Autophagy is a process that involves the bulk degradation of cytoplasmic components by the lysosomal/vacuolar system. In the yeast, *Saccharomyces cerevisiae*, an autophagosome is formed in the cytosol. The outer membrane of the autophagosome is fused with the vacuole, releasing the inner membrane structure, an autophagic body, into the vacuole. The autophagic body is subsequently degraded by vacuolar hydrolases. Taking advantage of yeast genetics, *apg* (autophagy-defective) mutants were isolated that are defective in terms of formation of autophagic bodies under nutrient starvation conditions. One of the *APG* gene products, Apg12p, is covalently attached to Apg5p via the C-terminal Gly of Apg12p as in the case of ubiquitylation, and this conjugation is essential for autophagy. Apg7p is a novel E1 enzyme essential for the Apg12p-conjugation system. In mammalian cells, the human Apg12p homolog (hApg12p) also conjugates with the human Apg5p homolog. In this study, the unique characteristics of hApg7p are shown. A two-hybrid experiment indicated that hApg12p interacts with hApg7p. Site-directed mutagenesis revealed that Cys572 of hApg7p is an authentic active site cysteine residue essential for the formation of the hApg7p/hApg12p intermediate. Overexpression of hApg7p enhances the formation of the hApg5p/hApg12p conjugate, indicating that hApg7p is an E1-like enzyme essential for the hApg12p conjugation system. Cross-linking experiments and glycerol-gradient centrifugation analysis showed that the mammalian Apg7p homolog forms a homodimer as in yeast Apg7p. Each of three human Apg8p counterparts, *i.e.* the Golgi-associated ATPase enhancer of 16 kDa, GABA<sub>r</sub> receptor-associated protein, and microtubule-associated protein light chain 3, coimmunoprecipitates with hApg7p and conjugates with mutant hApg7p<sup>CT728S</sup> to form a stable intermediate via an ester bond. These results indicate that hApg7p is an authentic protein-activating enzyme for hApg12p and the three Apg8p homologs.

Post-translational modifications regulate the functions and localization of target proteins, resulting in many significant intracellular events. One unique modification is the covalent attachment of modifier proteins, ubiquitin, ubiquitin-related proteins (SUMO-1/Smt3p and NEDD-8/RUB1), and Apg12p (for reviews, see Refs. 1–6). The enzymatic processes of these modifications have been intensively studied in ubiquitylation. Ubiquitin forms conjugates with a target protein via a three-step mechanism. First, ubiquitin is activated at its C-terminal Gly by the ubiquitin-activating enzyme (UBA1, E1 enzyme) to form a conjugate with the active site Cys in the E1 enzyme via a thiol ester bond. Next, ubiquitin is transferred from the E1 enzyme to one of several ubiquitin-conjugating enzymes (UBCs, E2 enzymes). In the last step, ubiquitin is attached to a Lys within the target protein via an isopeptide bond. This step is often catalyzed by a member of the ubiquitin-protein ligase family, an E3 enzyme. The reaction mechanism is basically common for each modifier protein.

Autophagy is a process of bulk degradation of cytoplasmic components by the lysosomal/vacuolar system (5, 7, 8). In the initial step of macroautophagy, a cup-shaped membrane sac surrounds cytosolic components to form an autophagosome (9). The outer membrane of the autophagosome fuses with a lysosome/vacuole (10). Taking advantage of yeast genetics, *apg* and *aut* mutants were isolated as autophagy-defective mutants in the yeast, *Saccharomyces cerevisiae* (12, 13). Surprisingly, most of the *apg* mutants overlap genetically with *cot* mutants, which have a defect in the cytoplasm-to-vacuole targeting of aminopeptidase I, indicating that these genes function in a unique transport system under vegetative growth conditions in addition to starvation conditions (14–16). A novel modifier protein, Apg12p, was discovered as an *APG* gene product (11). Apg12p shows little homology to ubiquitin, but it is covalently attached to Apg5p via the C-terminal Gly of Apg12p as in the case of ubiquitylation. In this conjugation reaction, Apg7p and Apg10p function as E1- and E2-like enzymes for Apg12p, respectively (11, 17–19). After the formation of the Apg12p-Apg5p conjugate, Apg16p attaches to Apg5p forming an Apg12p-Apg5p-Apg16p complex for autophagy (20). Unlike other modifier-conjugation systems, the unique character of

