Autophagy is a process for the bulk degradation of cytosolic compartments by lysosomes/vacuoles. The formation of autophagosomes involves a dynamic rearrangement of the membrane for which two ubiquitin-like modifications (the conjugation of Apg12p and the modification of a soluble form of MAP-LC3 to a membrane-bound form) are essential. In yeast, Apg10p is an E2-like enzyme essential for Apg12p conjugation. The isolated mouse APG10 gene product interacts with mammalian Apg12p dependent on mammalian Apg7p (E1-like enzyme), and facilitates Apg12p conjugation. The interaction of Apg10p with Apg12p is dependent on the carboxyl-terminal glycine of Apg12p. Mutational analysis of the predicted active site cysteine (Cys161) within mouse Apg10p shows that mutant Apg10pC161S, which can form a stable intermediate with Apg12p, inhibits Apg12p conjugation even in the presence of Apg7p, while overexpression of Apg7p facilitates formation of an Apg12p-Apg5p conjugate. Furthermore, the coexpression of Apg10p with Apg7p facilitates the modification of a soluble form of MAP-LC3 to a membrane-bound form, a second modification essential for autophagy. Mouse Apg10p interacts with MAP-LC3 in HEK293 cells, while no mutant Apg10pC161S forms any intermediate with MAP-LC3. Direct interaction between Apg10p and MAP-LC3 is also demonstrated by yeast two-hybrid analysis. The inability of mutant Apg10pC161S to form any intermediate with MAP-LC3 has ruled out the possibility that MAP-LC3 interacts with Apg10p as a substrate.

Ubiquitylation and ubiquitylation-like protein conjugation mechanisms are essential to many cell biological activities. Ubiquitin forms conjugates with target proteins via a three-step mechanism (1–3). First, ubiquitin is activated at its carboxyl-terminal glycine by the ubiquitin-activating (E1) enzyme to form a conjugate through the active cysteine in the E1 enzyme via a thiol ester bond. Next, ubiquitin is transferred from the E1 enzyme to one of several ubiquitin-conjugating (E2) enzymes. In the last step, ubiquitin attaches to a lysine residue on the target protein via an isopeptide bond. This step is often catalyzed by a member of the ubiquitin protein ligase (E3 enzyme) family. With regard to other modifiers, Nedd8, SUMOs, Apg12p, and Apg8p (Apg8p in yeast, and, MAP-LC3, GABARAP, and GATE-16 in mammals), each is modified by specific E1 and E2 enzymes, and conjugated with its target (4–10). Of these modifiers, Apg12p and MAP-LC3 (Apg8p in yeast) cooperatively play an essential role in the dynamic membrane formation of the autophagosome during autophagy.

Autophagy is a process for the bulk degradation of cytosolic components by lysosomes/vacuoles (6, 11, 12), and has a significant relationship with type II programmed cell death, several neurodegenerative disorders, and cardiomyopathies in addition to normal cell growth and differentiation (for reviews, see Refs. 6 and 11). In the initial step of macroautophagy, a cup-shaped membrane sac surrounds cytosolic components to form an autophagosome. The outer membrane of the autophagosome fuses with the lysosome/vacuole. Thereafter, intraluminal components are degraded by lysosomal lytic enzymes. The process of autophagy is essentially the same in yeast, plant, and animal cells, and the genes, which encode components of the autophagic system in several organisms, seem to be highly conserved. In mammalian cells, autophagic vacuoles are found in hepatocytes under conditions of starvation (13) and cell differentiation (14–16), as well as in several neuro muscular diseases (17–19).

In the formation of autophagosomes, two ubiquitin-like modifications, Apg12p conjugation and Apg8p/Aut7p processing, are essential (6, 9, 20). In yeast, Apg12p is covalently attached to Apg5p via its carboxyl-terminal glycine as in the case of ubiquitylation (7). In this conjugate reaction, Apg7p and specific E1 and E2 enzymes, and conjugated with its target (4–10). Of these modifiers, Apg12p and MAP-LC3 (Apg8p in yeast) cooperatively play an essential role in the dynamic membrane formation of the autophagosome during autophagy.

Received for publication, January 17, 2003, and in revised form, July 29, 2003 Published, JBC Papers in Press, July 30, 2003, DOI 10.1074/jbc.M300550200

The Mouse APG10 Homologue, an E2-like Enzyme for Apg12p Conjugation, Facilitates MAP-LC3 Modification*

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The Journal of Biological Chemistry Vol. 278, No. 41, Issue of October 10, pp. 39517–39526, 2003

This paper is available online at http://www.jbc.org

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E1-like enzyme, Apg7p, and a second E2-like enzyme, Apg3p, mediate the reaction.

