The Reversible Modification Regulates the Membrane-Binding State of Apg8/Aut7 Essential for Autophagy and the Cytoplasm to Vacuole Targeting Pathway

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Abstract. Autophagy and the Cvt pathway are examples of nonclassical vesicular transport from the cytoplasm to the vacuole via double-membrane vesicles. Apg8/Aut7, which plays an important role in the formation of such vesicles, tends to bind to membranes in spite of its hydrophilic nature. We show here that the nature of the association of Apg8 with membranes changes depending on a series of modifications of the protein itself. First, the carboxy-terminal Arg residue of newly synthesized Apg8 is removed by Apg4/Aut2, a novel cysteine protease, and a Gly residue becomes the carboxy-terminal residue of the protein that is now designated Apg8FG. Subsequently, Apg8FG forms a conjugate with an unidentified molecule "X" and thereby binds tightly to membranes. This modification requires the carboxy-terminal Gly residue of Apg8FG and Apg7, a ubiquitin E1-like enzyme. Finally, the adduct Apg8FG-X is reversed to soluble or loosely membrane-bound Apg8FG by cleavage by Apg4. The mode of action of Apg4, which cleaves both newly synthesized Apg8 and modified Apg8FG, resembles that of deubiquitinating enzymes. A reaction similar to ubiquitination is probably involved in the second modification. The reversible modification of Apg8 appears to be coupled to the membrane dynamics of autophagy and the Cvt pathway.

Key words: autophagy/Cvt pathway • Apg4/Aut2 • cysteine protease • ubiquitination • deubiquitination

Introduction

Autophagy is a major pathway for the bulk degradation of intracellular proteins in a lytic compartment, the lysosome/vacuole, and is conserved throughout eukaryotes (Kopitz et al., 1990; Dunn, 1994). In the yeast *Saccharomyces cerevisiae*, autophagy is induced when cells are starved of various nutrients and also upon inhibition of Tor-mediated signaling (Takeshige et al., 1992; Noda and Ohsumi, 1998). During autophagy, cytoplasmic components and organelles are sequestered in vacuoles by a nonclassical vesicular transport system (Baba et al., 1994). First, autophagosomes, double-membrane structures that surround part of the cytoplasm, are formed in the cytoplasm next to the vacuole. Then, the outer membrane of the autophagosome fuses with the vacuolar membrane and an autophagic body, consisting of the inner-membrane structure, is released into the lumen of the vacuole. Finally, the autophagic body is degraded by vacuolar hydrolases (Takeshige et al., 1992). In *Saccharomyces cerevisiae*, the cytoplasm to vacuole targeting (Cvt)[1] pathway is another nonclassical vesicular transport that delivers aminopeptidase I (API) from the cytosol to the vacuole (Harding et al., 1995; Baba et al., 1997; Scott et al., 1997). API is synthesized in the cytoplasm as a precursor, proAPI. The precursor is sequestered in a small double-membrane vesicle (Cvt vesicle) after its homooligomerization (Baba et al., 1997; Kim et al., 1997). The Cvt vesicle fuses with the vacuole, with the subsequent release into the vacuolar lumen of a single membrane structure (Cvt body) that contains proAPI. The entire membrane transport process of the Cvt pathway is topologically identical to autophagy (Baba et al., 1997;

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[1]*Abbreviations used in this paper:* ALP, alkaline phosphatase; API, aminopeptidase I; Cvt, cytoplasm to vacuole targeting; GST, glutathione-S-transferase; HSP, high-speed pellet peroxisomes; HSS, high-speed supernatants; LSP, low-speed pellet peroxisomes; NEM, *N*-ethylmaleimide; ORF, open reading frame; proAPI, precursor of API.

Scott et al., 1997) and both pathways share components that are encoded by the *APG/AUT/CVT* genes (Harding et al., 1996; Scott et al., 1996). The transport process in both pathways consists of two major steps; namely, formation of autophagosome or the Cvt vesicle and fusion of the vesicle to the vacuole. The targeting and fusion machinery that functions in the classical vesicular transport pathway also plays a role in the second step of autophagy and the Cvt pathway (Darsow et al., 1997; Sato et al., 1998; Fischer von Mollard and Stevens, 1999; Kim et al., 1999a; Kirisako et al., 1999). By contrast, details at the molecular level of the way in which the autophagosome and the Cvt vesicle are formed remain unclear, although it has been reported that Tlg2 (t-SNARE) and Vps45 (a homologue of Sec1) are required only for the first major step in the Cvt pathway (Abeliovich et al., 1999).

The yeast *apg* mutants have defects in autophagy (Tsukada and Ohsumi, 1993). The product of the *APG8/AUT7* gene is a hydrophilic protein (117 amino acids) that is required for the formation of autophagosomes and Cvt vesicles (Kirisako et al., 1999; Huang et al., 2000). The transcription of *APG8* is stimulated in response to nutrient starvation and inhibition of Tor-mediated signaling; namely, in response to the conditions that induce autophagy (Noda and Ohsumi, 1998; Kirisako et al., 1999). The intracellular localization of Apg8 changes dramatically after the shift to starvation conditions. Some Apg8 is concentrated selectively in autophagosomes and is transported to vacuoles with autophagic bodies. In spite of its hydrophilic nature, considerable amounts of Apg8 are bound to membrane under both growing and starvation conditions. Under starvation conditions, more Apg8 is localized on the membranes of autophagosome intermediates than on the membranes of mature autophagosomes or autophagic bodies. This localization suggests that Apg8 might function directly in the assembly of source membranes into the membranes of autophagosomes and, probably, Cvt vesicles (Kirisako et al., 1999).

A ubiquitination-like system appears to be essential for both autophagy and the Cvt pathway (Mizushima et al., 1998). Apg12, a modifier with no apparent similarity to ubiquitin, is conjugated to Apg5 via an isopeptide bond between the carboxy-terminal Gly residue of Apg12 and Lys149 of Apg5. This conjugation reaction requires two other factors that are essential for autophagy, Apg7 and Apg10, which function as the E1 and E2 enzymes for Apg12, respectively (Kim et al., 1999a; Shintani et al., 1999; Tanida et al., 1999). Since the discovery of ubiquitination (Hochstrasser, 1996; Varshavsky, 1997; Ciechanover, 1998; Hershko and Ciechanover, 1998), similar covalent modifications have been discovered in the various ubiquitin-related molecules, such as SUMO-1/Smt3 (Matunis et al., 1996; Johnson et al., 1997; Mahajan et al., 1997), NEDD-8/Rub1 (Lammer et al., 1998; Liakopoulos et al., 1998; Osaka et al., 1998), UCRP (Loeb and Haas, 1992), and Urm1 (Furukawa et al., 2000). Such discoveries suggest that this type of conjugation system serves as a regulatory mechanism in a variety of cellular processes. Ubiquitin and related modifiers, with the exception of Apg12 and Urm1, are synthesized as proproteins, and post-translational processing yields the active carboxyl terminus (Kamitani et al., 1997; Johnson et al., 1997; Ciechanover, 1998; Potter et al., 1999). Some of the proteases that are responsible for the processing of the proproteins are also able to reverse the conjugation to liberate the modifiers from the respective adducts (Wilkinson, 1997; Li and Hochstrasser, 1999; Suzuki et al., 1999). Such deconjugation reactions are also involved in numerous biological processes (Huang et al., 1995; Hegde et al., 1997; Wilkinson, 1997; Desterro et al., 1998; Li and Hochstrasser, 1999).

Lang et al. (1998) demonstrated that Aut7/Apg8 binds to Aut2/Apg4, another component that is essential for autophagy and the Cvt pathway. In this study, we clarified the relationship between Apg8 and Aut2/Apg4, demonstrating that Apg4 is a novel cysteine protease that cleaves newly synthesized Apg8. We also showed that the cleaved Apg8 (Apg8FG) undergoes reversible modification via reactions that resemble ubiquitination and deubiquitination, which appear to be essential for both autophagy and the Cvt pathway.

Materials and Methods

Yeast Strains, Media, and Construction of Plasmids

The yeast strains used in this study are listed in Table I. The media used in this study were described previously (Kirisako et al., 1999): YPD and SD+CA media were used for cell growth and SD(-N) medium was used for nitrogen starvation.

A BamHI site was inserted by PCR in the open reading frame (ORF) of the *APG8* gene just after the initiation codon or just before the termination codon, as described previously (Kirisako et al., 1999), and a BamHI–BamHI fragment containing a 3× myc sequence was inserted at each BamHI site. Each *APG8* fragment containing the promoter region and the inserted 3× myc sequence was cloned into a yeast centromeric vector, pRS316 (Sikorski and Hieter, 1989). Using the APG8 plasmid that encoded Apg8 connected with 3× myc at the carboxyl terminus, we generated APG8 plasmids that encoded Apg8-myc variants with mutations in the carboxyl terminal region of Apg8 using QuikChange™, as described in manufacturer's protocol (Stratagene). We used oligonucleotides 5′-GGAGAAAATACATTTAAGGGATCCTCTAG-3′, 5′-GAGAAAATACATTTGGCATGGGATCCTCTAGAGGTG-3′, and 5′-GGAGAAAATACATTTGCCAGGGGATCCTCTAGAGGTG-3′ for generation of the plasmids that encoded Apg8FR-myc, Apg8FGM-myc, and Apg8FAR-myc, respectively. For construction of the APG8 plasmids that encoded Apg8 variants with sequential truncation of individual amino acids from the carboxyl terminus and Apg8FA, we generated the fragments of the *APG8* ORF with mutations in the 3′-terminal region by PCR using the forward primer 5′-GGAATTCATGAAGTCTACATTTAAGTC-3′ and the following reverse primers that included a BamHI site: 5′-CGGGATCCTTAGCCAAATGTATTTTCTCCT-3′ (for Apg8FG), 5′-CGGGATCCTTAAAATGTATTTTCTCCTGAGT-3′ (for Apg8F), and 5′-CGGGATCCTTAGGCAAATGTATTTTCTCCT-3′ (for Apg8FA). The fragments were digested by AccI and BamHI and substituted for the original AccI–BamHI sequence of the ORF of *APG8* in which a BamHI site had been inserted just before the termination codon. The mutated ORFs of *APG8*, connected with the original authentic promoter of the *APG8* gene, were cloned into pRS316.