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the Apg12p-conjugation system is that it plays indispensable roles in the formation of membrane structures, including autophagosomes and Cvt-vesicles. Apg7p, an authentic E1-like enzyme essential for Apg12p, plays an indispensable role in the initial step of the conjugation system, whereas the enzyme shows slight homology to other E1 enzymes (18). Apg7p interacts with Apg8p/Aut7p and Aut1p/Apg3p in addition to Apg12p (21, 22). The dimerization of Apg7p via the C-terminal region is essential for these interactions, suggesting that Apg7p forms multimeric complexes with these proteins. Apg8p/Aut7p and Apg3p are corresponding E1- and E2-like enzymes (18). Apg7p interacts with two ER-to-Golgi v-SNAREs (Bet1p and Sec22p) and vacuolar t- and v-SNAREs (Vam3p and Nvy1p, Ref. 25). Furthermore, more recent findings suggest that Apg8p/Aut7p, Aut1p/Apg3p, and Apg7p comprise a second protein-conjugation system indispensable for autophagy and Cvt pathways (22, 26). The second modifier is Apg8p/Aut7p, and Apg7p and Aut1p/Apg3p are corresponding E1- and E2-like enzymes. These results suggest that Apg7p, because it is involved in two distinct conjugation systems, is a key enzyme for membrane formation and the targeting of autophagosomes and Cvt vesicles.

In mammalian cells, several homologs of yeast Apg7p have been reported. hApg12p conjugates with hApg5p (first identified as an apoptosis-specific protein), suggesting that the Apg12p conjugation system exists even in human cells (27, 28). There are three candidates for mammalian Apg8p/Aut7p homologs, GATE-16 (Golgi-associated ATPase enhancer of 16 kDa), GABARAP (GABA receptor-associated protein), and MAP-LC3 (microtubule-associated protein light chain 3) (25, 29–33). GATE-16 was first identified as a ganglioside expression factor, but was recently characterized as a soluble transport factor. GATE-16 interacts with NSF and the Golgi v-SNARE GOS-28 (33). The mRNA of GATE-16 is expressed ubiquitously but at significantly higher levels in brain tissue. GATE-16 is ubiquitously but at significantly higher levels in brain tissue. The interaction of yeast Apg8p/Aut7p with two ER-to-Golgi SNAREs, Bet1p and Sec22p, was confirmed by coimmunoprecipitation experiments. Moreover, experiments were carried out using the yeast Apg12p-conjugation system and the mammalian homolog, GABARAP, and GATE-16 (Golgi-associated ATPase enhancer of 16 kDa), GABARAP, polymerase chain reaction with high fidelity (36, 40). The amplified DNA fragment was cloned into the SalI-NotI site of pBluescriptII (SK+) and designated the resultant plasmid for pShhApg7p plasmid. To construct an expression plasmid as hApg7p, a KpnI-NotI fragment (~2.5 kilobase pairs) of the pShhApg7p plasmid was introduced into the pEGFP-N1 vector (CLONTECH) and designated pCMV-hApg7.

To obtain a DNA fragment containing an open-reading frame of the human Apg12 homolog, GATE-16, and GABARAP, polymerase chain reaction was performed with specific primers for their open-reading frames with high fidelity using a human brain cDNA library as a template, and the amplified fragment was introduced into pGEM-T vector (pGEM-hApg12, pGEM-hGATE-16, and pGEM-hGABARAP). The isolated DNA fragments were introduced into pEGFP-C1 to express GFP fusion proteins (pGFP-hApg12p, pGFP-hGATE-16, and pGFP-hGABARAP).

Cys572 within hApg7p was replaced by Ser, mutated by the Gene-Editor in vitro site-directed mutagenesis system (PROMEGA) with an oligonucleotide (hAPG7CS; 5’-CGGACCTTGGACACGAGG-3’ corresponding to residues 550–571 of hApg7p) according to the manufacturer’s protocol. The expression plasmid for mutant Apg7pC572S was constructed as in the case of pCMV-hApg7 and was designated pCMV-hApg7C572S.

