Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice

Masaaki Komatsu,1,3 Satoshi Waguri,2 Takashi Ueno,3 Junichi Iwata,3 Shigeo Murata,1 Isei Tanida,3 Junji Ezaki,3 Noboru Mizushima,4 Yoshinori Ohsumi,5 Yasuo Uchiyama,2 Eiki Kominami,3 Keiji Tanaka,1 and Tomoki Chiba1

1Department of Molecular Oncology, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613, Japan
2Department of Cell Biology and Neurosciences, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan
3Department of Biochemistry, Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113-8421, Japan
4Department of Bioregulation and Metabolism, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613, Japan
5Department of Cell Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan

Autophagy is a membrane-trafficking mechanism that delivers cytoplasmic constituents into the lysosome/vacuole for bulk protein degradation. This mechanism is involved in the preservation of nutrients under starvation condition as well as the normal turnover of cytoplasmic component. Aberrant autophagy has been reported in several neurodegenerative disorders, hepatitides, and myopathies. Here, we generated conditional knockout mice of Atg7, an essential gene for autophagy in yeast. Atg7 was essential for ATG conjugation systems and autophagosome formation, amino acid supply in neonates, and starvation-induced bulk degradation of proteins and organelles in mice. Furthermore, Atg7 deficiency led to multiple cellular abnormalities, such as appearance of concentric membranous structure and deformed mitochondria, and accumulation of ubiquitin-positive aggregates. Our results indicate the important role of autophagy in starvation response and the quality control of proteins and organelles in quiescent cells.

Introduction

There are two major protein degradation pathways in eukaryotic cells: the proteasome and the lysosome. The proteasome is a self-compartmentalized protease complex with catalytic activities inside its central proteinaceous chamber (Baumeister et al., 1998). It plays crucial roles in selective degradation of not only short-lived regulatory proteins but also abnormal proteins that should be eliminated from the cells (Goldberg, 2003). In contrast, the lysosome is a vesicle that contains many hydrolases, which are separated from the cytosol by the limiting membrane. In this lysosomal pathway, degradation of plasma membrane proteins and extracellular proteins is mediated by endocytosis, whereas degradation of cytoplasmic components is achieved through several pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy (Seglen and Bohley, 1992; Dunn, 1994; Klionsky and Emr, 2000; Massey et al., 2004).

Macroautophagy (hereafter referred to as autophagy) is the main route for sequestration of the cytoplasm into the lysosome. The initial step of autophagy is elongation of the isolation membrane. The isolation membrane initially enwraps cytoplasmic constituents such as organelles, and then its edges fuse with each other forming a double membrane structure called autophagosome. Finally, the outer membrane of the autophagosome fuses with the lysosome/vacuole and the sequestered cytoplasmic components are degraded by the lysosomal/vacuolar hydrolases, together with the inner membrane of the autophagosomes (Mizushima et al., 2002).

In mammals, autophagy is considered necessary for the turnover of cellular components, particularly in response to starvation or glucagon (Mortimore and Poso, 1987). Yeast deficient in autophagy rapidly die under nutrition-poor conditions (Tsukada and Ohsumi, 1993), suggesting its important roles in preservation of nutrient supply. Indeed, autophagy is necessary for survival in early neonatal starvation period in mice (Kuma et al., 2004). Furthermore, autophagy plays a role in cellular remodeling during differentiation and development of multicellular organisms, such as fly, worm, and slime mold (Levine and Klionsky, 2004), and cellular defense against invading streptococcus (Nakagawa et al., 2004). Plants deficient in autophagy show accelerated senescence (Hanaoka et al., 2002). In humans, autophagy has been implicated in several pathological conditions (Shintani and Klionsky, 2004), e.g., low levels of autophagy were described in some malignant tumors (Liang et al., 1999)
In contrast, elevated levels of autophagosome formation were reported in other human pathologies such as neurodegenerative diseases, myopathies, and liver injury (Mizushima et al., 2002; Perlmutter, 2002), and autophagy is implicated in the execution of cell death (Xue et al., 1999; Bursch, 2001). However, the high level of autophagosome formation does not necessarily reflect enhanced protein degradation because the formation of autophagosomes is increased in Danon cardiomyopathy, which is characterized by defective lysosomal degradation (Nishino et al., 2000; Tanaka et al., 2000). Thus, it is not clear whether increased levels of autophagosome formation reflect the activation or defective protein degradation.

