The carboxyl terminal 17 amino acids within Apg7 are essential for Apg8 lipidation, but not for Apg12 conjugation

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Abstract In the yeast, Saccharomyces cerevisiae, two ubiquitin-like modifications, Apg12 conjugation with Apg5 and Apg8 lipidation with phosphatidyethanolamine, are essential for autophagy and the cytoplasm-to-vacuole transport of aminopeptidase I (Cvt pathway). As a unique E1-like enzyme, Apg7 activates two modifiers (Apg12 and Apg8) in an ATP-dependent manner and, for this activity, the carboxyl terminal 40 amino acids are essential. For a better understanding of the function of the carboxyl terminus of Apg7, we performed a sequential deletion of the region. A mutant expressing Apg7AC17 protein, which lacks the carboxyl 17 amino acids of Apg7, showed defects in both the Cvt pathway and autophagy. Apg8 lipidation is inhibited in the mutant, while Apg12 conjugation occurs normally. A mutant expressing Apg7AC13 protein showed a defect in the Cvt pathway, but not autophagy, suggesting that the activity of Apg7 for Apg8 lipidation is more essential for the Cvt pathway than for autophagy. Mutant Apg7AC17 protein is still able to interact with Apg8, Apg12, and Apg3, and forms a homodimer, indicating that the deletion of the carboxyl terminal 17 amino acids has little effect on these interactions and Apg7 dimerization. These results suggest that the carboxyl terminal 17 amino acids of Apg7 play a specific role in Apg8 lipidation.

Key words: Apg7; Ubiquitin-like modification; Apg12-Apg5 conjugate; Apg8/Aut7 lipidation; Apg3; Homodimer

1. Introduction

Autophagy is a bulk degradation of intracellular proteins and organelles in the lytic organelles, lysosome and vacuole [1–4]. During autophagy, a cup-shaped membrane sac surrounds cytosolic proteins and organelles non-selectively to form an autophagosome. The autophagosomes finally fuse with the lysosome/vacuole, and the contents sequestered into autophagosomes are degraded by lytic enzymes in the lysosome/vacuole. In the initial step of the formation of autophagosomes, a sequential reaction of two ubiquitin-like modifications, Apg12 conjugation and Apg8/Aut7 lipidation, is essential in yeast (Fig. 1A) [5–8]. Apg12 is conjugated to Apg5 by Apg7/Cvt2/Gsi7, an E1-like enzyme, and Apg10, an E2-like enzyme, respectively (Fig. 1A, Apg12 conjugation) [9–13]. The Apg12-Apg5 conjugate is essential for the formation of an autophagosomal precursor [14]. Thereafter, Apg8 is conjugated to phosphatidyethanolamine (PE) by Apg7 and Apg3/Aut1, the same E1-like enzyme and a second E2-like enzyme, respectively [7,13,15–18]. The Apg8 lipidation is indispensable for the localization of Apg8 to preautophagosomes (Fig. 1A, Apg8 lipidation) [19].

These modifications are also essential for a unique transport, the cytoplasm-to-vacuole (Cvt) pathway of aminopeptidase I (Ape1) and α-mannosidase in yeast, Saccharomyces cerevisiae [20–23]. Ape1 is synthesized in the cytoplasm as a precursor (proApe1), and forms a homo dodecamer (Fig. 1B) [24]. ProApe1 is packaged into a double-membrane structure called Cvt vesicles (Fig. 1B, Cvt pathway) [25,26]. Cvt vesicles target the vacuole and fuse with it [25]. After the degradation of the inner membrane of the Cvt body by Cvt17/Aut5 lipase [27,28], proApe1 is finally processed to mature Ape1 by vacuolar proteases. The deletion of each of these APG/CVT/IAUT genes results in a defect of the Cvt pathway and autophagy, indicating that both pathways share the same molecular machinery to form both Cvt vesicles and autophagosomes [29]. However, there are differences between the Cvt pathway and autophagy. While the Cvt pathway is constitutive, autophagy is inducible. The Cvt pathway shows high cargo specificity, but autophagy is a bulk degradation of protein and organelle. The Cvt vesicle is smaller than the autophagosome (Fig. 1B) [23].