The *APG4* gene was cloned as reported previously (Kametaka et al., 1996). To construct the APG4 plasmids, we cloned the BglII–EcoRI fragment that included the ORF of *APG4* into pRS314, pRS316, pRS424, and pRS426 (Sikorski and Hieter, 1989). Using these plasmids, we generated plasmids that encoded variants of Apg4 using QuikChange™ with the oligonucleotides 5′-GATATTGGGTGGGGGTCTATGATAAGGACAGGAC-3′ (Apg4C159S) and 5′-CTGATATTGGGTGGGGGGCTATGATAAGGACAGGAC-3′ (Apg4C159A).

Preparation of Proteins and Apg4-specific Antibodies

To prepare glutathione-S-transferase (GST)-fused Apg4 and GST–fused Apg8-myc, we cloned the ORF of *APG4* and the ORF of *APG8* tagged with a 3× myc sequence at the 3′-terminus into pGEX4T and pGEX2T (Amersham Pharmacia Biotech), respectively. Each plasmid was introduced into *Escherichia coli*, XL1-blue. For preparation of GST-Apg4, transformants in LB medium were stimulated to produce GST-Apg4 by incubation with 0.1 mM IPTG at 30°C for 3 h. For preparation of GST-

Table I. Strains Used in this Study

Strain	Genotype	Source
SEY6210	*MATα leu2 ura3 his3 trp1 lys2 suc2-Δ9*	Darsow et al., 1997
KVY5	*MATα leu2 ura3 his3 trp1 lys2 suc2-Δ9 Δapg8::HIS3*	Kirisako et al., 1999
KVY13	*MATα leu2 ura3 his3 trp1 lys2 suc2-Δ9 Δapg4::LEU2*	This study
KVY15	*MATα leu2 ura3 his3 trp1 lys2 suc2-Δ9 Δapg8::HIS3 Δapg4::LEU2*	This study
KVY52	*MATα leu2 ura3 his3 trp1 lys2 suc2-Δ9 Δapg8::HIS3 Δapg4::LEU2 Δpho8::PHO8Δ60*	This study
KVY53	*MATα leu2 ura3 his3 trp1 lys2 suc2-Δ9 Δapg4::LEU2 Δpho8::PHO8Δ60*	This study
KVY54	*MATα leu2 ura3 his3 trp1 lys2 suc2-Δ9 Δapg8::HIS3 Δpho8::PHO8Δ60*	This study
KVY118	*MATα leu2 ura3 his3 trp1 lys2 suc2-Δ9 Δapg7::HIS3*	This study
KVY135	*MATα leu2 ura3 his3 trp1 lys2 suc2-Δ9 Δapg6::LEU2*	This study
NNY20	*MATa leu2 ura3 trp1 Δapg1::LEU2*	Matsuura et al., 1997
MT2-4-3	*MATa ura3 apg2*	Lab stock
TKD3	*MATa leu2 ura3 trp1 Δapg3::LEU2*	Lab stock
TID4-2D	*MATa leu2 ura3 trp1 Δapg4::LEU2*	Lab stock
SKD5-1D	*MATa leu2 ura3 trp1 Δapg5::LEU2*	Kametaka et al., 1996
SKD6-1d	*MATa leu2 ura3 trp1 Δapg6::LEU2*	Kametaka et al., 1998
TID7-1	*MATa leu2 ura3 trp1 Δapg7::LEU2*	Lab stock
CTD1	*MATa leu2 ura3 trp1 Δapg9::TRP1*	Noda et al., 2000
TFD10-L1	*MATa leu2 ura3 trp1 Δapg10::LEU2*	Shintani et al., 1999
NMY101	*MATα leu2 ura3 trp1 his3 Δapg12::HIS3*	Mizushima et al., 1998
TFD13-W3	*MATa leu2 ura3 trp1 Δapg13::TRP1*	Funakoshi et al., 1997
SKD14-1C	*MATa leu2 ura3 trp1 Δapg14::LEU2*	Kametaka et al., 1998
MT15-4-3	*MATa leu2 ura3 apg15*	Lab stock
NMY124	*MATα leu2 ura3 trp1 his3 Δapg16::HIS3*	Mizushima et al., 1998

Apg8-myc, transformants were incubated in LB medium with 0.5 mM IPTG at 30°C for 5 h. Cells were lysed by sonication, and then lysates were centrifuged at 15,000 *g* for 15 min. Recombinant proteins recovered in supernatant were allowed to bind to glutathione Sepharose 4B (Amersham Pharmacia Biotech) and eluted with 20 mM reduced glutathione (Kohjin). Fractions containing purified GST-Apg4 or GST-Apg8-myc were dialyzed overnight in a 1,000-fold volume of the reaction buffer described below. GST-Apg4 was used to immunize a rabbit, and Apg4-specific antibodies (antiserum) were obtained.

Assay of Alkaline Phosphatase Activity

Strains with SEY6210 background for assays of alkaline phosphatase (ALP) activity were obtained as reported previously (Noda et al., 1995). Preparation of cell lysates and measurement of ALP activity were also performed as described previously (Noda and Ohsumi, 1998; Kirisako et al., 1999). Cell lysates for assays were prepared from exponentially growing cells cultured in SD+CA medium and from cells that had been starved for 4.5 h in SD(-N) medium.

Subcellular Fractionation

Cells at the logarithmic phase of growth in YPD medium were converted to spheroplasts and lysed in lysis buffer (0.2 M sorbitol, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and Protease Inhibitor Cocktail™ (Boehringer). Lysates were generated by centrifugation of crude lysates at 500 *g* for 5 min. Low-speed pellet peroxisomes (LSP; 13,000 *g* pellet), high-speed pellet peroxisomes (HSP; 100,000 *g* pellet), and high speed supernatants (HSS; 100,000 *g* sup) were generated as described previously (Kirisako et al., 1999). We examined the distribution of Apg8 by immunoblotting with a 5,000-fold dilutes of the antibodies against Apg8 (Kirisako et al., 1999). Signals were detected as reported previously (Kirisako et al., 1999).

Solubilization of Apg8 from Pellet

Lysates were prepared as described above. They were centrifuged at 100,000 *g* for 1 h to generate a pellet without the centrifugation at 13,000 *g*. The pellet was suspended in the lysis buffer supplemented with 1 M NaCl or 1% deoxycholate. The LSP and HSP prepared from wild-type cells or Δ*apg4*Δ*apg8* cells that expressed Apg8FG were treated with 1 M NaCl in the lysis buffer. The LSP prepared from Δ*apg4*Δ*apg8* cells that expressed Apg8FG was also suspended in the lysis buffer supplemented with 2 M urea, 0.1 M Na_2CO_3, pH 11.5, 1% deoxycholate, or 2% Triton X-100. Suspensions were kept on ice for 30 min, and then centrifuged at 100,000 *g* for 1 h to generate supernatants and pellets. Proteins in each fraction were precipitated with 10% TCA and resuspended in a sample buffer for SDS-PAGE. The distribution of Apg8 was examined by immunoblotting as described above.

Assay of Cleavage of Apg8 In Vitro

Growing cells were harvested and lysed in reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) with glass beads, as described previously (Kirisako et al., 1999). Lysates were prepared from Δ*apg8*, Δ*apg4*Δ*apg8*, and Δ*apg4*Δ*apg*8 cells that expressed Apg8-myc and the concentration of protein in the lysates was adjusted to 6, 6, and 3 mg/ml, respectively. Lysates were incubated at 30°C for 10 min, and then the lysate that contained Apg8-myc was mixed with the lysate of Δ*apg8* or Δ*apg4*Δ*apg8* cells at a volume ratio of 1:2. Then the mixtures were incubated at 30°C for various periods of time. For assays of the effects of protease inhibitors, the lysate containing Apg8-myc was incubated with the lysate of Δ*apg8* cells (which expressed Apg4) at 30°C for 1 h in the presence of 1 mM *N*-ethylmaleimide (NEM), 1 mM PMSF, 1 mM pepstatin, or 10 mM 1,10-phenanthroline. For cleavage assay of recombinant Apg8 by recombinant Apg4, 300 ng of GST-Apg8-myc were incubated with 500 ng of GST-Apg4 in 30 μl of reaction buffer with or without 1 mM DTT at 30°C for 1 h. As a control, 300 ng of GST-Apg8-myc were incubated similarly with 500 ng of GST. Processing of Apg8-myc and GST-Apg8-myc was examined by immunoblotting with the Apg8-specific antibodies and a monoclonal myc-specific antibody, 9E10.

The LSP and HSP of Δ*apg4*Δ*apg8* cells that expressed Apg8FG and the HSS of Δ*apg4*Δ*apg8* and Δ*apg4*Δ*apg8* cells that expressed Apg4 or Apg4C159S from pRS426-based multicopy plasmid were prepared as described above. The LSP and HSP were washed once with lysis buffer and resuspended in 50 mM Tris-HCl, pH 7.5, at 3 and 6 mg protein/ml, respectively. The concentration of protein in each HSS was adjusted to 10 mg protein/ml in 50 mM Tris-HCl, pH 7.5. The suspension of the LSP was incubated with an equal volume of HSS at 30°C for 1 h, with subsequent centrifugation at 100,000 *g* for 1 h to generate a supernatant and a pellet. The

suspensions of the LSP and HSP were incubated with an equal volume of HSS containing Apg4, or not, with subsequent treatment with/without 1 M NaCl, and then separated to supernatants and pellets by the same way. The distribution of Apg8 was examined by immunoblotting as described above. The LSP was also incubated with 500 ng of recombinant GST-Apg4 or GST in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT, with subsequent centrifugation at 100,000 *g* for 1 h. The resulting supernatant was subjected to immunoblotting with the Apg8-specific antibodies.