Antibodies—A polyclonal antibody against a synthetic polypeptide (VVAQPDDSTRDRTL) corresponding to residues 550–571 of hApg7p was raised in Japanese white rabbits (anti-hApg7p). The antibody was also affinity chromatography on immobilized proteinA-Sepharose. For the preparation of antibody against murine Apg12p homolog (mApg12p), rabbits were immunized with a maltose-binding protein-mApg12p fusion protein. The antibody to mApg12p was purified by affinity chromatography on maltose-binding protein-mApg12p-immobilized-Sepharose. The polyclonal anti-GFP antibody was purchased from CLONTECH.

Cloning of hMAP-LC3 cDNA—The advanced BLAST search program from the National Center for Biotechnology Information was used to search for homologs in the human and mouse EST database. Based on the DNA sequence of EST clones, we performed rapid amplification of the 5’-cDNA ends in Marathon-Ready cDNA (CLONTECH) by polymerase chain reaction with high fidelity (40). The amplified DNA fragment was introduced into pGEM-T vector, and the DNA sequence was determined. The DNA sequences of all five independent clones were identical, and the predicted amino acids of the clones show significant homology to rat and murine MAP-LC3. To express GFP-hMAP-LC3 in HEK293 cells, the cloned DNA fragment was introduced into pEGFP-C1 vector (pGFP-hMAP-LC3).

Expression of hApg Proteins in HEK293 Cells—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. For transfection, 2 × 10⁶ cells were seeded on 60-mm dishes. After incubation for 24 h at 37°C, the cells were transfected with a mixture of 2.5 μg of plasmid DNA and 12 μl of FuGene-6. For cotransfection, 1 μg of each plasmid was used. The transfected cells were harvested after incubation for an additional 48 h. 1 × 10⁶ cells were washed with 1 ml of phosphate-buffered saline, and resuspended in 200
hApg7p interacts with the hApg12p in vivo. pGBD-hAPG7 (TRP1) and pGAD-hAPG12 (LEU2) were transformed into PJ69-4A cells (trp1 leu2 lys2:GAL1-HIS3) to express GAL4BD-hApg7p and GAL4AD-hApg12p, respectively. pGAD-C1 and pGBD-C1 were used as controls. Cells were plated on SD-Trp-Leu plate (positive control) and SD-Trp-Leu-His plate (selective condition) and incubated at 30 °C for 3 days. PJ69-4A cells strain counterclockwise from the right carried pGBD-C1 and pGAD-C1 (GBD GAD), pGBD-C1 and pGAD-hAPG12 (GBD GAD-hApg12p), pGBD-hAPG7 and pGAD-C1 (GBD-hApg7p GAD), pGBD-hAPG7 and pGAD-hAPG12 (GBD-hApg7p GAL4BD-hApg12p). A strain expressing both GAL4BD-hApg7p and GAL4AD-hApg12p grew well on the SD-Trp-Leu-His plate, indicating that hApg7p interacts with hApg12p.

