

Apg7p/Cvt2p: A Novel Protein-activating Enzyme Essential for Autophagy

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In the yeast *Saccharomyces cerevisiae*, the Apg12p–Apg5p conjugating system is essential for autophagy. Apg7p is required for the conjugation reaction, because Apg12p is unable to form a conjugate with Apg5p in the *apg7/cvt2* mutant. Apg7p shows a significant similarity to a ubiquitin-activating enzyme, Uba1p. In this article, we investigated the function of Apg7p as an Apg12p-activating enzyme. Hemagglutinin-tagged Apg12p was coimmunoprecipitated with c-myc-tagged Apg7p. A two-hybrid experiment confirmed the interaction. The coimmunoprecipitation was sensitive to a thiol-reducing reagent. Furthermore, a thioester conjugate of Apg7p was detected in a lysate of cells overexpressing both Apg7p and Apg12p. These results indicated that Apg12p interacts with Apg7p via a thioester bond. Mutational analyses of Apg7p suggested that Cys⁵⁰⁷ of Apg7p is an active site cysteine and that both the ATP-binding domain and the cysteine residue are essential for the conjugation of Apg7p with Apg12p to form the Apg12p–Apg5p conjugate. Cells expressing mutant Apg7ps, Apg7p^{G333A}, or Apg7p^{C507A} showed defects in autophagy and cytoplasm-to-vacuole targeting of aminopeptidase I. These results indicated that Apg7p functions as a novel protein-activating enzyme necessary for Apg12p–Apg5p conjugation.

INTRODUCTION

Autophagy is the process of bulk degradation of cytoplasmic components by the lysosomal/vacuolar system (Seglen and Bohley, 1992; Dunn, 1994). The phenomenon is dramatically enhanced under nutrient starvation conditions. In the initial step of the macroautophagy, a cup-shaped membrane sac surrounds cytosolic components to form an autophagosome (Baba *et al.*, 1994). The outer membrane of the autophagosome fuses with a lysosome/vacuole (Baba *et al.*, 1995). A transient single-membrane structure, the autophagic body, is released into the lumen and subsequently degraded in the lysosome/vacuole. Although biochemical and cell-biological approaches have revealed several aspects of autophagy in mam-

malian cells, the molecular mechanism of autophagy remains unclear.

In the yeast *Saccharomyces cerevisiae*, macroautophagy was first described by Takeshige *et al.* (1992), and the process is similar to that in higher eukaryotes (Baba *et al.*, 1994, 1995). Taking advantage of yeast genetics, autophagy-defective mutants (14 *apg* mutants and 9 *aut* mutants) have been isolated in two different laboratories (Tsukada and Ohsumi, 1993; Thumm *et al.*, 1994; Harding *et al.*, 1996). Surprisingly, most of the *apg* and *aut* mutants overlap genetically and phenotypically with some *cvt* mutants, which have defects in the cytoplasm-to-vacuole targeting of aminopeptidase I (API)¹. These results suggest that the *APG* and *AUT* gene products function even in a veg-

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¹ Abbreviations used: Ac.No., accession number; API, aminopeptidase I; GAL4AD, GAL4 activation domain; GAL4BD, GAL4 DNA binding domain; HA, hemagglutinin.

etative growth (APG1/CVT10/AUT3, APG7/CVT2, APG8/CVT5/AUT7, APG9/CVT7/AUT9, APG14/CVT12, APG15/CVT11, and CVT17/AUT5) (Harding *et al.*, 1996; Scott *et al.*, 1996). Several APG and AUT genes have been identified, and most encode novel proteins (APG1/CVT10/AUT3, APG5, APG6, APG7/CVT2, APG12, APG13, APG14, AUT1, AUT2, and AUT7) (Kametaka *et al.*, 1996; Funakoshi *et al.*, 1997; Matsuura *et al.*, 1997; Schlumpberger *et al.*, 1997; Straub *et al.*, 1997; Kametaka *et al.*, 1998; Lang *et al.*, 1998; Mizushima *et al.*, 1998a). Biochemical characterization of the gene products has revealed some molecular aspects of autophagy: Apg1p/Aut3p is a novel protein kinase (Matsuura *et al.*, 1997; Straub *et al.*, 1997). Apg6p/Vps30p and Apg14p form a protein complex (Kametaka *et al.*, 1998). Aut2p interacts with Aut7p/Apg8p, a homologue of rat microtubule-associated protein light chain 3 (Lang *et al.*, 1998).

Recently, we found that the Apg12p–Apg5p conjugation system is essential for autophagy (Mizushima *et al.*, 1998a). Apg12p has no significant homology to ubiquitin or ubiquitin-related modifiers; however, Apg12p is conjugated to Apg5p through an isopeptide bond between the C-terminal Gly residue of Apg12p and the Lys¹⁴⁹ residue of Apg5p. Ubiquitination is a posttranslational modification to present the degradation signal for proteolytic attack by 26S proteasomes (reviewed by Finley and Chau, 1991; Hershko and Ciechanover, 1992; Hershko, 1996; Hochstrasser, 1996a,b; Haas and Siepmann, 1997; Varshavsky, 1997). Ubiquitin is activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent manner in which a thioester bond is formed between the C terminus of ubiquitin and a Cys residue within the E1 enzyme (Ciechanover *et al.*, 1982; Haas *et al.*, 1982). The ubiquitin is transferred from the E1 enzyme to a Cys residue within a ubiquitin-conjugating enzyme (E2). Finally, ubiquitin is covalently attached to a target protein by an isopeptide linkage directly from E2 or by a ubiquitin-protein ligase (E3) (Hershko *et al.*, 1983; Bartel *et al.*, 1990; Scheffner *et al.*, 1993, 1995; Peters *et al.*, 1996; Zachariae *et al.*, 1996).

Recent discoveries have revealed that the ubiquitin-related modifiers other than ubiquitin play essential roles in eukaryotes (reviewed by Johnson and Hochstrasser, 1997; Saitoh *et al.*, 1997; Dolan, 1998; Hochstrasser, 1998). A mammalian ubiquitin-related protein, SUMO-1 [small ubiquitin-related modifier; also called GMP1, PIC1, UBL1, or sentrin (Boddy *et al.*, 1996; Matunis *et al.*, 1996; Okura *et al.*, 1996; Shen *et al.*, 1996)] is covalently attached to the RanGAP1 and PML proteins (Matunis *et al.*, 1996; Mahajan *et al.*, 1997; Muller *et al.*, 1998). This posttranslational modification affects the subcellular localization of these proteins (Mahajan *et al.*, 1998; Matunis and Blobel, 1998; Muller *et al.*, 1998). A yeast SUMO-1 homologue, Smt3p, is activated by an E1-like heterodimer Aos1p/Uba2p

(Dohmen *et al.*, 1995; Johnson *et al.*, 1997). The E2 enzyme for Smt3p and SUMO-1 are Ubc9p and its mammalian homologue (Gong *et al.*, 1997; Johnson and Blobel, 1997; Lee *et al.*, 1998; Schwarz *et al.*, 1998). Another family of ubiquitin-related proteins is RUB1 and NEDD8 (Kumar *et al.*, 1993; Callis *et al.*, 1995; Hochstrasser, 1996; Kamitani *et al.*, 1997; Lammer *et al.*, 1998; Liakopoulos *et al.*, 1998). A major substrate of RUB1/NEDD8 is Cdc53p/Cullin in yeast and mammalian cells, which play an essential role in regulating the cell cycle (Lammer *et al.*, 1998; Liakopoulos *et al.*, 1998; Osaka *et al.*, 1998). In *Arabidopsis thaliana*, the auxin response depends on the RUB1 modification of nuclear proteins (del Pozo *et al.*, 1998). RUB1 and NEDD8 are activated by E1-like heterodimers: Ula1p (Enr2p)/Uba3p in yeast, an APP-BP1 (a 59-kDa β -amyloid-protein-precursors-binding protein)/human UBA3 homologue in humans, and AXR1/ECR1 in *A. thaliana* (Leyser *et al.*, 1993; Chow *et al.*, 1996; del Pozo *et al.*, 1998; Liakopoulos *et al.*, 1998; Osaka *et al.*, 1998). The E2 enzyme for RUB1 and NEDD8 are Ubc12p and its mammalian homologue (del Pozo *et al.*, 1998; Liakopoulos *et al.*, 1998; Osaka *et al.*, 1998).

