Apg7p/Cvt2p, a protein-activating enzyme, is essential for both the Apg12p-Apg6p conjugation system and the Apg8p membrane targeting in autophagy and cytoplasm-to-vacuole targeting in the yeast Saccharomyces cerevisiae. Similar to the ubiquitin-conjugating system, both Apg12p and Apg6p are activated by Apg7p, an E1-like enzyme. Apg12p is then transferred to Apg10p, an E2-like enzyme, and conjugated with Apg5p, whereas Apg6p is transferred to Apg3p, another E2-like enzyme, followed by conjugation with phosphatidylethanolamine. Evidence is presented here that Apg7p forms a homodimer with two active-site cysteine residues via the C-terminal region. The dimerization of Apg7p is independent of the other Apg proteins and facilitated by overexpressed Apg12p. The C-terminal 123 amino acids of Apg7p (residues 508 to 630 out of 630 amino acids) are sufficient for its dimerization, where there is neither an ATP binding domain nor an active-site cysteine essential for its E1 activity. The deletion of its carboxyl 40 amino acids (residues 591–630 out of 630 amino acids) results in several defects of not only Apg7p dimerization but also interactions with two substrates, Apg12p and Apg8p and Apg12p-Apg5p conjugation, whereas the mutant Apg7p contains both an ATP binding domain and an active-site cysteine. Furthermore, the carboxyl 40 amino acids of Apg7p are also essential for the interaction of Apg7p with Apg3p to form the E1-E2 complex for Apg8p. These results suggest that Apg7p forms a homodimer via the C-terminal region and that the C-terminal region is essential for both the activity of the E1 enzyme for Apg12p and Apg8p as well as the formation of an E1-E2 complex for Apg8p.

Autophagy is responsible for the bulk of intracellular protein degradation in the lytic organelles, lysosome/vacuole (1, 2). When cells exist under conditions of nutrient starvation, the cytoplasmic components are nonselectively sequestered into autophagosomes, double-membrane structures, and are subsequently targeted to the lysosome/vacuole for degradation. The entire process is conserved through eukaryotes from yeast to mammals. Unique membrane dynamics are observed in the process of autophagy. In the case of the yeast, Saccharomyces cerevisiae, cytoplasmic components are nonselectively surrounded by membranes, which on expansion and completion, give rise to an autophagosome (3, 4). Autophagosomal membranes are morphologically distinct from any other known organelar membranes (3). The outer membrane of the autophagosome fuses with the vacuolar membrane (5). The inner membrane structure, which is referred to as an autophagic body, is released into the lumen (3, 4). Finally, the cytoplasmic components within an autophagic body are degraded in the vacuole (3). In the case of dynamic autophagosomal membrane-formation and fusion with the vacuole (lysosome in mammals), the molecular mechanism for this process remains unknown.

Several autophagy-defective (apg and aut) mutants have been isolated via the application of yeast genetics (6, 7). These apg and aut mutants genetically overlap with most cvt mutants, which have defects in the cytoplasm-to-vacuole targeting (Cvt) pathway of aminopeptidase I (Refs. 8–10; for a review, see Ref. 11), indicating that the mechanism for autophagy and the Cvt pathway share some common features. Recently, some of the characteristics of individual APG gene products have been elucidated. APG11/ AUT2 encodes a protein kinase (12, 13). Apg13p is phosphorylated and interacts with Apg1p and Vps8p (14, 15). Apg6p/Vps30p forms a complex with Apg14p and is localized on as-yet unidentified membrane structures (16). Aut9p/Apg9p is an integral membrane protein that is required for both the Cvt and autophagic pathways and is localized on large perivacuolar punctate structures (17, 18).

Of the APG gene products characterized thus far, the Apg12p modification system and the Apg8p/Aut7p1 membrane-targeting system have been the subjects of considerable attention in that they function as protein modifiers similar to ubiquitin (for reviews, see Refs. 19–22). In the case of autophagy, Apg12p binds covalently to Apg5p (23, 24). In this conjugation system, Apg7p and Apg10p function as E1 and E2 enzymes, respectively (24–26). After Apg12p-Apg5p conjugation, Apg16p is assembled with the conjugate, resulting in a high molecular weight Apg12p-Apg5p-Apg16p complex (27), which is essential for the subsequent formation of autophagosomes. A second

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1 The abbreviations used are: Apg8p, Aut7p/Apg5p; Apg3p, Aut1p/Apg6p; Apg5-myc, myc-tagged Apg5p; API, aminopeptidase I; proAPI, pro-form of API; Cvt, cytoplasm-to-vacuole; GAD, GAL4-activating domain; GBD, GAL4 DNA binding domain; HA, hemagglutinin epitope-tagged; PAGE, polyacrylamide gel electrophoresis; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein.
Apg7p also interacts with two ER-to-Golgi vesicular-soluble modifier, Apg8p, which is processed by a novel cysteine protease, Apg4p, and Apg3p/Aut1p is essential for Apg8p lipiddation (conjugation with phosphatidylethanolamine), suggesting that Apg7p and Apg3p are, respectively, E1 and E2 enzymes for Apg8p. The expression of Apg8p is enhanced by starvation, and Apg8p is associated with E1 and E2 enzymes for Apg8p (28–30). The expression of Apg7p, an interacting partner of Apg3p in the formation of an E1-E2 complex, is processed by a novel cysteine protease, Apg8p, which is associated with the C-terminal region is essential not only for interaction with Apg12p and Apg8p, but also for interaction with Apg3p in the formation of an E1-E2 complex.

