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Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast

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Autophagy is a cellular process for bulk degradation of cytoplasmic components. The attachment of Apg12p, a modifier with no significant similarity to ubiquitin, to Apg5p is crucial for autophagy in yeast. This reaction proceeds in a ubiquitination-like manner, and requires Apg7p and Apg10p. Apg7p exhibits a considerable similarity to ubiquitin-activating enzyme (E1) and is found to activate Apg12p with ATP hydrolysis. Apg10p, on the other hand, shows no significant similarity to other proteins whose functions are known. Here, we show that after activation by Apg7p, Apg12p is transferred to the Cys-133 residue of Apg10p to form an Apg12p–Apg10p thioester. Cells expressing Apg10p^{C133S} do not generate the Apg12p–Apg5p conjugate, which leads to defects in autophagy and cytoplasm-to-vacuole targeting of aminopeptidase I. These findings indicate that Apg10p is a new type of protein-conjugating enzyme that functions in the Apg12p–Apg5p conjugation pathway.

Keywords: autophagy/protein conjugation/protein-conjugating enzyme/*Saccharomyces cerevisiae*

Introduction

Proteins are often modified by a variety of moieties in cells: phosphates, carbohydrates, lipids or proteins, etc. Such chemical processes, called post-translational modifications, modulate protein function by altering protein activity, stability, subcellular localization or interaction with other molecules. A well characterized type of protein modification is ubiquitination, which is involved in selective degradation of short-lived proteins, quality control of endoplasmic reticulum proteins, turnover of plasma membrane proteins and so on (Varshavsky, 1997; Bonifacino and Weissman, 1998; Ciechanover, 1998; Hershko and Ciechanover, 1998). Ubiquitin, a highly conserved 76-amino-acid-residue protein, is transferred to target proteins to form isopeptide bonds between the C-terminal glycine of ubiquitin and the ε-amino group of a lysine residue in the target protein. Ubiquitination involves sequential enzymatic reactions by several classes of enzymes. The ubiquitin-activating enzyme, E1, hydrolyzes ATP and forms an intermediate of ubiquitin adenylate, followed by the binding of ubiquitin to its own active-site cysteine residue in a thioester linkage. Activated ubiquitin is next transferred to an active-site cysteine residue of a ubiquitin-conjugating enzyme, E2, to form the ubiquitin thioester in a similar fashion. At the final step, which is often catalyzed by ubiquitin-protein ligase, E3, ubiquitin is attached to a lysine residue of the target protein via an isopeptide bond (Varshavsky, 1997; Bonifacino and Weissman, 1998; Hershko and Ciechanover, 1998).

In recent years, the ubiquitin-related proteins have been discovered and some of them have been found to be covalently attached to other proteins (Johnson and Hochstrasser, 1997; Kamitani *et al.*, 1997; Saitoh *et al.*, 1997; Hochstrasser, 1998; Lammer *et al.*, 1998). RanGAP1 (Ran-specific GTPase-activating protein) (Matunis *et al.*, 1996, 1998; Mahajan *et al.*, 1997, 1998), PML (Kamitani *et al.*, 1998; Müller *et al.*, 1998), Sp100 (Sternsdorf *et al.*, 1997, 1999) and I-κBα (Desterro *et al.*, 1998) were found to be covalently modified by SUMO-1 (small ubiquitin-related modifier-1). The ligation of SUMO-1 to RanGAP1 is crucial for its association with RanBP2, a component of the nuclear pore complex. Modification of PML or Sp100 by SUMO-1 is also important for the assembly of subnuclear structures termed nuclear dots or PML nuclear bodies (Duprez *et al.*, 1999; Müller and Dejean, 1999). Smt3p, a yeast homolog of SUMO-1, is essential for viability (Johnson and Blobel, 1997) and is likely to contribute to cell cycle regulation (Li and Hochstrasser, 1999). The enzyme system involved in conjugation of SUMO-1/Smt3p to other proteins has been identified recently (Gong *et al.*, 1997, 1999; Johnson *et al.*, 1997; Schwarz *et al.*, 1998; Desterro *et al.*, 1999; Okuma *et al.*, 1999). SUMO-1/Smt3p is activated by an Aos1–Uba2 heterodimer and conjugated to proteins by Ubc9. NEDD8 (neural precursor cell-expressed developmentally down-regulated gene 8) and its yeast homolog Rub1p, showing ~50% identity to ubiquitin, are conjugated to cullin/Cdc53p, a common subunit of the multifunctional SCF ubiquitin ligase, in a ubiquitination-like manner. The NEDD8 conjugation pathway requires at least two enzymes: an E1-like heterodimeric activating enzyme, APP-BP–hUba3, and an E2-like conjugating enzyme, hUbc12 (Osaka *et al.*, 1998; Gong and Yeh, 1999). The activating and conjugating enzymes of the Rub1p conjugation pathway have also been identified as Ula1p–Uba3p and Ubc12p, respectively (Lammer *et al.*, 1998; Liakopoulos *et al.*, 1998). Although substrates of Ubc9 and Ubc12 are not ubiquitin, Ubc9 and Ubc12 are structurally similar to the ubiquitin E2s.