In mammals, these two modifications seem to be conserved; the Apg12p-Apg5p conjugate associates with an autophagosomal precursor in embryonic stem cells (29). After the dissociation of the conjugate according to extension of the precursor, modified, or lipidated form of MAP-LC3 (a mammalian Apg8p/Aut7p homologue) is localized to a cup-shaped preautophagosome and autophagosome (10, 20). As the apparent mobility of the soluble form of MAP-LC3 is smaller than that of the membrane-bound form in SDS-PAGE, we have designated the soluble and membrane-bound form as MAP-LC3-I and MAP-LC3-II, respectively, for simplicity (20). We have characterized mammalian Apg7p as an E1-like enzyme essential to both

FIG. 1. Comparison of the amino acid sequence of mouse Apg10p with that of yeast Apg10p. The amino acid sequence of mouse Apg10p (mouse APG10) was compared with that of yeast Apg10p (yeast APG10) using the ClustalW program. Asterisks, identical amino acids; dots, similar amino acids. The region containing the predicted active site cysteine is indicated in bold.

FIG. 2. The interaction between Apg10p and Apg12p is dependent on the presence of Apg7p in HEK293 cells. A, the Apg10p interaction with Apg12p is dependent on Apg7p. FLAGhApg12p and YFPmApg10p were expressed in the presence or absence of Apg7p in HEK293 cells. Lysates of cells expressing FLAGhApg12p and YFPmApg10p were immunoprecipitated with anti-FLAG antibody (IP: anti-FLAG) and anti-mApg10 antibody (IP: anti-mApg10p), respectively, and the immunoprecipitates were separated by SDS-PAGE. FLAGhApg12p and YFPmApg10p were recognized by immunoblotting with anti-FLAG (WB: anti-FLAG) and anti-mApg10 antibody (WB: anti-mApg10p), respectively. AG; pCMV-tag2B-hApg12/H9004G plasmid was transfected instead of pCMV-tag2B-hApg12 to express FLAGhApg12p/H9004G, a mutant that lacks the carboxy-terminal glycine essential for conjugation. The interaction of FLAGhApg12p with YFPmApg10p was dependent on the presence of Apg7p, while FLAGhApg12p/H9004G did not interact with YFPmApg10p. B, Apg10p interacts with Apg7p. YFPmApg10p and Apg7p were expressed in HEK293 cells (Expression, WB anti-mApg10p and anti-Apg7p). YFPmApg10p in the lysate of the transfec tant was immunoprecipitated with anti-mApg10p antibody, and the immunoprecipitate was separated by SDS-PAGE. Apg7p in the immunoprecipitates was recognized by immunoblotting with anti-Apg7p antibody (lane 1) when both YFPmApg10p and Apg7p were expressed.
mammalian Apg12p and three Apg8 homologues (MAP-LC3, GABARAP, and GATE-16), and mammalian Apg3p as an E2-like enzyme essential to three Apg6p homologues (10, 30, 31). However, there has been little progress in the biochemical characterization of mammalian Apg10p with regard to interacting proteins and cooperative function between Apg12p conjugation and MAP-LC3 modification. Mutational analysis showed that a mutant, Apg10pC161S, in which the active site cysteine was changed to serine, inhibits the conjugation of Apg12p even in the presence of Apg7p. Furthermore, we found that Apg10p facilitates the modification of MAP-LC3 (a second modification pathway), suggesting that the Apg12p conjugation mediated by Apg10p cooperates in the modification of a soluble form of MAP-LC3 to a membrane-bound form.

MATERIALS AND METHODS

Cloning of Mouse Apg10 cDNA—Two oligonucleotides (mAPG10-GSP1, 5′-GGCAGATGGCGTGGAAATGG-3′; mAPG10-GSP2, 5′-AGGCTG-GCTCCAGGGACCCTG-3′) were synthesized based on the DNA sequence of two EST clones (GenBankT accession numbers, AI125249 and AI159459). Using these primers, the 5′-rapid amplification of cDNA ends were performed by high fidelity PCR using mouse brain Marathon-ready cDNA as a template according to the manufacturer’s protocol (Clontech, Palo Alto, CA).

Plasmid Construction and Site-directed Mutagenesis—Based on the obtained DNA sequence of the mouse Apg10 homologue (GenBankT accession number, AB079383), we amplified an open-reading frame of the mouse Apg10 cDNA by PCR with high fidelity introducing a BglII site before the start codon, and a SalI site after the termination codon, cloned the fragment into pGEM-T, and designated the resultant plasmid the pGEMmAPG10 plasmid. To express YFPmApg10p under the control of the CMV promoter, a BglII-SalI fragment of the pGEMmAPG10 plasmid was introduced into a pEEYFP-C1 vector (Clontech, Palo Alto, CA), and designated pYFP-mApg10p. To express FLAGmAp10p in HEK293 cells, a BglII-SalI fragment of the pGEMmAPG10 plasmid was introduced into the BambHI-SalI site of a pCMV-Tag2B vector (Stratagene), and designated pCMV-tag2B-mApg10p. Mammalian expression vectors for each of the EGFP modifier fusion proteins and FLAG-tagged proteins have been described previously (10, 30, 31). To express Myc-tagged hApg5p, the BamHI-SalI fragment of the pGEMhAPG5 plasmid was introduced into the BamHI-SalI site of a pCMV-Tag3B vector (Stratagene), and designated pCMV-tag3B-hApg5p.

Cys161 within Apg10p was replaced by Ser and mutagenized using the Gene-Editor in vitro site-directed mutagenesis system (Promega) with an oligonucleotide (mAPG10CS; 5′-CAACCATTTTTTGTTCTA-CATCCAGTCCAGGATGATCATGCTG-3′) according to the manufacturer’s directions.