These findings strongly suggest that there must be E1- and E2-like enzymes for the Apg12p–Apg5p conjugation system in yeast. Candidates in the Apg12p conjugation system are Apg7p/Cvt2p and Apg10p. In *apg7* and *apg10* mutants, no Apg12p–Apg5p conjugate is observed, indicating that Apg7p and Apg10p play indispensable roles in the Apg12p conjugation system (Mizushima *et al.*, 1998a). A region of Apg7p (residues 322 to 407 out of 633 amino acids) shows significant homology to the corresponding region of a ubiquitin-activating enzyme, Uba1p, although the other regions show no homology (McGrath *et al.*, 1991; Mizushima *et al.*, 1998a). We took particular interest in Apg7p/Cvt2p and investigated functions of Apg7p through both biochemical and molecular biological techniques. In this study, we provide several lines of evidence showing that Apg7p is an Apg12p-activating enzyme.

MATERIALS AND METHODS

Strains, Media, Materials, and Genetic Techniques

Escherichia coli strain DH5 α as the host for plasmids and protein expression was grown in Luria Broth medium in the presence of antibiotics as required (Ausubel *et al.*, 1995). The *S. cerevisiae* strains and plasmids used in this study are listed in Table 1. All yeast strains were cultured in rich medium (YPD, pH 5.0: 1% yeast extract, 2% polypeptone, 2% glucose, 20 mg/l adenine, 20 mg/l tryptophan, 20 mg/l uracil, and 50 mM succinate/NaOH, pH 5.0), MVD medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, and 2% glucose), or SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and appropriate amino acids as described by Kaiser *et al.* [1994]). Nitrogen-starvation medium contained 0.17% yeast nitrogen base without amino acids and ammonium sulfate and 2% glucose. Solid medium contained 2% Bacto agar. Standard genetic and molecular biological techniques were performed as described by Kaiser *et al.* (1994) and

Table 1. Yeast strains and plasmids

	Genotype	Source
Yeast strains		
YW5-1B	<i>MATa leu2-3, 112 trp1 ura3-52</i>	Tsakada and Ohsumi (1993)
YIT701	<i>MATa leu2-3, 112 trp1 ura3-52 apg7Δ::LEU2</i>	This study
YIT702	<i>MATa leu2-3, 112 trp1 ura3-52 apg7Δ::LEU2</i> [pAPG7myc-314, pAPG12HA-316]	This study
YIT703	<i>MATa leu2-3, 112 trp1 ura3-52 apg7Δ::LEU2</i> [pAPG7myc-324, pAPG12HA-426]	This study
YIT704	<i>MATa leu2-3, 112 trp1 ura3-52 apg7Δ::LEU2</i> [pYO324, pAPG12HA-426]	This study
YIT7G333A	<i>MATa leu2-3, 112 trp1 ura3-52 apg7Δ::LEU2</i> [pAPG7G333Amyc-314, pAPG12HA-316]	This study
YIT7C507A	<i>MATa leu2-3, 112 trp1 ura3-52 apg7Δ::LEU2</i> [pAPG7C507 Amyc-314, pAPG12HA-316]	This study
YTS2	<i>MATa ura3-52 lys2-801 ade2-101 trp1 his3</i> <i>leu2 pho8::pho8Δ60 apg7::HIS3</i>	This study
PJ69-4A	<i>MATa trp1-901 leu2-3, 112 ura3-52 his3-200</i> <i>gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3</i> <i>met2::GAL7-lacZ</i>	James <i>et al.</i> (1996)
Plasmids		
pRS314	<i>CEN6 TRP1</i>	Sikorski and Hieter (1989)
pRS316	<i>CEN6 URA3</i>	Sikorski and Hieter (1989)
pYO324	<i>2μ TRP1</i>	Homma <i>et al.</i> (1998)
pAPG7myc-314	<i>CEN6 TRP1 APG7-3 × cmyc epitope</i>	This study
pAPG7myc-324	<i>2μ TRP1 APG7-3 × cmyc epitope</i>	This study
pAPG7G333Amyc-314	<i>CEN6 TRP1 APG7^{G333A}-3 × cmyc epitope</i>	This study
pAPG7C507A-314	<i>CEN6 TRP1 APG7^{C507A}-3 × cmyc epitope</i>	This study
pAPG12HA-316	<i>CEN6 URA3 3 × HA epitope-APG12</i>	Mizushima <i>et al.</i> (1998)
pAPG12HA-426	<i>2μ URA3 3 × HA epitope-APG12</i>	Mizushima <i>et al.</i> (1998)
pGAD-C1	<i>2μ LEU2 P_{ADHI}::GAL4 AD</i>	James <i>et al.</i> (1996)
pGAD-APG12	<i>2μ LEU2 P_{ADHI}::GAL4 AD-APG12</i>	This study
pGBD-C1	<i>2μ TRP1 P_{ADHI}::GAL4 BD</i>	James <i>et al.</i> (1996)
pGBD-APG7	<i>2μ TRP1 P_{ADHI}::GAL4 BD-APG7-3 × cmyc epitope</i>	This study
pGBD-APG7G333A	<i>2μ TRP1 P_{ADHI}::GAL4 BD-APG7^{G333A}-3 × cmyc epitope</i>	This study
pGBD-APG7C507A	<i>2μ TRP1 P_{ADHI}::GAL4 BD-APG7^{C507A}-3 × cmyc epitope</i>	This study

Ausubel *et al.* (1995). The PCR was performed with a program temperature control system PC-701 (ASTEC, Fukuoka, Japan). The DNA sequence was determined by an ABI 373A DNA sequencer (PE Applied Biosystems, Foster City, CA). Restriction enzymes were purchased from TOYOBO (Osaka, Japan) and New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by Sawady Technology (Tokyo, Japan) or ESPEC oligo-service (Ibaraki, Japan). pYO324 was a kind gift from Y. Ohya, pRS series vectors were kind gifts from P. Hieter, and pGAD-C1 vector, pGBD-C1 vector, and PJ69-4A strain were kind gifts from P. James (Sikorski and Hieter, 1989; James *et al.*, 1996; Homma *et al.* 1998). pGEX-3X and cyanogen bromide-activated Sepharose beads were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden); pGEM-T was from Promega (Madison, WI).

Gene Disruption of the APG7 Gene

Gene disruption with the PCR product was performed as described by Lorenz *et al.* (1995). Briefly, PCR was performed with APG7-pRSF primer (5'-GTCGTCAGAAAGGGTCTTAAGTTATGCACCAGCTTTTAAATCATTCTGGTATCACGAGGCCCTTTTCGTC-3'), APG7pRSR primer (5'-GCAATCTCATCAGATTCATCATCTTCCCATTCAAAAACATCGTTGCTAGGTGCGGTATTTACACCCG-3'), and pRS30X plasmids as a template. The amplified PCR product was transformed into a yeast strain. APG7 gene disruption was confirmed by PCR with APG7Bgl2ATG primer (5'-AGATCTATGTCTGTCAGAAAGGGTCTTAAG-3') and

APG7SalISTOP primer (5'-GTCGACTATTAAGCAATCTCATCAGATTCATC-3').