Mass Spectrometry

Protein samples were prepared from GST-Apg8-myc by incubation with thrombin and subsequent treatment with GST-Apg4. Matrix-assisted laser desorption ionization (MALDI)–mass spectrometry (MS) was performed using a Voyager Elite XL time-of-flight mass spectrometer. Protein samples were mixed with the matrix solution, the supernatant of a 50% acetonitrile solution saturated with α-cyano-4-hydroxycinnamic acid, and then air dried on the flat surface of a stainless steel plate. Ions were generated by irradiating the sample area with the output of a nitrogen laser at a wavelength of 337 nm.

Results

Cleavage of Newly Synthesized Apg8 Requires Apg4/Aut2

During our efforts to characterize Apg8, we constructed *apg8* null mutants that expressed two versions of Apg8 tagged with three consecutive myc epitopes at the carboxyl or amino terminus; namely, Apg8-myc and myc-Apg8, respectively. Each tagged Apg8 restored the autophagic activity of the *apg8* null mutants (data not shown). We attempted to detect the products of the gene constructs by immunoblotting with Apg8-specific antibodies and a monoclonal myc-specific antibody, 9E10. Lysates were prepared by breaking with glass beads, as described previously (Kirisako et al., 1999). In cells that expressed myc-Apg8, we detected the myc-tagged form of Apg8 with both types of antibody (Fig. 1 A, 3 and 5). No signal was detected with the myc-specific antibody in cells that expressed Apg8-myc (Fig. 1 A, 6). In the cells that expressed Apg8-myc, the Apg8-specific antibodies generated a band at the same position as endogenous Apg8 and not at the expected position (Fig. 1 A, 4). These results indicated that newly synthesized Apg8 had been cleaved in its carboxy-terminal region in vivo.

Using *apg* mutants that expressed Apg8-myc, we examined whether other Apg proteins might be required for the cleavage of newly synthesized Apg8. Only in *apg4* null mutant, Apg8-myc remained unprocessed, while the myc epitopes were completely removed in the other mutants (Fig. 1 B). The *APG4* gene has been shown to be allelic to the *AUT2* gene (Lang et al., 1998; Kirisako et al., 1999). Therefore, we concluded that Apg4/Aut2 is required for the cleavage that occurs near the carboxyl terminus of newly synthesized Apg8.

Apg4 Is a Novel Cysteine Protease

A lysate of yeast cells containing Apg8-myc but not Apg4 was mixed with a lysate that contained Apg4 but not Apg8, and the mixture was incubated at 30°C for the indicated times (Fig. 2 A). The level of the processed form of Apg8 increased in a time-dependent manner, with a parallel decrease in the level of Apg8-myc. No cleavage was detected in the absence of Apg4 (Fig. 2 A). Thus, the cleavage of Apg8 in vitro also depended on Apg4. We performed the same experiment in the presence of representative inhibitors of cysteine, serine, aspartic, and metallo-proteases (NEM, PMSF, pepstatin, and 1,10-phenanthroline, respectively). As shown in Fig. 2 B, cleavage was only inhibited in the presence of NEM, suggesting that cleavage of Apg8 was catalyzed by a cysteine protease. This cleavage proceeded even in the presence of the 3× myc sequence at the carboxyl terminus of Apg8. Thus, it is likely that the relevant protease has endopeptidase activity.

We next investigated the possibility that Apg4 itself might catalyze the processing of Apg8. GST-fused forms of Apg4 and Apg8-myc (GST-Apg4 and GST-Apg8-myc) were expressed in *E. coli* and purified on a column of glutathione-Sepharose. Then we incubated GST-Apg8-myc with GST-Apg4 at 30°C for 1 h in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT. Subsequent immunoblotting with the Apg8-specific antibodies revealed two bands with different mobilities (Fig. 2 C, left). The rapidly migrating band was not detected with the myc-specific antibody (data not shown). In addition, after the incubation of GST-Apg8-myc with GST, only the slowly migrating band was detected with the Apg8-specific antibodies (Fig. 2 C, right). These data provided strong evidence that GST-Apg4 cleaved GST-Apg8-myc directly in the carboxy-terminal region of Apg8. The reaction barely proceeded in the absence of a reducing reagent (DTT; Fig. 2 C, middle). Taken together, the results indicate that Apg4 is a novel cysteine endopeptidase that cleaves Apg8 in its carboxy-terminal region.

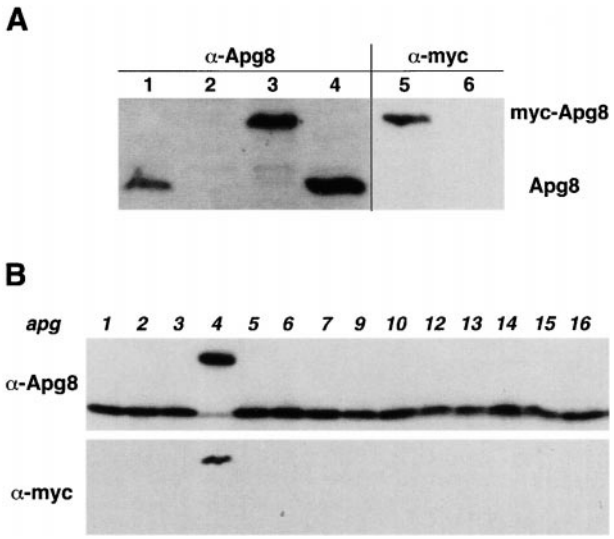

Figure 1. Processing of Apg8. (A) Removal of myc epitopes connected to the carboxyl terminus of Apg8. Lysates were prepared from logarithmically growing cultures of wild-type cells (SEY6210; 1), Δ*apg8* cells (KVY5; 2), and Δ*apg8* cells that expressed myc-Apg8 (3 and 5) or Apg8-myc (4 and 6) encoded by a centromeric plasmid. Lysates were subjected to immunoblotting with Apg8-specific antibodies (α-Apg8; 1–4) or a monoclonal antibody against myc, 9E10 (α-myc; 5 and 6). (B) Cleavage of Apg8-myc in *apg* mutant cells. The centromeric plasmid encoding Apg8-myc was introduced into the various lines of *apg* mutant cells. Lysates were prepared and subjected to immunoblotting as described above. Lane numbers correspond to the designations of the *apg* mutants. Note that endogenous Apg8 was expressed in these mutants.

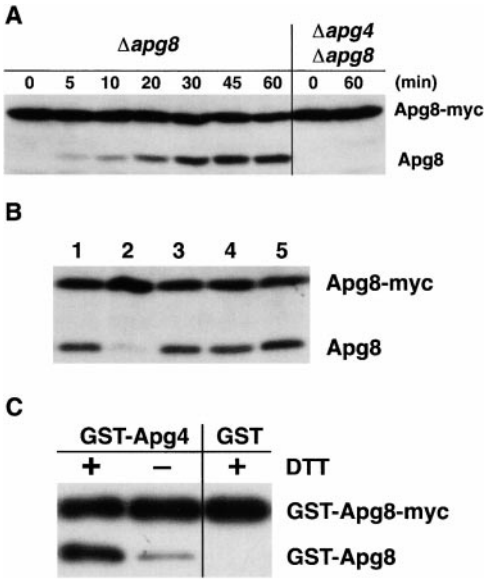

Figure 2. Cleavage of Apg8 by Apg4 in vitro. (A) Cleavage of Apg8-myc by Apg4 in vitro. Lysates were prepared from vegetative cultures of Δ*apg8* cells (KVY5), Δ*apg4*Δ*apg8* cells (KVY15), and Δ*apg4*Δ*apg8* cells that expressed Apg8-myc encoded by a centromeric plasmid as described in Materials and Methods. A lysate of Δ*apg4*Δ*apg8* cells that expressed Apg8-myc was incubated with a lysate of Δ*apg8* or Δ*apg4*Δ*apg8* cells at 30°C for the indicated times, and then subjected to immunoblotting with Apg8-specific antibodies. (B) Effects of protease inhibitors on cleavage of Apg8-myc. A lysate of Δ*apg4*Δ*apg8* cells that expressed Apg8-myc was incubated with a lysate of Δ*apg8* cells at 30°C for 1 h in the presence of various protease inhibitors, as follows, with the type of protease indicated in parentheses: 1 mM NEM (cysteine; 2), 1 mM PMSF (serine; 3), 1 mM pepstatin (aspartic; 4), and 10 mM 1,10-phenanthroline (metallo-; 5). The result of the reaction in the absence of inhibitors is shown in 1. Cleavage of Apg8-myc was examined by immunoblotting with Apg8-specific antibodies. (C) Cleavage of recombinant Apg8-myc by recombinant Apg4. GST-Apg8-myc, GST-Apg4, and GST were prepared as described in Materials and Methods. GST-Apg8-myc was incubated with GST-Apg4 in the presence of 1 mM DTT (+) or in its absence (−) at 30°C for 1 h. As a control, GST-Apg8-myc was treated with GST in the presence of 1 mM DTT at 30°C for 1 h. Cleavage of GST-Apg8-myc was examined as described above.