### EXPERIMENTAL PROCEDURES

**Strains, Media, Materials, and Molecular Biological Techniques—Escherichia coli strain DH5α, the host for plasmids and protein expression, was grown in Luria Broth medium in the presence of the required antibiotics (35). The S. cerevisiae apg7Δ mutants and P69-4A used in this study are listed in Table 1. The apg mutant strains have been described previously (6). All yeast strains were cultured in a rich medium (YPD: 1% yeast extract, 2% polypeptone, 2% glucose, 20 mg/liter adenine, 20 mg/liter tryptophan, 20 mg/liter uracil, and 50 mM succinate/NaOH, pH 5.0), MVD medium (0.67% yeast nitrogen base without amino acids, 2% glucose), or SD medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, and 2% glucose), or SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and appropriate amino acids) as described by Kaiser et al. (36). The nitrogen starvation medium contained 0.17% yeast nitrogen base without amino acids, ammonium sulfate, and 2% glucose. For the galactose-inducible expression of proteins in the yeast, SSG medium (0.67% yeast nitrogen base without amino acids, 2% galactose) was employed. The solid medium contained 2% Bacto agar. Standard genetic operations were performed with a program temperature control system PC-701 (ASTEC, Fukuoka, Japan). The DNA sequence was determined using a ABI 373A DNA sequencer (PE Applied Biosystems, Foster City, CA). Restriction enzymes were purchased from TOYOBO (Osaka, Japan) and New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by ESPEC oligo service (Ibaraki, Japan). Two-hybrid screening was performed using the system described by James et al. (37). The expression of a protein under the control of a galactose-inducible promoter was performed after the manufacturer's recommended protocol (Stratagene, La Jolla, CA). pRS series vectors were generous gifts from P. Hieter, and the pGAD-C1 vector, pGBD-C1 vector, and PJ69-4A (Stratagene, La Jolla, CA) were generous gifts from T. Tanida.}

**Plasmids**

pRS314
pAPG7-myc-314
pAPG7Ac40–314
pAPG12HA-316
pAPG12HA-426
pESC-MycAPG7
pESC-HAAPG7
pESC-MycAPG7ΔC40
pESC-HAAPG7ΔC40
pESC-HAAPG7ΔC40
pESC-UraAPG7
pESC-MycAPG7
pESC-FlagAPG8
pGAD-APG7
pGAD-APG7ΔN261
pGAD-APG7ΔN507
pGAD-APG7ΔC368
pGAD-APG7ΔC190
pGAD-APG7ΔC40
pGAD-APG7
pGAD-APG8
pGAD-C1
pGAD-C1

**TABLE I**

| Yeast strains          | Genotype                           | Source          |
|------------------------|------------------------------------|-----------------|
| YIT701                 | MATα leu2–3, 112 trp1 ura3–52Δapg7::LEU2 | Tanida et al. (26) |
| TID701                 | MATα leu2–3, 112 trp1 ura3–52Δapg7::TRP1 | This study      |
| PJ69-4A                | MATα ura3 leu2 his3 trp1 gal4Δ gal80ΔLYS2::GAL1-HIS GAL2-ADE met2::GAL7-lacZ | James et al. (37) |

**Plasmids**

pRS314
pAPG7-myc-314
pAPG7Ac40–314
pAPG12HA-316
pAPG12HA-426
pESC-MycAPG7
pESC-HAAPG7
pESC-MycAPG7ΔC40
pESC-HAAPG7ΔC40
pESC-HAAPG7ΔC40
pESC-UraAPG7
pESC-MycAPG7
pESC-FlagAPG8
pGAD-APG7
pGAD-APG7ΔN261
pGAD-APG7ΔN507
pGAD-APG7ΔC368
pGAD-APG7ΔC190
pGAD-APG7ΔC40
pGAD-APG7ΔC40
pGAD-APG7ΔC40
pGAD-APG7ΔC40
pGAD-APG7ΔC40
pGAD-APG3
pGAD-APG8
pGAD-C1
pGAD-C1

**Plasmid Construction**—Plasmids used in this study are listed in Table 1. For two-hybrid screening, the bait plasmid (pGBD-APG7),

