Intracellular Inclusions Containing Mutant $\alpha_1$-Antitrypsin Z Are Propagated in the Absence of Autophagic Activity*

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Mutant $\alpha_1$-antitrypsin Z ($\alpha_1$-ATZ) protein, which has a tendency to form aggregated polymers as it accumulates within the endoplasmic reticulum of the liver cells, is associated with the development of chronic liver injury and hepatocellular carcinoma in hereditary $\alpha_1$-antitrypsin (\(\alpha_1\)-AT) deficiency. Previous studies have suggested that efficient intracellular degradation of $\alpha_1$-ATZ is correlated with protection from liver disease in $\alpha_1$-AT deficiency and that the ubiquitin-proteasome system accounts for a major route, but not the sole route, of $\alpha_1$-ATZ disposal. Yet another intracellular degradation system, autophagy, has also been implicated in the pathophysiology of $\alpha_1$-AT deficiency. To provide genetic evidence for autophagy-mediated disposal of $\alpha_1$-ATZ, here we used cell lines deleted for the \(\text{Atg}5\) gene that is necessary for initiation of autophagy. In the absence of autophagy, the degradation of $\alpha_1$-ATZ was retarded, and the characteristic cellular inclusions of $\alpha_1$-ATZ accumulated. In wild-type cells, colocalization of the autophagosomal membrane marker GFP-LC3 and $\alpha_1$-ATZ was observed, and this colocalization was enhanced when clearance of autophagosomes was prevented by inhibiting fusion between autophagosome and lysosome. By using a transgenic mouse with liver-specific inducible expression of $\alpha_1$-ATZ mated to the GFP-LC3 mouse, we also found that expression of $\alpha_1$-ATZ in the liver \textit{in vivo} is sufficient to induce autophagy. These data provide definitive evidence that autophagy can participate in the quality control/degradative pathway for $\alpha_1$-ATZ and suggest that autophagic degradation plays a fundamental role in preventing toxic accumulation of $\alpha_1$-ATZ.

Human $\alpha_1$-antitrypsin (\(\alpha_1\)-AT), a monomeric 394-amino acid glycoprotein, is synthesized and secreted primarily by liver cells. It is a prototypic member of serine protease inhibitor (serpin) superfamily proteins and the most abundant of the circulating serpins. The principal role of $\alpha_1$-AT in serum is to protect lung tissues from destructive proteases (elastase, cathepsin G, and proteinase 3) released by neutrophils during inflammation. Some genetic alterations in $\alpha_1$-AT are responsible for defective secretion and thus cause serum $\alpha_1$-AT deficiency (1–3). The most common causative mutation found in Caucasian populations is the replacement of Glu-342 by Lys that characterizes the Z mutant of $\alpha_1$-AT ($\alpha_1$-ATZ). This substitution is sufficient to cause an abnormality in folding early in the secretory pathway with retention of the mutant $\alpha_1$-ATZ molecule in the ER of liver cells. Homozygotes for the $\alpha_1$-ATZ mutation (PIZZ) are characterized by serum levels of $\alpha_1$-AT that are ~10–15% of those in the general population and are susceptible to two major target organ injuries. Destructive lung disease/emphysema in adults is due to a loss-of-function mechanism. Chronic liver disease often first discovered in childhood, but also affecting adults, is due to a gain-of-toxic-function mechanism in which liver cell injury results from the hepatotoxic effects of retained $\alpha_1$-ATZ. However, only 8–10% of homozygotes develop clinically significant liver disease. This observation has led to the concept that mechanisms by which cells respond to the ER retention of mutant $\alpha_1$-ATZ play a role in determining which of these homozygotes develop liver disease and which are protected from it. Because previous studies have shown that a reduction in $\alpha_1$-ATZ disposal activity correlates with the presence of liver disease among deficient individuals (4), the mechanisms by which $\alpha_1$-ATZ is degraded are thought to be particularly important in determining the liver disease phenotype of patients with $\alpha_1$-AT deficiency.

A number of studies have addressed the determinants of the cellular fate of $\alpha_1$-ATZ, including retention in the ER and disposal by the quality control/degradative pathways of the ER. Seminal works by Lomas and co-workers (2, 5) have shown how the Z mutation confers an unstable polymerogenic intermediate conformation on $\alpha_1$-AT so that polymerization is promoted by a reactive loop;\(\beta\)-sheet A linkage reminiscent of the inhibitory interaction between serpins and cognate proteases. Recent studies of the ER-associated degradation system revealed that immature or misfolded glycoproteins are captured by ER chaperone proteins calnexin/calreticulin via the terminal glucose residue on asparagine-linked oligosaccharide side chains, which is reciprocally added or trimmed by UDP-glucose:glycoprotein glucosyltransferase or glucosidase II, respectively, according to the folding status of the glycoprotein (6, 7). Indeed, a stoichiometric interaction was observed between $\alpha_1$-ATZ and calnexin (4, 8). Terminally misfolded proteins are translocated from ER lumen to the cytoplasm and consequently degraded by
the ubiquitin-proteasome system putatively deployed at the cytoplasmic face of ER.