Antibodies—A polyclonal antibody against a synthetic polypeptide corresponding to residues 550–571 of hApg7p has been described previously (10). For the preparation of anti-serum against mouse Apg10p, rabbits were immunized with a glutathione S-transferase-Apg10p fusion protein. The antibody to mouse Apg10p was purified by affinity chromatography on glutathione S-transferase-Apg10p fusion protein-immobilized Sepharose. Then, the obtained antibody was passed through a column of glutathione S-transferase-immobilized Sepharose. The resultant antibody (anti-mApg10p antibody) recognized a recombinant YFPmApg10p in HEK293 cells. The monoclonal anti-green fluorescent protein (GFP) antibody was purchased from Clontech. The monoclonal anti-FLAG antibody (M2) was purchased from Sigma-Aldrich. The co-immunoprecipitation of interacting proteins has been described previously (10, 30, 31).

Cell Culture and Transfection—HEK293 cells were obtained from Health Science Research Resources Bank (Osaka, Japan). Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. HEK293 cells (5 × 10⁵ cells) were transfected with the indicated constructs using LipofectAMINE 2000 transfection reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). After 48 h, cells were harvested for further analyses.

Western Blotting and Immunoprecipitation—Cells were lysed with TBS/Dounce buffer (10 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 150 mM NaCl, and 1 mM EDTA) containing a Complete T protease inhibitor mixture tablet (Roche Applied Science). Cell lysates were separated by SDS-PAGE and transferred onto polyvinylidine difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat dried milk and primed with the indicated antibodies. The membranes were washed and then incubated with HRP-conjugated anti-mouse IgG or anti-rabbit IgG antibodies. Immunoreactivity was detected on x-ray film using Super Signal ULTRA chemiluminescent Substrate (Pierce). For immunoprecipitation, cell lysates were pre-absorbed with protein

![Fig. 3. Apg10p facilitates the formation of the Apg12p-Apg5p conjugate.](image)
A-agarose at 4 °C for 1 h. The pre-absorbed lysates were incubated with the indicated antibodies at 4 °C for 18 h. After incubation, unbound proteins were removed and the protein A-agarose was washed five times with TNE buffer. The immunoprecipitated proteins were eluted by boiling with Laemmli's sample buffer.

Animals—Male Wistar rats (250–300 g) were maintained in an environmentally controlled room (lights on 7:00 to 20:00) for at least 2 weeks before the experiments. All rats were fed a standard pelleted laboratory diet and tap water ad libitum during this period.

Preparation of Cell Lysates from Rat Tissues—To investigate the E2-like enzyme essential for the mammalian Apg12p conjugation system, we first isolated a cDNA to mouse Apg10p from a mouse brain cDNA library as a template (GenBank™ accession number, AB079383). The predicted amino acid sequence of the isolated clone (calculated molecular mass, 24.3 kDa) shows 10.0% identity and 22.5% similarity to yeast Apg10p (47.7% similarity). This result indicates that Apg10p interacts with Apg12p dependent on Apg7p.

RESULTS

Isolation and Identification of Mouse APG10 cDNA and Its Gene Product—To investigate the E2-like enzyme essential for the mammalian Apg12p conjugation system, we first isolated a cDNA to a mouse APG10 homologue. A BLAST search of the EST data base with the amino acid sequence of yeast Apg10p indicated a candidate mouse Apg10p homologue (GenBank™ accession numbers, A1152249 and A1594591). Based on the DNA sequences of two EST clones, a cDNA to mouse Apg10p was isolated by 5’-RACE using a mouse brain cDNA library as a template (GenBank™ accession number, AB079383). The predicted amino acid sequence of the isolated clone (calculated molecular mass, 24.3 kDa) shows 10.0% identity and 22.5% similarity to yeast Apg10p, and the region including the predicted active-site cysteine residue (Cys161) is significantly conserved between the isolated clones and yeast Apg10p (47.7% similarity) (Fig. 1).

Apg10p Interacts with Apg12p via the Carboxyl-terminal Glycine within Apg12p Dependent on Apg7p—Yeast Apg10p interacts with yeast Apg12p, its substrate, depending on yeast Apg7p, an E1-like enzyme for yeast Apg12p (23). If the isolated clone encodes an authentic E2-like enzyme essential to mammalian Apg12p, its gene product will interact with mammalian Apg12p. We then investigated whether its gene product (Apg10p) interacts with human Apg12p by immunoprecipitation. We expressed YFPmApg10p (pYFP-mApg10) and FLAGhApg12p (pCMV-tag2B-mApg10CS) in the presence of human Apg7p in HEK293 cells. When FLAGhApg12p in the lysate of the transfected cell was immunoprecipitated with anti-FLAG antibody, YFPmApg10p coprecipitated with FLAGhApg12p (Fig. 2A, lane 2, IP, anti-FLAG, WB, anti-FLAG and mApg10p). Apg12p also coprecipitated with Apg10p when the anti-mApg10p antibody was used (Fig. 2A, lane 2, IP, anti-mApg10p, WB, anti-FLAG). In the absence of Apg7p, little interaction occurred (Fig. 2A, lane 1). These results indicate that Apg10p interacts with Apg12p dependent on Apg7p.