**Genetic resource**

This study shows that Apg7p forms a homodimer via the C-terminal region with two active-site cysteines and that the C-terminal region is essential not only for interaction with Apg12p and Apg8p, but also for interaction with Apg3p in the formation of an E1-E2 complex.
which encodes the Apg7p fused in-frame to the C-terminal end of the GAL4-DNA binding domain, has been described previously (26). pGAD-
APG7AN containing Apg7p, which encodes the C-terminal half (amino acids 262–630 out of 630 amino acids), was included in the two-hybrid screening. Parts of the APG7-encoding deletion of the C- and N-terminal half of Apg7p was inserted just before the start codon of the gene, and the resulting DNA fragments were cloned into the pGEM-T vector. To construct galactose-inducible expression plasmids for these epistagged Apg7ps, the DNA fragments were introduced into the BamHI and SalI sites of pESC-LEU and pESC-URA vectors (Stratagene), resulting in pESC-HA-APG7, pESCL-MycAPG7, pESC-
HAAPG7, and pESC-MycAPG7, respectively. For the deletion of the C-terminal region of Apg7p, polymerase chain reaction was performed with appropriate primers, and the resulting product was introduced into the BamHI-SalI sites of pESC-LEU and pESC-URA vectors (Stratagene) to generate pESC-HAAPG7C40, pESC-MycAPG7C40, pESCL-HAAPG7C40, and pESC-MycAPG7C40, respectively.

The Apg3 and Apg8 open reading frames in which the stop codon was replaced by polylinker sequence were amplified from yeast genomic library and appropriate oligonucleotides that incorporated a BamHI site on the 5′-primer and a SalI site on the 3′-primer (5′-CTGATCCATATGATGATCATCTAC-3′, 5′-AGTCGACCAAC-CTTCCATGGTATAGT-3′ for Apg3, 5′-AGATCGACAGAGCTAGAGA-GTCTTACAT-3′, 5′-AGTCGACCTGGCAGATGTTTCTC-3′ for Apg8), and the amplified DNA fragments were cloned into pGEM-T vector (Promega), resulting in pGEM-APG3 and pGEM-APG8, respectively. The BamHI-SalI fragments of pGEM-APG3 and pGEM-APG8 were introduced into the BamHI-SalI sites of pGAD-C1, thus generating pGAD-APG3 and pGAD-APG8, respectively. For the expression of Myc-Apg3p, the BamHI-SalI fragments of pGEM-APG3 were inserted into the BamHI-SalI site of pESC-URA vector (Stratagene), resulting in pESC-HAAPG3Mye for the expression of the N-terminal FLAAG-Apg3p, the DNA sequence, which encodes for a FLAAG epitope tag, was inserted just before the start codon of the APG8 gene, and the resulting sequence was introduced into the BamHI and SalI sites of pESC-URA vector (Stratagene), resulting in pESC-FLAGAPG8.

Two-hybrid Experiment—The improved two-hybrid system was performed as described previously (26). A host strain, PJ69-4A (MATa trpl1 leu2 his3 gal4A gal80A GAL2-ADE2 LY22:GAL1-HIS3 met2:GAL7-UAS2) has been created that contains three easily assayed reporter genes, each under the control of a different inducible promoter. The ADE2 gene is under the control of the GAL2 promoter, and HIS3 gene is under the control of the GAL1 promoter. As a result, this strain is extremely sensitive to weak interactions and eliminates nearly all false positives using simple plate assays, i.e., a strong interaction of the expected protein(s) functions as targeting machinery in an Apg12p-dependent manner. To investigate the Apg12p-dependent Apg7p-interacting protein, a yeast two-hybrid screening was carried out using a tester strain that overexpressed HA-Apg12p on a 2-μm plasmid with Apg7p as the bait. Of 1 × 10⁶ independent clones, two positive candidates were isolated. DNA-sequencing analysis indicated that both inserts encode the C-terminal region of Apg7p (residues 262–630 out of 630 amino acids, Apg7pΔN261) (Fig. 1, Apg7pΔN261, PJ69-4A [APG12 2 μl]). An interaction between Apg7pΔN261 and Apg7p was observed without Apg12p being expressed (Fig. 1, Apg7pΔN261, PJ69-4A), although overexpressed Apg12p significantly enhanced the interaction. Similarly, the interaction of wild-type Apg7p with itself was also observed in the absence of overexpressed Apg12p (Fig. 1, APG7 wild type).

To confirm the interaction of Apg7p homooligomer, the co-immunoprecipitation with Myc- and HA-tagged Apg7p proteins was employed. Myc-Apg7p and HA-Apg7p were coexpressed in the apg7Δ mutant under the control of a galactose-inducible promoter, which suppresses both Apg7p and Cvt7p phenotypes of the apg7Δ mutant (data not shown). Cell lysates of the transformant were prepared, and HA-Apg7p was immunoprecipitated with an anti-HA antibody. When galactose was added to the medium, HA-Apg7p was immunoprecipitated with the anti-HA antibody (Fig. 2A, lane 4). At the same time, Myc-Apg7p was also coimmunoprecipitated (Fig. 2A, lanes 2). Essentially the same results were obtained by coimmunoprecipitation with an anti-Myc antibody (data not shown).