Cys159 of Apg4 Is Essential for Protolytic Activity

We performed BLAST searches to identify proteins homologous to Apg4 in higher eukaryotes and found several proteins that have not yet been characterized (Fig. 3 A). After aligning the amino acid sequences of Apg4 and these homologues, we identified several conserved regions in the primary structures of the proteins. In the most strongly conserved region, we recognized only a single invariant cysteine residue (corresponding to Cys159 of Apg4) (Fig. 3 A, arrow), which could be involved in formation of a catalytic active site. We then replaced Cys159 of Apg4 with Ser (Apg4C159S) or Ala (Apg4C159A) by site-directed mutagenesis. These variants of Apg4 were expressed in Δ*apg4* cells that produced Apg8-myc and cleavage of Apg8-myc was examined. Cleavage of Apg8-myc was detected in cells that expressed wild-type Apg4, but not in cells that expressed the variants (Fig. 3 B). Both variants were expressed from multicopy plasmids and were stable (Fig. 3 B). Thus, the defect in processing was presumably due to loss of proteolytic activity. We postulated that Cys159 of Apg4 is a catalytically active amino acid.

The Cvt pathway can be monitored by following the maturation of proAPI. We investigated the maturation of proAPI in Δ*apg4* cells that expressed wild-type Apg4 or the variants by immunoblotting with API-specific antibodies (a gift from Dr. D.J. Klionsky, University of California at Davis, Davis, CA). As shown in Fig. 3 B, proAPI did not undergo maturational processing in cells that expressed the protease-negative variants of Apg4. We also examined the effects of these variants on autophagy using the ALP assay system that was developed by Noda et al. (1995) to measure autophagic activity. The autophagic activity in Δ*apg4* cells that expressed Apg4C159S or Apg4C159A was reduced to the level of that in the *apg4* null mutant cells (Fig. 3 C). These results clearly demonstrated that the proteolytic activity of Apg4 was necessary both for autophagy and for the Cvt pathway.

Apg8 Exposes Gly at the Carboxyl Terminus by Apg4

Apg8-myc variants with a mutation in the carboxy-terminal region of Apg8 (Fig. 4 A) were expressed in Δ*apg8* cells. Hereafter, to facilitate identification of the various forms of Apg8, the carboxy-terminal sequence of Apg8 is written after Apg8. Thus, the wild-type newly synthesized Apg8 is written as Apg8FGR. Apg8FGM-myc, in which the carboxy-terminal Arg residue of Apg8 had been replaced with a Met residue, was cleaved as effectively as Apg8FGR-myc (Fig. 4 B, 1 and 2). However, when the second residue (Gly116) from the carboxyl terminus of Apg8 was deleted (Apg8FR-myc), no cleavage occurred (Fig. 4 B, 3). The replacement of Gly116 by Ala (Apg8FAR) also resulted in loss of cleavage (Fig. 4 B, 4). These results demonstrated that Gly116 was essential for the cleavage of Apg8 by Apg4. Since Apg4 cleaved Apg8FGM-myc in the normal manner (Fig. 4 B, 2), it appears that Apg4 recognizes the upstream region beyond Gly116 of Apg8FGR irrespective of the presence or absence of the carboxy-terminal Arg. This hypothesis is supported by the fact that Apg8 and its homologues have strong similarity in their upstream sequences from the conserved Gly residue, which corresponds to Gly116 of Apg8 (Fig. 4 C, arrowhead), whereas they have variable sequences in the downstream region.

The homology shown in Fig. 4 C also prompted us that Apg4 might cleaved just after Gly116 of Apg8. In addition, Meyers et al. (1998) reported that a polyprotein that included part of the sequence of LC3, a mammalian homologue of Apg8, was cleaved by a cellular protease after Gly120, which is corresponding to the invariant Gly residue in Apg8 and its homologues (Fig. 4 C, arrowhead). To determine the cleavage site of Apg8, we examined Apg8 generated by cleavage by Apg4 using mass spectrometry. GST-Apg8-myc was treated first with thrombin to remove GST, and then with GST-Apg4. MALDI-TOFMS gave a signal at 13,864.0 (Fig. 4 D, arrowhead). This value was well assigned to the predicted mass (13,864.0) of Apg8 terminating in Gly116 (the first Met was replaced with Gly-Ser-Pro-Gly-Ile-Leu). Thus, we confirmed that Apg8 was cleaved just after Gly116 by Apg4 and exposed the Gly at the COOH terminus.

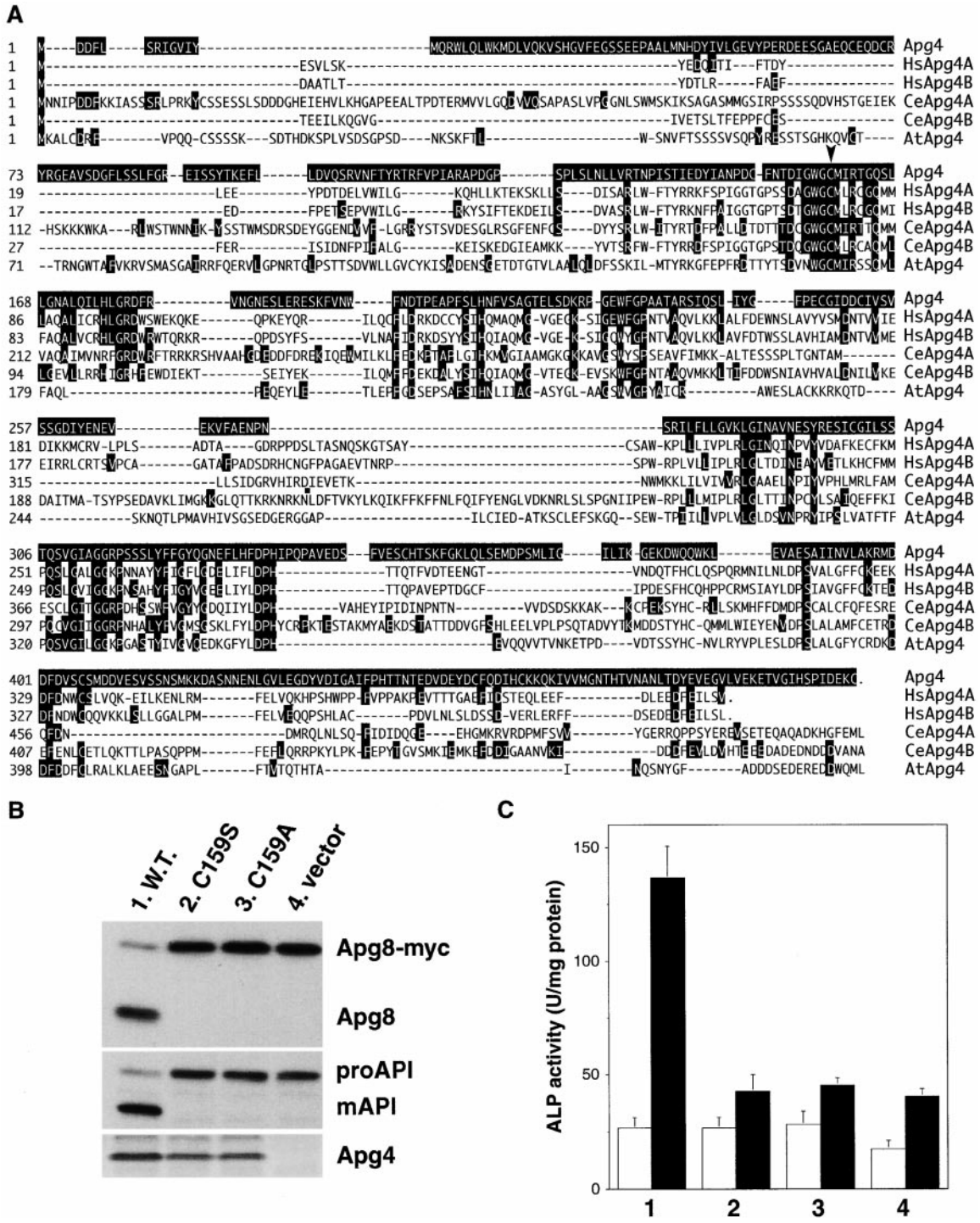

Figure 3. Identification of Apg4 as a novel cysteine protease. (A) Alignment of the amino acid sequences of Apg4 and its homologues from other organisms. An arrowhead indicates the most likely candidate for the cysteine residue at the active site of these enzymes. The homologues include two proteins from *Homo sapiens* (HsApg4A; deduced amino acid sequence based on an EST clone with GenBank No. W30741 and a genomic sequence from 889N15 with No. AL031177, and HsApg4B, No. AL080168), two proteins from *Caenorhabditis elegans* (CeApg4A and CeApg4B; Nos. Z68302 and AL110500) and one protein from *Arabidopsis thaliana* (AtApg4; No. AC004005). (B) Effects of the replacement of Cys159 of Apg4 on the cleavage of Apg8 and the Cvt pathway. (Top) Cleavage of Apg8-myc. Wild type of Apg4 (W.T.; 1), Apg4C159S (2), and Apg4C159A (3) were expressed from centromeric plasmids derived from pRS314 in Δ*apg4*Δ*apg8* cells (KVY15) that expressed Apg8-myc encoded by a centromeric plasmid. Lysates were prepared from exponentially growing cells and subjected to immunoblotting with Apg8-specific antibodies. (Middle) The Cvt pathway. Apg4, Apg4C159S, and Apg4C159A encoded by centromeric plasmids were expressed in Δ*apg4* cells (KVY13). Maturation of proAPI in growing cells was examined by immunoblotting with API-specific antibodies. (Bottom) Detection of Apg4 and its variants. Apg4 and its variants were expressed from pRS426-based 2-μl plasmids in Δ*apg4* cells (KVY13) and detected by immunoblotting with Apg4-specific antibodies. (4) Δ*apg4* cells that harbored the vector only. (C) Effects of mutation of Cys159 in Apg4 on autophagy. Apg4, Apg4C159S, and Apg4C159A were expressed from centromeric plasmids in Δ*apg4* cells (KVY53) that expressed Pho8Δ60. Cells at the logarithmic phase of growth in SD+CA medium were transferred to nitrogen-starvation medium, SD(-N), for 4.5 h. Lysates were prepared from growing (open bars) and starved (closed bars) cells. ALP activity in each lysate was measured as described previously (Noda and Ohsumi, 1998). Values shown are means ± SD of results from triplicates in each case. Numbering of columns corresponds to the numbering of lanes in B.