Recently, we found a novel modifier essential for autophagy (Mizushima *et al.*, 1998a). Autophagy is a cellular

process essential for bulk degradation of cytoplasmic components in eukaryotes. We demonstrated previously that cytoplasmic components including proteins, carbohydrates, lipids, nucleic acids and organelles are transported to the vacuole in yeast by macroautophagy in response to nutrient starvation (Takeshige *et al.*, 1992; Baba *et al.*, 1994, 1995). When cells face nutrient starvation, cytoplasmic components are sequestered non-selectively by double-membrane-bound structures termed autophagosomes and then targeted to the lysosome/vacuole to be degraded. Autophagy plays a central role in protein turnover, which may be important for cellular remodeling during development and differentiation. Taking advantage of yeast genetics, autophagy-deficient mutants of *Saccharomyces cerevisiae* (*apg* and *aut*) have been isolated independently by two laboratories (Tsukada and Ohsumi, 1993; Thumm *et al.*, 1994). Surprisingly, the *apg* and *aut* mutants partially overlap with *cvt* mutants that are based on defects in cytoplasm-to-vacuole targeting (Cvt) of aminopeptidase I (Harding *et al.*, 1996; Scott *et al.*, 1996). The Cvt pathway uses a similar mechanism to the autophagic pathway (Baba *et al.*, 1997). During analyses of *APG* gene products, we found a novel protein conjugation system that is indispensable for both autophagy and the Cvt pathway (Mizushima *et al.*, 1998a). Apg12p is a hydrophilic 186-amino-acid-residue protein with no similarity to ubiquitin or ubiquitin-related modifiers, which is covalently attached to Apg5p. The Apg12p–Apg5p conjugate further associates with Apg16p to form the Apg12p–Apg5p–Apg16p multimeric complex (Mizushima *et al.*, 1999). The manner of conjugation of Apg12p to Apg5p is ubiquitination-like: a covalent linkage of the C-terminal glycine of Apg12p to the lysine-149 side chain of Apg5p via an isopeptide bond. This reaction requires at least two proteins: Apg7p and Apg10p. Apg7p is similar to ubiquitin-activating enzyme in its amino acid sequence around the ATP binding site and the active-site cysteine residue, and has been found to be an E1-like Apg12p-activating enzyme (Mizushima *et al.*, 1998a; Tanida *et al.*, 1999). Apg10p, on the other hand, shows no significant similarity with other proteins whose functions are known. Here, we show that Apg10p is a new type of protein-conjugating enzyme that functions in the Apg12p conjugation pathway.

Results

apg10-1 shows a defect in autophagy

We have isolated and characterized an *apg10-1* mutant (Tsukada and Ohsumi, 1993). Wild-type cells accumulated autophagic bodies (ABs) in their vacuoles in a nitrogen-starvation medium containing 1 mM phenylmethylsulfonyl fluoride (PMSF), which were observed under a light microscope (Takeshige *et al.*, 1992 and Figure 1A, wild type). On the other hand, the *apg10-1* cells did not accumulate ABs (Figure 1A). Moreover, the *apg10-1* mutation severely reduced the viability of the cells under the starvation condition (Figure 1B). It was found that Apg12p is covalently attached to Apg5p via an isopeptide linkage in a ubiquitination-like manner and this conjugation is crucial for autophagy (Mizushima *et al.*, 1998a). The *apg10-1* mutant completely lost the ability to generate the Apg12p–Apg5p conjugate (Mizushima *et al.*, 1998a and Figure 1C). These data suggest that the defect of the *apg10-1* mutant in autophagy is due to loss of the Apg12p–Apg5p conjugate, and that Apg10p probably functions at the Apg12p-conjugating step.

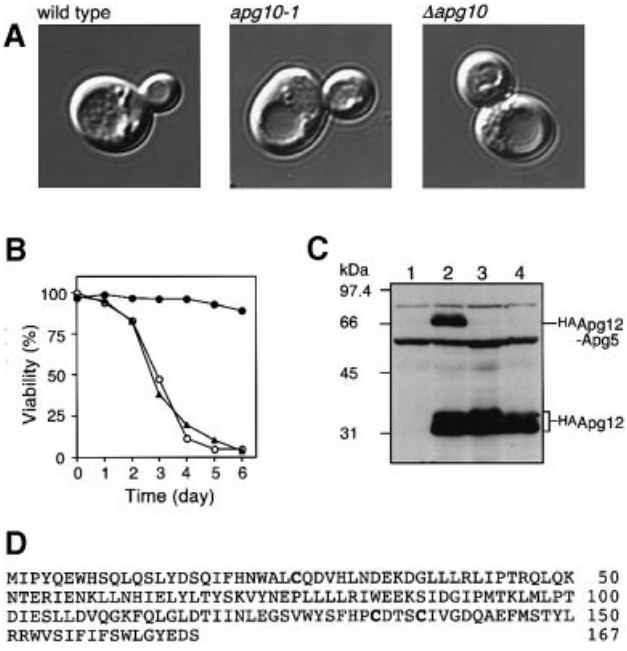

Fig. 1. Cloning of the *APG10* gene and phenotype of Δ*apg10* cell. (**A**) Wild-type (YW5-1B), *apg10-1* mutant (MT91-4-2) and Δ*apg10* (TFD10-L1) cells were incubated in SD(–N) medium containing 1 mM PMSF at 30°C. After incubation for 6 h, cells were observed under light microscopy (Nomarski images). (**B**) Wild-type (closed circles), *apg10-1* mutant (closed triangles) and Δ*apg10* (open circles) cells were grown to 1 OD_{600}/ml in YPD and then transferred to SD(–N) medium. After incubation at 30°C for the times indicated, their viability was determined by phloxine B staining (Tsukada and Ohsumi, 1993). (**C**) Cell lysates from wild-type cells with empty vector pRS316 (lane 1) or ^{HA}*APG12* (lane 2), and also *apg10-1* (lane 3) and Δ*apg10* cells with ^{HA}*APG12* (lane 4) were subjected to Western blotting analysis with anti-HA antibody (16B12). (**D**) Amino acid sequence of Apg10p. Cysteine residues are indicated in bold.