Detailed studies of proteasome-mediated α₁-ATZ disposal, including cell-free assays using ER-derived microsomes, have suggested the existence of one disposal pathway in which the α₁-ATZ-calnexin complex is ubiquitinated on calnexin and subsequently degraded (8–10). It is conceivable that the asparagine-linked Glc-Man₂GlcNAc₂ on α₁-ATZ determines the proteasomal degradation pathway via its physical interaction with calnexin and that diversion to other degradation pathways is brought about by further mannose trimming of the oligosaccharide chain during ER retention (11). An unknown protease activity sensitive to tyrosine phosphatase inhibitors has also been reported as a potential mechanism for nonproteasomal intramicrosomal degradation of α₁-ATZ (11).

Autophagy (synonymously used here as macroautophagy) is a major intracellular degradation pathway mediated by proteins of the evolutionarily conserved Atg family unique to this function (12–18). Autophagy is characterized by bulk sequestration of cytoplasmic constituents within a double-membrane-bound vesicle, called an autophagosome, and their subsequent degradation upon fusion of this vesicle with lysosomes. This process accounts for a major portion of the cellular turnover of long-lived proteins and organelles such as ER and mitochondria. In addition to constitutive bulk turnover at steady state, autophagic sequestration is induced by specific physiological perturbations, such as nutrient deprivation. Several lines of evidence have implicated autophagy as a physiological response to cope with accumulation of α₁-ATZ in the ER in vivo (19–21). The liver lesion in PIZZ patients as well as in the PiZ mouse model of α₁-ATZ deficiency is accompanied by a marked autophagic response as determined by ultrastructural studies. When cells engineered to express α₁-ATZ were treated with 3-methyladenine, an inhibitor of autophagy, the degradation of α₁-ATZ was attenuated (19).

Traditional methods for monitoring autophagy by means of ultrastructural criteria or the use of chemical inhibitors can be criticized because it is sometimes difficult to discriminate autophagic vacuoles from other organelles especially in degenerating cells, and because 3-methyladenine, a relatively nonspecific inhibitor of autophagy, could potentially suppress degradation pathways other than autophagy (14). Here we tried to provide molecular evidence for autophagy-mediated disposal of α₁-ATZ by two approaches. First, we used cell lines deleted for the Atg5 gene, a target molecule for the ubiquitin-like Atg12 conjugation that is necessary for the initial steps of autophagic sequestration (12). Second, we used GFP-LC3, a defined marker for autophagosome membrane (13, 22), for colocalization studies in cell lines and in the liver of novel mouse models of α₁-ATZ deficiency.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids, Antibodies, and Reagents**—The cDNAs corresponding to α₁-ATZ, Atg5, Atg5-K138R, and EYFP were subcloned into pCE-neo plasmid. This plasmid is a modified version of pCI-neo (Promega) in which the promoter region was replaced with a containing the cytomegalovirus enhancer and elongation factor promoter of pCE-FL plasmid (a kind gift from Dr. Sumio Sugano). Proteasome sensor vector pcDEF-Ub-G76V-EGFP (23) was kindly provided by Dr. Shigeo Murata (Tokyo Metropolitan Institute of Medical Science). The coding sequence for hemagglutinin-tagged canine Rab7 T22N was inserted into pcDNA3 (Invitrogen). The following antibodies were used: rabbit polyclonal anti-rat LC3 (13); anti-human Atg5 (14); anti-α₁-AT (DAKO); anti-GFP (Invitrogen); goat polyclonal anti-α₁-AT (Research Diagnostics, Inc.); mouse monoclonal anti-α-tubulin (clone B5-1-2; Sigma); and anti-KDEL (clone 10C3; Stressgen SPA-827). Lactacycin (Peptide Institute, Inc.) was prepared as 1 mM stock in distilled water; and MG115, MG132, and epoxomicin (Peptide Institute, Inc.) were prepared as 10 mM stock in Me₂SO. Bafilomycin A₁ was prepared as 250 μM stock in Me₂SO.