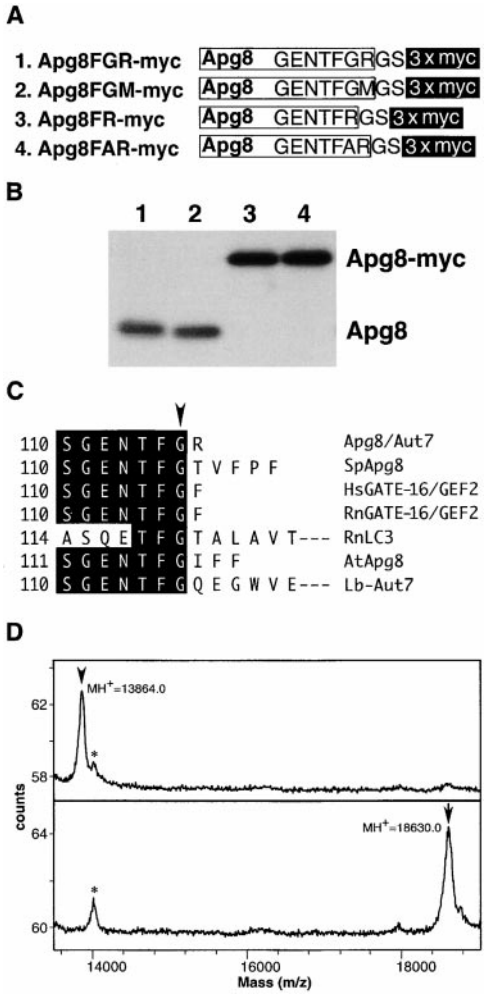

Figure 4. Cleavage of Apg8. (A) Schematic representation of Apg8-myc fusion proteins. The amino acid sequence of the Apg8-myc junction is shown in the single-letter code. (B) Cleavage of Apg8-myc and its variants. Lysates were prepared from the logarithmically growing Δ*apg8* cells (KVY5) that expressed the fusion proteins depicted in A from pRS316-based plasmids. Numbering of lanes corresponds to the numbering of the proteins in A. (C) Alignment of the amino acid sequences of carboxy-terminal segments of Apg8 and representative homologues. An arrowhead indicates the Gly residue at the cleavage site of Apg8 and, possibly, of the homologues. Apg8/Aut7 (*S. cerevisiae*), SpApg8 (*Schizosaccharomyces pombe*; GenBank No. AL032684), HsGATE-16/GEF2 (*H. sapiens*), RnGATE-16/GEF2 (*Rattus norvegicus*), RnLC3 (*R. norvegicus*), AtApg8 (*A. thaliana*; No. AC006220), and Lb-Aut7 (*Laccaria bicolor*; Kim et al., 1999b). (D) MALDI mass spectra of Apg8 (top) and Apg8-myc (bottom). Arrowhead, Apg8 generated by cleavage by Apg4; Arrow, Apg8-myc; *contaminant.

Carboxy-terminal Glycine of Apg8 Is Necessary for the Autophagy/Cvt Pathway

We investigated activities of variants of Apg8 with changes in the carboxy-terminal amino acid (Fig. 5 A) in autophagy and the Cvt pathway (Fig. 5, B and D). Apg8FGR allowed both maturation of proAPI and increased ALP activity in Δ*apg8* cells only when Apg4 was present. Apg8FG was also fully active in both pathways in Δ*apg8* cells even, to some extent, in the absence of Apg4. Apg8F did not suppress the defect in autophagy or in the Cvt pathway in Δ*apg8* cells irrespective of the presence or absence of Apg4. Furthermore, the replacement of the carboxy-terminal Gly of Apg8 (Apg8FA) resulted in the disruption of both pathways (Fig. 5, C and D). These data indicated that Apg8FG was functional in both pathways. The carboxy-terminal Gly residue of Apg8FG appears to have a crucial role in autophagy and the Cvt pathway.

Apg8FG Is Converted to a Tightly Membrane-associated Form

We examined the intracellular distribution of Apg8 and its variants (Fig. 5 A). More than half of each form was recovered in the pellet after centrifugation at 100,000 *g* for 1 h, and all pelleted Apg8 was almost completely solubilized by the detergent, deoxycholate at 1% (Fig. 6 A; DOC). These results suggested that all forms of Apg8 recovered in the 100,000 *g* pellet were associated with membranes. Next, we treated each pellet with 1 M NaCl (Fig. 6 A). In wild-type cells, in which most Apg8FGR was converted to Apg8FG by Apg4 (Fig. 1 A), about half of the Apg8 was solubilized and the rest remained pelletable (Fig. 6 A; Apg8FGR, wild type). By contrast, Apg8FGR expressed in *apg4* null mutant cells was completely solubilized by such treatment (Fig. 6 A; Apg8FGR, Δ*apg4*). The results demonstrated that Apg8 was converted to an NaCl-insoluble form only after cleavage by Apg4. Furthermore, we found that neither Apg8F nor Apg8FA was converted to NaCl-insoluble Apg8 irrespective of the presence or absence of Apg4 (Fig. 6 A; Apg8F and Apg8FA, Δ*apg8*, and data not shown). The data indicated that the carboxy-terminal Gly residue of Apg8FG was essential for the conversion. Because the converted form of Apg8FG (referred to hereafter as Apg8FG*) was solubilized by detergent and not by 1 M NaCl, Apg8FG* must have been bound tightly to membranes.

We next fractionated cells by centrifugation to generate a 13,000 *g* pellet (LSP), a 100,000 *g* pellet (HSP), and a 100,000 *g* supernatant (HSS). By this fractionation, marker proteins, such as ALP for the vacuole, Kex2p for Golgi apparatus, and alcohol dehydrogenase for the cytosol, were recovered in the LSP, HSP, and HSS, respectively (a small amount of Kex2p was detected in the LSP) (data not shown). In wild-type cells, Apg8 was distributed in all fractions, as reported previously (Fig. 6 A; Apg8FGR, wild type; Kirisako et al., 1999). By contrast, Apg8FGR (Δ*apg4*), Apg8F, and Apg8FA were recovered in the HSP and HSS, but not in the LSP (Fig. 6 A). These results indicated that the appearance of Apg8 in the LSP depended on the carboxy-terminal Gly and thereby Apg8 in the fraction represented Apg8FG*. In wild-type cells, although Apg8FG* occupied about half of the whole pelleted Apg8, only several percent of Apg8 were collected in the LSP (Fig. 6 A; Apg8FGR, wild type, compare left with right). This suggested that Apg8FG* might be recovered not only in the LSP but also HSP. The lysates of wild-type cells were separated to the LSP and the HSP. Both were treated with 1 M NaCl, with subsequent centrifugation at 100,000 *g* for 1 h to generate supernatants and pellets. As shown in Fig. 6 B, the LSP contained Apg8FG* only, whereas the HSP contained two forms of Apg8, Apg8FG and Apg8FG*.

Apg8FG and Apg12 exhibit weak homology in their carboxy-terminal regions and both terminate with Phe-Gly

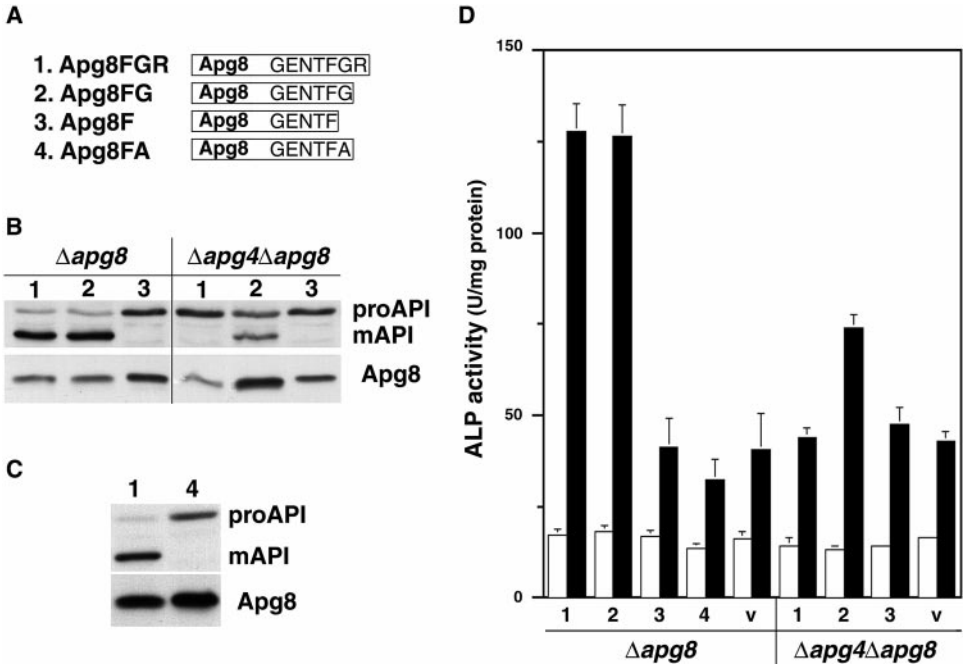

Figure 5. Autophagy and the Cvt pathway in the presence of carboxy-terminal variants of Apg8. (A) Schematic representation of the variants of Apg8. Carboxy-terminal sequences are shown. (B and C) The Cvt pathway. (B) Apg8 and its variants were expressed in Δ*apg8* cells (KVY5) and in Δ*apg4*Δ*apg8* cells from the pRS316 vector. (C) Apg8FGR and Apg8FA were expressed in Δ*apg8* cells (KVY5) from pRS316-based centromeric plasmids. Maturation of proAPI in cells at the logarithmic phase of growth was examined by immunoblotting with API-specific antibodies. Expression of each variant of Apg8 was detected with Apg8-specific antibodies. Numbering of lanes corresponds to the numbering of constructs in A. mAPI, mature API. (D) Autophagy (ALP assay). Each pRS316-based plasmid encoding Apg8 or a variant was introduced into mutants with SEY6210 background, namely, Δ*apg8* (KVY54) and Δ*apg4*Δ*apg8* (KVY52), which expressed Pho8Δ60. Cells were cultured in SD+CA medium to logarithmic phase and transferred to nitrogen-starvation medium, SD(-N), for 4.5 h. Lysates were prepared from growing (open bars) and starved (closed bars) cells. ALP activity in each lysate was measured as described previously. Values shown are means ± SD of results from triplicates in each case. Numbering of columns corresponds to the numbering of constructs in A. v, cells harboring the vector only.