We next employed a cross-linking experiment using a chemical cross-linker, disuccinimidyl suberate. The lysis of the apg7Δ cells, which express Myc-Apg7p, was prepared in the presence or absence of disuccinimidyl suberate and separated by SDS-PAGE, and the Myc-Apg7p was subsequently identified by immunoblotting with an anti-Myc antibody. In the absence of the cross-linker, a 78-kDa band was observed that corre-
sponds to monomeric Apg7p. When the cell lysate was treated with the cross-linker, the intensity of the monomer band decreased significantly, and a higher molecular mass band (about 160 kDa), the size of which corresponded to a dimer, appeared (Fig. 2B).

Glycerol density gradient ultracentrifugation also indicates that Apg7p forms a homodimer. Total cell lysates of the apg7D cells overexpressing Myc-Apg7p were subjected to a 10–40% glycerol density gradient centrifugation. The resulting fractions were analyzed by SDS-PAGE, and the Myc-Apg7p was subsequently identified by immunoblotting with an anti-Myc antibody. Myc-Apg7p was collected in fractions 12–15 and mainly sedimented with a sedimentation coefficient of 7.4 S (Fig. 2C, fraction 13). The overexpression of Apg12p along with the Apg7p resulted in a higher concentration of Apg7p in fraction 13 (data not shown).

The Apg7p-Apg7p interaction was first found in the presence of excess Apg12p. Thus, it is possible that an endogenous Apg12p mediates the interaction of Apg7p. To investigate this possibility, we examined the cross-linking experiment of Myc-Apg7p in the apg12D mutant. Even in the apg12D mutant, a higher molecular mass band corresponding to Apg7p homodimer (about 160 kDa) appeared in the presence of a chemical cross-linker as in wild-type (Fig. 2D). The dimerization of Apg7p was further investigated in other apg mutants. As in wild-type and the apg12D cells, a higher molecular weight band of Apg7p (about 160 kDa) appeared depending on the chemical cross-linker in other apg mutants (apg1c, apg2, apg3, apg4,

FIG. 1. Two hybrid analysis of self interaction of Apg7p. The domain structure of wild-type Apg7p is schematically represented on the top. The black box shows ATP binding domain (residues 331–336 out of 630 amino acids), and Cys-507 is the active-site cysteine. In a two-hybrid assay, a tester strain, PJ69-4A, contains two reporter genes under the control of a different inducible promoter, and its growth phenotype is shown according to the auxotrophy: ++ , cells grew well on the SD-Ade and SD-His plates (colony size was about 1.5 mm after incubation at 30 °C for 3 days on an SD-Ade plate); + , cells grew on SD-Ade and SD-His plate (colony size was about 0.5 mm after incubation at 30 °C for 3 days on an SD-Ade plate and about 1.5 mm after incubation at 30 °C for 3 days on an SD-His plate); − , cells did not grow on SD-Ade plate after incubation at 30 °C for 7 days. As host strains, a tester PJ69-4A strain overexpressing Apg12p (PJ69-4A [APG12, 2m]) and a tester PJ69-4A strain (PJ69-4A) were used. Constructs fused with GAD of wild type and five deletion mutants of Apg7p are shown. The number in the parentheses of each construct indicates the amino acid number of Apg7p.

FIG. 2. Homodimer formation of Apg7p. A, coimmunoprecipitation (IP) of Myc-Apg7p with HA-Apg7p. A total lysate of the apg7Δ cells expressing both Myc-Apg7p and HA-Apg7p was prepared, and HA-Apg7p was immunoprecipitated with the anti-HA antibody. The sediments were analyzed by immunoblotting (IB) using anti-Myc (lane 2) or anti-HA (lane 4) antibodies. B, Apg7p dimer formed by chemical cross-linking. Myc-Apg7p was expressed in the apg7Δ cells, and the cell lysate was treated with 5 mM disuccinimidyld suberate (DSS), as described under “Experimental Procedures.” Myc-Apg7p was recognized by immunoblotting with an anti-Myc antibody. C, sedimentation analysis of Apg7p. Cell lysate was prepared from the apg7Δ cells expressing Myc-Apg7p and separated by centrifugation through 10–40% glycerol gradients. Fractions were collected from the bottom of the gradients and assayed for the presence of Myc-Apg7p by immunoblotting using an anti-Myc antibody (9E10). The positions of marker proteins in the gradients are indicated on the top (both as molecular mass, kDa; and sedimentation values, S). D, cross-linking of Apg7p with DSS in the extracts prepared from several apg mutants.
Apg5, Apg6, Apg8, Apg9, Apg10, Apg13, and Apg14 mutants (representative data are presented in Fig. 2D, and the data on the other mutants are not shown). These results indicate that Apg7p forms a homodimer with itself without the participation of other APG gene products.

The C-terminal Region of Apg7p Is Essential for Apg7p Dimerization—The issue arises as to the nature of the essential domain for the formation of Apg7p-homodimer. Motif analysis of the amino acid sequence of Apg7p showed that no potential dimerization motifs such as a coiled-coil or a leucine zipper exist on the molecule. To determine the region of Apg7p that is essential for dimer formation, systematic deletion analyses were performed. The original clone isolated (Apg7pΔN261) lacks N-terminal 261 amino acid residues, suggesting that a region that is proximal to the C terminus may be important for dimerization. Since Apg7pΔN261 still possesses both an ATP-binding site and an active-site cysteine residue, we first deleted the catalytic domain and examined the resultant construct (Apg7pΔN507) to determine whether it is capable of binding to full-length Apg7p. Apg7pΔN507 interacts with full-length Apg7p, indicating that the C-terminal portion (residues 508–630 out of 630 amino acids), which contains neither an ATP binding domain nor an active-site cysteine, is sufficient for interaction with the full-length Apg7p (Fig. 1, APG7ΔC40).