Isolation and sequence analysis of APG10

The *APG10* gene was cloned by complementation of reduced viability of *apg10-1* mutant under starvation conditions as described in Materials and methods. A YCp50-based yeast genomic library (a gift from Dr Wada, Osaka University) was introduced into *apg10-1* cells, and screening of ~10 000 transformants yielded plasmid 5-5C containing an 8.0 kb genomic fragment. Subcloning of the fragment of 5-5C revealed that a 1.2 kb *Xba*I–*Hin*dIII fragment containing one open reading frame (ORF) YLL042c was sufficient for complementation. The YLL042c is a novel gene and encodes a hydrophilic protein of 167 amino acids with a predicted molecular mass of 19.8 kDa (Figure 1D).

To determine whether YLL042c was the authentic *APG10* gene, this ORF was disrupted by replacing with a *LEU2* gene. The disruptant exhibited the same phenotype as the *apg10-1* cell: (i) the autophagic bodies did not accumulate in the vacuole during starvation (Figure 1A); (ii) the viability of the disruptant decreased under the starvation condition (Figure 1B); and (iii) the Apg12p–Apg5p conjugate was not generated at all (Figure 1C). Sequence analysis of the *apg10-1* allele revealed that the 482nd nucleotide of the coding sequence was altered from

G to A, which led to translation termination at the 160th amino acid residue. Furthermore, a diploid cell obtained by crossing *apg10-1* and Δ*yll042c* cells is also defective in autophagy. We concluded therefore, that YLL042c was the authentic *APG10* gene.

The amino acid sequence of Apg10p did not provide any insight into its function. A BLAST search, however, revealed that it was homologous to a *Caenorhabditis elegans* protein of unknown function (DDBJ/EMBL/GenBank accession No. Z54282) but exhibited no significant similarity to ubiquitin-conjugating enzymes or ubiquitin-protein ligases.

Formation of an Apg12p–Apg10p thioester

To elucidate how Apg10p participates in the Apg12p–Apg5p conjugation pathway, we first examined whether Apg10p could interact with other components of this pathway. In a two-hybrid assay (James *et al.*, 1996), we observed that Apg10p physically interacts with Apg7p and Apg12p but not with Apg5p (Figure 2A). Next we performed coimmunoprecipitation to confirm the Apg10p–Apg12p interaction. Total cell lysates of Δ*apg5* cells with ^{HA}*APG10* and/or ^{Myc}*APG12* (3× Myc-tagged construct) on 2μ plasmids were immunoprecipitated with either anti-HA or anti-Myc antibody. The resulting precipitates were analyzed by Western blotting. When the cells expressed both ^{HA}Apg10p and ^{Myc}Apg12p, ^{Myc}Apg12p was coprecipitated by pulling ^{HA}Apg10p down with anti-HA antibody (Figure 2B, lane 17), and ^{HA}Apg10p was coprecipitated with ^{Myc}Apg12p (Figure 2B, lane 18). These results indicate that Apg10p interacts with Apg12p *in vivo*. Under non-reducing SDS–PAGE conditions, an additional 56 kDa band was also detected by both anti-HA and anti-Myc antibodies (Figure 2B, lanes 8 and 9), whereas it was not detected in cells harboring either ^{HA}*APG10* or ^{Myc}*APG12* plasmid. Furthermore, its molecular mass was shifted from 56 to 61 kDa by changing ^{Myc}*APG12* plasmid to a 6× Myc-tagged construct (data not shown). This difference in molecular mass just corresponds with a size of 3× Myc. These findings show that the 56 kDa band contains both ^{HA}Apg10p and ^{Myc}Apg12p, and these molecules conjugate 1:1 via a reducing reagent-sensitive bond. Apg12p has three cysteine residues. Because the substitutions of all three cysteine residues of Apg12p by alanine did not affect the formation of the Apg12p–Apg10p conjugate, the dithiothreitol (DTT)-sensitive bond is probably a thioester bond between the C-terminal glycine of Apg12p and a cysteine residue of Apg10p, but is not a disulfide bond. Another DTT-sensitive 67 kDa band was also detected by anti-Myc but not by anti-HA antibody (Figure 2B, lanes 6, 8 and 9), suggesting that it contained Apg12p but not Apg10p. We concluded, however, from several lines of evidence (described below) that the 67 kDa complex is not involved in the Apg12p–Apg5p conjugation pathway. Similar results were obtained with the cells expressing both ^{HA}Apg10p and ^{Myc}Apg12p from CEN plasmids (data not shown).

Next we examined the effects of Apg7p and Apg5p on formation of the Apg12p–Apg10p conjugate. Apg7p has already been found to function as an E1-like Apg12p-activating enzyme by Mizushima *et al.* (1998a) and Tanida *et al.* (1999). Apg7p probably activates Apg12p with hydrolysis of ATP and subsequent transfer to its own thiol