(Ichimura, Y., T. Kirisako, N. Ishihara, N. Mizushima, M. Ohsumi, T. Noda, and Y. Ohsumi, manuscript in preparation). Apg12 is covalently attached to Apg5 via the activating enzyme Apg7 (Mizushima et al., 1998; Kim et al., 1999a; Tanida et al., 1999). We examined whether Apg7 might similarly be involved in the generation of Apg8FG* (Fig. 6). In Δ*apg7* mutant, Apg8 was completely solubilized by the treatment with 1 M NaCl, and none was de-

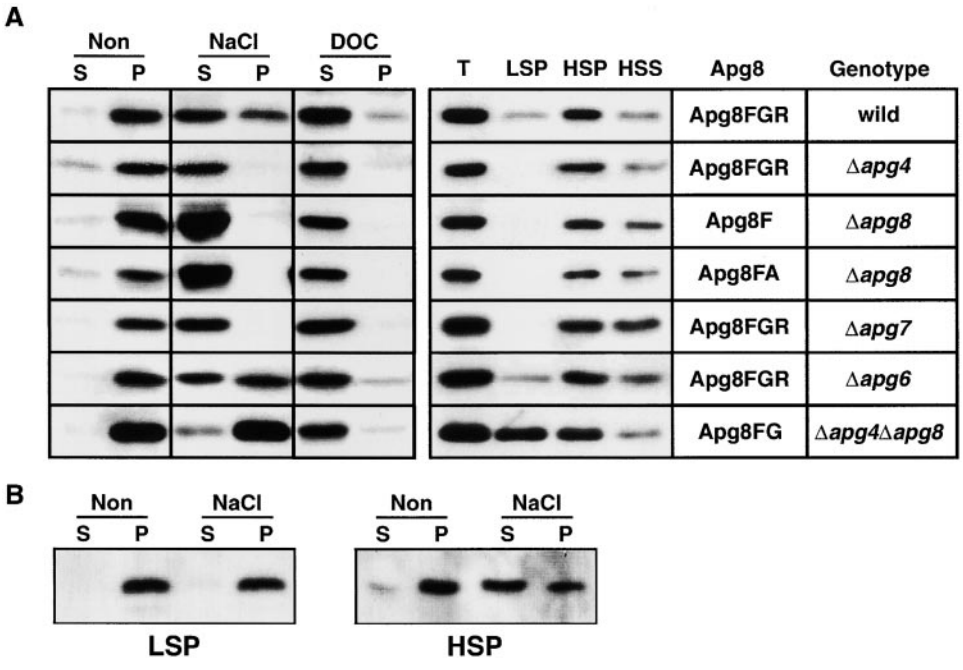

Figure 6. Distribution of Apg8. (A) Lysates were prepared from SEY6210 cells (Apg8FGR; wild type), KVY13 cells (Apg8FGR; Δ*apg4*), KVY5 cells that expressed Apg8F (Apg8F; Δ*apg8*), or Apg8FA (Apg8FA; Δ*apg8*), KVY 135 cells (Apg8FGR; Δ*apg6*), KVY118 cells (Apg8FGR; Δ*apg7*), and KVY15 cells that expressed Apg8FG (Apg8FG; Δ*apg4*Δ*apg8*), as described in Materials and Methods. (Left) Solubilization. Lysates were centrifuged at 100,000 *g* for 1 h to generate pellets. The pellets were treated with 1 M NaCl or 1% deoxycholate (DOC) or held untreated (Non) on ice for 30 min, and then centrifuged at 100,000 *g* for another 1 h to generate to supernatants (S) and pellets (P). (Right) Subcellular distribution. Lysates were centrifuged at 13,000 *g* for 15 min to generate supernatants and pellets (LSP). The resulting supernatants were then centrifuged at 100,000 *g* for 1 h to generate supernatants (HSS) and pellets (HSP). The distribution of Apg8 was examined by immunoblotting with Apg8-specific antibodies. (B) The LSP and the HSP of wild-type cells (SEY6210) were treated with 1 M NaCl or held untreated (Non) on ice for 30 min, and then centrifuged 100,000 *g* for 1 h to generate to supernatants (S) and pellets (P). Apg8 was detected as described above.

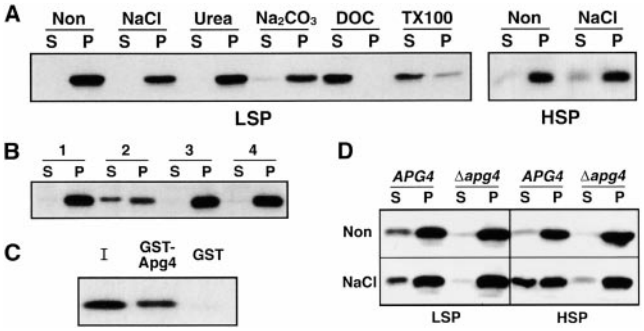

Figure 7. Cleavage of Apg8FG* by Apg4. Solubilization of the Apg8 recovered in the LSP. (Left) The LSP was prepared from the Δ*apg4*Δ*apg8* cells (KVY15) that expressed Apg8FG, as described in Fig. 6. It was treated with 1 M NaCl, 2 M Urea, 0.1 M Na_2CO_3, pH 11.5, 1% deoxycholate (DOC), or 2% Triton X-100 (TX100) or held untreated (Non) on ice for 30 min, with subsequent centrifugation at 100,000 *g* for 1 h to generate a supernatant (S) and a pellet (P). (Right) The HSP prepared from the Δ*apg4*Δ*apg8* cells (KVY15) that expressed Apg8FG was treated with NaCl or not, and then separated to a supernatant (S) and a pellet (P). The distributions of Apg8 were examined by immunoblotting with Apg8-specific antibodies. (B and C) Release of Apg8 from the LSP of Δ*apg4*Δ*apg8* cells (KVY15) expressing Apg8FG. (B) The LSP was incubated at 30°C for 1 h with the HSS fractions that were prepared from Δ*apg4* cells (KVY13; 1) and Δ*apg4* cells that expressed Apg4 (2 and 3) or Apg4C159S (4) from a multicopy vector (pRS426). The reaction with the HSS that contained Apg4 was performed in the presence (3) or absence (2) of 1 mM NEM. Then, reaction mixtures were centrifuged at 100,000 *g* for 1 h to separate to supernatants (S) and pellets (P). (C) The LSP was incubated with GST-Apg4 or GST at 30°C for 1 h, and then the reaction mixtures were centrifuged at 100,000 *g* for 1 h to generate supernatants. Apg8 was detected by immunoblotting with Apg8-specific antibodies. I, 5% of input. (D) Release of Apg8 from the HSP and LSP. The HSP and LSP were prepared from of Δ*apg4*Δ*apg8* cells (KVY15) expressing Apg8FG. Both were first treated with the HSS that was prepared from Δ*apg4* cells that expressed Apg4, and then with 1 M NaCl or held untreated (Non). The mixtures were separated to supernatants (S) and pellets (P) by centrifugation at 100,000 *g* for 1 h. The distribution of Apg8 was examined by immunoblotting with Apg8-specific antibodies.

tected in the LSP even though Apg4 had completely cleaved Apg8FGR (Fig. 1 B). In another *apg* mutant (Δ*apg6*), the distribution of Apg8 was similar to that in wild-type cells. These data indicated that the defects in the generation and recovery in the LSP of Apg8FG* were caused directly by the lack of Apg7. Thus, Apg7 appeared to be necessary for the conversion of Apg8FG to Apg8FG* (see Discussion).

Cleavage of Apg8FG by Apg4 Is Required for the Autophagy/Cvt Pathway*

The formation of Apg8FG* was apparently essential for autophagy and the Cvt pathway. Therefore, we expected that both pathways would proceed normally without Apg4 if Apg8FG was produced. However, as shown in Fig. 5, B and D, in Δ*apg4*Δ*apg8* cells that expressed Apg8FG, the activities of both pathways were reduced but not eliminated. These data suggested that Apg4 was required in another step in addition to the cleavage of newly synthesized Apg8 (Apg8FGR).

We investigated the distribution of Apg8 in Δ*apg4*Δ*apg8* cells that expressed Apg8FG. As shown in Fig. 6 (Apg8FG, Δ*apg4*Δ*apg8*), most of the pelletable Apg8 was in an NaCl-insoluble form and the amount of Apg8 in the LSP was drastically increased. The Apg8 that accumulated in the LSP could not be solubilized by 1 M NaCl (Fig. 7 A, left), demonstrating that all the Apg8 in the LSP was present as Apg8FG*. Apg8FG* in the LSP was not solubilized by 2 M urea or 0.1 M Na_2CO_3 either, but it was done by the detergents, 1% deoxycholate and 2% Triton X-100. (Fig. 7 A, left). This result suggested that Apg8FG* had acquired a feature similar to those of integral membrane proteins.