The location of the essential domain within the C-terminal 123 amino acids (residues 508–630) is also an open question. ClustalW analysis revealed that a C-terminal region containing 40 amino acids (residues 591–630) has a significant homology with the equivalent region of mammalian Apg7p homologs and that it shows a weak homology with the equivalent region of Uba1p (Fig. 3A) (40). The relevance of this region (C40 region) to Apg7p-Apg7p interaction was thus investigated. In a two-hybrid analysis, the deletion of the C40 region from Apg7p results in the complete loss of its ability to interact with full-length Apg7p (Fig. 1, APG7ΔC40), suggesting that the C40 region is essential for Apg7p dimerization. An attempt was made to determine whether the C40 region is sufficient for interaction with full-length Apg7p in a two-hybrid system. However, it was found that cells expressing only GBD-C40 were able to grow in selection plates. Therefore, further analyses on the C40 region itself were not pursued, and efforts were concentrated on Apg7pΔC40.

Apg7p Dimerization and E1-E2 Complex for Apg8p
confirmed the loss of interaction of Apg7p with Apg7pΔC40. Both wild-type HA-Apg7p and Myc-Apg7pΔC40 were coexpressed in the *Saccharomyces cerevisiae* strain *C40* cells. The cell lysate was immunoprecipitated with the anti-HA antibody, and Myc-Apg7p and Myc-Apg7pΔC40 were examined by immunoblot with the anti-Myc antibody. Myc-Apg7pΔC40 was expressed at a level similar to that of wild-type Myc-Apg7p in a galactose-dependent manner (Fig. 3B, lanes 4 and 2). Wild-type Myc-Apg7p was coimmunoprecipitated with HA-Apg7p (Fig. 3C, lane 2). In contrast, the mutant Apg7pΔC40, was not coimmunoprecipitated with HA-Apg7p (Fig. 3C, lane 6). These results indicate that the C40 region of Apg7p is required for the formation of the Apg7p homodimer.

The *C*-terminal Region Is Also Essential for Interactions of Apg7p with Two Substrates. Apg12p and Apg8p—What is the importance of the Apg7p-dimerization? Apg7pΔC40 still contains both an ATP binding domain and an active-site cysteine residue but has a defect relative to the formation of Apg7p homodimer. We hypothesized that the dimerization of Apg7p may be somehow correlated with the E1 activity of Apg7p for the Apg12p and Apg8p. It would be interesting to determine whether Apg7pΔC40 is able to bind to Apg12p and Apg8p. The interaction of Apg7pΔC40 with Apg12p was first investigated using a two-hybrid analysis. Surprisingly, the interaction of Apg7pΔC40 with Apg12p was completely abolished compared with the interaction of wild-type Apg7p and Apg12p (Fig. 4A, Prey Apg12). Furthermore, the loss of interaction of Apg7pΔC40 resulted in a defect in the formation of the Apg12p-Apg5p conjugate. No Apg12p-Apg5p conjugate was present in the Apg7pΔC40 mutant expressing Apg7pΔC40, whereas the conjugate was present in the mutant expressing wild-type Apg7p (Fig. 4B, lane 4 and 2). Similarly, a loss of interaction of Apg7pΔC40 with Apg8p was also detected using a two-hybrid assay (Fig. 4A, Prey Apg8). This was further confirmed via a coimmunoprecipitation experiment. A lysate of *Saccharomyces cerevisiae* strain *C40* expressing HA-Apg7pΔC40 and FLAG-Apg8p was prepared, and HA-Apg7p was immunoprecipitated with an anti-HA antibody, FLAG-Apg8p in the sediment was identified by immunoblot with the anti-FLAG antibody. No FLAG-Apg8p was coimmunoprecipitated with HA-Apg7pΔC40, whereas the FLAG-Apg8p was coimmunoprecipitated with wild-type HA-Apg7p (Fig. 4C, lanes 4 and 2). These results indicate that the C40 region of Apg7p is essential for the interaction of Apg7p with the two substrates. These defects in the interactions of Apg7pΔC40 with two substrates will result in pleiotropic defects of the autophagic...
and Cvt pathways. In yeast, autophagy is induced by a variety of starvation conditions, and its progression is easily monitored by means of light microscopy; autophagic bodies accumulate in the vacuoles of wild-type cells under conditions of nitrogen starvation, and the detection of these autophagic bodies is facilitated by phenylmethylsulfonyl fluoride, a protease inhibitor that blocks their degradation (3). In the vacuoles of apg7Δ cells expressing wild-type Apg7p, a significant accumulation of autophagic bodies was observed, whereas the apg7Δ cells expressing Apg7pΔC40 failed to accumulate autophagic bodies in the vacuole, as was the case of the apg7Δ cells carrying a control vector (Fig. 4, wild type, ΔC40, and apg7Δ). A defect in autophagy results in a loss of viability under conditions of starvation (6). The colony color of the apg7Δ mutant expressing Apg7pΔC40 turned a pink color under nitrogen starvation conditions, as evidenced by phloxine B-staining, similar to the apg7Δ mutant, whereas that of the apg7Δ mutant expressing wild-type Apg7p was not stained (Fig. 4E, ΔC40, apg7Δ, and wild type).

