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Murine Apg12p Has a Substrate Preference for Murine Apg7p over Three Apg8p Homologs

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Apg7p is a unique E1 enzyme which is essential for both the Apg12p- and Apg8p-modification systems, and plays indispensable roles in yeast autophagy. A cDNA encoding murine Apg7p homolog (mApg7p) was isolated from a mouse brain cDNA library. The predicted amino acid sequence of the clone shows a significant homology to human Apg7p and yeast Apg7p. Murine Apg12p as well as the three mammalian Apg8p homologs co-immunoprecipitate with mApg7p. Site-directed mutagenesis revealed that an active-site cysteine within mApg7p is Cys[567], indicating that mApg7p is an authentic E1 enzyme for murine Apg12p and mammalian Apg8p homologs. The mutagenesis study also revealed that Apg12p has a substrate preference for mApg7p over the three Apg8p homologs, suggesting that the Apg12p conjugation by Apg7p occurs preferentially in mammalian cells compared with the modification of the three Apg8p homologs. We also report here on the ubiquitous expression of human APG7 mRNA in human adult and fetal tissues and of rat Apg7p in adult tissues.

Key Words: **Apg12p; GATE-16; GABARAP; MAP-LC3; E1-like enzyme; Apg12p-conjugation system; autophagy; Apg8p; modifier.**

The process of bulk degradation of cytoplasmic components by the lysosomal/vacuolar system, autophagy, plays an important role in mammals and yeast (1–9). Clinical and biochemical analyses of a group of severe, inherited neurodegenerative disorders in humans, neuronal ceroid-lipofuscinosis, and cathepsin-D deficient mice suggest that lysosomal degradation *via* autophagy actively occurs during neuronal development (10–12). Furthermore, clinical, genetic and biochemical analyses of X-linked vacuolar cardiomyopathy, myopathy in humans and LAMP-2-deficiency mice indicate that autophagic processes play an indispensable role in normal mammalian bodies (8, 13). In addition, autophagy is activated by apoptotic signaling in sympathetic neurons (14). While the importance of autophagy in mammals is of considerable, current interest, the complete molecular mechanism of autophagy remains unclear. Recently, it has been shown that, in the initial step of autophagy, two ubiquitylation-like modification systems (the Apg12p-conjugation system and the Apg8p-modification system) are essential for autophagy in yeast and murine embryonic stem cells (15–24). Recent findings indicate that the two systems are involved in the dynamic formation of a pre-autophagosomal membrane sac in the cytosol (23).

Apg7p is a unique E1 enzyme which is essential for two modification systems in yeast (15, 17–21, 24). In the Apg12p-conjugation system, Apg12p is activated by an E1 enzyme, Apg7p, to form a conjugate with the active-site cysteine in the E1 enzyme *via* a thiol ester bond (19). Apg12p is then transferred from Apg7p to an E2 enzyme, Apg10p (18). In the last step, Apg12p is attached to a Lys within Apg5p *via* an isopeptide bond (16). The conjugate appears to be localized to pre-autophagosomal precursors (24–26). In the Apg8p-modification system, Apg8p is activated by the same E1 enzyme, Apg7p, and transferred to another E2 enzyme, Apg3p (21). In the last step, Apg8p is attached to a phosphatidylethanolamine. Both systems play an indispensable role in the formation of autophagosomal membranes.

