ape1 maturation in atg3Δ cells. The premature form of Ape1 (prApe1) is delivered into the vacuole by autophagic pathways: the Cvt pathway under nutrient-rich conditions, and autophagy under starvation conditions (29). In both pathways, prApe1 is processed into its mature form (mApe1) by vacuolar hydrolases. The conversion from prApe1 to mApe1 can be monitored by Western blotting with anti-Ape1 antiserum; two protein bands with molecular masses of ~60 and 50 kDa correspond to prApe1 and mApe1, respectively (30). Ape1 maturation was restored in atg3Δ cells carrying the prApe1 (ATG3) plasmid, regardless of culture conditions (Fig. 1A, lower panels), indicating that Atg3 expressed from prApe1 can complement the autophagic defect of atg3Δ cells.

Construction of Functional GFP-tagged Atg3 Protein—We examined N and C terminally GFP-tagged Atg3 constructs, but both failed to rescue defects in autophagy (data not shown). We concluded that a GFP tag at either terminus of Atg3 yielded a non-functional protein that would be inappropriate for use in this study. Therefore, we screened for permissive sites for GFP insertion, referring to structural information obtained previously for S. cerevisiae Atg3 (20). In particular, we evaluated three GFP insertion sites C-terminal to the handle region of Atg3: (I) Asp265/Gly266, (II) Asp269/Trp270, and (III) Asp276/Ile277 (Fig. 1B). Western blot analysis with anti-Atg3 antisera revealed that all three internally GFP-tagged Atg3 proteins were expressed in atg3Δ cells (Fig. 1C, upper panels), and monitoring of Ape1 maturation revealed that all three were functional. atg3Δ cells carrying plasmids (I) or (II) exhibited Ape1 maturation at levels comparable with the wild-type control (Fig. 1C, lower panels). We chose plasmid (I) for microscopic
analysis, and hereafter refer to the encoded protein as Atg3-GFP.

Atg3 Is Detected as a Dot on the Vacuolar Membrane during Autophagy—We investigated the subcellular localization of Atg3-GFP by fluorescence microscopy. Under nutrient-rich conditions, Atg3-GFP diffused throughout the cytoplasm (Fig. 2A). When autophagy was induced by treatment of cells with rapamycin, Atg3-GFP accumulated in a single dot in each cell (Fig. 2A). To determine the localization of the dot, the vacuolar membrane was stained with FM4–64, revealing that Atg3-GFP was present in a single punctate structure attached to the vacuolar membrane (Fig. 2B). Next, we observed the dynamics of Atg3-GFP over a time course following rapamycin treatment. Dot formation reached a peak at 45 min after rapamycin treatment, and then dramatically decreased to the basal level within 120 min (Fig. 2C).

Atg3 Accumulates at the Pre-autophagosomal Structure during Autophagy—During autophagy, Atg3-GFP localized as a dot near the vacuole, similar to most other Atg proteins (5).
is detected as a perivacuolar dot and serves as a center for autophagosome formation, we examined the spatiotemporal dynamics of Atg3-GFP and mCherry-tagged Atg8 (Ch-Atg8). First, we monitored the dynamics of Ch-Atg8 before and after rapamycin treatment. Under nutrient-rich conditions, Ch-Atg8 was detected as a dot in ~7% of cells (Fig. 3A), indicating that the Cvt pathway was active under these conditions (31). Following rapamycin treatment, Ch-Atg8 dot formation reached its peak at 60 min, and then drastically reduced to the basal level within 180 min (Fig. 3, A and B). This result suggests that Atg8 transiently accumulates at the PAS during autophagy, but this PAS-localized Atg8 is transferred to subsequent autophagic structures and ultimately delivered to the vacuole.

Next, we examined the subcellular localization of the Atg3 dot in cells treated with rapamycin for 45 or 60 min. As expected, the Atg3-GFP dot colocalized with the Ch-Atg8 dot (Fig. 3C). Among the 44 cells contained both Atg3-GFP and Ch-Atg8 dots, none of the cells containing both kinds of dots exhibited separate localizations. These results indicate that Atg3 localizes to the PAS during autophagy.