Apg7 is an E1-like enzyme essential for both Apg12 conjugation and Apg8 lipidation, interacting with its substrates, Apg12 and Apg8 (Fig. 1A, Apg7) [9,11–13]. Furthermore, a comprehensive analysis of yeast proteins and co-immunoprecipitation experiments revealed that Apg7 also interacts with Apg3 [13,16,30–33]. Apg7 forms an homodimer in yeast and mammals [13,33]. In yeast, S. cerevisiae, we have shown that the deletion of the carboxyl terminal 40 amino acids out of 630 amino acids of Apg7 (the C40 region) results in pleiotropic phenotypes including defects in Apg7 homodimerization, Apg12 conjugation, Apg8 lipidation, and interactions of Apg7 with Apg12, Apg8, and Apg3, while the active-site cysteine and ATP-binding domain within Apg7, which are essential for the activity of E1-like enzyme, are still present [33]. We are interested in the C40 region within Apg7, because there is...
no report about the significance of the carboxyl terminal region with regard to E1 and E1-like enzymes other than Apg7. Considering these pleiotropic defects derived from the deletion of the C40 region, it is possible that the region is important for regulation of the two modifications. Before this investigation, we hypothesized two possibilities with regard to the function(s) of the C40 region. One possibility is that the C40 region would be essential for all the functions. If the former is correct, a sequential deletion of the carboxyl terminus of the C40 region will result in a single defect of homodimerization, Apg12 conjugation or Apg8 lipida- tion. The other possibility is that the whole of the region homodimerization, Apg12 conjugation and Apg8 lipida- tion. The other possibility is that the whole of the region would be essential for all the functions. If the former is cor- rect, a sequential deletion of the carboxyl terminus of the C40 region will result in a single defect of homodimerization, Apg12 conjugation or Apg8 lipida- tion. To test this hypothesis, we performed a sequential deletion analysis of the carboxyl terminal region of Apg7 in detail. We found that the carboxyl terminal 17 amino acids (residues 614–630 out of 630 amino acids) of Apg7 play an indispensable role specific for Apg8 lipida- tion, but not Apg12 conjugation or homodimeri- zation.

2. Materials and methods

2.1. Strains, media, materials, and molecular biological techniques

Molecular biological and biochemical techniques were performed as described previously [34]. *Escherichia coli* strain DH5α, cells, the host for plasmids and protein expression, were grown in Luria broth me- dium in the presence of antibiotics as required. Yeast strains used in this study are listed in Table 1. To construct plasmids expressing the mutant Apg7pΔCx series (Fig. 2A), the polymerase chain reaction was performed with high fidelity.

2.2. Antibodies

Polyclonal antibody against yeast aminopeptidase I (anti-Ape1) was kindly provided by Dr. Daniel Klionsky. Monoclonal mouse anti-myc (9E10), monoclonal mouse anti-hemagglutinin (anti-HA) (F7), and polyclonal rabbit anti-HA (Y-11) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Other techniques

Chemical cross-linking for recognition of Apg7 homodimerization and co-immunoprecipitation was performed as described by Komatsu et al. [33]. Two-hybrid analysis was performed as described by James et al. [35] and Komatsu et al. [33] using strain PJ69-4A.

3. Results

3.1. The carboxyl terminal 17 amino acids are essential for the Cvt pathway and autophagy

To localize the minimal region within the carboxyl terminus of Apg7 essential for the Cvt pathway and/or autophagy, we constructed plasmids expressing mutant Apg7 proteins, which lack the carboxyl terminal amino acids as indicated in Fig. 2A. Each of the mutant proteins Apg7ΔC5, Apg7ΔC9, Apg7ΔC11, Apg7ΔC13, and Apg7ΔC17 was expressed under the control of their own promoter on a centromere-type plasmid in the *apg7Δ* mutant, which lacks the *APG7* gene on the chromosome. We first investigated whether these mutant pro- teins result in a defect in the Cvt pathway, i.e. processing of Ape1 under nutrient-rich conditions. Ape1 is synthesized in the cytosol as a precursor (proApe1) (Fig. 1B). Thereafter, proApe1 is processed to mature Ape1 by vac- uolar protease(s) in the vacuole. This Cvt pathway is constitutive. Contrary to the Cvt pathway, autophagy is significantly induced under conditions of starvation. During autophagy, proApe1 is en- wrapped into the autophagosomal vesicle together with cytosolic proteins and compart- ments. After fusion of the autophagosomal vesicle with the vacoule, proApe1 is processed to mature Ape1 by vacuolar protease(s).