In mammalian cells, several homologs of yeast *APG* gene-products have been reported. The human Apg12p homolog (hApg12p) conjugates with the human Apg5p homolog (15, 27). There are three candidates for mammalian Apg8p/Aut7p-homologs, GATE-16 (Golgi-

Abbreviations: GABARAP, GABAA receptor-associated protein; GATE-16, Golgi-associated ATPase enhancer of 16 kDa; GFP, green fluorescent protein; hApg5p, human Apg5p homolog; hApg7p, human Apg7p homolog; hApg12p, human Apg12p homolog; kbp, kilobase pairs; kDa, kilodalton; mApg5p, murine Apg5p homolog; mApg7p, murine Apg7p homolog; mApg12p, murine Apg12p homolog; MAP-LC3, microtubule-associated protein light chain 3; mGABARAP, murine GABARAP; mGATE-16, murine GATE-16; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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associated ATPase enhancer of 16 kDa), GABARAP (GABA receptor-associated protein), and MAP-LC3 (microtubule-associated protein light chain 3) (28–33). GATE-16 has been characterized as a soluble transport factor, and interacts with NSF and the Golgi v-SNARE GOS-28 (32). The mRNA of GATE-16 is expressed ubiquitously, but at significantly higher levels in brain tissue. GABARAP interacts with $GABA_A$ receptors, cytoskeleton, and gephyrin, suggesting its functional importance in brain or neuronal cells (30, 31, 34–37). MAP-LC3 co-polymerizes with tubulin, and is a component of the MAP-1 complex, which is composed of light chains 1, 2 and 3 and heavy chains (28). Rat MAP-LC3 is localized on autophagosomal membranes, suggesting that rat MAP-LC3 is also a functional Apg8p/Aut7p homolog (22). These results suggest that mammalian Apg8p/Aut7p homologs have divergent functions in mammalian cells, especially neuronal cells. Recent studies using murine *APG5* gene-knock out embryonic stem cells revealed that the hApg12p-conjugation system and MAP-LC3 are indispensable for mammalian autophagy (23). Regarding Apg7p, the human Apg7p homolog (hApg7p) functions as an E1 enzyme essential for multi-substrates, the human Apg8ps and human Apg12p (38). The isolation and characterization of human Apg3p homolog has recently been reported (39). For investigations of the function of Apg7p in mammalian tissues and development along with GATE-16 and GABARAP, a gene-disruption technique in mouse would be an effective approach, but murine Apg7p has not yet been identified and characterized. In this paper, we report on the isolation and characterization of the murine Apg7p homolog (mApg7p) as a multi-substrates E1-enzyme and studies of the affinity of mApg7p for four substrates. The expression of the mammalian Apg7p homolog was also carried out.

MATERIALS AND METHODS

Materials and molecular biological techniques. Molecular biological and biochemical techniques were performed as described previously (40). DNA plasmid was transfected into mammalian cells with the FuGene-6 transfection reagent according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). pcDNA3 was purchased from Invitrogen (Carlsbad, CA); pGEM-T was from PROMEGA (Madison, WI); pEGFP-C1 was from Clontech (Palo Alto, CA), pBluescriptII (SK+) was from Stratagene (La Jolla, CA). A multiple tissues Northern dot blot was purchased from Clontech (Palo Alto, CA).

Cloning of complementary DNA of murine APG7 homolog. Based on the DNA sequence of the four EST clones (GenBank accession numbers W75594, AA822955, AA399856, and AA546344), two oligonucleotides were synthesized (mAPG7-GSP1, 5′-GCGGTCATCACTGCTGCTGGCGACTGCG-3′; mAPG7-GSP2, 5′-GAACATTATCGAACCGTGACAGAAAACCCCGG-3′). Using these primers, 5′-rapid amplification of the cDNA ends (5′-RACE) was performed by high fidelity PCR with mouse brain Marathon-ready cDNA as a template according to the manufacturer's protocol (Clontech, Palo Alto, CA). The amplified DNA fragment was cloned into the pGEM-T vector. The DNA sequences of five independent clones were determined. Each of the four sequences were identical to the others. Furthermore, the determined sequences showed a significant homology with that of the human *APG7* homolog.