We next examined the effect of the C40 region of Apg7p on the Cvt pathway. Aminopeptidase I (API) is synthesized as the pro-form (proAPI) in the cytoplasm, transferred to the vacuole by a mechanism that is closely related to the autophagic pathway, and then processed to the mature form in the vacuole. In the case of the apg7Δ cells expressing wild-type Apg7p, proAPI was processed to the mature form (Fig. 4F, lane 2). In contrast, in the case of apg7Δ cells expressing mutant Apg7pΔC40, the mature form of API was not detected, and proAPI accumulated, similar to that of the apg7Δ cells (Fig. 4F, lane 3 and 1). Thus, the loss of function of Apg7p as the result of the deletion of the C40 region of Apg7p results in a defect in the Cvt pathway. These results indicated that the C40 region of Apg7p is essential for its E1 function in both the autophagic and Cvt pathways.

The C-terminal Region of Apg7p Is Necessary for the Formation of E1-E2 Complex for Apg8p—Recently, a comprehensive analysis of protein-protein interactions in the yeast *S. cerevisiae* by two-hybrid screening has indicated that Apg7p interacts not only with Apg12p and Apg8p but also with Apg3p (Fig. 5A) (39). Recent findings revealed that Apg3p is an E2 enzyme for Apg8p. Thus far, no report has appeared on the formation of an E1-E2 complex in a protein modification system similar to ubiquitylation. Then we first investigated whether Apg3p is coimmunoprecipitated with Apg7p. Both HA-Apg7p and Apg3p-Myc were coexpressed under the control of a galactose-inducible promoter in the *apg7Δ* cells, and the cell lysate was immunoprecipitated with an anti-HA antibody. Apg3p-Myc in the sediment was recognized by immunoblot with an anti-Myc antibody. Apg3p-Myc was coimmunoprecipitated with HA-Apg7p (Fig. 5B, lane 2). The coimmunoprecipitation of Apg3p with Apg7p occurs in the *apg12Δ* mutant (data not shown). This result indicates that the Apg7p interacts with Apg3p to form an E1-E2 complex.

Since the C40 region of Apg7p is essential for its interaction with the two substrates, it is probable that this region is essential for the interaction of Apg7p with Apg3p, too. To investigate this possibility further, a coimmunoprecipitation experiment was performed in the *apg7Δ* cells expressing both HA-Apg7pΔC40 and Apg3p-Myc. No Apg3p-Myc was coimmunoprecipitated with HA-Apg7pΔC40, whereas the Apg3p-Myc was coimmunoprecipitated with wild-type HA-Apg7p (Fig. 5B, lanes 4 and 2). This finding was confirmed by a two-hybrid analysis (Fig. 5A). Furthermore, as is the case with the interaction of Apg12p and Apg8p, Apg3p does not interact with Apg7p.

**FIG. 5. Effects of the deletion of C-terminal region of Apg7p on the formation of E1-E2 complex for Apg8p.** A, summary of interactions of Apg3p with Apg7p variants. Cells expressing both GBD fused Apg7p-variants (Bait) and GAD fused Apg3p (Prey) were examined for interaction-dependent activation of the *ADE2* gene and *HIS3* gene. B, inability of Apg3p to coimmunoprecipitate with mutant Apg7pΔC40. Total lysates from *apg7Δ* cells expressing either HA-Apg7p or HA-Apg7pΔC40 and Apg3p-Myc in the presence (+) or absence (−) of galactose were immunoprecipitated (IP) with an anti-HA antibody. The sediments were analyzed by immunoblotting (IB) using an anti-HA or anti-Myc antibody. The evidence presented herein indicates that Apg7p is a unique protein-activating enzyme that is capable of forming a homodimer and is essential for the two substrates (Apg12p and Apg8p). These characteristics have not been reported for other E1 enzymes. Furthermore, the Apg7p is able to form a stable E1-E2 complex. The dimerization occurs independently of other Apg gene products examined thus far, supporting the possibility that Apg7p interacts with itself without the need for any other factors. The deletion of the C40 region of Apg7p results in the loss of Apg7p dimerization. It is surprising that the C40 region is also essential for the interaction of Apg7p with two substrates, Apg12p and Apg8p, even though the mutant Apg7pΔC40 monomer still contains an ATP binding domain and an active site cysteine. The C40 region of Apg7p is also essential for the formation of the E1-E2 complex, Apg7pΔC40. The C-terminal 123 residues (residues 508–630), which contain the C40 region, are sufficient for interaction with full-length Apg7p. Combining these data, we conclude that homodimer formation via the C-terminal region is important for enzyme-substrate interaction and the formation of an E1-E2 complex.