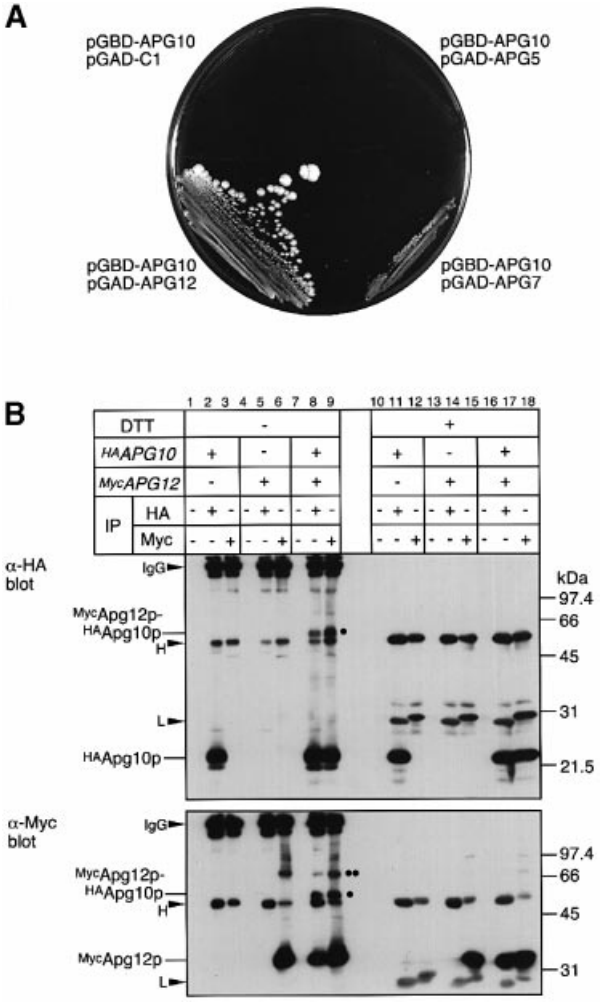

Fig. 2. *In vivo* interaction of Apg10p with Apg12p. (**A**) Two-hybrid assay for interactions of Apg10p with Apg5p, Apg7p and Apg12p. Yeast cells (PJ69-4A) harboring the indicated plasmids were streaked out on plates with medium lacking adenine to assay for interaction-dependent activation of the *ADE2* gene. (**B**) Total lysates from Δ*apg5* cells with ^{HA}*APG10* and/or ^{Myc}*APG12* on 2μ plasmid were immunoprecipitated with anti-HA (mAb 16B12) or anti-Myc (mAb 9E10) antibody. Precipitates were analyzed by Western blotting using anti-HA or anti-Myc antibody. Apg12p adduct of Apg10p is indicated by ● and an unidentified complex of 67 kDa by ●●. The positions of cross-reacting IgG heavy chain (H) and light chain (L) are indicated by arrowheads.

group of the Cys-507. By analogy with ubiquitination, an Apg12p covalently bound to the Cys-507 residue of Apg7p can be transferred to a cysteine residue of a hypothetical E2-like enzyme. Figure 3 shows that the Apg12p–Apg10p conjugate was not formed in Δ*apg7* cells, suggesting that Apg12p is tranferred to Apg10p after Apg7p functions (Figure 3, lanes 8 and 9). The non-covalent interaction between Apg10p and Apg12p was also lost completely in Δ*apg7* cells. Considering that Apg7p should activate the C-terminus of Apg12p, the Apg7p-dependent formation of the Apg12p–Apg10p conjugate suggests that Apg12p and Apg10p are linked via a thioester bond. As stated above, the interaction between Apg7p and Apg10p was confirmed in the two-hybrid assay. Taken together, these results suggest that an activated Apg12p is transferred directly from Apg7p to an active-site cysteine residue of Apg10p.

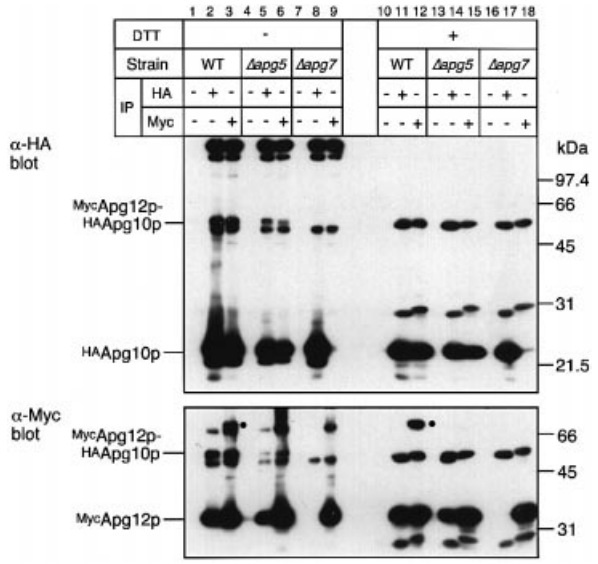

Fig. 3. Effects of the deletion of *APG5* or *APG7* on Apg12p–Apg10p thioester formation. Coimmunoprecipitation was performed with wild-type (KA311B), Δ*apg5* (YNM122) or Δ*apg7* (YTS12) cells harboring ^{HA}*APG10* and ^{Myc}*APG12* on 2μ plasmids. The resulting precipitates were analyzed by Western blotting. The DTT-resistant Apg12p–Apg5p conjugate is indicated by ●.