A possible hypothesis that explains the accumulation of Apg8FG* in the absence of Apg4 is that Apg4 maintains the level of Apg8FG*. To test this hypothesis, we performed in vitro cleavage assay against Apg8FG* using the LSP that accumulated Apg8FG*. The LSP was incubated in 50 mM Tris-HCl with HSS fractions from Δ*apg4*Δ*apg8* cells and from Δ*apg4*Δ*apg8* cells that expressed Apg4 or Apg4C159S at 30°C for 1 h, with subsequent centrifugation at 100,000 *g* for 1 h. As shown in Fig. 7 B, Apg8 was detected in the resulting supernatant in the presence of Apg4. No such release was observed in the presence of 1 mM NEM. A protease-negative form of Apg4 (Apg4C159S) also failed to release Apg8 from the LSP (Fig. 7 B) and was unable to suppress the partial defects in autophagy and the Cvt pathway in Δ*apg4*Δ*apg8* cells that expressed Apg8FG (data not shown). Furthermore, such release could be demonstrated with recombinant GST-Apg4 even in the absence of the yeast cytosol (~5% of inputted Apg8 was liberated from the pellet) (Fig. 7 C). These data indicated that Apg4 acted on Apg8FG* proteolytically.

Apg4 Attacks Apg8FG both in the LSP and in the HSP In Vitro*

Unexpectedly, Apg8 was released from the LSP without 1 M NaCl treatment (Fig. 7 B), although Apg8 itself has an affinity to membranes without the conversion to Apg8FG* (Fig. 6 A). We examined whether Apg8 remained as an NaCl-soluble form on the LSP treated with the HSS that contained Apg4. As shown in Fig. 7 D, the release from the LSP was little increased in the presence of 1 M NaCl. We found that Apg8FG* was also accumulated in the HSP of Δ*apg4*Δ*apg8* cells expressing Apg8FG (Fig. 7 A, right). Therefore, we performed the same experiment on the HSP. The HSP was treated with the HSS of Δ*apg4*Δ*apg8* cells that expressed Apg4. After treatment with 1 M NaCl, or not, the mixture was centrifuged at 100,000 *g* for 1 h. As shown in Fig. 7 D, Apg8 was little released from the pellet in the absence of 1 M NaCl, whereas nearly half of Apg8 was released in the presence of 1 M NaCl. These data clearly indicated that Apg4 attacked Apg8FG* both in the HSP and in the LSP in vitro. In addition, they suggested that Apg8 (not Apg8FG*) should have different affinities to the membranes in the HSP and in the LSP. The Apg8FG*-localized membranes in these two fractions might have different composition.

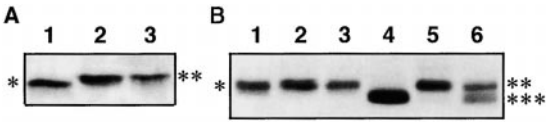

Figure 8. Mobilities of Apg8FGR, Apg8FG. and Apg8FG* (A) Standard SDS-PAGE. (1) Apg8 expressed in Δ*apg4* cells (Apg8FGR), (2) Apg8 generated by cleavage of Apg8-myc in vitro (Apg8FG; see Fig. 2 A), (3) Apg8 in Δ*apg7* cells (Apg8FG). (B) SDS-PAGE in the presence of urea. Each sample was resolved in standard sample buffer for SDS-PAGE and subjected to electrophoresis on a modified slab gel, with subsequent immunoblotting with Apg8-specific antibodies. The modified gel consisted of a standard stacking gel and a 13.5% polyacrylamide separating gel that contained the standard concentration of SDS and 6 M urea. (1–3) The same samples as in A (1–3), (4) Apg8FG* (Apg8 recovered in the LSP of Δ*apg4*Δ*apg8* cells that expressed Apg8FG), (5) Apg8 (Apg8FG) released from the LSP by cleavage in vitro by Apg4 (see Fig. 7 C), (6) Apg8 (Apg8FG and Apg8FG*) in wild-type cells. *Apg8FGR, **Apg8FG, ***Apg8FG*.

An Unidentified Molecule Is Conjugated to the Carboxy-terminal Gly Residue of Apg8FG

Anchorage of Apg8FG to membranes depended on its carboxy-terminal Gly residue (Figs. 6 A and 7 A). Apg4 acted on Apg8FG* proteolytically (Fig. 7, B and C). Furthermore, Apg4 was a protease that cleaved Apg8FGR just after Gly116 (Fig. 4). These observations suggested that Apg8FG might be attached to a component of membrane via the carboxy-terminal Gly residue and the resulting conjugate (Apg8FG*) was cleaved by Apg4 just after the Gly to regenerate Apg8FG.

We attempted to separate Apg8FGR, Apg8FG, and Apg8FG* by standard SDS-PAGE. Apg8FGR and Apg8FG (Apg8 generated from Apg8-myc by Apg4 in vitro) had clearly different mobilities (Fig. 8 A, 1 and 2), but Apg8FG* migrated close to them (data not shown). To separate Apg8FG* from Apg8FGR and Apg8FG, we modified the procedure for SDS-PAGE by including 6 M urea in the separating gel. As shown in Fig. 8 B (1, 2, and 4), Apg8FG* (Apg8 in the LSP prepared from Δ*apg4*Δ*apg8* cells that expressed Apg8FG) was then clearly distinguishable from Apg8FGR and Apg8FG, while Apg8FGR and Apg8FG became indistinguishable. Using this method, we were able to detect Apg8 in wild-type cells as doublet bands of Apg8FG (Fig. 8 B, 6, top) and Apg8FG* (bottom). In Δ*apg7* cells, newly synthesized Apg8 was completely cleaved by Apg4 (Fig. 1 B), but the cleaved Apg8 was not converted to the NaCl-insoluble form and was not detected in the LSP. Thus, these defects should explain the failure in the conversion of Apg8FG to Apg8FG*. Fig. 8, A and B (2–4), clearly shows that Apg8 in Δ*apg7* cells existed as Apg8FG and was not converted to Apg8FG*. Furthermore, the Apg8 released from the LSP prepared from Δ*apg4*Δ*apg8* cells that expressed Apg8FG had the same mobility as Apg8FG. This result indicates that Apg8FG* corresponds to Apg8FG conjugated with a small molecule at its carboxy-terminal Gly residue.

Discussion

On the basis of our results, we propose a model for the serial modification of Apg8 (Fig. 9). First, newly synthesized Apg8 (Apg8FGR) is cleaved just after Gly116 by Apg4, a novel cysteine protease, and is converted to Apg8 with a Gly residue exposed at its carboxy terminus (Apg8FG). Subsequently, by a ubiquitination-like mechanism, Apg8FG is conjugated to an unidentified molecule "X" via its carboxy-terminal Gly residue (see below). The resulting conjugate, designated Apg8FG-X (Apg8FG*), is tightly bound to membranes. Finally, the Apg8FG-X conjugate is cleaved by the proteolytic action of Apg4 and Apg8FG is released from membranes or returned to loosely membrane-bound. The liberated Apg8FG from X are probably recycled and participate in a new conjugation reaction. Alternatively, it might be entrapped in an autophagosome or a Cvt vesicle, and then transported to a vacuole, as described previously (Kirisako et al., 1999).

Apg4 efficiently cleaved Apg8FGR in logarithmically growing cells (Figs. 1 and 3 B). The carboxy-terminal Arg residue of Apg8FGR seems to have no physiological significance because expression of Apg8FG overcame defects

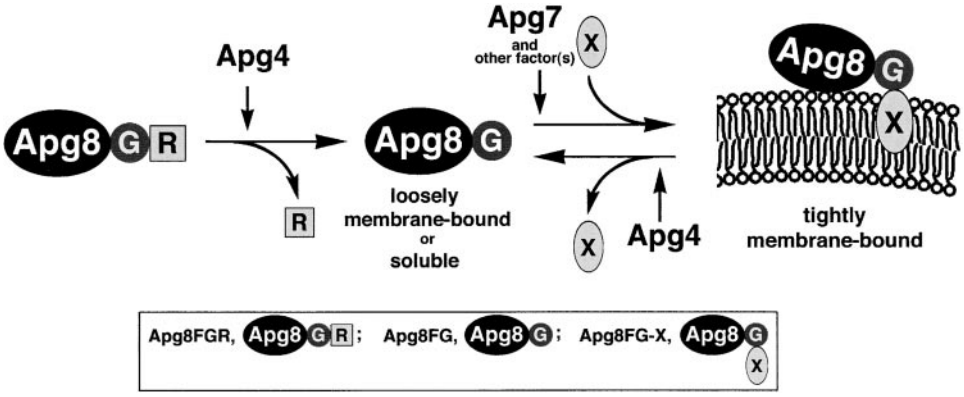

Figure 9. Schematic model of the serial modification of Apg8. First, Apg8FGR (newly synthesized Apg8) is converted to Apg8FG as a result of proteolytic cleavage by Apg4. The intracellular site of this cleavage remains to be determined. Second, Apg8FG is converted to Apg8FG-X via a reaction that resembles ubiquitination and becomes tightly membrane bound. The molecule X might be buried in a membrane. Finally, Apg8FG-X is reversed to soluble or loosely membrane bound Apg8FG by proteolytic cleavage at the junction of Apg8FG-X by Apg4. Some of the reversed Apg8FG might be recycled in a subsequent conjugation reaction.

in both the autophagy and the Cvt pathway in Δ*apg8* cells similarly to Apg8FGR (Fig. 5, B and C). This first cleavage appears not to be a rate-limiting step in autophagy and in the Cvt pathway, but it seems to be prerequisite for the next modification of Apg8FG. The second modification is essential for autophagy and the Cvt pathway, and the third one is required for the normal progression of both pathways (Fig. 5). The reversible modification of Apg8FG that determines its membrane-binding state might be tightly linked both to autophagy and to the Cvt pathway.