**DISCUSSION**

The evidence presented herein indicates that Apg7p is a unique protein-activating enzyme that is capable of forming a homodimer and is essential for the two substrates (Apg12p and Apg8p). These characteristics have not been reported for other E1 enzymes. Furthermore, the Apg7p is able to form a stable E1-E2 complex. The dimerization occurs independently of other Apg gene products examined thus far, supporting the possibility that Apg7p interacts with itself without the need for any other factors. The deletion of the C40 region of Apg7p results in the loss of Apg7p dimerization. It is surprising that the C40 region is also essential for the interaction of Apg7p with two substrates, Apg12p and Apg8p, even though the mutant Apg7pΔC40 monomer still contains an ATP binding domain and an active site cysteine. The C40 region of Apg7p is also essential for the formation of the E1-E2 complex, Apg7pΔC40. The C-terminal 123 residues (residues 508–630), which contain the C40 region, are sufficient for interaction with full-length Apg7p. Combining these data, we conclude that homodimer formation via the C-terminal region is important for enzyme-substrate interaction and the formation of an E1-E2 complex.
During starvation-induced autophagy, Apg8p became localized on the forming autophagosomal membranes (31). Our recent findings suggest that both Apg7p and Apg3p function as E1 and E2 enzymes, respectively, which is necessary for Apg8p to target the autophagosomal membranes (29, 30). We therefore reason that multimer complexes that are formed during the two enzymatic reactions catalyzed by the Apg7p homodimer may play a key role in autophagy. An Apg7p-related scheme is shown in Fig. 6A. First, Apg7p undergoes homodimer formation (Fig. 6A, I). After Apg7p dimerization, Apg12p(s) is activated by the Apg7p homodimer (IIa). Subsequent transfer of Apg12p to Apg10p results in the formation of the Apg12p-Apg10p intermediate. Finally, Apg12p is covalently attached to Apg5p via an isopeptide bond (IIIa). When the Apg7p homodimer interacts with Apg8p, Apg8p is activated by the Apg7p homodimer (IIb). Apg3p transiently interacts with Apg7p homodimer to effectively form Apg8p-Apg3p intermediate. Finally, Apg8p targets to an autophagosome (IIIb). PE, phosphatidylethanolamine. B, Box V is proposed as the essential domain for the homodimerization of Apg7p and Uba1p. The box V, which contains a cluster of acidic amino acids, is essential for homodimerization of Apg7p and the formation of E1-E2 complex. The C-terminal region of Uba1p has a similarity with the box V within Apg7p, and Uba1p will also then form a homodimer. UBI, ubiquitin.
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IIIb). Apg7p, which forms an enzyme-substrate conjugate with Apg8p, also functions as an E1 enzyme, which is essential for subsequent Apg8p-phophatidyethanolamine conjugation. The interaction of Apg3p (E2) with Apg7p (E1) may facilitate the effective targeting of Apg8p to autophagosomal membranes as well as to ER-to-Golgi vesicles (Fig. 6A, IIIb) (28–32). How do these interactions correlate with the functions of these APG gene products? A key to revealing this question would be an interaction of Apg12p with Apg7p, which has been reported by a comprehensive two-hybrid screening (39). This suggests that Apg12p plays an important role in Apg8p targeting on autophagosomal membranes. At present, it is difficult to completely explain the functional correlation between Apg12p modification system and Apg8p-membrane targeting. Further analyses to clarify Apg12p versus Apg3p interaction will be necessary.

The human Apg7p/Cvt2p/Gsa7p homolog has been reported (41). The issue of whether or not the mammalian homolog forms a homodimer is of great interest. Several lines of observations suggest that the mammalian Apg7p homolog also is capable of entering into a homodimer formation. (i) The Apg7p conjugation system is conserved from yeast through mammalian cells (42). (ii) The human Apg7p homolog is an authentic protein-activating enzyme for human Apg12 (43). (iii) The C40 region of S. cerevisiae Apg7p is highly conserved among human Apg7p and mouse Apg7p (Fig. 4A). We are now investigating the possibility of homodimer formation in mammalian Apg7p homologs by means of cross-linking experiments and glycerol density gradient centrifugation.

Several E1 enzymes, Uba1p for ubiquitin, Aos1p-Uba2p for Smt3p, Ula1p-Uba3p for Rub1p, ApgP for Apg12p, and Uba4p for Ure1p have been characterized in yeast (26, 44–51). Additional E1 enzymes, Apg7p, which forms an enzyme-substrate conjugate with Smt3p, Ula1p-Uba3p for Rub1p, Apg7p for Apg12p, and Uba4p for Ure1p have been characterized in yeast (26, 44–51).