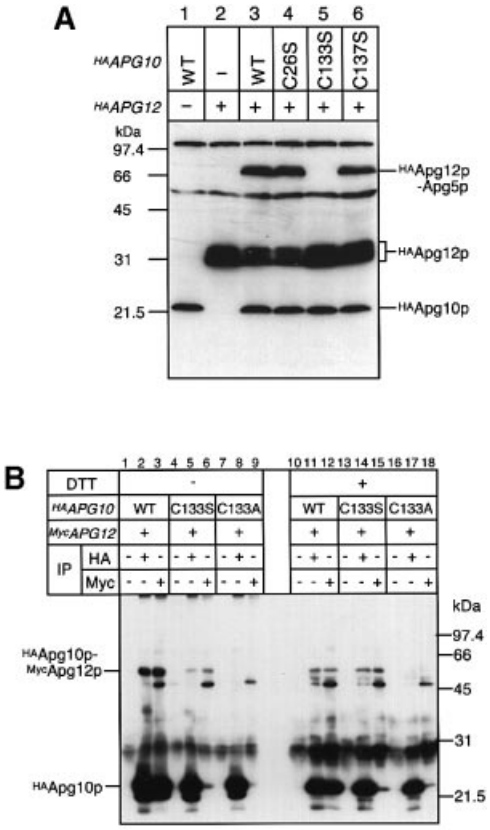

Fig. 4. Apg12p is transferred to the Cys-133 residue of Apg10p. (**A**) Cell lysates from Δ*apg10* cells (TFD10-L1) harboring ^{HA}*APG12* and each mutant form of ^{HA}*APG10* on CEN plasmids were subjected to Western blotting analysis with anti-HA antibody (mAb 16B12). (**B**) Cell extracts from the wild-type cells (KA311B) harboring ^{Myc}*APG12* and mutated ^{HA}*APG10* were subjected to immunoprecipitation and the resulting precipitates were analyzed by Western blotting with the rabbit anti-HA antibody.

When coimmunoprecipitation was carried out using wild-type cells, Apg12p–Apg10p thioester was generated as in the Δ*apg5* cells, i.e. the deletion of Apg5p, a target molecule of the Apg12p conjugation, did not promote accumulation of the Apg12p–Apg10p conjugate (Figure 3, lanes 2 and 3). Overexpression of Apg5p also had no effect on the formation of the Apg12p–Apg10p thioester (data not shown). These results indicate that the amount of Apg5p would not change the kinetics of Apg12p–Apg10p thioester formation. Moreover, in wild-type cells, the Apg12p–Apg5p conjugate was precipitated with anti-Myc but not with anti-HA antibody (Figure 3B, lanes 3 and 12) providing evidence that Apg10p no longer associates with Apg12p after Apg12p is transferred to Apg5p. The interaction between Apg10p and Apg5p was not detected in the two-hybrid assay (Figure 2A). These data suggest that Apg12p is not transferred directly from Apg10p to Apg5p, and other factor(s) may be necessary for the Apg10p-to-Apg5p transfer of Apg12p.

The DTT-sensitive 67 kDa band was even detected in the Δ*apg7* cells when immunoprecipitation was carried out by anti-Myc antibody (Figure 3, lane 9), suggesting that the 67 kDa complex could be generated without activation of Apg12p. Moreover, the band appeared even when an *APG12ΔG* (C-terminal Gly deletion) construct, which could conjugate to Apg5p no longer, was used for immunoprecipitation instead of a wild-type *APG12* construct (data not shown). Therefore, we concluded that the 67 kDa complex did not participate in the Apg12p–Apg5p conjugating reaction.

Apg12p is attached to the Cys-133 residue of Apg10p

There are three cysteine residues in Apg10p (Figure 1D). To determine which cysteine residue contributes to thioester formation, each cysteine residue of Apg10p was substituted by serine using site-directed mutagenesis. First, we examined the abilities of the substitutes to generate the Apg12p–Apg5p conjugate by Western blotting. The Apg12p–Apg5p conjugate of 70 kDa was formed in Δ*apg10* cells harboring ^{HA}*APG10* and ^{HA}*APG12* on CEN plasmids (Figure 4A, lane 3). The substitution of Cys-26 or Cys-137 by Ser (C26S or C137S, respectively) in Apg10p did not affect the generation of the conjugate at all, whereas the substitution of Cys-133 by Ser (C133S) resulted in a complete loss of the conjugation, although Apg10p^{C133S} was detected in an amount comparable to that of wild-type Apg10p (Figure 4A).

To investigate whether Apg12p was covalently attached to Cys-133 of Apg10p by a thioester bond, coimmunoprecipitation was carried out using the C133S or C133A (the substitution of Cys-133 by Ala) mutants (Figure 4B). As compared with the positive control (Figure 4B, lanes 2 and 3), quite a small amount of Apg12p–Apg10p^{C133S} conjugate was detected (Figure 4B, lanes 5 and 6). This conjugate was found to be resistant to the reducing reagent (Figure 4B, lanes 14 and 15), which suggested that an ester bond is formed between Apg12p and Apg10p^{C133S} instead of a thioester bond. The C133A mutation caused a complete loss of the Apg12p–Apg10p conjugate even in the absence of the reducing reagent (Figure 4B, lanes 8 and 9). These results indicate that Cys-133 is an active center of Apg10p and contributes to thioester formation.