Plasmid Construction and Site-directed Mutagenesis

A *Bam*HI fragment (3.9 kb) containing the APG7 gene (ORF YHR171w) was introduced into the *Bam*HI site of pRS314 and pYO324 (pAPG7-314 and pAPG7-324, respectively). To construct an expression plasmid for c-myc-tagged Apg7p, *Sac*I and *Apa*I sites were introduced just before the termination codon of the APG7 gene by nested PCR with APG7SAF primer (5'-GAATCTGATGAGATTGCTGAGCTCGGGCCCTAATATTTGTCATATAATAGC-3'), APG7SAR primer (5'-GCTATTATATGCAAAATATTAGGGCCCCGAGCTCAGCAATCTCATCAGATTC-3'), M13-47 primer (5'-CGCCAGGGTTT-TCCCAGTCCACGAC-3'), and M13-RV primer (5'-GAGCGGATAA-C AATTTCCACACAGG-3'). The amplified DNA fragment was cloned into pGEM-T Vector (pAPG7SA-GEMT). A *Stu*I-*Sac*I fragment (0.12 kb) of pAPG7-GEMT, a *Sac*I-*Apa*I fragment (0.16 kb) of pMPY-3xMYC, and an *Apa*I-*Sall* fragment (1.0 kb) of pAPG7-GEMT were replaced with the *Stu*I-*Sall* region of pAPG7-314 and pAPG7-324 (pAPG7myc-314 and pAPG7myc-324). The junction and PCR-amplified region were confirmed by DNA sequencing. The plasmid complemented the *apg7Δ* mutation.

Site-directed mutagenesis of the APG7 gene (Gly³³³ and Cys⁵⁰⁷ changed to Ala) was performed by nested PCR with APG7E1928F primer (5'-CTTTAAAAATTGCTGACCAATCCGTGG-3'), APG7X2298F primer (5'-GAGCATTAAATAAAAGAGCATG-3'), APG7G333AR primer (5'-CA-

ACCTAGTGTAGCAGCACCTAGTAGTAG-3'), APG7C507AR primer (5'-CTAGTACTGTGGCCATTGATCC-3'), APG7S2855R primer (5'-CCTGCTTTATGACTGACAAACCGC-3'), and pAPG7-314 as a template. The amplified *EcoRI*-*SstI* fragment (0.8 kb) was replaced with the same region of pAPG7myc-314. The mutation site and PCR-amplified region were confirmed by DNA sequencing. The resultant plasmids were designated pAPG7G333Amyc-314 and pAPG7C507Amyc-314.

For the two-hybrid experiment, a *Bgl*II site was introduced just before the start codon of the *APG7* gene by PCR with APG7Bgl2ATG primer, APG7SallSTOP primer, and pAPG7-314 as a template. The PCR product was cloned into pGEM-T. The *Afl*II-*Sall* fragment (~1.9 kb) of the clone was replaced with the *Afl*II-*Sall* fragment (~3.8 kb) of pAPG7myc-314 (pAPG7BS-GEMT). The *Bgl*II-*Sall* fragment of pAPG7BS-GEMT was subcloned into the *Bam*HI-*Sall* sites of the pGBD-C1 vector (pGBD-APG7). The *Afl*II-*Sall* fragment of pGBD-APG7 was replaced with the *Afl*II-*Sall* fragment from pAPG7G333Amyc-314 or pAPG7C507Amyc-314 to construct pGBD-APG7G333A and pGBD-APG7C507A, respectively. The whole *APG12* coding region was PCR-amplified and then introduced into the *Pst*I site of pGAD-C1 (pGAD-APG12).

Antibodies

To express the GST-Apg7p fusion protein in *E. coli*, an *EcoRV* fragment (0.6 kb) and an *EcoRV*-*EcoRI* fragment (1.6 kb) of pAPG7-316 were subcloned into the *Sma*I and *Sma*I-*EcoRI* sites of pGEX-3X, respectively (pGEX-APG7N and pGEX-APG7C). GST-Apg7p fusion proteins were expressed in DH5 α cells carrying pGEX-APG7N and pGEX-APG7C according to manufacturer's protocol (Amersham Pharmacia Biotech). The GST-C-terminal region of Apg7p was expressed in cells carrying pGEX-APG7C and purified on glutathione Sepharose 4B. The GST-N-terminal region of Apg7p was expressed in cells carrying pGEX-APG7N but was included in the inclusion bodies. The protein was extracted from the inclusion bodies with extraction buffer (8 M urea, 50 mM Tris-Cl, pH 9.5), subjected to SDS-PAGE, and excised from the gel. Polyclonal antibodies against the carboxyl and amino terminal fragments of Apg7p were raised in Japanese white rabbits using purified proteins as antigens (α Apg7C and α Apg7N, respectively). The IgG fraction was precipitated with 50% saturated ammonium sulfate and dissolved in TBS buffer (150 mM NaCl, 20 mM Tris-Cl, pH 7.5). Anti-Apg7p antibodies were purified on a GST-Apg7p Sepharose column. A polyclonal anti-API antibody was a kind gift from D. J. Klionsky. An anti-hemagglutinin (HA) mouse mAb (16B12) was purchased from Berkeley Antibody Company (Berkeley, CA), an anti-c-myc mouse mAb (9E10)-Agarose conjugate was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-yeast 3-phosphoglycerate kinase and anti-yeast Dol-P-Man synthase mouse mAbs were from Molecular Probes (Eugene, OR).

Immunoprecipitation

Cells (OD₆₀₀ = 10) grown to early logarithmic phase in MVD or YPD pH 5.0 medium were harvested and converted to spheroplasts in spheroplasting solution (1% yeast extract, 2% polypeptone, 0.5% glucose, 1 mg/ml Zymolyase 100T). The spheroplasts were harvested in 1.3 M sorbitol as a cushion, lysed with lysis buffer (1% SDS, 150 mM NaCl, 20 mM sodium phosphate, pH 7.5), vortexed, boiled for 5 min, and chilled on ice. When indicated, 1 mM DTT was added to the lysate before boiling. Ten volumes of IP buffer (2% Triton X-100, 150 mM NaCl, 20 mM sodium phosphate, pH 7.5) were added to the cell lysate, and the mixture was centrifuged at 10,000 \times g for 5 min at 4°C to remove debris. The supernatant was precleared with 50 μ l Protein A-Agarose (20% slurry, Santa Cruz Biotechnology). Fifty microliters of Agarose beads conjugated with anti-c-myc antibody (9E10) (20% slurry, Santa Cruz Biotechnology) were added to the lysate, and the mixture was incubated for 2 h at 4°C. The immunoprecipitate-bead complex was washed six times with ice-cold RIPA buffer (10 mM Tris-Cl, pH 7.4, 1% Nonidet P40,

0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA). Proteins were eluted from the beads with 0.1N glycine-HCl, pH 2.5, and precipitated by incubation with 10% TCA on ice for 1 h. The sediment was washed twice in cold acetone, subjected to reducing SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and analyzed by Western blotting. All solutions contained a protease-inhibitor mixture for use with fungal and yeast extracts (Sigma, St. Louis, MO).

Other Techniques

Two-hybrid analysis was performed as described by James *et al.* (1996). A biochemical assay monitoring autophagy in yeast by alkaline phosphatase processing was performed as described by Noda *et al.* (1998). Subcellular fractionation of yeast cells was performed as described by Huang and Chiang (1997). Advanced BLAST search was performed on the National Center for Biotechnology Information web site (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple alignment of amino acids sequences was performed using a CLUSTAL W program by the computer laboratory in the National Institute for Basic Biology.

RESULTS

Apg12p Interacts with Apg7p via a Thioester Bond

A significant homology between Apg7p and Uba1p suggests that Apg7p functions as an Apg12p-activating enzyme. In this case, Apg12p should first form a transient enzyme-substrate complex with Apg7p as demonstrated with Uba1p and ubiquitin. To examine this possibility, we constructed a strain of yeast expressing both c-myc-tagged Apg7p and HA-tagged Apg12p (YIT702 strain), and immunoprecipitated the c-myc-tagged Apg7p with anti-c-myc antibody. Western analysis using α Apg7N antibody recognized c-myc-tagged Apg7p (~78 kDa) in the immunoprecipitates (Figure 1A, WB α Apg7N). HA-tagged Apg12p was also detected in the same immunoprecipitate with anti-HA antibody (Figure 1A, WB α HA). The results correspond to those of the two-hybrid experiment (Figure 2). PJ69-4A strain (*trp1-901 leu2-3112 gal4 Δ gal80 Δ GAL2-ADE2 GAL1-HIS3*) expressing both GAL4AD-Apg12p and GAL4BD-Apg7p grew well on SD-Ade-His-Trp-Leu plate, whereas strains expressing GAL4AD and GAL4BD, GAL4AD-Apg12p and GAL4BD, or GAL4AD and GAL4BD-Apg7p did not. These results suggest that Apg12p interacts with Apg7p.