**Cell Culture and Transfection**—Wild-type and Atg5⁻/⁻ ES cells were established previously (14). Atg5⁻/⁻ mouse embryonic fibroblast (MEF) cells were established (24) and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mM l-glutamine, and antibiotics in 5% CO₂ incubator at 37 °C. Wild-type and Atg5⁻/⁻ MEF cells were engineered for stable expression of α₁-ATZ, Ub-G76V-EGFP, and GFP-LC3, exactly as described previously (25). Transient transfection was carried out using Lipofectamine2000 reagent (Invitrogen) according to the manufacturer’s protocol.

**Pulse-Chase Experiments**—Cell lines engineered for expression of α₁-ATZ either by transient or stable transfection were subjected to pulse-chase studies. The transiently transfected cell lines were studied 24 h after transfection. Separate monolayers were incubated in serum- and methionine/cysteine-free medium for 1 h at 37 °C followed by pulse labeling with 150 μCi/ml of [³⁵S]-labeled EasyTag Express protein labeling mix (NEG-772; PerkinElmer Life Sciences) for 2 h at 37 °C. Cells were then rinsed with chase medium and chased for several different time periods. Cells were lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 3 mg/ml BSA, 1 mM PMSF, and protease inhibitor mixture (Roche Applied Science). Cell lysates were subjected to immunoprecipitation using anti-α₁-AT polyclonal antibody and protein G-Sepharose 4F (Amersham Biosciences), followed by analysis with SDS-PAGE (10%) and autoradiography using LAS-3000 bioimager analyzer (Fuji Film).

**Western Blotting**—Cells were collected, rinsed with PBS, and lysed in PBS containing 1% Triton X-100, 1 mM PMSF, and protease inhibitor mixture (Roche Applied Science) on ice for 30 min. Triton X-100-soluble and -insoluble fractions were obtained by centrifuging cell lysates at 15,000 rpm for 10 min at 4 °C. Alternatively, cells were rinsed with PBS and directly lysed in SDS sample buffer. Samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk in 0.1% Tween 20/Triton-buffered saline and then incubated with primary antibodies. Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibodies (The Jackson Laboratories) and luminol solution (1.25 mM luminol, 65 mM Tris-HCl (pH 8.0), 0.2 mM coumaric acid, 0.01% H₂O₂).

**Endoglycosidase-H Digestion**—Cell lysates were subjected to immunoprecipitation using goat anti-α₁-AT polyclonal antibody and protein G-Sepharose 4F (Amersham Biosciences). Immunoprecipitates were boiled in 1% SDS, 1% 2-mercaptoethanol, and supernatants were added to the reaction containing 50 mM sodium citrate pH 5.0, 1% Triton X-100, 1 mM PMSF, 10 μM pepstatin A, and 0.25 units/ml endoglycosidase-H (Seikagaku Kogyo, Inc.). The reaction was carried out at 37 °C for 16 h.

**Immunofluorescence Microscopy and Flow Cytometry**—For immunofluorescence microscopy, cells cultured on coverslips were fixed with 4% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100/PBS, and blocked with 3% BSA/PBS. Primary antibodies were diluted 1:100, and secondary antibodies were diluted 1:200 in 1% BSA, 0.1% Triton X-100/PBS. Coverslips were successively incubated with primary antibodies, Alexa-conjugated secondary antibodies (Invitrogen), and 1 μg/ml Hoechst 33342/PBS (Sigma) with intervening washes with PBS. Samples were examined by using Olympus FV1000 confocal microscopy. For flow cytometry, cells were collected, rinsed with PBS, and
fixed with 4% paraformaldehyde/PBS. Cells in suspension were stained using anti-α1-AT polyclonal antibody and Alexa 488-conjugated secondary antibody as in immunofluorescence microscopy samples and analyzed by BD FACScan.

Mice—GFP-LC3 transgenic mice were described previously (22). Z mice, in which expression of human α1-ATZ is induced only in liver parenchymal cells upon removal of doxycycline, were produced by using the Tet-Off gene expression system and TAlap 2 mice (26). These mice were crossed to produce Z × GFP-LC3 mice. Piz mice with constitutive expression of α1-ATZ (27) were also crossed to produce PiZ × GFP-LC3 mice. Liver sections were viewed by confocal microscopy. Quantitative morphometry was carried out by counting green vacuoles in 10 cells with many red globules and 10 cells with few or no red globules in three random areas of the liver each from two different liver sections. The results were analyzed using the MetaMorph software program.