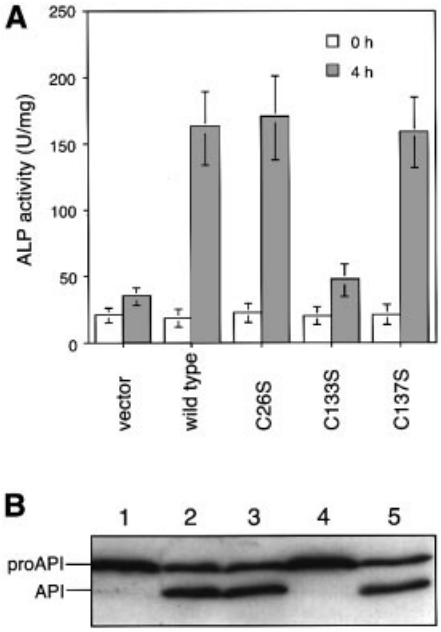

Fig. 5. The cysteine-133 residue of Apg10p is essential for autophagy and the Cvt pathway. (**A**) Autophagic activities were measured by an ALP assay (Noda and Ohsumi, 1998). YTS3 cells (Δ*apg10 PHO8::pho8Δ60*) harboring each mutant *APG10* on CEN plasmids were grown to 1 OD_{600}/ml in SC medium lacking tryptophan and then transferred to SD(–N) medium. Lysates from the cells after incubation for 0 and 4 h were used for assay. Error bars indicate the SD of three independent experiments. (**B**) Cell lysates from Δ*apg10* cells (TFD10-L1) harboring empty vector, pRS316 (lane 1), wild-type $^{HA}APG10$ (lane 2), $^{HA}APG10^{C26S}$ (lane 3), $^{HA}APG10^{C133S}$ (lane 4) or $^{HA}APG10^{C137S}$ (lane 5) on CEN plasmids were subjected to Western blotting analysis with anti-API antiserum.

Both mutations prevented the interaction between the free forms of Apg12p and Apg10p (Figure 4B, lanes 6 and 9).

Next we asked whether the Cys-133 mutation of Apg10p affected autophagy and Cvt of proaminopeptidase I (pro-API). Autophagic activities were measured using a biochemical assay monitoring autophagy-dependent processing of alkaline phosphatase Pho8Δ60 (Noda *et al.*, 1995). In wild-type cells, Pho8Δ60 was activated by transferring the cells from a rich medium to a nitrogen-depleted medium. The Δ*apg10* cells expressing $Apg10p^{C133S}$ did not activate Pho8Δ60 even in the starvation medium, while those expressing $Apg10p^{C26S}$ or $Apg10p^{C137S}$ showed normal autophagic activity (Figure 5A). These data indicate that the C133S mutation in Apg10p causes a defect in autophagy. ProAPI is synthesized in the cytoplasm and imported directly to the vacuole by a mechanism closely related to the autophagic pathway, and then processed to the mature form by proteinase B. As expected, proAPI was not processed in the Δ*apg10* cells expressing $Apg10p^{C133S}$ (Figure 5B), implying that the formation of a thioester intermediate is also essential for API transport.

Discussion

Apg12p is a modifier protein with no significant similarity to ubiquitin and ubiquitin-related modifiers. The covalent binding of Apg12p to Apg5p is essential for autophagy in yeast. The Apg12p–Apg5p conjugating reaction is initiated by Apg7p, an Apg12p-activating enzyme, with ATP hydrolysis (Mizushima *et al.*, 1998a; Tanida *et al.*, 1999). Here, we have characterized Apg10p as an Apg12p-conjugating enzyme acting after Apg7p in the Apg12p–Apg5p conjugation pathway. Coimmunoprecipitation experiments revealed that a reducing reagent-sensitive covalent adduct was formed between Apg12p and Apg10p in an Apg7p-dependent manner (Figures 2B and 3). This adduct is likely to be a thioester linked between the C-terminal carboxy group of Apg12p and the active-site cysteine-133 of Apg10p (Figure 4). These findings suggest that the high-energy thioester bond between Apg12p and Apg7p is transferred to Apg10p through transacylation. The Apg12p transfer from Apg7p to Apg10p certainly occurs directly because the interaction between Apg7p and Apg10p was observed in the yeast two-hybrid assay (Figure 2A). As shown above, Apg10p functions as an 'E2' in the Apg12p–Apg5p conjugation system.

On the other hand, we speculate that the transfer of Apg12p from Apg10p to Apg5p, a final target molecule of Apg12p, requires other protein(s) on the grounds of several lines of evidence: (i) the interaction between Apg10p and Apg5p was not detected by a two-hybrid assay (Figure 2A); (ii) Apg12p did not interact with Apg5p in the Δ*apg7* or Δ*apg10* cells with a two-hybrid assay but did in wild-type cells (data not shown); (iii) Apg10p no longer associated to the Apg12p–Apg5p conjugate (Figure 3, lanes 2, 3, 11 and 12), which suggests that neither Apg10p nor Apg12p contributes to the recognition of Apg5p; and (iv) moreover, the changes in expression level of Apg5p did not affect the kinetics of the Apg12p–Apg10p thioester formation (Figure 3). The other factor(s) that recognizes Apg5p may be essential for the transfer of Apg12p from Apg10p to Apg5p. Our model for an enzyme system of the Apg12p–Apg5p conjugation pathway is shown in Figure 6. Apg7p hydrolyzes ATP and forms the high-energy thioester bond between its active-site cysteine and the C-terminus of Apg12p. The activated Apg12p is transferred directly from Apg7p to the active-site cysteine-133 of Apg10p to form the thioester bond. Then Apg12p is transferred to the target protein Apg5p, which may require the additional protein(s).

In the coimmunoprecipitation experiment, the non-covalent interaction between Apg12p and Apg10p was also observed, which required both Apg7p and Apg10p activity (Figures 3 and 4B). This suggests that the non-covalent association is dependent on Apg12p–Apg10p thioester formation. Therefore, the non-covalent complex may actually exist as one of the intermediate forms of the reaction during transfer of Apg12p from Apg10p to Apg5p. Alternatively, the non-covalent complex may be due to the unexpected cleavage of the thioester bond during the preparation of samples.