Although autophagy has been extensively studied, little was known about its molecular mechanism until the recent discovery of ATG genes in budding yeast (Tsukada and Ohsumi, 1993). Of the many ATG genes, seven uniquely compose two ubiquitin-like conjugation systems: ATG12 and ATG8 conjugation systems (Mizushima et al., 1998; Ichimura et al., 2000; Ohsumi, 2001). The ubiquitin-like protein Atg12p covalently attaches to Atg5p in a reaction similar to ubiquitination. In this process, Atg12p is activated by an E1-like enzyme, Atg7p (Tanida et al., 1999), and transferred to an E2-like enzyme, Atg10p (Shintani et al., 1999), and then finally conjugates to Atg5p. Atg8p, another ubiquitin-like protein, is unique among other ubiquitin-like molecules, as it conjugates to phosphatidylyl-ethanolamine (Ichimura et al., 2000). Atg8p is activated by Atg7p, which is common to the Atg12 conjugation system, and is transferred to Atg5p, an E2-like enzyme (Ichimura et al., 2000). In mammals, there exist at least three Atg8 homologues that can all be activated by Atg7 (Tanida et al., 2001), GATE-16, GABARAP, and LC3 (Ohsumi, 2001), and they localize to the autophagosome (Kabeya et al., 2000, 2004).

Here, we generated conditional knockout mice of Atg7 and analyzed the roles of autophagy in neonates and adult liver. Autophagosome formation and starvation-induced degradation of proteins and organelles was impaired in Atg7-deficient mice and adult livers. We also found an important role for autophagy in constitutive turnover of cytoplasmic components, and its loss resulted in accumulation of abnormal organelles and ubiquitinated proteins. Our results suggest that autophagy is important for clearance of ubiquitin-positive aggregates.

**Results**

**Generation of Atg7 conditional knockout mice**

To investigate the physiological roles of autophagy in mammals, we generated Atg7 conditional knockout mice. Mouse Atg7 gene is encoded by 17 exons that span 216-kb long genomic DNA. The active site cysteine residue essential for activation of the substrates is encoded by exon 14 and the targeting vector is designed to conditionally disrupt this exon by Cre-loxP technology. The targeted exon 14 was modified so that it could express Atg7 even in the presence of neo-resistant gene cassette in intron 14 (Fig. 1 A). Mice homozygous for the Atg7<sup>fluo</sup> allele (referred to as Atg7<sup>fluo</sup> mice), which were expected to express intact Atg7, were born healthy and fertile without any noticeable pathological phenotypes. Fig. 1 B shows Southern blots of mice with the indicated genotypes. Immuno blot analysis revealed the presence of Atg7 protein in Atg7<sup>fluo</sup> mouse embryonic fibroblasts (MEFs; Fig. 1 C), suggesting that Atg7 is efficiently expressed from the Atg7<sup>fluo</sup> allele.

The phenotype of Atg7-deficient mice

To examine the Atg7-deficient phenotype, we bred Atg7<sup>fluo</sup> mice with a line of transgenic mice that express the Cre recombinase under the control of the Zp3 promoter in the oocyte (Lewandoski et al., 1997). The heterozygous mice (referred to as Atg7<sup>+/−</sup>) were obtained from female Atg7<sup>fluo</sup>-Zp3 mice. Atg7<sup>+/−</sup> mice were born healthy and fertile without any noticeable pathological phenotypes for 1 yr. The Atg7<sup>−/−</sup> mice, obtained by breeding Atg7<sup>−/−</sup> mice, were born at Mendelian frequency (+/+: +/−: −/− = 21: 38: 19). The results of PCR genotyping are shown in Fig. 2 A. Neither Atg7 mRNA nor protein was detected in the homozygous mice (Fig. 2, B and C). We also tested the loss of Atg7 activity by examining the ATG conjugation systems in the neonate liver. A 56-kD protein, equivalent to Atg5-Atg12 conjugate, was detected by Atg5 antibody in the control Atg7<sup>+/−</sup> but not Atg7<sup>−/−</sup> liver (Fig. 2 C). In contrast, free Atg5 of 30 kD, which was faintly observed in the Atg7<sup>+/−</sup> liver, increased in Atg7<sup>−/−</sup> liver (Fig. 2 C). Mammalian Atg8p homologue LC3 has two forms (i.e., LC3-I and LC3-II; Kabeya et al., 2000). It is generally accepted that LC3-I is the free mature form whereas LC3-II is the lipiddated form, in analogy to yeast Atg8p (Ichimura et al., 2000; Kabeya et al., 2000). Both forms were detected in Atg7<sup>+/−</sup> liver whereas only the LC3-I form was detected and increased in Atg7<sup>−/−</sup> liver (Fig. 2 C). When crossed with GFP-LC3 transgenic mice (Mizushima et al., 2004), the punctate structures representing autophagosomes were detected in Atg7<sup>+/−</sup> but not in Atg7<sup>−/−</sup> heart (Fig. 2, D and E). These results indicate that Atg7 is essential for ATG conjugation systems and autophagosome formation in mice.