We next determined whether this interaction is mediated by a thioester bond. When the lysate was treated with 1 mM DTT before immunoprecipitation, no Apg12p coimmunoprecipitated with the c-myc-tagged Apg7p (Figure 1B, DTT+). In contrast, Apg12p coimmunoprecipitated with c-myc-tagged Apg7p when the lysate was not treated with DTT (Figure 1B, DTT-). Furthermore, when a lysate of cells overexpressing both Apg7p and Apg12p (YIT703 strain) was analyzed on a nonreducing gel, two bands of Apg7p were detected by Western analysis using α Apg7N antibody (Figure 1C, pAPG7myc-324/pAPG12HA-

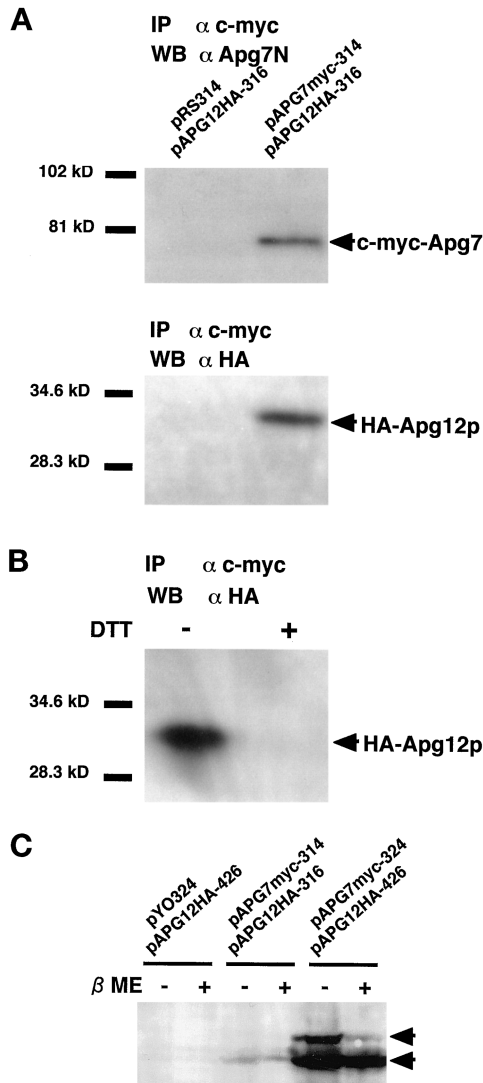
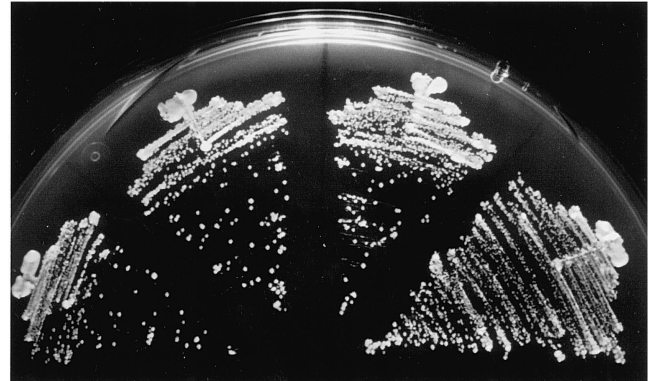
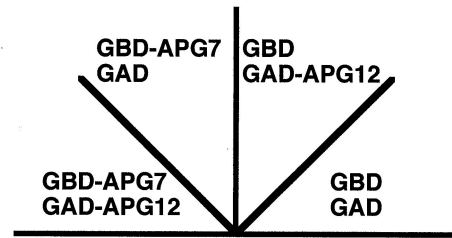
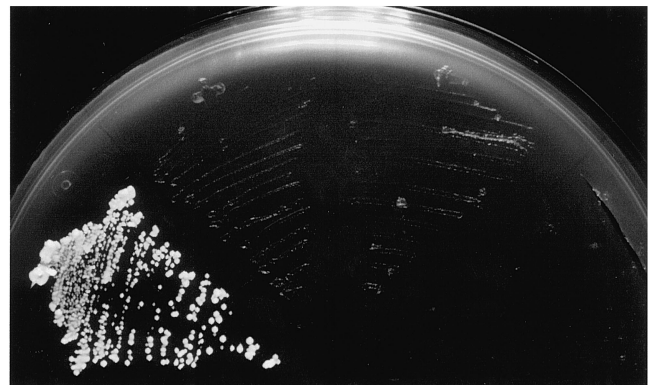


Figure 1. Apg12p conjugates with Apg7p via a thioester bond. (A) Apg12p was coimmunoprecipitated with Apg7p. pAPG7myc-314 plasmid (CEN) was transformed into *apg7 Δ* cells producing HA-tagged Apg12p to express c-myc-tagged Apg7p. A transformant was designated as the YIT702 strain. The pRS314 vector was used as a control. Cell lysates were prepared as described in MATERIALS AND METHODS. A c-myc-tagged Apg7p was immunoprecipitated with Agarose beads conjugated with anti-c-myc mAb (9E10). The immunoprecipitates were subjected to SDS-PAGE on a 10% gel and transferred to a polyvinylidene difluoride membrane. Proteins were detected by Western blotting with α Apg7N antibody (for Apg7p) and anti-HA mouse mAb (16B12; for HA-tagged Apg12p). (B) The coimmunoprecipitation of Apg12p with Apg7p is sensitive to DTT. A cell lysate of the YIT702 strain was prepared as described above. The lysate was boiled with (DTT+) or without 1 mM DTT (DTT-). Immunoprecipitation and Western blotting were performed as described above. (C) The higher molecular weight band of Apg7p in cells overexpressing Apg7p and Apg12p is sensitive to β -mercaptoethanol. Cells grown to early logarithmic phase in MVD medium were harvested and converted to spheroplasts in spheroplasting solution. The spheroplasts were harvested in 1.3 M sorbitol as a cushion, lysed with a 4 \times SDS sample buffer (Ausubel *et al.*, 1995) with a protease-inhibitor mixture (Sigma). The lysate was boiled for 5 min in the presence (β ME+) or absence (β ME-) of 3% β -mercaptoethanol. SDS-PAGE on a 7% gel and Western blotting were



SD-Trp-Leu



SD-His-Ade-Trp-Leu

Figure 2. Apg7p interacts with Apg12p in vivo. pGBD-APG7 (*TRP1*) and pGAD-APG12 (*LEU2*) were transformed into PJ69-4A strain (*gal4 Δ gal80 Δ trp1 leu2 GAL2-ADE2 GAL1-HIS3*) to express GAL4BD-Apg7p and GAL4AD-Apg12p, respectively. pGAD-C1 and pGBD-C1 were used as controls. Cells were plated on SC-Trp-Leu plate (positive control) and SC-Ade-His-Trp-Leu plate, and incubated at 30°C for 3 d. PJ69-4A strain counterclockwise from the bottom carried pGBD-C1 and pGAD-C1 (GBD GAD), pGBD-C1 and pGAD-APG12 (GBD GAD-APG12), pGBD-APG7 and pGAD-C1 (GBD-APG7 GAD), and pGBD-APG7 and pGAD-APG12 (GBD-APG7 GAD-APG12). A strain expressing both GAL4BD-Apg7p and GAL4AD-Apg12p grew well on SC-Ade-Trp-Leu plate, indicating that Apg7p interacts with Apg12p.

Figure 1 (cont). performed as described above. pYO324/pAPG12HA-426: strain YIT704 cells; pAPG7myc-314/pAPG12HA-316: strain YIT702 cells; pAPG7myc-324/pAPG12HA-426: strain YIT703 cells.