Plasmid construction and site-directed mutagenesis. Based on the obtained DNA sequence of the murine *APG7* homolog, we amplified an open-reading frame of the murine *APG7* cDNA by high fidelity PCR, introducing the *Bgl*II site before the start codon and the *Sal*I site after the termination codon, cloned the fragment into a pGEM-T and designated the resultant plasmid as the pGEMmAPG7 plasmid. To express mApg7p under the control of a CMV promoter, a *Bgl*II-*Sal*I fragment of the pGEMmAPG7 plasmid was introduced into a pcDNA3, designated as pCMVmAPG7. Mammalian expression vectors for each of the GFP-modifier fusion proteins (GFPmApg12p, GFPhMAP-LC3, GFPmGATE-16, GFPmGABARAP) were described previously (38), designated as pGFPmAPG12, pGFPhMAP-LC3, pGFPmGATE16, and pGFPmGABARAP respectively. Cys^{567} within mApg7p was replaced by Ser, mutagenized by the Gene-Editor *in vitro* site-directed mutagenesis system (PROMEGA, Madison, WI) with an oligonucleotide (mAPG7CS; 5′-CTGGACCAGCAGAGCACAGTGAGCCGCCC-3′) according to the manufacturer's protocol. The expression plasmid for the mutant $mApg7p^{C567S}$ was constructed in a manner to that for pCMVmAPG7, and is designated as pCMVmAPG7CS.

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 labitum during this period.

Antibodies and immunoprecipitation. A polyclonal antibody against a synthetic polypeptide corresponding to residues 550–571 of hApg7p has been described previously (38). The antibody was cross-reacted with murine and rat Apg7p. A monoclonal antibody against GFP was purchased from Clontech (Palo Alto, CA).

Other techniques. An advanced BLAST search was performed on the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/BLAST/). The multiple alignment of amino acids sequences was performed using a CLUSTAL W program using MacVector software on a Macintosh computer (Oxford Molecular Biology, Madison, MI).

RESULTS AND DISCUSSION

Isolation of complementary DNA of murine APG7 homolog. A BLAST search of human *APG7* on murine EST (expressed sequence tagged) database indicated that the predicted amino acid sequence of four EST-clones (Genbank accession numbers W75594, AA822955, AA399856, and AA546344) shows significant homology with that of the carboxyl terminus of human Apg7p. To isolate a murine *APG7* homolog, we performed a 5′-rapid amplification of the complementary DNA ends according to the sequences of the clones by polymerase chain reaction with high fidelity. The nucleotide sequence of the open reading frame of the isolated clone shows an 86.2% identity with that of human *APG7*, and the predicted amino acid sequence of the clone shows a significant homology to human Apg7p (99% homology, 92% identity) and yeast Apg7p (67% homology, 37% identity). CLUSTAL-W analysis among the amino acid sequences of yeast, human, and murine Apg7p indicated that a predicted active-site cysteine would be Cys^{567} within the murine clone (Fig. 1, arrow-head), and that a predicted ATP-binding domain (358–363 out of 698 amino acids; **GAGTLG**) is conserved among all molecules (Fig. 1, underline).