When mutant FLAGhApg12pC161S, which lacks the carboxyl-terminal glycine of wild-type FLAGhApg12p was expressed in HEK293 cells, little interaction of YFPmApg10p with Apg7p alone was expressed, little interaction occurred (Fig. 2A, lane 5 versus lane 4). These results indicate that the interaction of Apg10p with Apg12p in the presence of Apg7p is dependent on the carboxyl-terminal glycine of Apg12p that is essential for the conjugation reaction (Fig. 2A, lane 5).

Yeast Apg10p also interacts with yeast Apg7p, an E1-like enzyme (23). Considering that Apg10p interacts with Apg12p dependent on Apg7p (Fig. 2A), it is likely that mammalian Apg10p would also interact with Apg7p. We next studied whether mammalian Apg10p interacts with Apg7p in HEK293 cells. YFPmApg10p was expressed together with human Apg7p, and immunoprecipitated with anti-mApg10p antibody. When both YFPmApg10p and Apg7p were expressed, Apg7p coprecipitated with YFPmApg10p using the anti-mApg10p antibody (Fig. 2B, lane 1). When Apg7p alone was expressed, little Apg7p was immunoprecipitated with this antibody (Fig. 2B, lane 2). These results indicate that Apg10p interacts with Apg7p and Apg12p in mammals, as is the case in yeast.

Mutant Apg10pC161S Forms a Stable Intermediate with Apg12p Dependent on Apg7p—As previously reported (31–33), when an active site cysteine within E2 (or E2-like) enzyme is changed to serine, a stable intermediate between substrate and enzyme is formed in the presence of the respective E1 (or
E1-like) enzyme. Therefore, if the isolated mouse Apg10p is an authentic E2-like enzyme essential for Apg12p conjugation in cooperation with Apg7p, a mutant Apg10p (Apg10pC161S), in which the predicted active site cysteine positioned at 161 has been changed to serine by site directed mutagenesis, will form a stable intermediate with Apg12p in the presence of Apg7p. Then, we expressed both a mutant YFPmApg10pC161S and FLAGhApg12p in the absence or presence of Apg7p in HEK293 cells (Fig. 3A, lanes 1 and 2). Apg7p, YFPmApg10pC161S, and FLAGhApg12p were expressed well in the cells (Fig. 3A, Apg7p, YFPmApg10pC161S, and FLAGhApg12p). When Apg7p was overexpressed, a stable intermediate was recognized by immunoblotting with anti-FLAG, anti-mApg10p, and anti-Apg7p antibodies, respectively. The modification of a soluble form of MAP-LC3 to a membrane-bound form was analyzed by gel mobility shift of MAP-LC3. When FLAG-MAP-LC3 and Apg7p were expressed in HEK293 cells, a soluble form of MAP-LC3 was modified to a membrane-bound form (lane 2 versus lane 4). When YFPmApg10pC161S was expressed together with FLAG-MAP-LC3 and Apg7p, MAP-LC3 modification was facilitated (lane 1). The facilitation is dependent on the presence of Apg7p (lane 3 versus lane 1). To confirm that the modified form was the membrane-bound form, we further performed cell fractionation (Cell Fractionation) of lysates of these transformants. A soluble form of MAP-LC3 (18 kDa) (soluble form of LC3) was processed to a membrane-bound form (16 kDa) (membrane-bound form of LC3). Cell lysates were fractionated into the pellet (P) and soluble (S) fractions by centrifugation at 100,000 × g as described previously (31).

**Figure 5.** Apg10p facilitates MAP-LC3 modification. HEK293 cells were transfected with pYFP-mApg10p, pcDNA-hApg7, and pCMV-tag2B-MAP-LC3. After 48 h, cell lysates were subjected to SDS-PAGE. A soluble form of FLAG MAP-LC3 (soluble form of LC3 (LC3-I)), a membrane-bound form FLAG MAP-LC3 (membrane-bound form of LC3 (LC3-II)), YFPmApg10p, and Apg7p were recognized by immunoblotting with anti-FLAG, anti-mApg10p, and anti-Apg7p antibodies, respectively. The modification of a soluble form of MAP-LC3 to a membrane-bound form was analyzed by gel mobility shift of MAP-LC3. When FLAG-MAP-LC3 and Apg7p were expressed in HEK293 cells, a soluble form of MAP-LC3 was modified to a membrane-bound form (lane 2 versus lane 4). When YFPmApg10p was expressed together with FLAG-MAP-LC3 and Apg7p, MAP-LC3 modification was facilitated (lane 1). The facilitation is dependent on the presence of Apg7p (lane 3 versus lane 1). To confirm that the modified form was the membrane-bound form, we further performed cell fractionation (Cell Fractionation) of lysates of these transformants. A soluble form of MAP-LC3 (18 kDa) (soluble form of LC3) was processed to a membrane-bound form (16 kDa) (membrane-bound form of LC3). Cell lysates were fractionated into the pellet (P) and soluble (S) fractions by centrifugation at 100,000 × g as described previously (31).