RESULTS

The Degradation Rate of α1-ATZ Is Attenuated in Atg5−/− Cells—A previous study demonstrated that disruption of the Atg5 gene in an ES cell line resulted in complete abrogation of autophagy, as confirmed by both morphological and biochemical analyses (14). To investigate whether α1-ATZ is degraded via the autophagic pathway, we first carried out pulse-chase analysis in wild-type and Atg5−/− ES cells transiently transfected with the α1-ATZ expression plasmid. Twenty four hours after transfection, cells were metabolically radiolabeled for 2 h with [35S]methionine/cysteine and chased for 0, 4, 6, and 8 h. Cell lysates were subjected to immunoprecipitation using anti-α1-AT antibody, and immunoprecipitated samples were resolved by SDS-PAGE followed by autoradiography. A semi-logarithmic plot of α1-ATZ-specific signals against time indicated that there was more than a 2-fold decrease in the degradation rate of α1-ATZ in Atg5−/− cells in comparison with that in wild-type cells (Fig. 1A). Cell culture fluid from these cells was also subjected to immunoprecipitation and autoradiography at the same time, but secreted α1-ATZ was barely detectable in either wild-type or Atg5−/− cells (data not shown), indicating that secretion is not the cause for more rapid disappearance of α1-ATZ in wild-type cells. The intracellular half-life of α1-ATZ was calculated as 122 min (r² = 0.99) and 274 min (r² = 0.97) in wild-type and Atg5−/− cells, respectively. The value in wild-type ES cells is well within the range of values that have been described previously in transfected fibroblasts and hepatoma cell lines (8, 10). Next, we examined the fate of α1-ATZ by pulse-chase experiments in wild-type and Atg5−/− MEF cells engineered for stable expression of α1-ATZ (Fig. 1B). The results show that there is a delay in the disappearance of α1-ATZ in the Atg5−/− cells compared with the wild-type MEFs. The difference in degradation of 52-kDa precursor α1-ATZ in the presence and absence of autophagic activity in stably transfected MEFs was almost identical to that in transiently transfected ES cells. A trace amount of mature 55-kDa α1-ATZ was secreted into extracellular fluid. Most interestingly, the abrogation of autophagy was associated with a slight increase in the secretion of α1-ATZ in these cell lines, the significance of which is not yet known.

To compare the relative contribution of autophagy and the proteasomal pathway in α1-ATZ degradation in these cells, we performed pulse-chase analysis in the presence of various proteasome inhibitors. Twenty four hours after transient transfection with the α1-ATZ expression plasmid, cells were preincubated with Met/Cys-free medium for 1 h, metabolically labeled for 2 h, and chased for 4 h in the presence of proteasome inhibitors MG115 (20 μM), epoxomicin (10 μM), lactacystin (10 or 30 μM), or vehicle control. The results show that degradation of α1-ATZ was inhibited by all of the proteasome inhibitors in the wild-type cells but not in the Atg5−/− cells. The α1-ATZ degradation rate in wild-type cells treated with proteasome inhibitor was almost same as that in Atg5−/− cells treated with vehicle control (Fig. 1C), indicating that the inhibitory effects of autophagy and the proteasomal pathway were nearly equivalent in these experiments. To exclude the possibility that delayed α1-ATZ degradation in Atg5−/− cells is secondary to proteasome inhibition in these cells, we analyzed proteasome activity by using Ub-G76V-EGFP, a model substrate for the ubiquitin-fusion degradation pathway (23). Wild-type and Atg5−/− MEF cell lines were engineered for stable expression of Ub-G76V-EGFP and then the cell lines were subjected to flow cytometric analysis for the fluorescent signal. The results showed that there were no differences between the two cell lines in the absence or presence of MG132. Furthermore, transient transfection of the α1-ATZ expression plasmid had no effect on the levels of the proteasomal substrate (Fig. 1D). There was also no difference in the level of polyubiquitinated proteins in the two cell lines as determined by Western blot analysis for ubiquitin (data not shown). These data indicate that expression of α1-ATZ does not inhibit proteasomal activity and furthermore that inhibition of proteasomal activity cannot be an explanation for the delayed degradation in the Atg5-deficient background. Thus, the results of Fig. 1 provide definitive evidence that autophagy can contribute to degradation of α1-ATZ.

