Degradation of proteins that are retained in the quality control apparatus of the endoplasmic reticulum (ER) has been attributed to a third proteolytic system, distinct from the lysosomal and the cytoplasmic ubiquitin-dependent proteasomal proteolytic pathways. However, several recent studies have shown that ER degradation of a mutant membrane protein, CFTRΔF508, is at least in part mediated from the cytoplasmic side by the 26 S proteasome. In this study, we examined the possibility that ER degradation of mutant secretory protein α1-antitrypsin (α1-AT) Z, the mutant protein associated with infantile liver disease and adult-onset emphysema of α1-AT deficiency, is mediated by the proteasome. The results show that a specific proteasome inhibitor, lactacycin, inhibits ER degradation of α1-AT in transfected human fibroblast cell lines and in a cell-free microsomal translocation system. Although it is relatively easy to conceptualize how a transmembrane protein like CFTRΔF508 might be accessible on the cytoplasmic aspect of the ER membrane for ubiquination and degradation by the proteasome, it is more difficult to conceptualize how this might occur for a luminal polypeptide. The results show that, once within the lumen of the ER, α1-ATZ interacts with the transmembrane molecular chaperone calnexin and specifically induces the polyubiquitination of calnexin. The results, therefore, provide evidence that the proteasome, from its cytoplasmic localization, induces the degradation of the luminal α1-ATZ molecule by first attacking the cytoplasmic tail of calnexin molecules that are associated with α1-ATZ.

Homozygous PiZZ α1-antitrypsin (α1-AT) deficiency is a well recognized cause of emphysema/destructive lung disease (1). A subgroup of individuals with this deficiency also develop chronic liver disease and hepatocellular carcinoma (2). In fact, it is the most common genetic cause of liver disease in children. Although lung injury is due to the decrease in elastase inhibitor capacity ordinarily provided by α1-AT, liver injury is due to the hepatotoxic effect of the abnormally folded α1-ATZ molecule (Glu342 to Lys) that is retained in the ER (2). Recently, we found that most α1-AT-deficient individuals are "protected" from liver injury because of relatively efficient degradation of α1-ATZ in the ER ("protected hosts"), whereas in a subgroup of α1-AT-deficient individuals affected by severe liver disease ("susceptible hosts"), there is a lag in ER degradation of α1-ATZ (3). This results in greater net retention of hepatotoxic α1-ATZ within the ER. In one susceptible host, the lag in ER degradation of α1-ATZ is associated with a failure to interact with the ER molecular chaperone calnexin, raising the possibility that this interaction is necessary for entry into the degradative pathway (3). Examination of the biochemical characteristics of relatively efficient ER degradation of α1-ATZ in protected hosts (3) suggests similarities with those described for other proteins that are retained/degraded in ER, including, for example, T-cell receptor α subunit, asialoglycoprotein receptor F2, and hydroxymethylglutaryl-CoA reductase and CFTRΔF508 (4–7).

Because recent studies have indicated that ER degradation of CFTRΔF508 is at least in part mediated by the proteasome (8, 9), we examined the possibility that ER degradation of α1-ATZ is also mediated by the proteasome.

**EXPERIMENTAL PROCEDURES**

*Materials—* Rabbit reticulocyte lysate was purchased from Promega, Inc. (Madison, WI). [35S]Methionine was purchased from Amersham Corp. (Arlington Heights, IL) in purified form and from ICN Radiochemical (Irvine, CA) in crude form. Tran35S-label. m7G(5′G)ppp(5′G) was obtained from Pharmacia (Uppsala, Sweden). Canine pancreatic microsomal vesicles were gifts from Dr. Dennis Shields (Albert Einstein College of Medicine, New York) and later purchased from Promega, Inc.. Endoglycosidase H, GSSG and castanospermine were purchased from Sigma. N-Acetyl-NYT-amide was kindly provided by Dr. Steve Adams (Boston, MA). Antibody to α1-antitrypsin was purchased from Dako (Santa Barbara, CA). Antibody to calnexin (SPA-860) was obtained from StressGen (Victoria, British Columbia, Canada). Antibody to ubiquitin was purchased from Sigma. Preparation of lactacycin has been described previously (10). All other chemical products were purchased from Sigma.

**Cell Lines—** Fibroblast cell lines which had been transduced with amphotropic recombinant retroviral particles bearing α1-ATZ cDNA have been described previously (3, 11).

**Metabolic Labeling, Immunoprecipitation, and Analytical Gel Electrophoresis—** Cell lines were subjected to pulse-chase radiolabeling. For the pulse period, the cells were incubated at 37°C for 2 h in Tran35S-label 250 μCi/ml in Dulbecco’s modified Eagle’s medium lacking methionine. The cells were then rinsed rigorously and incubated in Dulbecco’s modified Eagle medium with excess unlabeled methionine for time intervals up to 10 h as the chase period. At the end of each chase period, the extracellular medium was harvested and the cells lysed in phosphate-buffered saline, 1% Triton X-100, 0.5% deoxycholic acid, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride. The radiolabeled cell lysates were then subjected to clarification, immunoprecipitation, and immunoprecipitates analyzed by SDS-PAGE/fluorography exactly as described previously (3, 11). Aliquots of the radiolabeled cell lysates were also subjected to trichloroacetic acid precipitation and scintillation counting of the trichloroacetic acid precipitates to ensure that there was
equivalent incorporation between cell lines under comparison (3, 11). Results were quantified by densitometric scanning of fluorograms on a laser densitometer (2222 ultrasan XL, LKB Instruments, Inc., Houston, TX), and quantification is reported as mean ± one standard deviation.