As described above, the reaction mechanism for Apg12p–Apg5p conjugation is closely related to those for the ubiquitin or ubiquitin-related modifier system, and Apg10p catalyzes the E2-like reaction in the Apg12p–Apg5p conjugation pathway. A large number of E2s have been identified widely in eukaryotes and comprise the Ubc (ubiquitin-conjugating enzyme) family that is characterized by conserved sequences, termed 'E2 motifs', containing the active-site cysteine and a conserved His–Pro–Asn tripeptide (Haas and Siepmann, 1997). It has been found that some Ubcs are used for ubiquitin-related

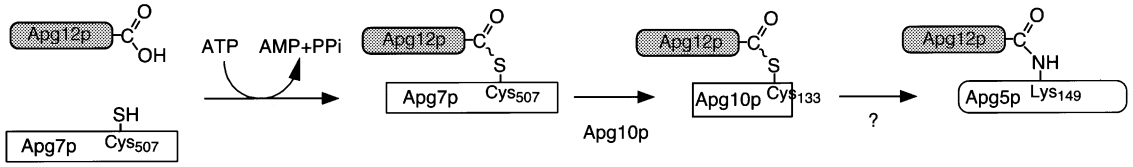

Fig. 6. Model of the enzymatic pathway for the Apg12p–Apg5p conjugation system. Apg12p is activated by Apg7p with ATP hydrolysis; the carboxy group of the C-terminal glycine of Apg12 is conjugated to the thiol group of the cysteine-507 residue of Apg7p via a high-energy thioester bond (~). Subsequent transfer of Apg12p to the thiol group of the cysteine-133 residue of Apg10p results in the formation of the Apg12p–Apg10p thioester. Finally, the C-terminus of Apg12p is covalently attached to the lysine-149 residue of Apg5p via an isopeptide bond, which may require hypothetical Apg5p-recognizing protein(s).

Table I. Yeast strains used in this study

Strain	Genotype	Source
YW5-1B	*MAT***a** *ura3 leu2 trp1*	Y.Wada
MT91-4-2	*MAT***a** *ura3 apg10-1*	Tsukada and Ohsumi (1993)
TFD10-L1	*MAT***a** *ura3 leu2 trp1 Δapg10::LEU2*	this study
KA311B	*MATα ura3 leu2 his3 trp1*	Irie *et al.* (1993)
YNM122	*MATα ura3 leu2 his3 trp1 Δapg5::HIS3*	Mizushima *et al.* (1998a)
YTS12	*MATα ura3 leu2 his3 trp1 Δapg7::HIS3*	this study
TN125	*MAT***a** *ura3 leu2 his3 trp1 ade2 lys2 PHO8::pho8Δ60*	Noda *et al.* (1998)
YTS3	*MAT***a** *ura3 leu2 his3 trp1 ade2 lys2 PHO8::pho8Δ60 Δapg10::LEU2*	this study
PJ69-4A	*MAT***a** *ura3 leu2 his3 trp1 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James *et al.* (1996)

modifier, e.g. Ubc9 and Ubc12 for SUMO-1/Smt3p and NEDD8/Rub1p, respectively (Gong *et al.*, 1997; Johnson and Blobel, 1997; Lee *et al.*, 1998; Liakopoulos *et al.*, 1998; Osaka *et al.*, 1998; Schwarz *et al.*, 1998). In spite of the similarity of the reaction mechanisms between the Apg12p and ubiquitin systems, the Apg12p system uses the protein with no significant sequence similarity to Ubcs as an E2. Because the structure of Apg12p does not resemble that of ubiquitin or ubiquitin-related modifier at all, except for the C-terminal glycine, the protein structurally unrelated to Ubcs, namely Apg10p, must be necessary for the recognition of Apg12p and/or Apg7p. This is the first example showing that the protein quite different from Ubcs functions as an E2-like protein-conjugating enzyme.

A BLAST search revealed that Apg10p has a potential *C.elegans* homolog of unknown function. In addition, the EST fragments that are closely related to Apg10p are also found in human (DDBJ/EMBL/GenBank accession No. AA448080) and mouse (DDBJ/EMBL/GenBank accession No. AA673136). The sequences around the cysteine-133 residue are well conserved among these proteins, supporting the suggestion that the cysteine-133 residue of Apg10p is the active site. Considering that the Apg12p–Apg5p conjugation system is conserved from yeast to human (Mizushima *et al.*, 1998b), Apg10p should also be widely distributed in eukaryotes. Recently, it was reported that the *Pichia pastoris* homolog of Apg7p, Gsa7, is required for microautophagy of peroxisomes and is able to complement the defects in macroautophagy and API transport in *S.cerevisiae* (Kim *et al.*, 1999; Yuan *et al.*, 1999). These findings suggest that Apg10p may function in peroxisome degradation as well as macroautophagy and the Cvt pathway.

Materials and methods

Strains and media, genetic and molecular biological techniques

The *S.cerevisiae* strains used in this study are listed in Table I. Yeast cells were grown in yeast extract–peptone–dextrose (YPD) or synthetic complete (SC) media. SD(–N) medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate and 2% glucose) was used for nitrogen starvation. Standard genetic manipulations were performed as described by Adams *et al.* (1998). DNA manipulations were performed using standard methods (Sambrook *et al.*, 1989).

Cloning, disruption and epitope tagging of APG10

APG10 was cloned by complementation of reduced viability of *apg10-1* in the SD(–N) medium as described previously (Funakoshi *et al.*, 1997; Kametaka *et al.*, 1998). A yeast mutant MT91-4-2 was transformed with a YCp50-based yeast genomic library and Ura^+ transformants were replica-plated onto SD(–N) plates containing 10 μg/ml phloxine B, on which dead cells were stained red (Tsukada and Ohsumi, 1993). White colonies were picked up and subjected to a light microscopic observation to confirm the accumulation of autophagic bodies in their vacuoles.