α1-ATZ Accumulation in Atg5−/− Cells Is Augmented Over Time—Next, we examined steady-state levels of α1-ATZ in wild-type and Atg5−/− cells (Fig. 2A). The results show a significant increase in levels of α1-ATZ in the Atg5−/− cells. α1-ATZ degradation in Atg5−/− cells was restored by cotransfection of wild-type Atg5 but not mutant Atg5K130R (14), indicating that α1-ATZ disposal requires functional Atg5 that is covalently modified by Atg12 to form autophagosomes and to promote the conversion of LC3-I to faster migrating LC3-II (13, 16) (Fig. 2A). To examine whether the degradation of any exogenously expressed protein would be inhibited in the autophagy-deficient background, we employed EYFP as a control cytosolic protein. When α1-ATZ and EYFP were cotransfected in wild-type and Atg5−/− cells, α1-ATZ accumulated in Atg5−/− cells, but there was no difference in the amounts of EYFP in wild-type as compared with Atg5−/− cells (Fig. 2B). At least a portion of the increased α1-ATZ levels was found in Triton X-100-insoluble fractions, recapitulating the previous observations that polymorphic α1-ATZ forms detergent-insoluble aggregates (10, 25, 28) (Fig. 2B).

To examine the accumulation profile of α1-ATZ over time, wild-type and Atg5−/− MEF cells were transiently transfected with the α1-ATZ expression plasmid and then examined 24, 48, and 72 h after transfection. Cells were directly lysed into SDS sample buffer, and whole cell lysates were analyzed for the amount of α1-ATZ by Western blotting and densitometry (Fig. 2C). Atg5−/− cells contained higher amounts of α1-ATZ than wild-type cells at each time point. The total level of α1-ATZ peaked around 24 h and then gradually decreased in both wild-type and Atg5−/− cells. The difference in accumulation of α1-ATZ in Atg5−/− cells compared with that in wild-type cells was progressively greater at later times, possibly reflecting the differential degradation rate. A previous study demonstrated that the conversion of cytosolic LC3-I to membrane-bound LC3-II represents a good marker for cellular autophagic activity (13, 16). Although in this experiment LC3-II levels were not significantly changed between mock- and α1-ATZ-transfected wild-type MEF cells (Fig. 2C), in vivo studies revealed autophagic induction in the liver following α1-ATZ expression (as demonstrated below).

In both wild-type and Atg5−/− cells, α1-ATZ migrated as a single 52-kDa band during chase periods (Fig. 1) and longer time intervals of
expression (Fig. 2C), suggesting that α1-ATZ accumulates as an ER-glycosylated form in either the presence or the absence of autophagic activity. This was further confirmed by the endoglycosidase-H sensitivity assay. α1-ATZ expressed in Atg5−/− cells was immunoprecipitated and digested with endoglycosidase-H, followed by immunoblot analysis using anti-α1-AT. The 52-kDa polypeptide was cleaved to 46 kDa, indicating that it represents the ER-glycosylated form of α1-ATZ (Fig. 2D) (29). These data indicate that glycosylation and the degradative intermediate profile of α1-ATZ are not altered in the absence of autophagy.

To exclude the possibility that the reduction in the level of α1-ATZ over time is due to a reduction in the percentage of cells expressing α1-ATZ, flow cytometric analysis was performed on MEF cells 24, 48, and 72 h after transfection with the α1-ATZ expression plasmid (Fig. 2E). The results show that there is a decrease in α1-ATZ-positive cells in both cases over time with greater α1-AT fluorescence in the Atg5−/− than in the wild-type background at each time point. The results were almost identical to the steady-state levels of α1-ATZ determined by densitometric analysis as shown above. These data also indicated that initial transfection efficiencies achieved similarly high levels of expression of α1-ATZ in wild-type and Atg5−/− cells. Because transfected wild-type and Atg5−/− cells divided at a nearly equivalent rate during the 72-h time course (data not shown), the more rapid disappearance of α1-ATZ in wild-type cells could not be attributed to the propagation of untransfected cells or the dilution of α1-ATZ because of more rapid cell division. The data were consistent with the idea that α1-ATZ is synthesized at a roughly equivalent rate but is degraded at a slower rate in

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Atg5 cells compared with parental wild-type cells, confirming the pivotal role of autophagic pathway in α1-ATZ disposal.

α1-ATZ Inclusion Formation Is Accelerated in Atg5−/− Cells—The accumulation profile of α1-ATZ in transiently transfected cell lines over time revealed that the net amount of α1-ATZ progressively decreased after 24 h and that α1-ATZ disposal was significantly delayed in Atg5−/− cells. Next, we compared α1-ATZ localization in wild-type to Atg5−/− MEF cells 24 and 72 h after transfection. We used immunostaining with anti-KDEL antibody as a marker of the ER, because it recognizes a series of ER-soluble proteins that have the C-terminal KDEL tetrapeptide ER retrieval signal, and immunostaining for cotransfected GFP-LC3 as a marker for autophagosomes. The results showed that 24 h after transfection in both wild-type and Atg5−/− cells and 72 h after transfection in wild-type cells, α1-ATZ primarily localized to the ER as evidenced by its colocalization with anti-KDEL staining (Fig. 3A, left panel). This ER localization of α1-ATZ is consistent with that described in previous studies. Seventy two hours after transfection of Atg5−/− cells, α1-ATZ was also prominently localized to cytoplasmic inclusion body-like structures distinct from the original ER pattern. The size and morphology of these inclusions ranged from small dots to large aggregates. Although these structures did not colocalize with anti-KDEL staining (Fig. 3A, middle and right panels), they did colocalize with an ER membrane chaperone protein calnexin (Fig. 3B), which directly interacts with α1-ATZ (4, 8).