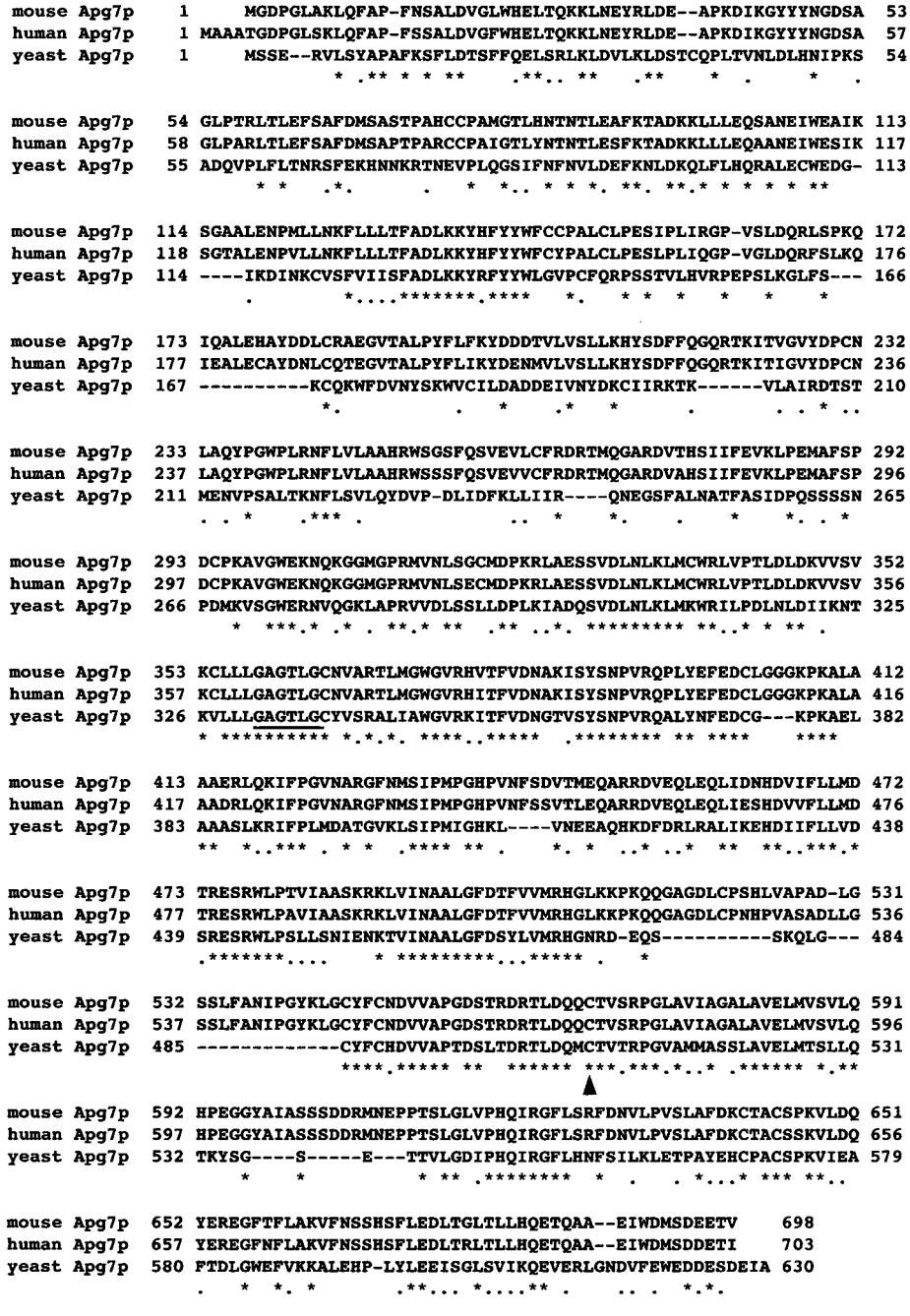

FIG. 1. Comparison of murine Apg7p, human Apg7p, and yeast Apg7p. A complementary DNA of murine Apg7p was isolated by a 5′-rapid amplification of complementary DNA using a mouse cDNA library and high-fidelity PCR. The predicted amino acids of the isolated clone were compared with that of human and yeast Apg7p using the CLUSTAL-W program. ATP-binding domain (***underlined***, GXGXXG) and active-site cysteine (**arrowhead**) are significantly conserved. The **asterisks** represent identical amino acids, and the **dots**, a similar one.

These results suggest that the isolated cDNA clone encodes a candidate of human and yeast Apg7ps.