μl of phosphate-buffered saline containing Complete protease inhibitor mixture (Roche Diagnostics). The cell suspension was lysed by sonication for 10 s at 4 °C. Proteins in the lysate were separated by reducing or nonreducing SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Immunoblot analysis was performed with anti-hApg7p and -GFP antibodies (CLONTECH), and the blots were developed by an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Glycerol Gradient Centrifugation—Livers were isolated from Wistar male rats, passed through a stainless steel mesh, and suspended in 5 volumes of 5 mM Tes-NaOH, pH 7.5, 0.3 M sucrose. The homogenate was centrifuged at 100,000×g for 1 h, and the supernatant was used as the cytosol fraction. Cytosol (0.4 ml) was loaded onto an 11.5-ml linear glycerol gradient (10–40%) in 20 ml Tes-NaOH, pH 7.5, 0.15 M NaCl and centrifuged at 151,000×g for 15 h (Beckman SW-41 rotor). Fractions of 0.7 ml were collected from the bottom of the tubes. Rat Apg7p was immunoprecipitated from each fraction with anti-hApg7p and subjected to immunoblotting analysis, because hApg7p cross-reacts with hApg12p (Amersham Pharmacia Biotech). The precipitate was analyzed by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Immunoblot analysis was performed with anti-hApg7p and -GFP antibodies (CLONTECH), and the blots were developed by an enhanced chemiluminescence system (Amersham Pharmacia Biotech).
Cys\(^{572}\) within hApg7p to Ser by site-directed mutagenesis and expressed both the mutant hApg7p\(^{572\text{Cys}\to\text{Ser}}\) and GFP-fused hApg12p (GFP-hApg12p) in HEK293 cells (Fig. 2B). Cell lysates expressing both proteins were prepared and analyzed by SDS-PAGE. hApg7p was recognized by immunoblot with anti-hApg7p antibody. Wild-type hApg7p and mutant hApg7p\(^{572\text{Cys}\to\text{Ser}}\) were both expressed well in HEK293 cells (Fig. 2B, wild and C572S, hApg7p \(-80\) kDa). When both hApg7p\(^{572\text{Cys}\to\text{Ser}}\) and GFP-hApg12p were expressed in HEK293 cells, a higher molecular mass band consistent with a stable GFP-hApg12p-hApg7p\(^{572\text{Cys}\to\text{Ser}}\) intermediate \((-140\) kDa) appeared in addition to the band of \(-80\) kDa for free hApg7p (Fig. 2B, C572S). This higher molecular mass band was also recognized by immunoblotting with anti-GFP antibody in the absence or absence of reducing reagent (data not shown). These results indicate that hApg12p is an authentic substrate for hApg7p.

If hApg7p is an E1-like enzyme in the hApg12p-conjugation system, it is possible that the overexpression of hApg7p will influence the conjugation of hApg12p with hApg5p. To investigate this possibility, we expressed both hApg7p and GFP-hApg12p in HEK293 cells, metabolically labeled the cells with \(^{35}\text{S}\)S-labeled Met and Cys and prepared a cell lysate. hApg12p was immunoprecipitated with anti-mApg12p antibody, and the precipitates were analyzed by SDS-PAGE and autoradiography. When GFP-hApg12p alone was expressed, GFP-hApg12p itself was immunoprecipitated with anti-mApg12p antibody (Fig. 2C, vector). In cells expressing both GFP-hApg12p and hApg7p, a high molecular weight peptide corresponding to the hApg5p-GFP-hApg12p conjugate was immunoprecipitated in addition to GFP-hApg12p with anti-mApg12p antibody (Fig. 2C, pCMV-hAPG7). The formation of the hApg5p-GFP-hApg12p conjugate was further confirmed by a second immunoprecipitation using anti-GFP antibody (data not shown). The overexpression of mutant hApg7p\(^{572\text{Cys}\to\text{Ser}}\) did not enhance the conjugation (data not shown). These results indicate that hApg7p is an authentic protein-activating enzyme essential for the human Apg12p-conjugation system.

hApg7p Forms a Homodimer—Komatsu et al. \(^5\) have found that yeast Apg7p forms a homodimer via the C-terminal region. Considering the functional homology between yeast and human Apg7p, it is likely that hApg7p will also form a homodimer. To investigate this possibility, we conducted a cross-linking experiment. A HEK293 cell lysate expressing hApg7p was prepared and treated with a noncleavable cross-linker, disuccinimidyl suberate. After cross-linking, the lysate was analyzed by SDS-PAGE, and hApg7p was detected by immunoblotting with anti-hApg7p antibody. Before treatment with the cross-linking reagent, hApg7p was detected in the cell lysate as a band corresponding to \(-80\) kDa (Fig. 3A, pCMV-hApg7, DSS\(^{-}\)). After cross-linking, the amount of this \(-80\) kDa band was decreased, and a broad band at \(-160\) kDa appeared (Fig. 3A, pCMV-hApg7, DSS\(^{+}\)). We next analyzed endogenous Apg7p in rat liver cytosol by gel-cylinder density gradient ultracentrifugation using the cross-reactivity of the anti-hApg7p antibody with rat Apg7p. The cytosolic fraction of a rat liver homogenate was prepared and subjected to a 10–40% glycerol gradient centrifugation. Rat Apg7p was immunoprecipitated with anti-rat Apg7p antibody. The cytosolic fraction of a rat liver was prepared and subjected to ultracentrifugation through a 10–40% glycerol gradient. Fractions were collected from the bottom of the gradient and assayed for the presence of rat Apg7p by immunoblotting using anti-hApg7p antibody. The positions of marker proteins in the gradient are indicated above the blot. kDa, molecular mass; S, sedimentation value.