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tern and persist after the disappearance of α₁-ATZ from the ER localization.

To confirm the apparent tendency for Atg5/−/− cells to bear α₁-ATZ inclusions, we used morphometric analysis to quantify the inclusions in wild-type and Atg5/−/− MEF cells transfected with α₁-ATZ expression plasmid. Seventy two hours after transfection, cells were stained for α₁-ATZ, and a series of confocal fluorescent images was obtained by using parameters of acquisition that were kept constant (Fig. 3D).

α₁-ATZ-positive cells were counted (Fig. 3E, blue bars) and grouped according to predominant localization of α₁-ATZ to either ER (green bars) or inclusions (red bars). The numbers are expressed as percentage of total cells. More than 480 cells were counted in a series of equally acquired images.

GFP-LC3-labeled Autophagosomes Colocalize with α₁-ATZ—By using GFP-LC3 as a marker for autophagosomes (13, 16, 22), we next examined the possibility that α₁-ATZ is sequestered within autophagosomes. We already observed in Atg5/−/− cells that α₁-ATZ forms inclusions in the cytoplasm that have very little overlap with staining by anti-KDEL and do not colocalize with GFP-LC3 (Fig. 3C, lower panel). In wild-type cells α₁-ATZ inclusions were much less frequent, and there was very little colocalization with anti-KDEL staining or staining with GFP-LC3 (Fig. 3C, upper panel). Here we found in wild-type MEF cells a few concentrated areas in the cytoplasm in which staining for α₁-ATZ was localized with anti-KDEL and GFP-LC3 staining (Fig. 4A), suggesting the possibility that these are autophagosomes sequestering α₁-ATZ.

To confirm this, we examined whether the inhibition of fusion between autophagosome and lysosome resulted in an accumulation of autophagosomes containing α₁-ATZ; once the fusion occurs, they disappear by degradation. The small GTPase Rab7 is known to play a role in autophagosome-lysosome fusion as well as in vesicular transport to late endosomes and biogenesis of lysosomes so that the GDP-form mutant...
Rab7 T22N has a dominant-negative effect on the fusion event (30, 31). In the presence of Rab7 T22N, the colocalization between GFP-LC3 and α₃-ATZ was readily detected in wild-type MEF cells 36 h after transfection (Fig. 4B). Similar results were obtained when cells were treated with vacuolar H⁺-ATPase inhibitor bafilomycin A1, another means to inhibit autophagosome-lysosome fusion (32) (data not shown). Because such colocalization is not found in Atg5⁻/⁻ cells (Fig. 4C), the data suggest that the colocalizing structures in the wild-type cells represent genuine autophagosomes. Together, these results not only demonstrate the sequestration of α₃-ATZ into autophagosomes but also further confirm that the autophagic pathway contributes to the efficient disposal of α₃-ATZ.

Previous studies have shown that there is a statistically significant increase in autophagosomes in liver cells in mouse models of α₁-AT deficiency as well as in liver cells of biopsy specimens from patients with α₁-AT deficiency (19). To determine whether the autophagic response is specifically induced by accumulation of α₁-ATZ in the ER of liver cells in vivo, we generated the Z × GFP-LC3 mouse model by mating the Z mouse with liver-specific inducible expression of α₁-ATZ to the GFP-LC3 mouse, which renders autophagosomes green under fluorescent microscopy. Separate groups of Z × GFP-LC3 mice that had aged to 3 months of age with doxycycline in their drinking water (α₁-ATZ gene expression suppressed) or without doxycycline (α₁-ATZ gene expression induced) were sacrificed, and their livers were examined under fluorescent microscopy (Fig. 5, C and D, respectively) in comparison to the liver of 3-month-old GFP-LC3 mice that were either fed or starved for 24 h prior to sacrifice (Fig. 5, A and B, respectively). The results show that withdrawal of doxycycline and thereby induction of the mutant protein is sufficient to elicit GFP-LC3-labeled structures (Fig. 5, D compared with C). This pattern of labeling was almost identical to that seen in the GFP-LC3 mouse but only after starvation (Fig. 5, B compared with A), indicating that the labeled structures were indeed autophagosomes. These data provide further confirmation of the induction of the hepatic autophagic response in α₁-AT deficiency and, moreover, that the expression of the mutant α₁-ATZ molecule is sufficient to induce hepatic autophagy in vivo.