**Table:**

| pCMV-tag2B-MAP-LC3 |  |  |  |  |
|-------------------|---|---|---|---|
| +                 | + | + | + | + |
| pYFP-mApg10p      | + | - | + | - |
| pcDNA-Apg7        | + | + | - | - |

**Diagram:**

- soluble form of LC3 (LC3-I)
- membrane-bound form of LC3 (LC3-II)

**Figure 5.** Apg10p facilitates MAP-LC3 modification. HEK293 cells were transfected with pYFP-mApg10p, pcDNA-hApg7, and pCMV-tag2B-MAP-LC3. After 48 h, cell lysates were subjected to SDS-PAGE. A soluble form of FLAG MAP-LC3 (soluble form of LC3 (LC3-I)), a membrane-bound form FLAG MAP-LC3 (membrane-bound form of LC3 (LC3-II)), YFPmApg10p, and Apg7p were recognized by immunoblotting with anti-FLAG, anti-mApg10p, and anti-Apg7p antibodies, respectively. The modification of a soluble form of MAP-LC3 to a membrane-bound form was analyzed by gel mobility shift of MAP-LC3. When FLAG-MAP-LC3 and Apg7p were expressed in HEK293 cells, a soluble form of MAP-LC3 was modified to a membrane-bound form (lane 2 versus lane 4). When YFPmApg10p was expressed together with FLAG-MAP-LC3 and Apg7p, MAP-LC3 modification was facilitated (lane 1). The facilitation is dependent on the presence of Apg7p (lane 3 versus lane 1). To confirm that the modified form was the membrane-bound form, we further performed cell fractionation (Cell Fractionation) of lysates of these transformants. A soluble form of MAP-LC3 (18 kDa) (soluble form of LC3) was processed to a membrane-bound form (16 kDa) (membrane-bound form of LC3). Cell lysates were fractionated into the pellet (P) and soluble (S) fractions by centrifugation at 100,000 × g as described previously (31).

**Cell fractionation**

- soluble form of LC3 (LC3-I)
- membrane-bound form of LC3 (LC3-II)

**Figure 5.** Apg10p facilitates MAP-LC3 modification. HEK293 cells were transfected with pYFP-mApg10p, pcDNA-hApg7, and pCMV-tag2B-MAP-LC3. After 48 h, cell lysates were subjected to SDS-PAGE. A soluble form of FLAG MAP-LC3 (soluble form of LC3 (LC3-I)), a membrane-bound form FLAG MAP-LC3 (membrane-bound form of LC3 (LC3-II)), YFPmApg10p, and Apg7p were recognized by immunoblotting with anti-FLAG, anti-mApg10p, and anti-Apg7p antibodies, respectively. The modification of a soluble form of MAP-LC3 to a membrane-bound form was analyzed by gel mobility shift of MAP-LC3. When FLAG-MAP-LC3 and Apg7p were expressed in HEK293 cells, a soluble form of MAP-LC3 was modified to a membrane-bound form (lane 2 versus lane 4). When YFPmApg10p was expressed together with FLAG-MAP-LC3 and Apg7p, MAP-LC3 modification was facilitated (lane 1). The facilitation is dependent on the presence of Apg7p (lane 3 versus lane 1). To confirm that the modified form was the membrane-bound form, we further performed cell fractionation (Cell Fractionation) of lysates of these transformants. A soluble form of MAP-LC3 (18 kDa) (soluble form of LC3) was processed to a membrane-bound form (16 kDa) (membrane-bound form of LC3). Cell lysates were fractionated into the pellet (P) and soluble (S) fractions by centrifugation at 100,000 × g as described previously (31).

Mouse Apg10p Facilitates Apg12p-Apg5p Conjugate Formation—Considering the above results, the mammalian Apg10p would facilitate Apg12p-Apg5p conjugation. When FLAGhApg12p and myc-Apg5p were expressed in HEK293 cells, a small amount of the FLAGhApg12p-myc-Apg5p conjugate was recognized (Fig. 3B, lane 5). In the presence of human Apg7p, FLAGhApg12p conjugates to myc-Apg5p significantly as described previously (Fig. 3B, lane 3, Apg12p-Apg5p) (10, 30). When FLAGmApg10p was expressed in addition to human Apg7p, the formation of the FLAGhApg12p-myc-Apg5p conjugate was facilitated significantly (Fig. 3B, lane 1). Without overexpressed Apg7p, a significant reduction in the amount of conjugate occurred even in the presence of Apg10p (Fig. 3B, lane 3). These results indicate that the cysteine positioned at 161 is an authentic active-site cysteine for the E2-like reaction of hApg12p via the carboxyl-terminal glycine within hApg12p.
A mutant Apg12p (FLAGhApg12p/H9004G) hardly conjugated to mychApg5p even in the presence of Apg7p and Apg10p, because the carboxyl-terminal glycine of Apg12p is essential for the conjugation (Fig. 3B, lane 4). These results indicate that Apg10p facilitates Apg12p-Apg5p conjugation dependent on human Apg7p.