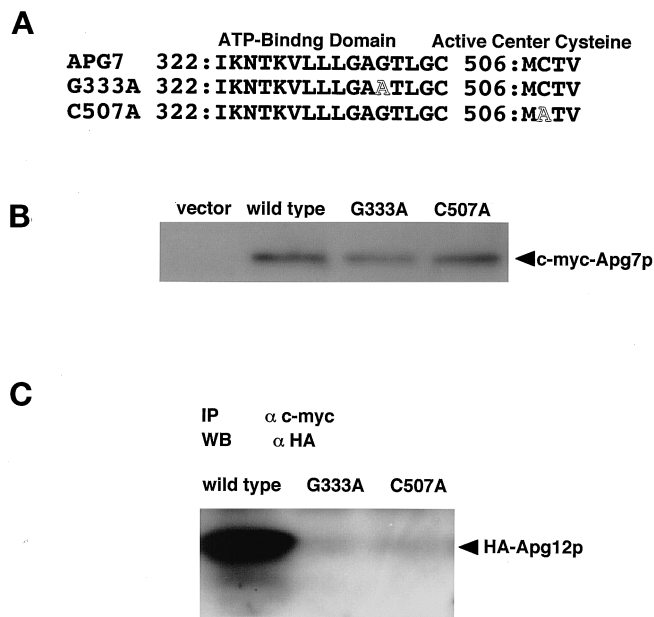


Figure 3. The ATP-binding domain and active site cysteine of Apg7p are essential for conjugation with Apg12p via a thioester bond. (A) Point mutation sites are schematically represented. Gly³³³ in a predicted ATP-binding domain of Apg7p was changed to Ala by site-directed mutagenesis, and Cys⁵⁰⁷ in Apg7p was also changed to Ala. (B) Apg7p^{G333A} and Apg7p^{C507A} are expressed in yeast cells at similar levels to Apg7p. The *apg7Δ* strain carrying pRS314 (vector), strain YIT702 (wild type), strain YIT7G333A (G333A), and strain YIT7C507A (C507A) cells were grown in MVD medium and lysed. c-myc-tagged Apg7p proteins were immunoprecipitated as described in Figure 1A. (C) No Apg12p is coimmunoprecipitated with Apg7p^{G333A} and Apg7p^{C507A}. c-myc-tagged Apg7p in the cell lysate of YIT702 (wild type), YIT7G333A (G333A), and YIT7C507A (C507A) strains were immunoprecipitated, and the coimmunoprecipitates were detected by Western blotting with anti-HA antibody as described above.

426, βME⁻). When a sample was treated with a thiol-reducing reagent, β-mercaptoethanol, the upper band disappeared, indicating that the upper band is a thioester conjugate of Apg7p. The conjugates were hardly detected in a lysate of cells expressing Apg7p and Apg12p on centromere-type plasmids (Figure 1C, pAPG7myc-314/pAPG12HA-316, YIT702 strain). This is probably due to the low amount of the conjugate form of Apg7p in the strain. These results indicate that Apg12p binds with Apg7p via a thioester bond.

The ATP-binding Domain of Apg7p Is Essential for the Interaction with Apg12p to Form the Apg5p–Apg12p Conjugate

Apg12p–Apg5p conjugation was reconstituted in an ATP-dependent manner in vitro (Mizushima *et al.* 1998a). A Uba1p-homologous region in Apg7p contains a putative ATP-binding domain (GXGXXG; residues 331 to 336) (Figure 3A) (Wierenga and Hol, 1983;

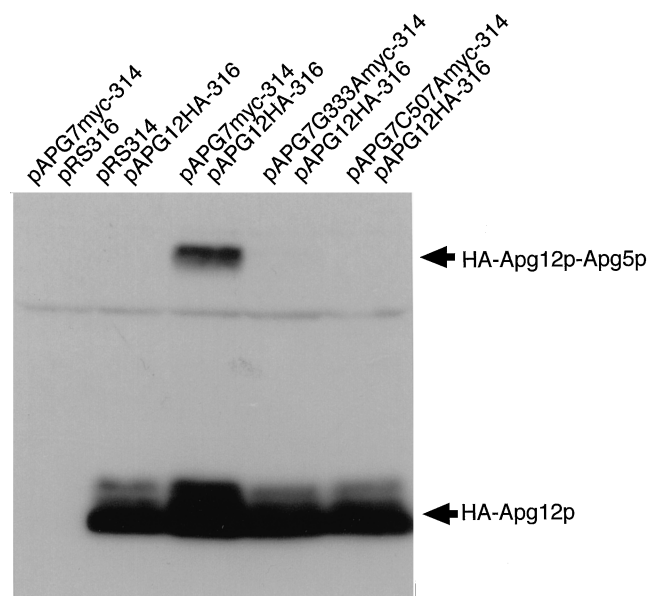


Figure 4. Apg7p^{G333A} and Apg7p^{C507A} show significant defects in the formation of the Apg12p–Apg5p conjugate. The conjugate in cell lysates expressing HA-tagged Apg12p was detected by Western blotting using αHA antibody as described previously (Mizushima *et al.* 1998a). pAPG7myc-314/pRS316: *apg7Δ* cells expressing c-myc-tagged wild-type Apg7p only; pRS314/pAPG12HA-316: *apg7Δ* cells expressing HA-tagged Apg12p only; pAPG7myc-314/pAPG12HA-316: strain YIT702 cells; pAPG7G333Amyc-314/pAPG12HA-316: strain YIT7G333A cells; pAPG7C507Amyc-314/pAPG12HA-316: strain YIT7C507A cells.

McGrath *et al.*, 1991). According to Wierenga and Hol (1983), the second Gly of the ATP-binding domain will be essential for function. To investigate whether the ATP-binding domain is essential for Apg7p function, we used the site-directed mutagenesis to change the Gly³³³ of Apg7p to Ala (Figure 3A). The mutant protein was expressed in an amount quite similar to wild-type Apg7p (Figure 3B); however, it could not precipitate HA-tagged Apg12p (Figure 3C). This result indicates that the ATP-binding domain of Apg7p is essential for the conjugation of Apg12p with Apg7p.

If Apg7p functions as an activating enzyme for Apg12p–Apg5p conjugation, the loss of conjugation of Apg7p^{G333A} with Apg12p may result in a defect in the formation of the Apg12p–Apg5p conjugate. In wild-type cells expressing HA-tagged Apg12p, the HA-tagged Apg12p–Apg5p conjugate was recognized, whereas no conjugate was recognized in *apg7Δ* cells expressing HA-tagged Apg12p, as described previously (Figure 4, pAPG7myc-314/pAPG12HA-316 and pRS314/pAPG12HA-316) (Mizushima *et al.*, 1998a). In cells expressing Apg7p^{G333A}, very little conjugate was recognized (Figure 4, pAPG7G333Amyc-314/pAPG12HA-316). These results suggest the ATP-binding domain of Apg7p is essential for the formation of the Apg12p–Apg5p conjugate.

Cys⁵⁰⁷ of Apg7p Is an Active Center Cysteine Essential for Its E1 Function

The active center cysteine (Cys⁶⁰⁰) of yeast Uba1p is essential for the function of a ubiquitin-activating enzyme. If Apg7p is a protein-activating enzyme for Apg12p, a Cys residue of Apg7p will be essential for Apg12p–Apg7p conjugation. The active center cysteine of Apg7p is strongly suggested to be Cys⁵⁰⁷, because the amino acid sequence of a neighboring region of Apg7p (MCTV) is identical to the corresponding regions in wheat UBA1 (MCTV; GenBank accession number [Ac.No.] P20973) and *A. thaliana* UBA1 (MCTV; GenBank Ac.No. U80808), and homologous to the corresponding regions in yeast Uba1p (LCTL; GenBank Ac.No. X55386), human UBA1 (ICTL; GenBank Ac.No. M58028), and mouse UBA1 (ICTL; GenBank Ac.No. D10576) (Hatfield *et al.*, 1990, 1997; Hatfield and Vierstra, 1992; Handley *et al.*, 1991; McGrath *et al.*, 1991; Imai *et al.*, 1992).