Finally, we examined the localization of autophagosomes in the liver of the Z × GFP-LC3 mouse relative to α₁-ATZ. It is well known that mutant α₁-ATZ can be detected by immunostaining in the liver of patients with α₁-AT deficiency or in mouse models of α₁-AT deficiency, but wild-type α₁-AT cannot be detected in the normal human liver (1, 33). This has been presumed to be due to the fact that the mutant

FIGURE 4. GFP-LC3-labeled autophagosomes colocalize with α₁-ATZ upon inhibition of autophagosome maturation. A, wild-type MEF cells stably expressing GFP-LC3 were transiently transfected with the α₁-ATZ expression plasmid and 36 h later immunostained using anti-α₁-AT (Alexa 568; magenta) and anti-KDEL (Alexa 647; cyan). Bar, 10 μm. Lower panels, the area indicated by the white rectangle was optically sectioned along the vertical z axis, and the x-z and y-z planes were constructed with FV1000 software. Bar, 1 μm. B, transient transfection was carried out with expression plasmids for α₁-ATZ and dominant-negative Rab7-T22N and immunostaining done 36 h later. C, as a control experiment, Atg5⁻/⁻ MEF cells stably expressing GFP-LC3 were processed in the same way as in A and B. Bars, 10 μm.
protein accumulates in liver cells to levels that are well beyond those that occur for the wild-type protein and to levels that reach the threshold for detection by immunostaining. It is also well known that mutant α₁-ATZ forms large globules in the ER of some, but not all, hepatocytes (27). The globule-devoid hepatocytes are thought to be progenitor, or at least relatively immature, hepatocytes that express α₁-ATZ but not to the extent that there is enough accumulation to form intracellular globules (27, 34). In Fig. 5, E and F, we immunostained liver from the Z/GFP-LC3 mouse shown in Fig. 5D for α₁-ATZ by using a red fluorophore together with anti-GFP (E and F). An area having hepatocytes with many α₁-ATZ globules is shown in E, and one with hepatocytes having few or no α₁-ATZ globules is shown in F. Several areas in which green autophagosomes adjacent to red globules are particularly apparent are indicated by arrowheads.

DISCUSSION

In the present study we provide the first genetic evidence that autophagy constitutes a major pathway for degradation of ER-retained α₁-ATZ. This is also the first report to show that autophagosomes can segregate and concentrate a mutant protein α₁-ATZ as cargo by using GFP-LC3, the most reliable marker for autophagosomes currently available (Figs. 4 and 5). Although it has been widely accepted that autophagic sequestration is a nonspecific bulk process, our data suggest that α₁-ATZ is delivered to autophagosomes in a more effective manner than a control cytosolic protein EYFP (Fig. 2B). Our recent study also demonstrated that invading pathogenic group A streptococci were spe-
cifically enclosed by autophagosomes to be delivered and killed in lysosomes, further indicating that autophagic sequestration can occur in a substrate-specific manner (35). Previous morphometric studies demonstrated that administration of phenobarbital induced the proliferation of ER membranes in rat liver, and following the cessation of this drug excess ER membranes were removed in parallel with a specific increase in the volume and number of autophagosomes containing ER membranes (36). There has also been a report showing that ER membrane proteins, phenobarbital-inducible cytochrome P450, and NADPH-cytochrome P-450 reductase were segregated by autophagy to be degraded in lysosomes without leakage into the cytosol fraction (37). A similar mode of autophagic sequestration could facilitate the specificity by which ER-retained α₃-ATZ is degraded.

Previous studies have shown that autophagic activity is increased in the α₃-ATZ-expressing liver of the PiZ transgenic mouse model in the absence of starvation and that the increase reaches levels that are comparable with the levels of autophagic activity induced in the liver of wild-type mice by starvation (19, 20). Moreover, in those studies starvation did not elicit any further increase in autophagic activity in the liver of the PiZ mouse (20), which could mean that autophagic activity is already at saturable levels or that starvation leads to increased clearance and well as induction of the formation of autophagosomes. Most interestingly, we found that autophagic activity was not increased in transiently transfected cell lines, as judged by LC3 modification (Fig. 2C), but was clearly induced in the Z × GFP-LC3 mouse by 3 months of age (Fig. 5). The most likely explanation is that induction of autophagy requires a duration of accumulation of α₃-ATZ that is longer than the 3-day duration of a transiently transfected system. One attractive alternative possibility is that new autophagic activity is only induced when existing "constitutive" autophagy is saturated with mutant-aggregated protein. However, it is also possible that there are differences in the milieu in vivo or that retention of α₃-ATZ in the ER has complex effects on both formation and clearance of autophagosomes.