An Active-site Cysteine Mutant, Apg10pC161S, Inhibits the Effect of Overexpression of Apg7p on Apg12p Conjugation—Considering the reaction of the E2-like enzyme, it is probable that a mutant Apg10pC161S inhibits the Apg12p conjugation even in the presence of endogenous Apg10p in mammalian cells. To investigate whether Apg10pC161S inhibits the formation of the Apg12p-Apg5p conjugate, YFPmApg10pC161S was expressed together with FLAGhApg12p and mychApg5p, in the presence or absence of Apg7p. When YFPmApg10p was expressed with FLAGhApg12p and mychApg5p, the YFPmApg10pC161S conjugate was formed (Fig. 4, lane 1, WB, anti-Myc, FLAGhApg12-mychApg5). When YFPmApg10pC161S was expressed instead of wild-type YFPmApg10p together with FLAGhApg12p, and mychApg5, little conjugate was recognized (Fig. 4, lane 2). The overexpression of human Apg7p stimulated the Apg12p conjugation reaction, as shown above (10). However, when mutant Apg10pC161S was expressed instead of wild-type Apg10p, the formation of the Apg12p-Apg5p conjugate was almost completely inhibited even in the presence of Apg7p. Essentially the same result was obtained, when FLAGmApg10pC161S was expressed instead of wild-type FLAGmApg10p together with GFPhApg12p, mychApg5p, and Apg7p (Fig. 4B, lane 5 versus lane 6, WB, anti-Myc, GFPhApg12-mychApg5). These results indicate that the overexpression of mutant Apg10pC161S inhibits the formation of the Apg12p-Apg5p conjugate even in the presence of Apg7p.

Overexpression of Mouse Apg10 Facilitates the Modification of a Soluble Form of MAP-LC3 to a Membrane-bound Form—A cytosolic MAP-LC3 (a soluble form of MAP-LC3, MAP-LC3-I) is modified to a membrane-bound form (MAP-LC3-II) to form autophagosomes after the Apg12p-Apg5p conjugate associates with autophagosomal precursors (20). The modification of the soluble form of MAP-LC3 to the membrane-bound form following the Apg12p-Apg5p conjugation is essential for mammalian autophagy (29). Recently, we showed that Apg3p, an E2-like enzyme essential for the modification of MAP-LC3, facilitates the conjugation of Apg12p-Apg5p, suggesting functional cross-talk between Apg12p conjugation and MAP-LC3 modification (31). Therefore, it is possible that Apg10p influences the modification of MAP-LC3 in reverse. We then investigated whether the overexpression of mammalian Apg10p affects the modifica-
When FLAGMAP-LC3 only was expressed, FLAG-MAP-LC3 was present as a soluble form (Fig. 5, lane 4, anti-FLAG, soluble form of LC3). When human Apg7p was expressed together with FLAGMAP-LC3, the modification of the soluble form of MAP-LC3 to the membrane-bound form was slightly facilitated by Apg7p (Fig. 5, lane 2, anti-FLAG, membrane-bound form of LC3). When YFPmApg10p was overexpressed together with Apg7p, the modification of the soluble form of MAP-LC3 to the membrane-bound form was further enhanced significantly (Fig. 5, lane 1 versus lane 2). The facilitation of the modification leading to the membrane-bound form of MAP-LC3 by Apg10p is dependent on Apg7p (Fig. 5, lane 1 versus lane 3). To confirm that the modified form of MAP-LC3 is a membrane-bound form, we performed subcellular fractionation. The modified form of MAP-LC3 was recognized mainly in the pellet fraction, although little of the soluble form of MAP-LC3 was recognized in the pellet fraction (Fig. 5, bottom, cell fractionation panel). A significant increase in the amount of the membrane-bound form of MAP-LC3 in the membrane fraction was observed when Apg10p was overexpressed together with Apg7p (Fig. 5, lane 1, P). These results indicate that the overexpression of Apg10p facilitates the modification of the soluble form of MAP-LC3 to the membrane-bound form.