To investigate whether the Cys residue is essential for Apg7p function, we changed the Cys⁵⁰⁷ of Apg7p to Ala by site-directed mutagenesis and expressed both c-myc-tagged Apg7p^{C507A} and HA-tagged Apg12p in the *apg7Δ* mutant with centromere-type plasmids (YIT7C507A strain) (Figure 3A). Immunoprecipitation using anti-c-myc antibody showed that no HA-tagged Apg12p was coimmunoprecipitated with c-myc-tagged Apg7p^{C507A} (Figure 3C), although Apg7p^{C507A} was stably expressed in the YIT7C507A strain (Figure 3B). We further examined the formation of the Apg12p–Apg5p conjugate. No HA-tagged Apg12p–Apg5p conjugate was recognized in the lysate of the YIT7C507A strain (Figure 4, pAPG7C507Amyc-314/pAPG12HA-316). These results indicate that Cys⁵⁰⁷ of Apg7p is an active site cysteine essential for the Apg12p-activating enzyme to form the Apg12p–Apg5p conjugate.

The ATP-binding Domain and Active Site Cysteine of Apg7p Are Essential for Autophagy

If the formation of the Apg12p–Apg5p conjugate via Apg7p is essential for autophagy in yeast, cells expressing Apg7p^{G333A} and Apg7p^{C507A} will show defects in autophagy. We first examined the accumulation of autophagic bodies in cells under starvation conditions in the presence of PMSF. Nomarski optics showed that no autophagic bodies accumulated in YIT7G333A and YIT7C507A cells cultured in nitrogen-starvation medium in the presence of PMSF similar to the result seen with *apg7Δ* cells (Figure 5A, b–d). In contrast, many autophagic bodies were seen in *apg7Δ* cells expressing wild-type Apg7p under the same conditions (Figure 5A, a).

We next investigated Apg7p^{G333A} and Apg7p^{C507A} for defects in autophagy using a biochemical assay monitoring autophagy-dependent alkaline phosphatase

processing (Noda and Ohsumi, 1998). The principle of this assay is as follows. A modified version of vacuolar alkaline phosphatase, Pho8Δ60p, remains in the cytosol as a proform (inactive form). When autophagy is enhanced, Pho8Δ60p in the cytosol is transferred to the vacuole and processed to the active form. This processing is monitored by assaying the activity of alkaline phosphatase in the cell lysate.

pAPG7G333Amyc-314 and pAPG7C507Amyc-314 were introduced into an *apg7Δ* tester strain (YTS2; *apg7Δ pho8::pho8Δ60*). Alkaline phosphatase activity was measured in the transformants under rich or nitrogen-starvation conditions. Under rich conditions, the alkaline phosphatase activities in cells expressing Apg7p^{G333A} and Apg7p^{C507A} were as low as that of wild-type Apg7p, indicating that autophagy was suppressed (Figure 5B, +N). When *apg7Δ* cells expressing wild-type Apg7p were incubated in nitrogen-starvation medium for 4 h at 30°C, the activity increased drastically (Figure 5B, –N), indicating that autophagy is induced by nitrogen starvation; however, the activities in *apg7Δ* cells expressing Apg7p^{G333A} and Apg7p^{C507A} were not enhanced, indicating that autophagy is suppressed in the mutant cells.

A defect in autophagy results in a loss of cell viability under nutrient starvation conditions (Tsukada and Ohsumi, 1993). We further examined the loss of viability of YIT7G333A and YIT7C507A strains using phloxine B. Phloxine B specifically stains dead cells. The colonies of YIT7G333A and YIT7C507A cells turned red (gray in monochrome) on nitrogen-starvation plates containing 10 μg/ml phloxine B, whereas the color of *apg7Δ* mutant colonies expressing wild-type Apg7p was pink (white in monochrome) (Figure 5C). The results indicate that the viability of YIT7G333A and YIT7C507A cells was markedly decreased under nitrogen-starvation conditions. From these results, we conclude that the ATP-binding domain and Cys⁵⁰⁷ are essential for the function of Apg7p in autophagy.

The ATP-binding Domain and Active Site Cysteine of Apg7p Are Essential for Cytoplasm-to-Vacuole Targeting of API

The *apg7* mutant also has a defect in the cytoplasm-to-vacuole targeting of API. API is synthesized in the cytoplasm as a precursor form (Klionsky *et al.*, 1992). After targeting to the vacuole, the precursor is processed to the mature form in the vacuole (Klionsky *et al.*, 1992). We examined whether Gly³³³ and Cys⁵⁰⁷ in Apg7p are also essential for the targeting. We prepared lysates of cells in logarithmic phase and detected the precursor and mature forms of API by Western blotting using αAPI antibody. In wild-type cells, the mature form of API was detected in addition to the precursor, indicating that the precursor trans-

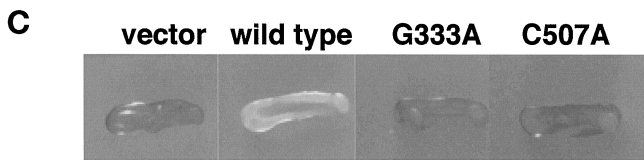
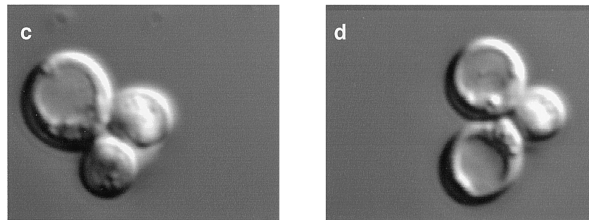
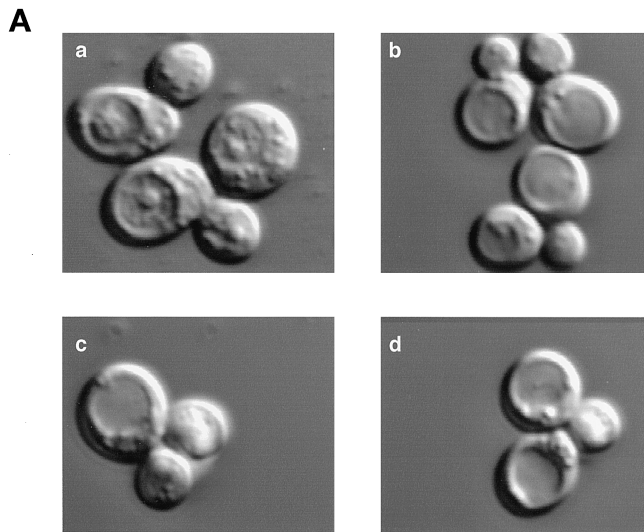


Figure 5. The ATP-binding domain and active site cysteine of Apg7p are essential for autophagy. (A) Gly³³³ and Cys⁵⁰⁷ in Apg7p are essential for the accumulation of autophagic bodies under nitrogen-starvation conditions. Cells grown to early-logarithmic phase in MVD+Ura medium were transferred to nitrogen-starvation medium in the presence of PMSF and incubated for 8 h at 30°C. Representative Nomarski images of the cells are shown. Autophagic bodies accumulated in vacuoles of *apg7Δ* cells carrying pAPG7myc-314 (a). Few autophagic bodies accumulated in vacuoles of *apg7Δ* cells carrying pRS314 (b), pAPG7G333Amyc-314 (c), and pAPG7C507Amyc-314 (d). (B) Autophagy in *apg7Δ* cells expressing Apg7p^{G333A} and Apg7p^{C507A} was monitored by an alkaline phosphatase processing assay. We constructed strain YTS2 (*pho8::pho8Δ60 apg7::HIS3 trp1*) and transformed it with plasmids pRS314 (vector), pAPG7myc-314 (wild type), pAPG7G333Amyc-314 (G333A), and pAPG7C507Amyc-314 (C507A). Cells growing logarithmically in MVD+Ade+Ura medium (N+) were transferred to nitrogen-starvation medium and incubated for 4 h at