Although homozygous mice seemed normal at birth (Fig. 2 F) and had no apparent developmental defect by histological analyses (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200412022/DC1), the mean body weight of Atg7<sup>−/−</sup> mice (0.983 ± 0.0763 g, ± SD, n = 9) was significantly lower than that of wild-type and heterozygote mice (1.20 ± 0.116 g, n = 29; P < 0.01), and Atg7<sup>−/−</sup> mice died within 1 d after birth (n = 19). We considered that Atg7<sup>−/−</sup> mice could survive in utero by virtue of the nutrients supplied through the placenta but could not survive when the supply terminates after birth, as recently reported (Kuma et al., 2004). We tested the survival time of Atg7<sup>−/−</sup> neonates under starvation condition after caesarean delivery. Wild-type and heterozygote mice died at 21.7 ± 3.3 h after birth, whereas Atg7<sup>−/−</sup> mice died at 13 ± 2.0 h (P < 0.01; Fig. 2 G). To further test whether the cause of earlier death correlates with lower nutrient supply, we measured amino acid concentrations in plasma at 10 h after caesarean delivery. Essential and branched-chain amino acid concentrations in the sera of Atg7<sup>−/−</sup> mice were lower than those of wild-type mice (essential amino acids: 1.536 ± 0.087 vs. 1.291 ± 0.166 mmol/L, P < 0.05; branched-chain amino acids: 0.375 ± 0.038 vs. 0.268 ± 0.015 mmol/L, P < 0.01, respectively). The same
results were also obtained using MEF cells from \( \text{Atg7}^{-/-} \) mice (unpublished data). Together, these results indicate that \( \text{Atg7} \) is crucial for the recycling of amino acids in cells and survival of newborn mice under starvation condition.

**Starvation response in adult mice liver**

To delete \( \text{Atg7} \) gene in the adult mice, we bred the \( \text{Atg7}^{F/F} \) mice with Mx1-Cre transgenic mice that express the Cre recombinase in response to interferon \( \gamma \) or its chemical inducer, polyinosinic acid–polycytidylic acid (pIpC). The Mx1-Cre transgenic mice can excise Flox allele completely in the liver and spleen and partially in the kidney and heart (Kuhn et al., 1995). Intraperitoneal injections of pIpC resulted in effective recombination of the \( \text{Atg7}^{Flox} \) allele in the liver and spleen (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200412022/DC1; and not depicted). No \( \text{Atg7} \) transcript, protein, and activity were detected, similar to \( \text{Atg7}^{-/-} \) mice (Fig. S2). Next, we tested the autophagosome formation under fasting condition. 1-d fasting resulted in induction of typical autophagosomes in control \( \text{Atg7}^{+/+} \) mice (Fig. 3, A–D and I). In contrast, no such induction of autophagosome formation was noted in the liver of fasted \( \text{Atg7}^{F/F} \) mice (Fig. 3, E, F, and I). Although some autophagosome-like structures were occasionally observed both in fed and fasted mutant mice livers (Fig. 3, G and H), they...
tended to be smaller than those observed in fasted control liver and hardly contained large cytoplasmic organelles (compare with Fig. 3, C and D). The number of autophagosomes per hepatocyte was counted and the mean values are shown in Fig. 3 I. The mutant hepatocytes lacked typical glycogen area, in contrast to the fed hepatocytes (Fig. 3, A and E); however, well-developed glycogen granules (α granules) were observed between numerous smooth endoplasmic reticula (Fig. 3 E, inset). Immunofluorescent analysis also revealed the presence of many cup-shaped and ringlike structures representing autophagosomes in the control hepatocytes (Fig. 3, J and K). Although several LC3-positive dots were observed in the mutant hepatocytes, they were not induced in response to starvation and did not form cup-shaped and ringlike structures (Fig. 3, L and M).