Apg10p Interacts with MAP-LC3, a Modifier of the Second Modification System by Apg7p and Apg3p—In yeast, systematic and comprehensive analyses of protein-protein interaction have been performed (34–36), but there is no report of the interaction of yeast Apg10p with Apg8p, a yeast homologue of MAP-LC3. However, considering the facilitation of MAP-LC3 modification by the overexpression of mammalian Apg10p (Fig. 5, lane 1 versus lane 2), the facilitation of the modification leading to the membrane-bound form of MAP-LC3 by Apg10p is dependent on Apg7p (Fig. 5, lane 1 versus lane 3). To confirm that the modified form of MAP-LC3 is a membrane-bound form, we performed subcellular fractionation. The modified form of MAP-LC3 was recognized mainly in the pellet fraction, although little of the soluble form of MAP-LC3 was recognized in the pellet fraction (Fig. 5, bottom, cell fractionation panel). A significant increase in the amount of the membrane-bound form of MAP-LC3 in the membrane fraction was observed when Apg10p was overexpressed together with Apg7p (Fig. 5, lane 1, P). These results indicate that the overexpression of Apg10p facilitates the modification of the soluble form of MAP-LC3 to the membrane-bound form.
5), it is possible that mammalian Apg10p would interact with MAP-LC3. To investigate whether mouse Apg10p interacts with MAP-LC3, we expressed YFPmApg10p and FLAGMAP-LC3 in HEK293 cells. YFPmApg10p and FLAGMAP-LC3 were expressed well in HEK293 cells (Fig. 6, Expression, WB, anti-Apg10p and anti-FLAG). When FLAGMAP-LC3 was immunoprecipitated with anti-FLAG antibody, YFPmApg10p coimmunoprecipitated with FLAGMAP-LC3 (Fig. 6, lane 3, IP anti-FLAG). When YFPmApg10p was immunoprecipitated with anti-GFP antibody, FLAGMAP-LC3 coimmunoprecipitated with YFPmApg10p (Fig. 6, lane 3, IP anti-GFP). As previously shown in Fig. 2A, the function of wild-type Apg10p in Apg12p conjugation. The carboxyl-terminal glycine of Apg12p is activated by Apg7p in an ATP-dependent manner to form an active thiol ester bond between the glycine of Apg12p and active-site cysteine in Apg7p. Then, Apg12p is transferred to Apg10p to form a thiol ester bond between the glycine of Apg12p and active-site cysteine at position 161 in Apg10p. Finally, the glycine in Apg12p conjugates to a lysine in Apg5p to form a stable Apg12p-Apg5p conjugate. B, the inhibition of Apg10p conjugation by the overexpression of mutant Apg10pC161S. When mutant Apg10pC161S was overexpressed, a stable O-ester bond between the glycine of Apg12p and the serine at position 161 in Apg10pC161S was formed. Therefore, further reaction is significantly inhibited by the overexpression of mutant Apg10pC161S even in the presence of excess Apg7p.

MAP-LC3 Is Not a Substrate of Apg10p but of Apg3p, while MAP-LC3 Interacts with Apg10p—MAP-LC3 is a modifier mediated by Apg7p and Apg3p in mammals (10, 20, 31). Considering the interaction of Apg10p with MAP-LC3 and the facilitation of MAP-LC3 modification by Apg10p, it would be possible that MAP-LC3 is a substrate of Apg10p in addition to hApg12p. If so, mutant Apg10pC161S will also form a stable intermediate with MAP-LC3 as well as hApg12p in the presence of Apg7p. As shown in Fig. 3A, when FLAGhApg12p and YFPmApg10pC161S were expressed in HEK293 cells, a stable intermediate of YFPmApg10pC161S-FLAGhApg12p was formed dependent on Apg7p (Fig. 7, WB, anti-Apg10 and anti-Apg12, lane 5 versus lanes 1 and 9). When FLAGMAP-LC3 was expressed together with YFPmApg10pC161S, no intermediate was formed even in the presence of Apg7p (Fig. 7, WB, anti-Apg10 and anti-LC3, lanes 2, 6, and 10). FLAGMAP-LC3 can form a stable intermediate via its carboxyl-terminal glycine with GFPhApg3pC264S, in which the active site cysteine position at 264 was changed to serine by site-directed mutagenesis (31), dependent on Apg7p (Fig. 7, WB, anti-Apg3 and anti-LC3, lane 8 versus lanes 4 and 12). These results indicate that Apg10p interacts with MAP-LC3, facilitates its modification, but does not recognize MAP-LC3 as a substrate.

**Tissue Distribution of Apg10p**—To investigate the tissue-specific distribution of endogenous Apg10p, we immunoprecipitated Apg10p from rat tissues. Tissues isolated from a male Wistar rat were homogenized with a glass/Teflon homogenizer.
After removal of the cell debris, the lysates were centrifuged at 100,000 × g for 1 h to separate the cytosolic and membrane fractions (Fig. 8, S and P). The Apg10p in each fraction was immunoprecipitated with an anti-mApg10p antibody. The immunoprecipitates were separated by SDS-PAGE, and Apg10p in the precipitates was recognized by immunoblotting using an anti-mApg10p antibody (Fig. 8). Apg10p was expressed ubiquitously in all tissues examined. In most tissues, it was more abundant in the cytosol than in the membrane fraction. Thus, the results correspond well to those for Apg7p and Apg3p (10, 31). In spleen, more Apg10p was recognized in the membrane fraction than in the cytosol.