DISCUSSION

In this study, we showed that the human Apg7p homolog is an authentic E1-like enzyme for the hApg12p conjugation system and that hGATE-16, hGABARAP, and hMAP-LC3 are substrates for hApg7p. GATE-16 as a soluble transport factor interacts with NSF and GOS-28, is localized in the Golgi, and is expressed in the largest amount in brain (33). GABARAP is the GABA\(_\text{A}\) receptor-associated protein that colocalizes with the GABA\(_\text{A}\) receptor in cultured cortical neurons and interacts with gephyrin (31, 32, 34). MAP-LC3 is localized on autophagosomal membranes (35). Considering the divergent functions...
and intracellular localizations of the three Apg8p homologs, it is surprising that all three human Apg8p homologs are substrates for hApg7p. Because yeast Apg7p plays an indispensable role in autophagy and the Cvt transport of aminopeptidase I, mammalian Apg7p must also be essential for autophagy and other forms of membrane transport, common phenomena involving the formation of cup-shaped and/or elongated membrane structures.

Because MAP-LC3 is localized on autophagosomal membranes in rat liver as in yeast Apg8p (35), at least two substrates, MAP-LC3 and hApg12p, play major roles in autophagy in mammalian cells. At present, there has been no report of a mammalian Cvt-like pathway. Considering the strong expression of GATE-16 and GABARAP in brain and neuronal cells, a Cvt-like pathway and/or other membrane transport pathways in which GATE-16 and GABARAP function as protein modifiers may also exist in these tissues. It is difficult to explain how hApg7p distinguishes the four substrates and regulates the multiple interactions among the substrates. There must be some regulatory factors associated with the hApg7p homodimer to form multimeric complexes. Further candidates related to hApg7p will be sought by a two-hybrid experiment using a human brain cDNA library and coimmunoprecipitation of rat Apg7p with anti-hApg7p antibody in several rat tissues.

At present, the target proteins of GATE-16, GABARAP, and MAP-LC3 remain unknown. There is no report that these proteins conjugate with other proteins. We have recognized no targeting protein with which MAP-LC3 forms a conjugate. Kabeya et al. (35) reported that MAP-LC3 forms a conjugate. Kabeya et al. (35) reported that MAP-LC3 forms a conjugate. Ka-

**Fig. 4.** GATE-16, GABARAP, and MAP-LC3 are also substrates of hApg7p. A, communoprecipitation of hApg7p with GATE-16, GABARAP, and MAP-LC3. Expression plasmids for hApg7p and GFP fusion proteins were cotransfected into COS7 cells. Cell lysates were prepared, and the GFP fusion proteins were immuno-precipitated (IP) with anti-GFP antibody (α-GFP). The precipitates were analyzed by SDS-PAGE, and hApg7p was recognized by immunoblot with anti-hApg7p antibody. All GFP fusion proteins immunoprecipitated well (data not shown). GFP-GATE-16, pGFP-hGATE-16 plasmid; GFP-GABARAP, pGFP-hGABARAP plasmid; GFP-MAP-LC3, pGFP-hMAP-LC3 plasmid; GFP, pGFP-C1 vector, hApg7p wild, pCMV-hApg7p plasmid. Pairs of plasmids for cotransfection are indicated as plus (+). Whereas hApg7p did not commuinoprecipitate with GFP alone, it co-

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The Human Homolog of Saccharomyces cerevisiae Apg7p Is a Protein-activating Enzyme for Multiple Substrates Including Human Apg12p, GATE-16, GABARAP, and MAP-LC3

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