Next, we examined the fasting response of LC3 and other homologues, GABARAP and GATE-16, by immunoblotting (Fig. 3 N). LC3 is known to be up-regulated and recruited to the autophagosome upon starvation and degraded in the lysosomes (Kabeya et al., 2000). Fasting slightly increased the modification of LC3 in heterozygous liver. In the mutant liver, no modification of LC3 was noted and LC3-I increased in response to fasting. These results suggest that LC3 is up-regulated, but its modification and degradation are impaired in mutant mice. The level of GABARAP did not change upon fasting in the mutant liver, whereas it decreased in the heterozygous liver, suggesting that GABARAP is not up-regulated but its degradation after modification is impaired in mutant mice. Although GATE-16 was hardly detected in the heterozygous liver under both fed and fasting conditions, it was clearly detected...
and increased upon fasting in the mutant liver. These results suggest that GATE-16 may be constitutively degraded even at fed condition in heterozygous mice and up-regulated in response to fasting under defective Atg7. The levels of all LC3 homologues were elevated even at fed condition in the mutant liver, suggesting their marked stabilization in autophagy-deficient condition. However, the possibility that their transcripts are up-regulated at basal level due to Atg7 deficiency cannot be excluded. We sought to determine their localizations in the cells. However, our antibodies for these molecules were not applicable for immunofluorescent analyses, and those localizations remain to be clarified. In conclusion, all Atg8 homologues respond to fasting, although in a different manner, and their levels are affected by the absence of Atg7.

Atg7 is indispensable for fasting-induced degradation of cytosolic proteins and organelles in the mouse liver

Given that autophagosome formation was impaired in Atg7-deficient liver, we next examined its effects on the bulk degradation of proteins and organelles under fasting condition. After 1-d fasting in control Atg7<sup>F/F</sup>:Mx1 and mutant Atg7<sup>F/F</sup>:Mx1 mice, the liver was dissected and the amount of total protein per whole liver was measured. The amount of total liver proteins decreased to ~66% by 1-d fasting in the control liver (Fig. 4 A). In contrast, fasting did not significantly decrease the amount of total liver proteins in the mutant liver. Moreover, the amount of total proteins in the mutant liver was 1.5-fold that of control. These results indicate that the decrease of total proteins is dependent on Atg7 and autophagosome formation.

We also examined whether or not fasting causes the degradation of cellular organelles such as mitochondria in the livers of mice. To quantify the amount of the mitochondria, we first measured the activity of mitochondrial enzyme succinate dehydrogenase (SDH) in total liver extracts. In the control livers, fasting was associated with a significant decrease of SDH activity, and the reduction was proportional with the decrease in the amount of total protein (Fig. 4 B). In contrast, fasting was not associated with any change in SDH activity in the mutant livers. Similar to total protein, the basal SDH activity in mutant liver was significantly higher than in control. The effect of fasting on the amount of the mitochondria was also assessed by immunoblot analysis of mitochondrial protein cytochrome c (Fig. 4 C). When equal amounts of proteins were loaded, the level of cytochrome c was equivalent in the two genotypes at either fed or fasting conditions, suggesting that the ratio of mitochondria versus total protein is not altered by fasting in both genotypes. Considering that the total protein amounts decreased by fasting in the control liver (Fig. 4 A), these results suggest that the mitochondria and cytoplasmic proteins are proportionally degraded upon fasting in heterozygous mice. However, such degradation is impaired in Atg7-deficient liver because the levels of both proteins and mitochondria are unchanged and kept at a higher level.

Next, we investigated the effect of autophagy deficiency on protein turnover. To quantify the turnover of long-lived protein, after each control and mutant hepatocytes had been labeled with [14C]leucine for 24 h and chased for 2 h, the release of TCA-soluble [14C]leucine was measured for 4 h. In control hepatocytes, nutrient deprivation significantly induced protein degradation, and such degradation was suppressed by the addition of lysosomal inhibitors such as monomethylamine and E64d and pepstatin (E/P) or epoxomicin (epoxo) was added as indicated. Data are the mean ± SD of triplicate experiments.