DISCUSSION

In this study, we characterize biochemically the mammalian APG10 gene product as an E2-like enzyme essential for Apg12p conjugation. The biochemical properties of the isolated Apg10p are consistent with a recent report of the cloning of the mouse APG10 gene by Mizushima et al. (37), and the amino acid sequence of the Apg10p isolated from a mouse brain cDNA library in this study (211 amino acids) shows 95% homology with that isolated from a whole mouse cDNA library (215 amino acids). Eight amino acids in the amino-terminal region differ (37), a difference that is probably due to alternative splicing. Furthermore, mutation analysis of mammalian Apg10p showed that a mutant, Apg10pC161S, in which the active site cysteine at position 161 is changed to serine, inhibits Apg12p-Apg5p conjugation even in the presence of Apg7p (Fig. 9A). We also found that the overexpression of Apg7p and Apg10p facilitates the modification of a soluble form of MAP-LC3 to a membrane-bound form, suggesting that there is cross-talk between Apg10p, the first E2-like enzyme essential for Apg12p conjugation, and MAP-LC3 modification, the second ubiquitin-like modification essential for autophagy. In Fig. 9 we present a working hypothesis for the finding that mutant Apg10pC161S (mutant E2-like enzyme) inhibits the Apg12p conjugation even in the presence of Apg7p (wild-type E1-like enzyme). In general, when the active site cysteine residue of an E2 enzyme is changed to serine, a stable O-ester bond instead of a thiol ester bond is formed between the E2 enzyme and substrate. Our observation that Apg10pC161S has an inhibitory effect on Apg12p-Apg5p conjugation can be attributed to the formation of the stable intermediate with a much slower rate of turnover toward Apg12p-Apg5p conjugation (Fig. 9B). With regard to UBC9, an E2 enzyme for sumoylation, it has been reported that UBC9 C93S functions as a dominant negative mutant. Mo et al. (32) revealed that the UBC9 C93S mutant inhibits the sumoylation of topoisomerase I, and simultaneously reduces the nucleolar delocalization of topoisomerase I. Considering these results, the mutant Apg10pC161S will provide a useful tool for further analyses of the Apg12p-Apg5p conjugation system.

We found that Apg10p facilitates the modification of a soluble form of MAP-LC3 to a membrane-bound form, which is also essential for the formation of autophagosomes after Apg12p-Apg5p conjugation. We also showed that mammalian Apg10p interacts with MAP-LC3, while there is no report of any interaction between yeast Apg10p and Apg8p (a yeast MAP-LC3 homologue). Interestingly, the interaction of Apg10p with MAP-LC3 is independent of Apg7p, while the interaction of Apg10p with Apg12p is dependent on Apg7p (Figs. 2 and 6). Furthermore, MAP-LC3 is not recognized as a substrate by Apg10p (Fig. 7). We have reported that mouse Apg12p shows a preference for mouse Apg7p rather than MAP-LC3 as a substrate (30). Using an active-site mutant Apg7pC572S protein, we investigated whether the overexpression of Apg10p affects the preference of MAP-LC3 for Apg7p, although there is little change in the substrate preference for Apg7p in HEK293 cells. Using active site mutant Apg7pC572S and Apg3pC264S proteins, we further investigated whether the overexpression of Apg10p facilitates the reactions of MAP-LC3 with Apg7p and Apg9p. However, Apg10p had little effect on the formations of E1-like or E2-like enzyme-substrate intermediates between MAP-LC3 and Apg7pC572S or Apg3pC264S, respectively. Therefore, it seems that the interaction of Apg10p with MAP-LC3 does not facilitate the reaction of MAP-LC3 with Apg7p and Apg3p, i.e. E1- and E2-like reactions in MAP-LC3-modification. In Apg12p conjugation and Apg8p (MAP-LC3 in mammals) modification, there are no reports of any E3-like enzyme in yeast or mammals. One possible explanation for these results is that Apg10p functions as an E3-like enzyme. Namely, the interaction between Apg10p and MAP-LC3 plays an indispensable role in facilitating the recognition of target MAP-LC3 by phospholipid. We have shown that Apg7p forms a homodimer (10, 22), and that Apg3p, a second E2-like enzyme for MAP-LC3 modification, interacts with Apg7p and Apg12p (31). The overexpression of Apg3p facilitates the formation of the Apg12p-Apg5p conjugate (31). When the Apg12p-Apg5p conjugate accumulates in the presence of Apg12p and Apg5p, MAP-LC3 modification is inhibited even in the presence of Apg7p and Apg10p (38). Considering the precise formation of autophagosomes, cross-talk between the two ubiquitin-like modifications via two E2-like enzymes would be indispensable for regulating the formation of autophagosomes correctly when required during autophagy. We have tried to reconstitute Apg12p conjugation and MAP-LC3 modification in vitro, but have not been successful so far. Therefore, at present, it is difficult to perform more precise analyses on the relationship between the two ubiquitin-like modifications in vitro. Apg10p was expressed ubiquitously in all tissues examined. Both Apg7p, an E1-enzyme for Apg12p conjugation and MAP-LC3 processing to the membrane-bound form, and Apg3p, an E2-enzyme for MAP-LC3 processing, are also expressed ubiquitously (10, 31). These findings suggest that the two modification systems play important roles in normal mammalian tissues. Further analyses using mutant Apg10pC161S and APG10-gene knockout mice will lead to a new understanding of the role of mammalian Apg10p in cell growth and differentiation.

Acknowledgments—We thank Drs. Y. Ohsumi, T. Noda, N. Mizushima, and Y. Ichimura (National Institute for Basic Biology, Okazaki, Japan), Dr. T. Yoshimori (National Institute of Genetics, Mishima, Japan) for significant discussion and information, and Drs. K. Ishidoh, J. Ezaki, D. Muno, and M. Komatsu (Juntendo University, Tokyo, Japan) for helpful discussion. We also thank Dr. M. Dooley-Ohoto for language editing of the manuscript.

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