Murine Apg7p, an authentic E1 enzyme for mammalian Apg12p and Apg8p homologs. If the isolated clone encodes an authentic murine Apg7p homolog, its gene-product, which was designated as mApg7p, would be expected to interact with murine Apg12p (mApg12p) and the three Apg8p-homologs, i.e. GATE-16, GABARAP, and MAP-LC3. To investigate these interactions, we employed co-immunoprecipitation using an anti-hApg7p antibody that cross-reacts with murine Apg7p (Fig. 2, Expression, WB anti-hApg7p). GFPmApg12p, GFPmGATE-16, GFPmGABARAP, and GFPhMAP-LC3 were individually expressed together with mApg7p in HEK293 cells (Fig. 2, Expression, WB anti-GFP). Murine Apg7p in a lysate of the transfec-

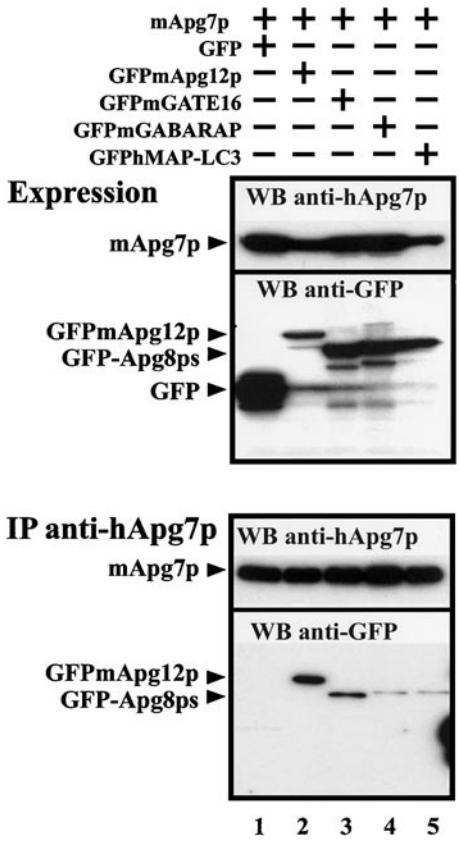

FIG. 2. Murine Apg7p interacts with murine Apg12p and three Apg8p homologs, MAP-LC3, GABARAP, and GATE-16. Plasmids were transfected into HEK293 cells to express mApg7p (**mApg7p**), and each of GFPmApg12p (**GFPmApg12p**), GFP-Apg8p homologs (**GFP-Apg8ps**). **pcDNA3** and **pEGFP-C1** were used as controls. mApg7p was expressed well in the cells (**Expression**, **WB**: **anti-hApg7p**). GFPmApg12p, GFPmGATE16, GFPmGABARAP, and GFPhMAP-LC3 were also expressed respectively (**Expression**, **WB**: **anti-GFP**, **GFPmApg12p**, and **GFP-Apg8ps**). When both mApg7p and GFP-fused proteins were expressed in HEK293 cells, mApg7p in the cell lysate was immunoprecipitated with the anti-hApg7p antibody, and GFPmApg12p and GFP-Apg8p homologs co-immunoprecipitated with mApg7p (**IP**: **anti-hApg7p**, **WB**: **anti-hApg7p**, and **anti-GFP**, lanes 2–5). When mApg7p and GFP were expressed, no GFP co-immunoprecipitated with mApg7p using anti-hApg7p antibody (**IP**: **anti-hApg7p**, **WB**: **anti-GFP**, lane 1). Arrowheads are **mApg7p**, murine Apg7p homolog; **GFPmApg12p**, GFP-tagged mApg12p; **GFPmGATE16**, GFP-tagged murine GATE-16, **GFPmGABARAP**, GFP-tagged murine GABARAP, **GFPhMAP-LC3**, GFP-tagged human MAP-LC3, and **GFP**, GFP only.

tant was immunoprecipitated with the anti-hApg7p antibody (Fig. 2, IP anti-hApg7p, WB anti-hApg7p). Each of the GFP-fused proteins co-immunoprecipitates with mApg7p, while little GFP alone does so (Fig. 2, IP anti-hApg7p, WB anti-GFP). These results indicate that mApg7p interacts with mApg12p and three Apg8p homologs.