Figure 4. Fasting response of Atg7-deficient liver. [A and B] Livers from Atg7<sup>F/F</sup>:Mx1 and Atg7<sup>F/F</sup>:Mx1 mice fed ad libitum (Fed) or fasted for 1 d (Fast) at 20 d after pIpC injection were dissected, and the amount of total protein (A) and SDH activity (B) per liver were measured. Data are mean ± SD values of five mice in each group; *, P < 0.01. [C] Cytochrome c levels in the cytosolic and mitochondria/lysosomal fractions of the liver at 20 d after injection. Equal amount of PNS fractions were centrifuged at 8,000 g for 10 min and the pellets were used as the mitochondrial/lysosomal fraction (ML). The supernatants were further centrifuged at 100,000 g for 1 h and the supernatant was used as the cytosolic fraction (C). Actin was blotted as control. Data shown are representative of two separate experiments. [D] Turnover of long-lived protein. Hepatocytes from Atg7<sup>F/F</sup>:Mx1 and Atg7<sup>F/F</sup>:Mx1 mice were isolated and labeled with [14C]leucine for 24 h, and degradation of long-lived protein in deprived or nondeprived condition was measured. Monomethylamine (MA) and/or E64d and pepstatin (E/P) or epoxomicin (epoxo) was added as indicated. Data are the mean ± SD of triplicate experiments.
at nondeprived condition in the control hepatocytes, such inhibition was not significant in the mutant hepatocytes (Fig. 4 D), indicating that significant amounts of proteins are constitutively degraded in the lysosome via autophagic pathway. Together, these results suggest that autophagy plays a significant role in turnover of long-lived protein.

Loss of Atg7 in the liver leads to hepatomegaly and accumulation of abnormal organelles in hepatic cells

We further chased the phenotypes of the mutant mice for up to 90 d after pIpC injection. Gross anatomy revealed severe enlargement of the liver, filling up most of the abdominal cavity (Fig. 5 A). Other major organs were normal histologically (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200412022/DC1). The mean liver weights of control and mutant mice at 90 d after pIpC injection were 1.39 ± 0.24 and 6.10 ± 2.06 g, respectively (n = 5 each). Histological analysis revealed disorganized hepatic lobules and cell swelling in the mutant liver (Fig. 5, B and C). No hepatocellular proliferation or regeneration was detected (unpublished data). Vacuolated hepatic cells were occasionally observed and those were associated with hepatic cell death, which is consistent with the leakage of alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase in the mutant mice sera (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200412022/DC1).

Although most hepatocytes were still alive in the mutant liver, ultrastructural analysis revealed the appearance of aberrant concentric membranous structures (Fig. 6, A and B), which were also observed as early as 20 d after pIpC-injected liver (not depicted). These structures surrounded various cytoplasmic constituents such as mitochondria, lipid droplets, and vesicular structures (Fig. 6 A). Their membranous elements were continuous with the rough ER (Fig. 6 B, arrowheads), and the corresponding structures were positive for calreticulin, an ER protein marker (not depicted), indicating that these structures originated from the rough ER. Accumulation of peroxisomes (Fig. 6 C) and deformed mitochondria (Fig. 6, C and D) was also observed in the mutant liver. These results suggest the important role of autophagy in turnover of organelles, and its defect results in accumulation of abnormal organelles.

Formation of ubiquitin-positive inclusions in Atg7-deficient hepatocytes

Autophagy has been implicated in not only organelle turnover but also in elimination of protein aggregates (Kopito, 2000). Protein aggregates are often ubiquitinated. In the next step, we immunostained the liver with an ubiquitin antibody to examine the presence of such aggregates. Several ubiquitin-positive par-
articles of various sizes were detected in the Atg7\textsuperscript{−/−}:Mx1 but not in Atg7\textsuperscript{+/+}:Mx1 hepatic cells at both 10 and 90 d after pIpC injection (Fig. 7, A and B; and Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200412022/DC1). The immunoblots of the liver lysates revealed the accumulation of high-molecular mass polyubiquitinated proteins in the mutant liver (Fig. 7 G and Fig. S5), suggesting that the ubiquitin particles are aggregates of polyubiquitinated proteins. To further determine the localization of ubiquitin-positive dots, analysis of immunoelectron micrographs was performed. Numerous particles of colloidal gold, indicative of ubiquitin, were detected on lipid dropletlike structures, membranous structures, and amorphous substances in the cytoplasm (Fig. 7, C–F). Such signals were not observed in the wild-type liver (unpublished data).

The accumulation of ubiquitin-positive inclusions in the cytoplasm prompted us to examine the effect of autophagy deficiency on proteasome function. Immunoblots of proteasome subunits (p112/Rpn2, Mss1/Rpt1, and α5) showed that their relative amounts were not affected in the mutant liver (Fig. 7 G). Furthermore, the activities of the proteasome, measured by Suc-LLVY-MCA as substrate, were also comparable between wild-type and mutant livers (unpublished data). These results indicate the accumulation of ubiquitin-positive aggregates in autophagy-deficient hepatocytes despite the apparently normal proteasome function.

Discussion

Autophagy is a bulk protein degradation pathway, which is conserved in eukaryotes, essential for the survival of unicellular organisms under nutrient-poor condition and for cellular remodeling of multicellular organisms (Mizushima et al., 2002; Levine and Klionsky, 2004). In the present study, we generated conditional knockout mice of Atg7 gene, which is an essential gene for autophagy in budding yeast, and analyzed its roles in mice.