Another interesting result of this study was the induction of autophagosomes in globule-devoid as well as globule-containing hepatocytes. Previous studies have suggested that globule-devoid hepatocytes do express α₃-AT but to apparently lesser levels than globule-containing hepatocytes, presumably because they are progenitor or younger cells (27, 33, 34). Thus, the most likely explanation for the presence of autophagosomes in both of these cell populations is that the threshold for induction of autophagy is reached at the lower level of α₃-ATZ in the globule-devoid hepatocytes. If this explanation is correct, then it is possible that the induced autophagy could in turn be responsible for limiting the formation of globules in the globule-devoid hepatocytes. However, there are a number of other explanations for the presence of autophagosomes in both cell populations, including the possibility that the accumulation of α₃-ATZ to higher levels has an effect on the clearance of autophagosomes and the remote possibility that either cell population has a "trans" effect on induction of autophagy in the other cell population.

Immunofluorescence studies showed that many characteristic inclusion body-like structures were formed by α₃-ATZ in Atg5⁻/⁻ cells (Fig. 3A). These inclusions immunolabeled for ER membrane protein calnexin (Fig. 3B) but not for KDEL-containing proteins. This labeling pattern could be explained by the formation of an α₃-ATZ-calnexin complex that is not dissociated during the formation of inclusions, whereas KDEL-possessing soluble proteins are not associated with, or become dissociated from, α₃-ATZ before evolution into inclusions within the ER lumen or during movement out of the ER. These structures share some characteristics with the recently described ER quality control compartment or concentric membranous body, including membranous morphology and colocalization of ER membrane chaperone calnexin and mutant substrate proteins (38, 39). Because proper function of the ER appears to be maintained while mutant proteins accumulate within these structures, they may constitute reservoirs of aberrant proteins similar to what has been attributed to aggresomes (40, 41). However, it should be noted that the overall morphology of α₃-ATZ inclusions and the lack of specific localization within the cytoplasm indicate that they are not structurally cognate with aggresomes, which converge at pericentriolar region by retrograde transport on microtubule network.

The tendency for formation of α₃-ATZ inclusions in autophagy-deficient cells may also bear on the apparent lack of additive or synergistic effects of proteasomal inhibition in Atg5⁻/⁻ cells (Fig. 1C). By using the probesensor construct Ub-G76V-EGFP reported by Dantuma et al. (23), we did not detect differences in the proteasome activity in wild-type and Atg5⁻/⁻ cells, in the absence or presence of proteasome inhibitors (Fig. 1D). Proteasome activity was not altered following the expression of α₃-ATZ, and Atg5⁻/⁻ cells contained a normal amount of polyubiquitinated proteins. Another recent study has also reported that proteasome activity is unperturbed in the absence of autophagy (42). However, it could be hypothesized that the α₃-ATZ sequestered into inclusions cannot be a substrate for retrograde translocation out of the ER, if that is a necessary step in the proteasomal mechanism that characterizes the ER-associated degradation pathway for α₃-ATZ. Accordingly, the preferential formation of α₃-ATZ inclusions in Atg5⁻/⁻ cells might decrease the contribution of proteasomes to the overall cellular disposal of α₃-ATZ. If so, autophagy should be particularly important in the degradation of aggregated α₃-ATZ, and therefore it becomes increasingly important as the level of mutant protein accumulates in the ER.

In conclusion, this study demonstrates that autophagy is indispensable for efficient disposal of ER-retained α₃-ATZ. Although it is still not entirely clear how protein aggregates found in conformational diseases such as α₃-AT deficiency result in tissue pathology or whether the pathobiology reflects specific characteristics of the protein that aggregates in each of these diseases, there is a great deal of circumstantial evidence that the cellular accumulation of α₃-ATZ aggregates is an important determinant of hepatic injury in α₃-AT deficiency. In this regard, future studies of the detailed molecular mechanisms by which ER-retained α₃-ATZ induces autophagy and is sequestered in autophagosomes are likely to have clinical significance as well as important implications for the mechanism and function of the autophagic pathway itself.

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