References

1. Seglen, P. O., and Bohey, P. (1992) *Experientia (Basel*) 48, 158–172
2. Dunn, W. A., Jr. (1994) *Trends Cell Biol.* 4, 139–143
3. Takeshige, K., Baba, M., Tsuboi, S., Noda, T., and Ohsumi, Y. (1992) *J. Cell Biol.* 119, 301–311
4. Baba, M., Takeshige, K., Baba, N, and Ohsumi, Y. (1994) *J. Cell Biol.* 124, 903–913
5. Baba, M., Osumi, M., and Ohsumi, Y. (1995) *Cell Struct. Funct.* 20, 465–471
6. Tsukada, M., and Ohsumi, Y. (1993) *FEBS Lett.* 333, 169–174
7. Thumm, M., Egner, R., Koch, B., Schlumpberger, M., Straub, M., Veenhuis, M., and Wolf, D. H. (1994) *FEBS Lett.* 349, 275–280
8. Harding, T. M., Hefner-Gravink, A., Thumm, M., and Klionsky, D. J. (1996) *J. Biol. Chem.* 271, 17621–17624
9. Scott, S. V., Nice, I. I. D. C., Nau, J. J., Weismann, L. S., Kamada, Y., and Ohsumi, M. (1996) *FEBS Lett.* 399, 5509–5516
10. Baba, M., Osumi, M., Scott, S. V., Klionsky, D. J., and Ohsumi, Y. (1997) *J. Cell Biol.* 139, 1657–1659
11. Klionsky, D. J., and Ohsumi, Y. (1999) *Annu. Rev. Cell Dev. Biol.* 15, 1–32
12. Matsuura, A., Tsukada, M., Wada, Y., and Ohsumi, Y. (1997) *Gene* 192, 245–250
13. Saitoh, M., Bredeschneider, M., and Thumm, M. (1997) *J. Bacteriol.* 179, 3875–3883
14. Funakoshi, T., Matsuura, A., Noda, T., and Ohsumi, Y. (1997) *J. Bacteriol.* 179, 207–213
15. Scott, S. V., Nau, J. J., Weismann, L. S., Kamada, Y., and Fricker, G. (1996) *J. Bacteriol.* 178, 9320–9328
16. Kametaka, S., Okano, T., Ohsumi, M., and Ohsumi, Y. (1998) *J. Bacteriol.* 170, 229–230
17. Noda, T., Kim, J., Huang, W.-P., Baba, M., Tokunaga, C., Ohsumi, Y., and Klionsky, D. J. (2000) *J. Cell Biol.* 146, 465–480
18. Lang, T., Reiche, S., Straub, M., Bredeschneider, M., and Thumm, M. (2000) *J. Bacteriol.* 182, 2125–2133
19. Varshavsky, A. (1997) *Trends Biochem. Sci.* 22, 383–387
20. Benfatto, J. S., and Weissman, A. M. (1998) *Annu. Rev. Cell Dev. Biol.* 14, 19–57
21. Ciechanover, A. (1998) *EMBO J.* 17, 7151–7160
22. Herskoe, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* 67, 425–479
23. Katsumata, S., Matsuura, A., Wada, Y., and Ohsumi, Y. (1996) *Gene* 179, 139–143
24. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Itoh, T., George, M. D., and Ohsumi, Y. (1996) *Nature* 382, 496–499
25. Shiota, K., Mizushima, N., Ogawa, Y., Matsuura, A., Noda, T., and Ohsumi, Y. (1999) *EMBO J.* 18, 5234–5241
26. Tanida, I., Mizushima, N., Kiyosaka, M., Ohsumi, Y., Ueno, T., Ohsumi, Y., and Komemiu, K. (1999) *Mol. Cell. Biol.* 19, 1367–1379
27. Mizushima, N., Noda, T., and Ohsumi, Y. (1999) *EMBO J.* 18, 3888–3896
28. Lang, T., Schaeffeler, E., Berruezo, D., Bredeschneider, M., Wolf, D. H., and Thumm, M. (1998) *EMBO J.* 17, 3597–3607
29. Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000) *J. Cell Biol.* 151, 365–375
30. Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shiminishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Komemiu, K., Ohsumi, M., Noda, T., and Ohsumi, Y. (2000) *Nature* 408, 488–492
31. Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., and Ohsumi, Y. (1999) *J. Cell Biol.* 147, 435–446
32. Huang, W.-P., Scott, S. V., Kim, J., and Klionsky, D. J. (2000) *J. Biol. Chem.* 275, 946–951
33. Legesse-Miller, A., Sagiv, Y., Gluzman, R., and Elazar, Z. (2000) *J. Biol. Chem.* 275, 32966–32973
34. Kim, J., Dalton, V. M., Eggerton, K. P., Scott, S. V., and Klionsky, D. J. (1999) *Mol. Cell. Biol.* 19, 1353–1366
35. Aos1p-Uba2p and Ula1p-Uba3p suggest that they are functional when the exist as a heterodimer with only one active-center center core. It is therefore important to understand how the divergence in molecular composition of protein-activating enzyme (E1) can be correlated with functional divergence in various cellular activities.

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The C-terminal Region of an Apg7p/Cvt2p Is Required for Homodimerization and Is Essential for Its E1 Activity and E1-E2 Complex Formation
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