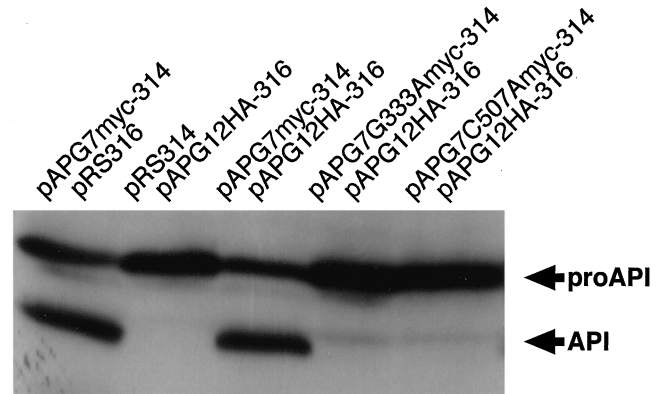


Figure 6. The ATP-binding domain and active center cysteine of Apg7p are essential for cytoplasm-to-vacuole targeting of API. The processing of API in cells growing logarithmically was measured as described by Mizushima *et al.* (1998a). Lanes correspond to those in Figure 4.

ferred from the cytoplasm to the vacuole to be processed into the mature form (Figure 6, pAPG7myc-314/pRS316 and pAPG7myc-314/pAPG12HA-316). In contrast, the processing of API was markedly decreased in cells expressing Apg7p^{G333A} and completely inhibited in cells expressing Apg7p^{C507A}, as in the case of *apg7Δ* cells (Figure 6, pAPG7G333Amyc-314/pAPG12HA-316, pAPG7C507Amyc-314/pAPG12HA-316, and pRS314/pAPG12HA-316). These results indicate that the ATP-binding domain and Cys⁵⁰⁷ of Apg7p are essential for the cytoplasm-to-vacuole targeting of API under rich conditions.

Apg7p Is Present in Cytosol

It has been shown that most free Apg5p, Apg5p–Apg12p conjugate, and more than half of Apg12p are present in the 100,000 × *g* pellet (Mizushima *et al.*, 1998a). To determine the intracellular localization of Apg7p, a lysate of wild-type cells was fractionated by centrifugation as described previously (Huang and Chiang, 1997). Apg7p in each fraction was detected by Western blotting using αApg7C antibody. Apg7p in vegetatively growing cells was present mainly in the 100,000 × *g* supernatant (Figure 7, wild type, Vegetative Growth). Under nitrogen-starvation conditions, the amount and localization of Apg7p remained unchanged (Figure 7, wild type, Nitrogen Starvation).

Figure 5 (cont). 30°C (N–). Alkaline phosphatase activity in cells under rich or nitrogen-starvation conditions was measured as described by Noda *et al.* (1998). (C) Gly³³³ and Cys⁵⁰⁷ in Apg7p are essential for cell viability under nitrogen-starvation conditions. Cells were plated on nitrogen-starvation medium containing 10 μg/ml phloxine B and incubated at 30°C for 3 d. Nonviable cells were stained red (gray in monochrome), whereas viable cells were not stained (white in monochrome).

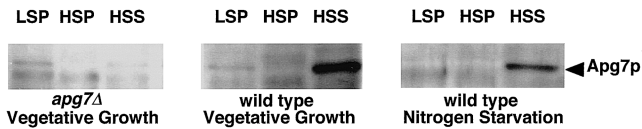


Figure 7. Apg7p is present in the cytosol. A wild-type strain (YW5-1B) was cultured in MVD+Ura+Trp medium, transferred to nitrogen-starvation medium, and incubated for 30 min. Cells growing vegetatively were converted to spheroplasts in rich buffer (1% yeast extract, 2% polypeptone, 0.5% glucose, and 0.7 M sorbitol), whereas nitrogen-starved cells were converted to spheroplasts in nitrogen-starvation buffer (0.15% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, and 0.9 M sorbitol). Cells were lysed and fractionated by centrifugation as described by Huang and Chiang (1997). LSP, 15,000 \times g pellet; HSP, 100,000 \times g pellet; HSS, 100,000 \times g supernatant.

Similar results were obtained with the immunoprecipitation of fractions prepared from cells expressing c-myc-tagged Apg7p. These results indicate that Apg7p is mainly present in the cytoplasm.

DISCUSSION

In this article, we have characterized Apg7p as an Apg12p-activating enzyme and demonstrated its indispensable role in the yeast autophagy. Apg12p, a novel modifier protein, binds to Apg7p via a thioester bond. This binding requires both the active site cysteine (Cys⁵⁰⁷) and the ATP-binding domain. Accordingly, both the cysteine residue and the ATP-binding domain are essential for Apg12p–Apg5p conjugation and for autophagy as well. From these results, we conclude that Apg7p is a novel E1-like enzyme that activates Apg12p and is essential for autophagy.

Although we clearly showed the E1-like function of Apg7p, only the C-terminal region of Apg7p (residues 322 to 407) shows homology to Uba1p. According to the similarity boxes within other E1-like enzymes as described by Johnson *et al.* (1997) and Liakopoulos *et al.* (1998), Apg7p has an ATP-binding domain within box I and an active site cysteine within box III that are essential for E1 function (Figure 8A); however, box III of Apg7p is not similar to those of Uba1p, Uba2p, or Uba3p. Also, neither similarity box II nor IV is present within Apg7p. Phylogenetic analysis of the regions containing box I and box III in the E1 enzymes also suggested that the relationship of Apg7p to the corresponding regions in UBA1, UBA2, and UBA3 family proteins is distant (Figure 8B) (Hatfield *et al.*, 1990, 1997; Handley *et al.*, 1991; McGrath *et al.*, 1991; Imai *et al.*, 1992; Dohmen *et al.*, 1995; Liakopoulos *et al.*, 1998; Mizushima *et al.*, 1998a; Osaka *et al.*, 1998; Yuan *et al.*, 1998). The comparison of the similarity boxes and phylogenetic analysis showed that Apg7p is distinct from other E1-like enzymes. It is of interest to know whether Apg7p functions as a heterodimer, because

the Aos1p/Uba2p and Ula1p/Uba3p complexes function as heterodimers (del Olmo *et al.*, 1997; Johnson *et al.*, 1997; Liakopoulos *et al.*, 1998). At present, we have no answer.

A working hypothesis of the Apg12p conjugation system consistent with these results is proposed in Figure 9. The C-terminal Gly of Apg12p is activated by Apg7p in an ATP-dependent manner. The Apg12p–Apg7p conjugate forms via the C-terminal Gly of Apg12p and Cys⁵⁰⁷ of Apg7p. The reaction probably occurs in the cytosol or the cytoplasmic side of an Apg12p-associated compartment(s), because Apg7p is mainly present in the cytoplasm (Figure 7) (Kim *et al.*, 1999). By analogy to ubiquitination and related modifications, there may be novel E2 and E3 enzymes for the conjugation system. One such candidate for E2 is Apg10p, because the Apg12p–Apg5p conjugate is not observed in an *apg10* mutant. Cloning of the *APG10* gene and biochemical analysis will reveal the function of Apg10p. Screening other candidates will be performed using an Apg12p-affinity column, an Apg7p-affinity column, or two-hybrid screening.

It remains to be determined at which step in autophagy the Apg12p conjugation system works. Apg7p is mainly present in the cytosol, whereas more than half of Apg12p is localized in membrane compartment(s) or a large complex. The Apg12p–Apg7p binding probably occurs in the cytosol or the cytoplasmic side of an Apg12p-associated structure(s). Recently, Yuan *et al.* (1999) suggested that a quite similar conjugation system is required for microautophagy in *Pichia pastoris*. Microautophagy is the sequestration of cytoplasmic components (peroxisomes in this case) by an invagination of the vacuolar membrane. In a conjugation-deficient mutant (*gsa7*), the sequestration is not accomplished completely, probably because of defects in the membrane fusion step. *Gsa7p*, a *P. pastoris* homologue of Apg7p, was shown to be conjugated to a small protein through a thioester bond. Although a modifier and substrate(s) have not been identified, it strongly suggests that the Apg12p conjugation system is essential for microautophagy. Because the process of microautophagy is quite different from that of macroautophagy, it would be interesting if similar machinery is involved in both processes.