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body. In the \textit{apg7}Δ mutant that lacks the \textit{APG7} gene, little mature Ape1 was recognized by immunoblotting using anti-Ape1 antibody, while proApe1 was recognized (Fig. 2B, \textit{apg7}Δ). In the \textit{apg7}Δ mutant expressing each of the \textit{Apg7}ΔC13 and \textit{Apg7}ΔC17 proteins, little mature Ape1 was recognized, indicating that the Ape1 processing was significantly impaired, as was the case for the \textit{apg7}Δ mutant (Fig. 2B, \textit{Apg7}ΔC13 and \textit{Apg7}ΔC17). In the other \textit{apg7}Δ mutants expressing each of the \textit{Apg7}ΔC9 and \textit{Apg7}ΔC11 proteins, mature Ape1 was recognized well, as was the case for the wild type (Fig. 2B,C). These results indicate that the carboxyl terminal 13 amino acids (residues 618–630 out of 630 amino acids) are essential for the function of Apg7 for the Cvt pathway.

Under conditions of starvation, Ape1 is transported to the vacuole via autophagy (Fig. 1B, Autophagy). If \textit{Apg7}ΔC13 and \textit{Apg7}ΔC17 proteins show a functional defect in autophagy in addition to the Cvt pathway, proApe1 will not be processed to mature Ape1 in the \textit{apg7}Δ mutants expressing each of the \textit{Apg7}ΔC9 and \textit{Apg7}ΔC11 proteins even under conditions of starvation. To investigate this issue, Ape1 processing was examined under conditions of starvation (Fig. 2C). In the \textit{apg7}Δ mutant expressing \textit{Apg7}ΔC13 protein, proApe1 was processed to mature Ape1 well under the conditions (Fig. 2C, \textit{Apg7}ΔC13, Starvation +), indicating that Ape1 is transferred to the vacuole via autophagy. In the \textit{apg7}Δ mutant expressing \textit{Apg7}ΔC17 protein, little proApe1 was processed even under the starvation conditions, as was the case for the \textit{apg7}Δ mutant (Fig. 2C, Starvation +, \textit{Apg7}ΔC17 vs. \textit{apg7}Δ).

To confirm that the \textit{apg7}Δ mutant expressing \textit{Apg7}ΔC17 protein has a defect in autophagy, we next investigated the accumulation of autophagic bodies under conditions of starvation. When wild type cells were incubated under nitrogen-starved conditions in the presence of phenylmethylsulfonyl fluoride (PMSF), autophagic bodies were significantly accumulated under conditions of starvation (Fig. 2C). In the \textit{apg7}Δ mutant expressing \textit{Apg7}ΔC13 protein, autophagic bodies were accumulated in the vacuole, as was the case for the wild type (Fig. 2D, \textit{Apg7}ΔC13). These results indicate that the minimal carboxyl terminal region essential for both the Cvt pathway and autophagy is the domain containing 614–630 out of 630 amino acids within \textit{Apg7}. This region was designated the C17 domain.