In mammals, Atg7 was indeed essential for ATG12 conjugation, LC3 modification systems, and autophagosome formation (Fig. 2, Fig. 3, and Fig. S2). Immunofluorescent analyses revealed that LC3-positive dots appeared but did not form cup-shaped and ringlike structures in Atg7\textsuperscript{−/−}:Mx1 livers (Fig. 3). The LC3-I form is usually present at the S100 fraction and the LC3-II form at the P100 fraction (Kabeya et al., 2000). In the mutant liver, LC3-I was present in both S100 and P100 fractions (unpublished data), suggesting that the LC3-positive dots in the mutant hepatocytes are indeed the LC3-I form. These results suggest that LC3 may be recruited to the dot structures independent of the modification (Fig. 3). In mammals, LC3 has at least two homologues, GABARAP and GATE-16, which share common biochemical characteristics (Tanida et al., 2001) and localize to autophagosome in response to fasting (Kabeya et al., 2004). Indeed, the modification and levels of these molecules under fasting condition were affected in the mutant liver (Fig. 3 N). However, these LC3 homologues have at least two homologues, GABARAP and GATE-16, which share common biochemical characteristics (Tanida et al., 2001) and localize to autophagosome in response to fasting (Kabeya et al., 2004). Indeed, the modification and levels of these molecules under fasting condition were affected in the mutant liver (Fig. 3 N). However, these LC3 homologues have been identified in a different biological pathway and may have diverse functions (Ohsumi, 2001). Thus, how their functions and localizations are affected in Atg7-deficient cells remains to be clarified.

Although Atg7\textsuperscript{−/−} mice were born at Mendelian ratio, and the major organs were almost normal histologically (Fig. S1), they had reduced body weight and died within 1 d after birth. Atg7\textsuperscript{−/−} mice had lower amino acid level and died earlier compared with wild type under nonsuckling condition after caesarean delivery (Fig. 2 G), suggesting that Atg7 is important for survival during the early neonatal starvation period, similar to recently reported Atg5\textsuperscript{−/−} mice phenotypes (Kuma et al., 2004). However, because suckling Atg7\textsuperscript{−/−} mice also died within 1 d after birth (unpublished data), the cause of death may not be only due to low level of amino acids. The reason for the reduced body size is also unclear and may be related to placental function or due to inefficient reutilization of nutrients during embryogenesis. It is of note that a lower level of autophagy occurs during embryogenesis (Mizushima et al., 2004) even when nutrients are supplied from the placenta. Furthermore, Atg7 null mice possess several ubiquitin-positive inclusions in some
organisms at the time of birth (unpublished data). This phenotype might be related to the earlier death of mutant. Further analysis of Atg7−/− mice is required to unravel the roles of autophagy, and such analysis is currently under way by breeding the Atg7+/− mice with several Cre-transgenic mice.

Starvation-induced autophagosomes appeared to sequester the cytoplasm randomly (Fig. 3). Consistent with this notion, the amount of mitochondria decreased in proportion with reduction in the amount of total protein (Fig. 4, A–C). These results suggest that mitochondria are degraded nonselectively under fasting condition. In Atg7-deficient liver, no autophagosome formation was noted and the degradation of proteins and organelles under fasting condition was largely impaired. These results suggest that the rapid reduction of proteins and organelles upon fasting is dependent on Atg7 and autophagosome formation.

Although autophagy can be induced by starvation, this pathway may take place even at feeding condition at basal level. This constitutive pathway may be important for turnover of organelles and cytoplasmic proteins. Indeed, the degradation of long-lived protein was inhibited in mutant hepatocytes irrespective of nutrient deprivation (Fig. 4 D), and multiple abnormalities of organelles (e.g., the presence of concentric membranous structure and accumulation of deformed mitochondria) were observed in Atg7-deficient hepatocytes (Fig. 6). Unexpectedly, the morphologically abnormal mitochondria appear to retain their function, as judged by the normal membrane potentials and the absence of cytochrome c leakage in the cytosol (unpublished data). In contrast to starvation-induced autophagy, whether or not constitutive autophagy eliminates abnormal and excess organelles in a degree of selectivity remains to be clarified.