Considering these interactions, mApg7p would be expected to react with mApg12p and the three Apg8p homologs as an E1-enzyme. To investigate the issue of whether mApg7p is an authentic E1 enzyme for mApg12p and three Apg8p homologs, we examined the formation of an E1-substrate intermediate. In the modification steps, an active site-cysteine within an E1 enzyme temporally conjugates to a substrate to form an E1 · substrate-intermediate *via* thiol-ester bond. Due to the rapid turn-over of an E1 reaction, it is difficult to recognize such an intermediate in sufficient quantity. When an active-site cysteine residue of mApg7p is changed to serine, a stable *O*-ester bond instead of a thiol-ester bond would be formed between the enzyme and substrate(s). As a result, Cys^{567} within mApg7p, that is, the predicted active-site cysteine, was changed to Ser by site-directed mutagenesis. Mutant $mApg7p^{C567S}$ was expressed together with each of GFPmApg12p, GFPmGATE-16, GFPmGABARAP, and GFPhMAP-LC3. Cell lysates expressing both proteins were prepared and analyzed by SDS-PAGE. Both wild type and mutant mApg7ps were recognized by immunoblot with the anti-hApg7p antibody, and GFP-fusions were performed with the anti-GFP antibody (Fig. 3). When both $mApg7p^{C567S}$ and GFP-modifiers were expressed in HEK293 cells, a higher molecular mass band consistent with a stable intermediate appeared in addition to the band corresponding to about 80 kDa for free mApg7p (Fig. 3, lanes 2, 4, 6, 8; 7 · 12 intermediates and 7 · 8 intermediates). When both $mApg7p^{C567S}$ and GFP were expressed in HEK293 cells, no higher molecular mass band was evident (Fig. 3, lane 10). These results indicate that mApg7p is an authentic E1-like enzyme for mApg12p, GATE-16, GABARAP, and MAP-LC3, and that the Cys^{567} residue of mApg7p is an authentic active-site cysteine.

Murine Apg12p has a substrate preference for mApg7p over three Apg8p homologs. It has been reported that the formation of the murine Apg12p · Apg5p conjugate is essential for the sequential formation of the pre-autophagosomal membrane sac, in which MAP-LC3 participates (23). Considering precedence of the mApg12p-conjugation system over MAP-LC3-modification, mApg7p may have different affinity for mApg12p compared with those for Apg8p homologs. The co-immunoprecipitation data shown in Fig. 2 indicated that GFP-mApg12p interacts with mApg7p in preference to Apg8p homologs (Fig. 2, lane 2). The mutational analysis in Fig. 3 also indicates that GFP-mApg12p reacts preferentially with $mApg7p^{C567S}$ to form an intermediate as compared with mammalian Apg8p homologs (Fig. 3, lanes 2, 4, 6, and 8; 7–12 intermediate and 7–8 intermediates). There is little difference between the three mammalian Apg8p homologs with regard to the formation of intermediates with $mApg7p^{C567S}$. These results indicate that murine Apg12p has a substrate-preference for mApg7p over the three Apg8p homologs.