3.2. The C17 domain within \textit{Apg7} is essential for \textit{Apg8} lipidation, but not \textit{Apg12} conjugation

\textit{Apg7} is an E1-like enzyme essential for \textit{Apg12} conjugation and \textit{Apg8} lipidation, and the sequential reaction of two modifications plays an indispensable role in the Cvt pathway and autophagy (Fig. 1A). Which modification is impaired in the \textit{apg7}Δ mutant expressing \textit{Apg7}ΔC17 protein, \textit{Apg8} lipidation only, \textit{Apg12} conjugation only, or both? We first investigated \textit{Apg8} lipidation, which is essential for the formation of a cup-shaped preautophagosome. To recognize \textit{Apg8} in each \textit{apg7}Δ mutant, HA epitope-tagged \textit{Apg8} (HA-\textit{Apg8}) was expressed under the control of its own promoter on a centromere-type plasmid. \textit{Apg8} lipidation in the wild type strain was recognized as a mobility shift of \textit{Apg8} on SDS-PAGE, as described previously (Fig. 3A, upper panel, wild type, \textit{Apg8}-PE) [15,19]. Little \textit{Apg8}-PE was recognized in the \textit{apg7}Δ mutant expressing \textit{Apg7}ΔC17 protein together with HA-\textit{Apg8} (Fig. 3A, upper and lower panels, \textit{Apg7}ΔC17), while \textit{Apg8}-PE in the \textit{apg7}Δ mutant expressing each of the \textit{Apg7}ΔC5, \textit{Apg7}ΔC9, and \textit{Apg7}ΔC11 proteins together with HA-\textit{Apg8} was recognized like that in wild type (Fig. 3A, upper and lower panels). A small amount of \textit{Apg8}-PE was recognized in the \textit{apg7}Δ mutant expressing \textit{Apg7}ΔC13 and HA-\textit{Apg8} (Fig. 3A, upper panel, \textit{Apg7}ΔC13). We next investigated the amount of \textit{Apg8}-PE under the starvation conditions. Even under the starvation conditions, little \textit{Apg8}-PE was recognized in the \textit{apg7}Δ mutant expressing \textit{Apg7}ΔC17 protein together with HA-\textit{Apg8}, and a small amount of \textit{Apg8}-PE was

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recognized in the \textit{apg7A} mutant expressing Apg7\textit{ΔC13} and HA-Apg8 (Fig. 3A, middle and lower panels). These results indicate that the C17 domain of Apg7 (614-630 out of 630 amino acids) is essential for Apg8 lipidation. With regard to mutant Apg7\textit{ΔC13} protein, it showed a defect in the Cvt pathway, but not autophagy (Fig. 2B-D). Therefore, the residual activity of Apg7 for Apg8 lipidation is sufficient for autophagy, while more activity is required for the Cvt pathway than for autophagy.

We next investigated the formation of the Apg12-\textit{Apg5} conjugate in the \textit{apg7A} mutants expressing Apg7\textit{ΔC17} and HA epitope-tagged Apg12 (HA-Apg12) proteins. HA-Apg12 was expressed in the strain under the control of its own promoter on a centromere-type plasmid. The HA-Apg12-\textit{Apg5} conjugate (\textasciitilde 70 kDa) was recognized by immunoblot analysis using mouse monoclonal anti-HA (F7) antibody in the wild type, while little conjugate was detected in the \textit{apg7A} mutant lacking the \textit{APG7} gene (Fig. 3B, Apg7\textit{Δ-myc} and \textit{apg7A}). In the \textit{apg7A} mutant expressing Apg7\textit{ΔC17} protein, the HA-Apg12-\textit{Apg5} conjugate was recognized well. Therefore, we conclude that the C17 domain is essential for Apg8 lipidation, but not Apg12 conjugation.