Plasmids were rescued from the candidate cells and one plasmid, named 5-5C, was used for further analysis. Subcloning identified a 1.2 kb *Xba*I–*Hin*dIII fragment as the minimal region with complementing activity.

The 2.4 kb *Xba*I–*Kpn*I fragment was subcloned into pBluescript II KS+ (Stratagene) to generate pKSAPG10. The 2.0 kb *Sma*I–*Pst*I *LEU2* fragment from pJJ282 (Jones and Prakash, 1990) was cloned into *Pst*I–*Eco*RV-digested pKSAPG10 to generate pKSapg10::LEU2. The 4.24 kb *Nhe*I–*Nco*I fragment from pKSapg10::LEU2 was used for transformation of YW5-1B and TN125. The disruption of the *APG10* gene was confirmed by PCR.

C-terminal epitope-tagged *APG10* (^{HA}*APG10*) was constructed by inserting the DNA sequence encoding a repeated hemagglutinin (HA)-epitope tag (2× HA) just before the stop codon. The tagged ^{HA}*APG10* was confirmed to be functional by complementation of the *apg* phenotype of the *apg10-1* cell.

Plasmid construction

^{HA}*APG10* was cloned into the *Xba*I and *Eco*RI sites of pRS316 and pRS426 (Sikorski and Hieter, 1989) to generate pHA-APG10-316 and pHA-APG10-426, respectively. pHA-APG12-314 was described previously (Mizushima *et al.*, 1998a) and pMyc-APG12-424 was generated by subcloning of the DNA fragment encoding 3× Myc-tagged Apg12p (Mizushima *et al.*, 1998a) into the *Spe*I and *Xho*I sites of pRS424. For the two-hybrid assay, the entire *APG10* ORF was amplified by PCR and inserted into the *Eco*RI site of pGBD-C1 (James *et al.*, 1996) to generate pGBD-APG10 that expresses the DNA binding domain of Gal4p fused with Apg10p. pGAD-APG5 was created by insertion of the entire *APG5* ORF into the *Bam*HI and *Pst*I sites of pGAD-C1 to fuse Apg5p to the C-terminal end of the Gal4p activation domain. pGAD-APG7 contains the same APG7 fragment as pGBD-APG7 (Tanida

et al., 1999). pGAD-APG12 was described previously (Mizushima *et al*., 1999).

Site-directed mutagenesis

Site-directed mutagenesis of *APG10* was performed by PCR (Ho *et al.*, 1989). The following primers were used: C26S-Fw, 5′-CTGGGCCCTTT-CCCAAGATGTCC-3′; C26S-Rv, 5′-GACATCTTGGGAAAGGGC-CCAGT-3′; C133S-Fw, 5′-TTCCATCCATCCGATACATCATGTATA-3′; C133S-Rv, 5′-CATGATGTATCGGATGGATGGAA-3′; C133A-Fw, 5′-TTCCATCCAGCCGATACATCATGTATA-3′; C133A-Rv, 5′-CATG-ATGTATCGGCTGGATGGAA-3′; C137S-Fw, 5′-GATACATCATCTA-TAGTAGGTGAC-3′; C137S-Rv, 5′-GTCACCTACTATAGATGATG-TATC-3′. The mutated *APG10* fragments were inserted into the *Xba*I and *Eco*RI sites of pRS316 or pRS426. All mutations were verified by dideoxy sequencing.

Immunoprecipitations

Yeast cells harboring ^{HA}*APG10* and/or ^{Myc}*APG12* on 2μ plasmid were broken with glass beads in IP buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF and 1× protease inhibitor mixture (Complete™, Boehringer Mannheim)]. Cell debris was removed by centrifugation at 5000 *g* for 5 min, and the protein concentration of the resultant lysate was determined by Bradford's method. After Nonidet P-40 was added to the lysate to a final concentration of 1%, it was incubated with protein G–Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) at 4°C for 1 h for preabsorption. The preabsorbed lysate containing 5 mg of protein was incubated with or without 1 μl of mouse monoclonal anti-HA (16B12, BAbCo) or anti-Myc (9E10) antibody at 4°C for 2 h and then 20 μl of protein G–Sepharose (50% slurry) were added to it. After incubation at 4°C for 2 h, unbound proteins were removed by three washes in 1 ml of IP buffer containing 1% Nonidet P-40. The immunoprecipitated proteins were eluted by boiling in SDS gel-loading buffer with or without 100 mM DTT. Proteins were separated by SDS–PAGE and analyzed by immunoblotting with anti-HA (16B12), anti-Myc antibody (9E10) or rabbit polyclonal anti-HA antibody (BAbCo).

Western blotting analysis of whole yeast lysate

Cells were resuspended in 100 μl of 0.2 N NaOH, 0.5% 2-mercapto-ethanol. After incubation for 15 min on ice, 1 ml of ice-cold acetone was added and further incubated for 30 min at –20°C. After centrifugation at 10 000 *g* for 5 min, the resulting pellets were resuspended in the appropriate volume of SDS loading buffer and boiled for 5 min. Lysates equivalent to 0.5 OD_{600} cells were separated by SDS–PAGE and electrotransferred to a polyvinylidene difluoride membrane (Millipore). Mouse monoclonal anti-HA antibody (16B12) or rabbit anti-aminopeptidase I antibody (a gift from Dr Klionsky) was used for immunodetection. Development was performed by the ECL detection methods (Amersham Pharmacia Biotech).

Other methods

For measurement of autophagic activity, the alkaline phosphatase (ALP) assay was performed as described previously (Noda and Ohsumi, 1998). The two-hybrid assay was performed as described by James *et al*. (1996).

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