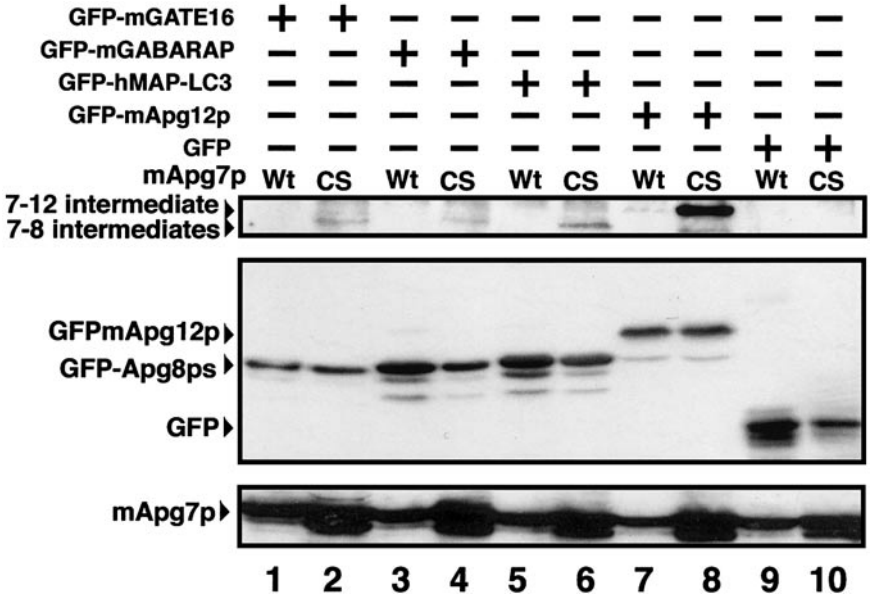

FIG. 3. Mutant mApg7p^{C567S} forms an intermediate with each of the murine Apg12p and three Apg8p homologs. Murine Apg12p (**GFPmApg12p**) and three Apg8p homologs (**GFPmGATE16**, **GFPmGABARAP**, and **GFPhMAP-LC3**) were expressed as GFP-fusion proteins together with mApg7p in HEK293 cells. A lysate of the transfectant was analyzed by SDS–PAGE. GFP-fused proteins were recognized by immunoblot using an anti-GFP antibody, and mApg7p was recognized by immunoblot using an anti-hApg7p antibody. mApg7p-GFPmApg12p intermediate and mApg7p-Apg8p intermediates were recognized by immunoblot with anti-hApg7p antibody, and confirmed by immunoblot with anti-GFP antibody. +, Expression of GFP only or GFP-fused protein; **mApg7p**, murine Apg7p; **Wt**, expression of wild type mApg7p; **CS**, expression of mutant mApg7p^{C567S}; **7–12 intermediate**, mApg7p-GFPmApg12p intermediate; **7–8 intermediates**, mApg7p intermediates with each of GFPmGATE16, GFPmGABARAP, and GFPhMAP-LC3. **GFPmApg12p**, GFP-fused mApg12p: **GFP-Apg8ps**, GFPmGATE16, GFPmGABARAP, or GFPhMAP-LC3.

Expression of human APG7 mRNA and rat Apg7p in tissues. MAP-LC3, one of the substrates of mammalian Apg7p, has been characterized as an essential factor in the formation of pre-autophagosomal membrane cooperatively with the mApg12p · mApg5p conjugate in autophagy. GATE-16 and GABARAP have been isolated from brain. We next investigated the expression of transcripts of the human *APG7* gene by Northern analysis using a multiple human adult and fetal tissue-blot with a DNA-fragment of the human *APG7* open reading frame as a probe (Fig. 4). Human *APG7* mRNA was expressed ubiquitously, especially in kidney, liver, lymph nodes, and bone marrow in adult tissues. The mRNA of human *APG7* is expressed ubiquitously even in fetal tissues. Considering that in autophagy, a significant relation to programmed cell death in the developmental stages of mammals exists, the ubiquitous expression of human Apg7p in fetal tissues seems reasonable.

Using the cross-reactivity of the anti-hApg7p antibody with rat Apg7p, we further investigated the expression of rat Apg7p in rat tissues. Cell-lysates derived from various rat tissues were prepared, and Apg7p in the lysate was immunoprecipitated with the anti-hApg7p antibody. The immunoprecipitate was separated by SDS-PAGE, and Apg7p in the precipitate was detected by immunoblot with the anti-hApg7p antibody. Rat Apg7p was expressed in all normal adult tissues examined, i.e. brain, lung, heart, liver, spleen, kidney, and skeletal muscle. The result coincides with the expression pattern of human *APG7* mRNA. Considering the possible divergent functions of GABARAP and GATE-16 that have been reported (30–32, 34–37), in addition to MAP-LC3 for autophagy, mammalian Apg7p would be expected to play an important role in other cell-biological functions of several tissues.