Modification of Apg8FG Resembles Ubiquitination

During SDS-PAGE in the presence of urea, the mobility of Apg8FG* was distinguishable from that of Apg8FG and Apg8FGR and, furthermore, Apg8 generated from Apg8FG* by the cleavage catalyzed by Apg4 was detected at the same position as Apg8FG (Fig. 8 B). These observations indicate that Apg4 cleaves Apg8FG* just after Gly116, as in the initial cleavage of Apg8FGR. Therefore, some molecule must be attached to the carboxy-terminal Gly residue of Apg8FG. This hypothesis is supported by the fact that the carboxy-terminal Gly residue was essential for the conversion of Apg8FG to Apg8FG* (Fig. 6). Ubiquitin and related molecules are conjugated to target molecules via their carboxy-terminal Gly residue. In addition, ubiquitination and the conjugation of SUMO-1/Smt3 are accompanied by the deconjugation of the adducts (Wilkinson, 1997; Li and Hochstrasser, 1999; Suzuki et al., 1999). Thus, in some sense, the modification of Apg8FG resembles ubiquitination and related conjugation reactions. We postulated that a ubiquitination-like system might participate in the modification of Apg8FG. As shown in Figs. 6 and 8, Apg7 was necessary for the modification of Apg8FG. Recently, we obtained direct evidence that Apg7 functions as the E1 enzyme for Apg8FG (Ichimura, Y., T. Kirisako, N. Ishihara, N. Mizushima, M. Ohsumi, T. Noda, and Y. Ohsumi, manuscript in preparation). Furthermore, a molecule corresponding to the E2 enzyme for Apg8FG has also been identified (Ichimura, Y., T. Kirisako, N. Ishihara, N. Mizushima, M. Ohsumi, T. Noda, and Y. Ohsumi, manuscript in preparation). Thus, a novel ubiquitination-like reaction seems certain to be involved in the conversion of Apg8FG to Apg8FG* (hereafter referred to as Apg8FG-X).

Apg8FG and Apg8FG-X migrated similarly during SDS-PAGE. Apg8FG-X was bound to membranes similarly to integral membrane proteins in spite of the fact that Apg8 has no membrane-spanning region (Figs. 6 and 7 B). These observations suggest that the molecule X might be a small molecule inserted in membranes. The target molecules of ubiquitin and related molecules are proteins, so an obvious candidate for X is a protein or peptide. However, we know of no examples of a small protein or peptide serving as an anchor in the membrane for a soluble protein. The target molecule of Apg8 might be another kind molecule buried in membrane.

Sites at which the Reversible Modification of Apg8 Occurs

In wild-type cells, the LSP contained Apg8FG-X exclusively, whereas the HSP contained both Apg8FG and Apg8FG-X (Fig. 6 B). The in vitro cleavage of Apg8FG-X clearly showed that Apg8FG (generated from Apg8FG-X) had less affinity to the membrane in the LSP, while it had high affinity to the membrane in the HSP (Fig. 7 D). Thus, at least two kinds of membranes to which Apg8 is bound should exist in the cells.

On the basis of the fact described above and the data that Apg8FG was recovered in the HSP without the conversion to Apg8FG-X (Fig. 6 B), Apg8FG might undergo conjugation to X on membrane structures recovered in the HSP. X might be present either in the membrane at the site of attachment of Apg8FG, or it might be recruited from the other structures. Immunofluorescence microscopy revealed that Apg8 is localized on tiny dot structures in the cytoplasm during vegetative growth (Kirisako et al., 1999). It is possible that some of the dots represent the sites at which Apg8FG is converted to Apg8FG-X. On the other hand, the cleavage of Apg8FG-X occurs on the membranes recovered in the HSP and in the LSP in vitro (Fig. 7 D), but the primary site at which the cleavage proceeds in vivo remains to be elucidated. We reported previously that little Apg8 was detected on the membranes of mature autophagosomes and autophagic bodies, while much was detected on the membranes of premature autophagosomes and their intermediate membrane structures (Kirisako et al., 1999). The deconjugation of Apg8FG-X may occur on the premature autophagosomes (and possibly the Cvt vesicles) and their intermediates.

Role of the Reversible Modification of Apg8 in Autophagy and the Cvt Pathway

Apg8 is required for the formation of autophagosomes and the Cvt vesicles (Kirisako et al., 1999; Huang et al., 2000). The conjugation of Apg8FG to X is crucial for autophagy and the Cvt pathway. Thus, this conjugation reaction is essential for the formation of autophagosomes and the Cvt vesicles. We propose two possible roles for the modification of Apg8FG in autophagy and the Cvt pathway.

One possible role for the modification is the production of Apg8FG-X, which plays an essential role in the formation of autophagosomes and the Cvt vesicles. We proposed previously that this process is accompanied by the assembly of certain membrane structures to autophagosomal membranes and involves the recruitment and the fusion of membranes (Kirisako et al., 1999). In fact, it was reported that fusion machinery is required before or at the time of formation of the Cvt vesicles (Abeliovich et al., 1999). A mammalian homologue of Apg8, GATE-16, was reported to function in intra-Golgi transport at or near the docking/fusion stage (Sagiv et al., 2000). Apg8, probably in the form of Apg8FG-X, was found to be concentrated on and around the intermediate membranes of autophagosomes (Kirisako et al., 1999). Thus, Apg8FG-X might function as a component of the membrane-docking and fusion machinery. Alternatively, Apg8FG-X might function during the budding of vesicles that are destined for the autophagosomal membrane from certain source membrane structures, just as ubiquitination is used during the internalization of plasma membrane proteins in the endocytic pathway (Hicke and Riezman, 1996; Hicke, 1999).

It is also possible that the conjugation reaction itself might play an essential role in vesicle formation. For example, Apg8FG- and X-carrying structures might be

formed separately. The conjugation of Apg8FG to X might serve to bind together the two kinds of structures that are destined to participate in the formation of autophagosomes or the Cvt vesicles. In addition, X will remain in the membranes of the autophagosomes and the Cvt vesicles after the deconjugation of Apg8FG-X. Recruitment of X might be crucial for the formation of autophagosomes and the Cvt vesicles.

Loss of the ability to deconjugate of Apg8FG-X led to a partial defect in autophagy and in the Cvt pathway (Fig. 5). This defect is probably due to the accumulation of Apg8FG-X on membranes as a result of loss of the second proteolytic action of Apg4 (Fig. 6). Overproduction of Apg8FG did not restore autophagic activity in Δ*apg4*Δ*apg8* cells (data not shown). Therefore, a decrease in the level of free Apg8FG cannot explain the defect. The accumulation of Apg8FG-X might itself interfere with some steps in the membrane dynamics in the two pathways.

Apg4 Is a Novel Cysteine Protease

Apg4 appears to be a novel cysteine protease that cleaves both Apg8FGR and Apg8FG-X irrespective of the sequence downstream of Gly116. This property of Apg4 resembles those of deubiquitinating enzymes (DUBs) and the related enzyme that acts on SUMO-1/Smt3 conjugates (Wilkinson, 1997; Li and Hochstrasser, 1999; Suzuki et al., 1999). These are two families of DUBs: ubiquitin carboxy-terminal hydrolases (UCHs) and ubiquitin-specific processing proteases (UBPs). UCHs are generally proteins of 20–30 kD and their primary structures are well conserved. By contrast, most UBPs are larger, 40–150 kD, and their primary structures exhibit little homology, with the exception of some conserved regions that include Cys and His boxes. Apg4 and its homologues have a well-conserved region that corresponds to the region from amino acids 154–172 of Apg4) and includes the conserved cysteine residue, which is probably part of the active site (Fig. 3 A). This region seems to correspond to the Cys box found in members of the UBP family. In addition, alignment of the amino acid sequences of Apg4 and its homologues revealed another conserved region (corresponding to the region from residue 307 to 336 of Apg4) that includes one conserved histidine residue. This region might be equivalent to the His box of members of the UBP family. Apg4 and its homologues might form a fourth family of cysteine proteases that deconjugates adducts via cleavage adjacent to carboxy-terminal Gly residue. The first three families are the UCH, UBP, and ULP (hydrolases for SUMO-1/Smt3 conjugates) families. Deconjugation of ubiquitin and Smt3 conjugates is required for a variety of cellular processes, such as progression of the cell cycle and development (Huang et al., 1995; Hegde et al., 1997; Wilkinson, 1997; Desterro et al., 1998; Li and Hochstrasser, 1999). The requirement for deconjugation of Apg8FG-X in both autophagy and the Cvt pathway also confirms the general importance of this type of deconjugation reaction.

Apg4, Apg7, and Apg8 are well conserved from yeast to higher eukaryotes. It is likely that the homologues of Apg8 undergo similar modifications, which play important roles in autophagy, as Apg8. Furthermore, since there are at least two homologues of Apg4 and several homologues of Apg8 in mammals (Wang et al., 1999), it is possible that, in higher eukaryotes, this kind of system is involved in a variety of intracellular processes. The precise role of Apg8 remains to be elucidated, but there is no doubt that the reversible modification of Apg8FG is essential for change of its membrane-binding state and is necessary for the nonclassical vesicular transport that is involved in autophagy and the Cvt pathway. The unique membrane dynamics that must operate formation of double membrane vesicles might require a sophisticated set of reactions as demonstrated by the present study.

The authors are grateful to Dr. D.J. Klionsky for the gift of API-specific antibodies, and Dr. M. Matsubae (Osaka University, Suita, Japan) for technical support in MS analysis.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan.

Submitted: 16 March 2000
Revised: 24 August 2000
Accepted: 30 August 2000

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