Beclin 1, a human homologue of ATG6/VPS30 essential for autophagy in yeast, was recently identified as a tumor suppressor gene, and autophagy has been implicated in the regulation of cellular proliferation (Liang et al., 1999). Indeed, heterozygous disruption of mouse Beclin 1 led to enhanced tumorigenesis (Qu et al., 2003; Yue et al., 2003). Atg7 deficiency led to hepatomegaly (Fig. 5 A), suggesting that cell proliferation or malignant transformation might be induced in the Atg7-deficient cells. However, neither tumorigenesis nor enhanced cell proliferation was detected as tested by BrdU incorporation at 90 d after pIpC injection in the mutant liver compared with control mice (unpublished data). The hepatomegaly observed in the mutant mice was likely due to increased cellular volume rather than cell number, which is also supported by the swollen appearance of hepatocytes (Fig. 5, D and E).

In Atg7-deficient liver, we detected numerous ubiquitin-positive particles indicative of protein aggregates (Fig. 7 and Fig. S5). It has been reported that proteasome inhibition leads to aggregate formation. Conversely, the formation of protein aggregates inhibits the proteasome (Bence et al., 2001), resulting in a malignant cycle of aggregate formation and proteasome inhibition. In the mutant liver, failure of the proteasome was postulated; however, no impairment of proteasome function, in terms of its expression or peptidase activities, was noted (Fig. 7 G and not depicted). Our results suggest that the ubiquitinated proteins eventually aggregate even in the presence of functional proteasomes. Considering that such ubiquitinated aggregates must be difficult to unfold, and proteasomes need to unfold their substrate before degradation (Baumeister et al., 1998), it is likely that elimination of ubiquitin-positive aggregates in the cells is largely dependent on the autophagic process. Protein ubiquitination may also occur after protein aggregation. In either case, we propose the possibility that protein ubiquitination may serve as a signal to the autophagic process in addition to the proteasomes pathway. In this context, it is worth noting that sperm mitochondria are known to be ubiquitinated before degradation during fertilization (Sutovsky et al., 1999). It is now well established that ubiquitin regulates not only proteasomal degradation, but also lysosomal degradation. Thus, it is conceivable that ubiquitin could also regulate the autophagic pathway.

A growing number of disease-associated proteins have been found to accumulate in aggregates, including huntingtin, parkin, α-synuclein, and peripheral myelin protein 22 (Netterpeck et al., 1999; Ciechanover and Brundin, 2003). The aggregation of these proteins is thought to be involved in the pathogenesis of Huntington’s disease, Parkinson’s disease, and peripheral neu-
opathies, respectively. Enhanced autophagosome formation is prevalent in most of these diseases (Mizushima et al., 2002), and autophagy has also been considered as a caspase-independent cell death pathway (Xue et al., 1999; Bursch, 2001). Our Atg7 mutant mice should be useful for examining the role of autophagy in the cell death pathway or in a cellular defense mechanism in the pathogenesis of these diseases.

Materials and methods

Generation of Atg7
deficient mice

The targeting vector was constructed by insertion of a loxP sequence within introns 13 and 14 of Atg7 gene. Exon 14 was fused to a CDNA fragment encoded by exons 15, 16, and 17 (ca 1786–2097) and polyA signal sequence was added after the stop codon. Neo resistant gene cassette (mcl-neoA) was ligated behind the polyA sequence followed by the second loxP sequence, splicing acceptor site, and exon 14 with stop codon preceding the active site. We electroporated the targeting vector into mouse TT2 E5 cells, selected with 200 μg/ml G418 (GIBCO BRL), and then screened for homologous recombinants by PCR and Southern blot analyses. PCR primers were as follows: 5′-TGCTGACCTTCTC-GGAACGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
Bursch, W. 2001. The autophagosomal-lysosomal compartment in programmed cell death. Cell Death Differ. 8:569–581.

Ciechanover, A., and P. Brundin. 2003. The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. Neuron. 40:427–446.

Dunn, W.A., Jr. 1994. Autophagy and related mechanisms of lysosome-mediated protein degradation. Trends Cell Biol. 4:139–143.

Goldberg, A.L. 2003. Protein degradation and protection against misfolded or damaged proteins. Nature. 426:895–899.

Gronostaevski, R.M., and P. Brundin. 1984. Protein degradation in 3T3 cells and tumor necrosis factors 3T3 cells. J. Cell. Physiol. 119:127–132.

Hanaoka, H., T. Noda, Y. Shirano, T. Kato, H. Hayashi, D. Shibata, S. Tabata, and Y. Ohsumi. 2002. Leaf senescence and starvation-induced chlorosis are accelerated by the disruption of an Arabidopsis autophagy gene. Plant Physiol. 129:1181–1193.