Previous studies have shown that monomeric RFP-tagged prApe1 (mRFP-prApe1), which is detected as a perivacuolar dot, is a useful marker for the PAS (27). Therefore, we asked whether the Atg3-GFP dot colocalized with the dot of mRFP-prApe1. Indeed, in cells treated with rapamycin for 45 min, Atg3-GFP and mRFP-prApe1 were colocalized (Fig. 3D). This observation supports the finding, described above, that Atg3 resides at the PAS.

**Fine Localization Analysis Reveals That Atg3 Is Localized to the IM during Autophagosome Formation**—Recently, we reported that overexpression of prApe1 enables visualization of the IM as a cup-shaped structure under the fluorescence microscope (10). Using this technique, localization of Atg proteins can be categorized into three patterns: a dot at the junction between the IM and the vacuolar membrane, termed the “VICS (vacuole-isolation membrane contact site)” pattern; a cup-shaped pattern overlapping extensively with the Ch-Atg8-labeled IM, termed the “IM” pattern; and two or three dots associated with the IM edge, termed the “IM edge” pattern. In prApe1-overexpressing cells, Atg3-GFP clearly exhibited the IM pattern 3 h after rapamycin treatment (Fig. 4A). This result indicates that Atg3 is localized to the IM during autophagosome formation, and raises the possibility that Atg3 plays a role in autophagosome formation at the IM.

The finding that Atg3-GFP did not label the vacuolar lumen during autophagy (Fig. 2, A and B) suggested that Atg3 dissociates from the IM upon completion of autophagosome formation. Therefore, we examined whether Atg3-GFP was localized...
to complete autophagosomes by fluorescence microscopy. Ypt7, a Rab GTPase, is required for the fusion of autophagosomes to the vacuole (9). To accumulate autophagosomes, ypt7Δ cells were used. Because prApe1 is efficiently enclosed in autophagosomes during autophagy (29), we used mRFP-prApe1 as an autophagosome marker in this experiment. During autophagy, mRFP-prApe1 was detected as several dots, indicating that autophagosomes accumulated in these cells (Fig. 4B). GFP-Atg8, another well described autophagosome marker (2), also formed multiple dots in ypt7Δ cells (Fig. 4B), and these dots were well colocalized with mRFP-prApe1 dots (Fig. 4, B and C). By contrast, Atg3-GFP dots were very rarely detected on mRFP-prApe1 dots (Fig. 4, B and C), suggesting that Atg3 does not concentrate at autophagosomes. Notably, ~7% of Atg3-GFP dots were still colocalized with mRFP-prApe1 dots (Fig. 4C). This is probably because Atg3 is colocalized with mRFP-prApe1 at the PAS (Fig. 3D). Taken together, our data demonstrate that Atg3 is localized to the IM during autophagosome formation but released into the cytoplasm upon completion of autophagosome formation.

**DISCUSSION**

The Atg8 lipidation system plays an indispensable role in the formation of autophagosomes. However, the role of Atg8 lipidation in the development of IM remains unresolved. In this study, we found that Atg3 is localized to the PAS during autophagy. Furthermore, fine-localization analysis revealed that Atg3-GFP is localized to the IM during autophagosome formation but absent from complete autophagosomes. Thus, this study provides a novel insight into the role of Atg3 during IM expansion.