### 3.3. Mutant Apg7\textit{ΔC17} protein can interact with Apg8, while it causes a defect of Apg8 lipidation

The deletion of the C17 domain results in a defect of Apg8 lipidation, while the active-site cysteine (Cys\textsuperscript{507}) and ATP-binding domain are still present within Apg7\textit{ΔC17} protein. However, it is likely that the loss of interaction of mutant Apg7\textit{ΔC17} protein with Apg8 would cause the defect in Apg8 lipidation, because the interaction of E1-like enzyme (Apg7) with a substrate (Apg8) is indispensable for a further reaction. To investigate this possibility, we examined the interaction of Apg7 with Apg8 by two-hybrid analysis [35]. GAL4 DNA binding domain (GBD)-Apg8 (bait) and mutant GAL4-activating domain (GAD)-Apg7\textit{ΔC17} (prey) were expressed in a tester strain, PJ69-4A, and cell growth was examined as described by Komatsu et al. [33]. The strain expressing GAD-Apg7\textit{ΔC17} and GBD-Apg8 grew on the selective medium as well as did the strain expressing wild type GAD-Apg7 (Table 2, Apg7-\textit{Apg8} interaction), indicating that Apg7\textit{ΔC17} protein interacts with Apg8. We further investigated the interactions of Apg7\textit{ΔC17} protein with Apg12 and Apg3 by two-hybrid analyses. However, there is no difference between the carboxyl terminal 17 amino acids (the C17 domain) are essential for both the Cvt pathway and autophagy. A: Schematic representation of the deleted region of mutant Apg7\textit{ΔC} proteins lacking the carboxyl terminal region. Cys\textsuperscript{507} within yeast Apg7 is an authentic active-site cysteine. ATP-binding domain and Cys\textsuperscript{507} within Apg7 are essential for the activity of E1-like enzyme. Apg7\textit{ΔC17} protein lacks the region of 614-630 residues out of 630 amino acids of Apg7. B: Ape1 processing under nutrient-rich conditions. Each mutant protein (Apg7\textit{CS}, Apg7\textit{C9}, Apg7\textit{C11}, Apg7\textit{C13}, and Apg7\textit{C17}) was expressed in the \textit{apg7A} mutant lacking the \textit{APG7} gene (Fig. 3B, Apg7\textit{Δ-myc} and \textit{apg7A}). In the \textit{apg7A} mutant expressing Apg7\textit{ΔC17} protein, the HA-Apg8 lipidation, while more activity is required for the Cvt pathway than for autophagy.

3.3. Mutant Apg7\textit{ΔC17} protein can interact with Apg8, while it causes a defect of Apg8 lipidation

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wild type Apg7 and mutant Apg7ΔC17 (Table 2, Apg7–Apg12 and Apg7–Apg3 interactions).

Another possible defect is that homodimerization of Apg7 will be significantly impaired in the apg7Δ mutant expressing Apg7ΔC17 protein. We then investigated the homodimerization of mutant Apg7ΔC17 protein using a non-cleavable cross-linking reagent as described previously [33]. Mutant Apg7ΔC17 protein formed a homodimer as in the case of wild type Apg7 protein (Table 2, homodimerization). Therefore, we conclude that there is little defect in Apg7 homodimerization and the interactions of Apg7 with two substrates (Apg8 and Apg12) and an E2-like enzyme.

4. Discussion

We showed that the C17 domain within Apg7 is essential
for Apg8 lipidation, but not Apg12 conjugation, resulting in defects of the Cvt pathway and autophagy. However, mutant Apg7ΔC17 protein still contains an active-site cysteine and ATP-binding domain that are indispensable for the activity of the E1-like enzyme, and interacts with Apg8, Apg12, and Apg3 (Fig. 3 and Table 2). Considering that the Apg12–Apg5 conjugate is present in the apg7Δ mutant expressing Apg7ΔC17 protein, the activity of the E1-like enzyme will remain in the mutant Apg7ΔC17 protein. One possibility is that the C17 domain would affect the interaction of Apg3 with its target, PE. Apg8 lipidation is a quite unique ubiquitin-like modification; the target of Apg8 is not a protein, but a phospholipid, PE. Therefore, the C17 domain would have a specific function for Apg3 to recognize PE or to localize to an autophagosomal precursor. Another possibility is that the C17 domain is essential for Apg8 lipidation, but not Apg12 conjugation. A: Apg8 lipidation in the apg7Δ mutant expressing Apg7ΔC17 protein under the nutrient-rich (upper panel) and starvation (middle panel) conditions. The activity of Apg12–Apg5 conjugate was recognized in the apg7Δ mutant expressing Apg7ΔC17 protein, while the conjugate was recognized in the apg7Δ mutant expressing Apg7ΔC13 protein. Only a small amount of Apg8-PE was recognized in the apg7Δ mutant and Apg7ΔC13 mutant expressing Apg7ΔC17 protein.

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