Ichinura, Y., T. Kirisako, T. Takao, Y. Satomi, Y. Shimomishii, N. Ishihara, N. Mizushima, I. Tanida, E. Kominami, M. Ohsumi, et al. 2000. A ubiquitin-like system mediates protein lipidation. Nature. 408:488–492.

Kabeya, Y., N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, and T. Yoshimori. 2000. LC3, a mammalian homologue of yeast Atg8p, is localized in autophagosome membranes after processing. EMBO J. 19:5720–5728.

Kabeya, Y., N. Mizushima, A. Yamamoto, S. Oshitani-Okamoto, Y. Ohsumi, and T. Yoshimori. 2004. Lcs2, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J. Cell Sci. 117:2805–2812.

Klionsky, D.J., and S.D. Emr. 2000. Autophagy as a regulated pathway of cellular degradation. Science. 290:1717–1721.

Komatsu, M., I. Tanida, T. Ueno, M. Ohsumi, Y. Ohsumi, and E. Kominami. 2001. The C-terminal region of an Atg7p/Cvt2p is required for homodimerization and is essential for its E1 activity and E1-E2 complex formation. J. Biol. Chem. 276:9846–9854.

Kopito, R.R. 2000. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol. 10:524–530.

Kuhn, R., F. Schwenk, M. Aguet, and K. Rajewsky. 1995. Inducible gene targeting in mice. Science. 269:1427–1429.

Kuma, A., M. Hatanou, M. Matsui, A. Yamamoto, H. Nakaya, T. Yoshimori, Y. Ohsumi, T. Tokuhisa, and N. Mizushima. 2004. The role of autophagy during the early neonatal starvation period. Nature. 432:1032–1036.

Levine, B., and D.J. Klionsky. 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev. Cell. 6:463–477.

Lewandoski, M., K.M. Wassarman, and G.R. Martin. 1997. Zp3-cre, a transgenic mouse line for the activation or inactivation of loxp-flanked target genes specifically in the female germ line. Curr. Biol. 7:148–151.

Li, X.H., S. Jackson, M. Seaman, K. Brown, B. Kempkes, H. Hibshoosh, and B. Levine. 1999. Induction of autophagy and inhibition of tumorigenesis by beclin1. Nature. 402:672–676.

Massey, A., R. Kiffin, and A.M. Cuervo. 2004. Pathophysiology of chaperone-mediated autophagy. Int. J. Biochem. Cell Biol. 36:2420–2434.

Mizushima, N., T. Noda, T. Yoshimori, Y. Tanaka, T. Ishii, M.D. George, D.J. Klionsky, and Y. Ohsumi. 1998. A protein conjugation system essential for autophagy formation. Nature. 395:395–398.

Mizushima, N., A. Yamamoto, M. Hatanou, Y. Kobayashi, Y. Kabeya, K. Suzuki, T. Tokuhisa, Y. Ohsumi, and T. Yoshimori. 2001. Dissection of autophagosome formation using Atg5-deficient mouse embryonic stem cells. J. Cell Biol. 152:657–668.

Mizushima, N., Y. Ohsumi, and T. Yoshimori. 2002. Autophagosome formation in mammalian cells. Cell Struct. Funct. 27:421–429.

Mizushima, N., A. Yamamoto, M. Matsui, T. Yoshimori, and Y. Ohsumi. 2004. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol. Biol. Cell. 15:1101–1111.

Morimoto, G.E., and A.R. Poso. 1987. Intracellular protein catabolism and its control during nutrient deprivation and supply. Annu. Rev. Nutr. 7:539–564.

Murata, S., H. Udono, N. Tanahashi, N. Hamada, K. Watanabe, K. Adachi, T. Yamano, K. Yu, N. Kobayashi, M. Kasahara, et al. 2001. Immuno-proteasome assembly and antigen presentation in mice lacking both PA28alpha and PA28beta. EMBO J. 20:5898–5907.

Nakagawa, I., A. Amano, N. Mizushima, A. Yamamoto, H. Yamaguchi, T. Kamimoto, A. Nara, J. Funao, M. Nakata, K. Tsuda, et al. 2004. Autophagy defends cells against invading group A Streptococcus. Science. 306:1037–1040.

Nishino, I., J. Fu, K. Tanji, T. Yamada, S. Shimojo, T. Koori, M. Mora, J.E. Riggs, S.J. Oh, Y. Koga, et al. 2000. Primary LAMP-2 deficiency causes X-linked vascular cardiomypathy and myopathy (Danon disease). Nature. 406:906–910.