*Functional Atg3-GFP Is Constructed by Insertion of GFP into Just After the Handle Region—*To determine the in vivo localization of Atg3, we constructed functional Atg3-GFP for use in microscopic visualization. Initially, as is conventional, we tested N- and C-terminal GFP insertions, but these proteins were not functional (data not shown). Eventually, we found that proteins containing GFP insertions immediately after the handle region were functional (Fig. 1, B and C) and able to produce fluorescent signals sufficient for detection (Fig. 2, A and B). GFP tagging at the N terminus might obstruct the interaction of this
domain with PE, resulting in functional impairment (32). Although it is not clear whether the C-terminal domain of Atg3 has an interaction partner, this domain is located very close to the E2-like domain of Atg3 (20). In the Atg3/Atg7 complex, the flexible region of Atg3 is rearranged to interact with a distal groove in the N-terminal domain of Atg7 (33). Therefore, insertion of GFP after the C-terminal domain might obstruct translocation of the flexible region of Atg3 upon interaction with Atg7. We show here that insertion of GFP just after the handle region yielded Atg3-GFP that retained its activity, but this result does not exclude the possibility of generating functional Atg3-GFP constructs by insertions in other locations within the protein. Recently, localization of functional Atg3-GFP has been visualized by inserting GFP between Gln117 and Ser118 in the flexible region of Atg3.3

Atg3-GFP Associates with the IM—Time course analysis revealed that the number of the Atg3-GFP and Ch-Atg8 dots peaked 45–60 min after induction of autophagy by rapamycin. The accumulation curve of Atg3-GFP and Ch-Atg8 dots underwent a steep drop right after the peak (Figs. 2C and 3A), suggesting that they departed from the PAS. Subsequently, Ch-Atg8 was transported into the vacuole via autophagosomes (Fig. 3B). Given that a previous study demonstrated that autophagic bodies emerge within the vacuolar lumen ~60 min after starvation (8), it is conceivable that the reduction in Atg8 dots after 60 min corresponds to the onset of autophagosome formation. Although Atg3-GFP was localized to the IM (Fig. 4A), the vacuolar lumen was not stained with Atg3-GFP during autophagy (Fig. 2, A and B), suggesting that Atg3 is not selectively enclosed in mature autophagosomes. This hypothesis was supported by colocalization analysis of Atg3-GFP with mRFP-prApe1 using ypt7Δ cells, which revealed that Atg3 does not accumulate at complete autophagosomes (Fig. 4, B and C). It is very likely that Atg3 dissociates from the IM when autophagy.

3 Sakoh-Nakatogawa, M., Kirisako, H., Nakatogawa, H., and Ohsumi, Y. (2015) Localization of Atg3 to autophagy-related membranes and its enhancement by the Atg8-family interacting motif to promote expansion of the membranes. FEBS Lett. 0.1016/j.febslet.2015.02.003.
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gosome formation has been completed. We note that this behavior of Atg3 is similar to that of the Atg16-Atg5-Atg12 complex, which acts as the E3-like enzyme in Atg8 lipiddation (17). This complex is localized to the IM, and dissociates when autophagosome formation is completed (10, 34). Thus, during IM expansion, Atg3 might catalyze the lipiddation of Atg8 along with the Atg16-Atg5-Atg12 complex.

On the basis of these observations, we propose a model for the dynamics of Atg3 in the formation of autophagosome. Upon induction of autophagy, Atg3-GFP accumulates to the PAS along with Ch-Atg8, suggesting that Atg8 lipiddation is facilitated at the PAS. The peak formation of Atg3-GFP and Ch-Atg8 dots suggests that Atg8-PE stays at the PAS until it reaches a threshold level needed for autophagosome formation. Then Atg3-GFP is localized to the IM during IM expansion. Upon completion of autophagosome formation, Atg3 is released into the cytoplasm, whereas Atg8 (along with other cytoplasmic materials) is delivered to vacuole.

In this study, we visualized the cellular distribution of GFP-tagged Atg3, and showed that Atg3 exhibits the IM pattern during autophagosome formation. Further analysis will be required to confirm the cellular site where Atg3 catalyzes the lipiddation of Atg8.

Acknowledgments—we thank Dr. Yoshinori Ohsumi for materials. We also thank Dr. Takahiro Negishi, Yang Wang, Karen Kubo, and Shohei Yoshitake for technical assistance.

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