We have identified a human Apg12p homologue and have observed that the Apg12p homologue also conjugates with a human Apg5p homologue, which has been identified as an apoptosis-specific protein (Hammond *et al.* 1998; Mizushima *et al.* 1998b). A BLAST search of Apg7p in the Expressed Sequence Tag database showed potential mammalian homologues of Apg7p. Recently, an Apg7p homologue in *P. pastoris*, *Gsa7p*, and a human *Gsa7p*/Apg7p homologue were identified by Yuan *et al.* (1999). The human *Gsa7p*/Apg7p homologue is expressed in various tissues as revealed by Northern analysis

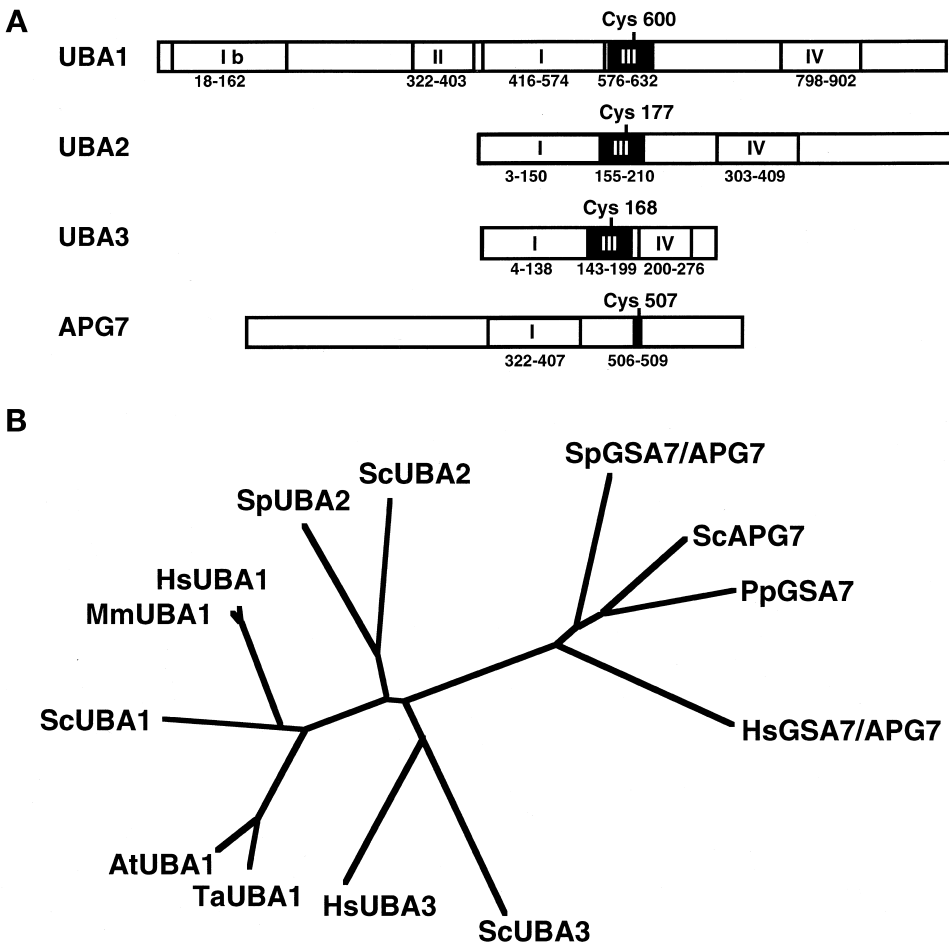


Figure 8. Apg7p is distantly related to Uba1p and its relatives. (A) Schematic representation of the similarity domains among UBA1, UBA2, UBA3, and APG7. The putative active site cysteines are located within similarity box III (UBA domain), as indicated. The essential ATP-binding domains are located within similarity box I. UBA1 contains another ATP-binding motif within similarity box Ib, which is nonessential for function. The similarity boxes correspond to those of Johnson *et al.* (1997) and Liakopoulos *et al.* (1998), except box Ib. (B) The phylogenetic tree of E1 enzymes. The homologous regions of E1-like enzymes containing the ATP-binding domain and active site cysteine were multi-aligned and analyzed by a CLUSTAL W multiple alignment program (Thompson *et al.*, 1994). Shown are ScAPG7 (residues 321 to 520 of *S. cerevisiae* Apg7p; PID: g731742), PpAPG7 (residues 322 to 521 of *P. pastoris* Gsa7p), HsAPG7/GSA7 (residues 235 to 434 of *Homo sapiens* APG7/GSA7), SpAPG7/GSA7 (residues 332 to 531 of *Schizosaccharomyces pombe* Apg7p/Gsa7p; PID: g2894280), HsUBA1 (residues 465 to 664 of *H. sapiens* UBA1; PID: g340072), MmUBA1 (residues 465 to 664 of *Mus musculus* UBA1; PID: g220629), ScUBA1 (residues 431 to 630 of *S. cerevisiae* Uba1p; PID: g4715), AtUBA1 (residues 489 to 688 of *A. thaliana* UBA1; PID: g1750376), TaUBA1 (residues 459 to 658 of *Triticum aestivum* UBA1; PID: g136632),

ScUBA2 (residues 18 to 219 of *S. cerevisiae* Uba2p; PID: g1717852), SpUBA2 (residues 22 to 221 of *S. pombe* Uba2p; PID: g2956755), HsUBA3 (residues 45 to 244 of *H. sapiens* UBA3; PID: g3342564), and ScUBA3 (residues 1 to 200 of *S. cerevisiae* Uba3p; PID: g2980755) (Hatfield *et al.*, 1990; Handley *et al.*, 1991; McGrath *et al.*, 1991; Imai *et al.*, 1992; Dohmen *et al.*, 1995; Hatfield *et al.*, 1997; Liakopoulos *et al.*, 1998; Mizushima *et al.*, 1998a; Osaka *et al.*, 1998; Yuan *et al.*, 1999).

(Tanida and Kominami, unpublished observations). These findings suggest that the Apg12p conjugation system generally functions in eukaryotes. By analogy to the Apg5p homologue, an Apg7p homologue may play a significant role in the autophagic and apoptotic pathways in mammalian cells. Cloning and biochemical analyses of Apg7p homologues will reveal the function of the novel protein conjugation system in mammalian cells.

Apg7p is distantly related to UBA1, UBA2, and UBA3 family proteins (Figure 8A). Nevertheless, Apg7p functions as an E1 enzyme for Apg12p–Apg5p conjugation. These findings suggest that the ubiquitin-like protein modification system will form a ubiquitous regulatory system in eukaryotes, not specific to ubiquitin and ubiquitin-like proteins. Further analyses of potential E1 enzymes may reveal a novel regulatory system by posttranslational modification.

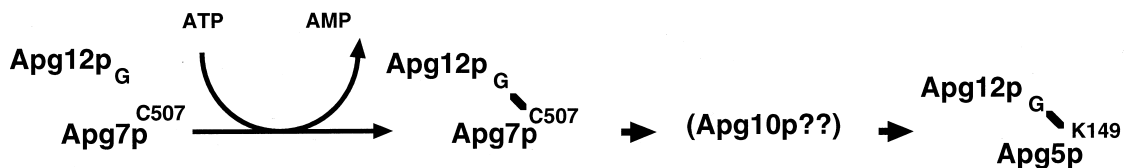


Figure 9. Working hypothesis for the function of Apg7p in the Apg12p conjugation system. Apg12p associates with Apg7p. The C-terminal Gly of Apg12p conjugates with Cys⁵⁰⁷ in Apg7p via a thioester bond in an ATP-dependent manner. The reaction occurs in the cytoplasm or on the cytoplasmic side of an Apg12p-associated compartment(s). Although the E2 enzyme has not been identified, one candidate is Apg10p.

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