To better understand the cell-biological and developmental functions of mammalian Apg7p cooperative with mammalian Apg12p and three Apg8p homologs, the next step is to construct a *APG7*-gene deficient mouse. Considering the ubiquitous distribution of mammalian Apg7p and the potential functional divergency of the three Apg8p homologs, a redundancy in the murine *APG7* gene would be expected. However, genomic southern analysis of mouse genomic DNA using a complementary DNA of an open reading frame of murine *APG7* gene indicates that no such redundancy exists, including pseudo-gene (M. Komatsu, I. Tanida, and E. Kominami, unpublished results). Therefore, the functional divergency of three Apg8p homologs will be dependent on the other regulatory factor(s). One of the candidates for this is an E2 enzyme for three Apg8p homologs, murine Apg3p. We have now isolated and characterized, Apg3p, and further screened the other interacting proteins with mApg7p. With regard to constructing a *APG7* gene-deficient mouse, we have suc-

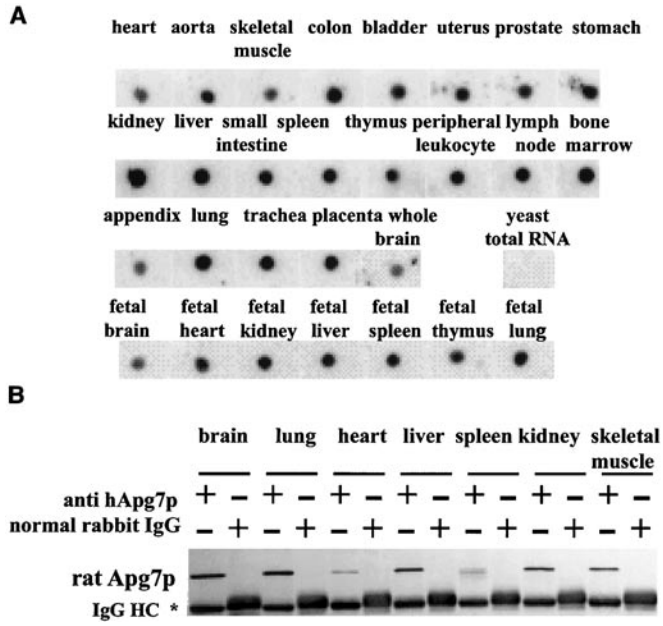

FIG. 4. Mammalian Apg7p is expressed ubiquitously in mammalian tissues. (**A**) Northern dot blot analysis of human poly A+ RNA in human tissues. Northern analysis was preformed using human RNA master blot (Clontech) and a complementary DNA containing an open-reading frame for mApg7p according to the manufacturer's protocol. RNA dot blot containing a normalized loading of 89–514 ng of each poly A+ RNA per dot from different human tissues. The cause of death of each of the RNA sources was trauma. Yeast total RNA (100 ng) was used as a negative control. (**B**) Immunoprecipitation of murine Apg7p from normal adult rat tissues. Rat tissues isolated from a male Wistar rat were homogenized with a glass/Teflon homogenizer. After removing the cell debris, mApg7p was immunoprecipitated from the cell lysate with an anti-hApg7p antibody (**anti-hApg7p**, **+**). Normal rabbit IgG was used as a negative control (**normal rabbit IgG**, **+**). The immunoprecipitates were recognized by immunoblot with anti-hApg7p. Rat Apg7p was immunoprecipitated using anti-hApg7p antibody (**rat Apg7p**). **IgG HC**, IgG heavy chain.

cessfully isolated a genomic DNA fragment containing the entire sequence of murine *APG7* reported in this paper, and are in the process of constructing a *APG7* gene-deficient mouse. Further results will be reported in the near future.

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