Cell-free Translation and Translocation—The pGEM-4Z vector (Promega, Inc.) containing either α₁-ATM cDNA or α₁-ATZ cDNA was linearized beyond the 3' end of the cDNA using HindIII. SP6 RNA polymerase was used for in vitro transcription in the presence of m⁷G(5’ppp)5’G to generate 3’G-capped mRNAs following the protocol provided by Promega. α₁-ATM and α₁-ATZ polypeptides were synthesized in the reticulocyte lysate cell-free system according to the protocol in the Promega technical manual. The cell-free reaction mixture (50 μl) contained 35 μl of micrococcal nuclease-treated rabbit reticulocyte lysate supplemented with the following final concentrations of additional components: 20 μM of 19-amino acid mixture minus methionine, 0.8 μg/μl of RNase inhibitor RNasin, 0.8 μCi/μl of [³⁵S]methionine, 4 A₂₆₀/ml of canine pancreas microsomal vesicles, and 20 μg/ml of the appropriate mRNAs. The cell-free translation and translocation assay was performed for 1 h at 30 °C. Where indicated, endoglycosidase H (Endo H) digestion of cell-free translated products was carried out as described previously (3). In addition, where indicated, proteinase K digestion of cell-free translated products was performed as described (12). The products were analyzed on 10% SDS-PAGE under reducing conditions and visualized by fluorography.

Proteolysis Assay in the ER Vesicles—After the translation reaction, the microsomal vesicles that contained either α₁-ATM or α₁-ATZ polypeptide were isolated by centrifugation at 15,000 g for 15 min at 4 °C. The pelletted microsomal vesicles were resuspended in fresh proteolysis primed lysate contained in a final volume of 50 μl: 40 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 mM diithiothreitol, 0.5 mM ATP, 10 mM phosphocreatine, 1600 μg/ml creatine phosphokinase, and fresh reticulocyte lysate, according to Ref. 13, and incubated at 37 °C for the indicated time intervals.

Western Blotting of Endogenous Canine Pancreatic Microsomal Calnexin—Canine pancreatic microsomal membranes were incubated at 22 °C for 30 min in the presence of DTT or GSSG in several different concentrations (our cell-free translocation/pulse-chase assay is done in 2 mM DTT). The vesicles were then subjected to SDS-PAGE and Western blot analysis with anti-calnexin antibody. The blot was developed with the ECL system (Amersham Corp.).

RESULTS AND DISCUSSION

Effect of Lactacystin on Degradation of α₁-ATZ in Fibroblast Cell Lines—First, we examined the effect of a specific, irreversible inhibitor of the proteasome, lactacystin (14), on degradation of α₁-ATZ in fibroblast cell lines from protected hosts that had been transduced with the α₁-ATZ gene. The α₁-ATZ polypeptide is retained in the ER of these cells as a 52-kDa ER-resident high-mannose glycoprotein, but is then degraded in a relatively efficient manner (11). Our previous studies had shown that degradation of α₁-ATZ in these cell lines was inhibited by several synthetic proteasome inhibitors known to inhibit the proteasome, albeit in a relatively less specific manner (3). These cell lines were preincubated for 2 h in the absence or presence of 10 μM lactacystin and then subjected to biosynthetic radiolabeling in a pulse-chase experiment. The results were analyzed by immunoprecipitation and the immunoprecipitates subjected to SDS-PAGE/fluorography (Fig. 1a).

In the absence of lactacystin, α₁-ATZ progresses decays over 2 h and completely disappears between 2 and 4 h of the chase period. In the presence of lactacystin, the α₁-ATZ polypeptide disappears only minimally over the entire 10-h chase period. In Fig. 1b, we examined the effect of lactacystin on degradation of α₁-ATZ in another protected cell line. Again, lactacystin mediated a marked decrease in rate of α₁-ATZ degradation. In this cell line, a slightly faster migrating ~50-kDa polypeptide was retained in the presence of lactacystin. The immunoprecipitation of this polypeptide was completely blocked by unlabeled purified α₁-ATZ (data not shown), indicating that it is an α₁-ATZ intermediate. At ~50 kDa, this cannot be an underglycosylated or deglycosylated form of α₁-ATZ (46 kDa) and therefore probably represents an endoproteolytic intermediate. Nevertheless, its biochemical nature is as yet unknown. Lactacystin inhibited the rate of degradation of α₁-ATZ in five separate experiments in three different cell lines (data not shown). The t½ for degradation of α₁-ATZ was 1.75 and 5.25 h in the absence and presence of lactacystin, respectively.

Degradation of α₁-ATZ in a Cell-free System—In order to further characterize the degradation of α₁-ATZ, we examined the possibility that it could be recapitulated in a cell-free microsomal translocation system. α₁-AT wild type (M) and α₁-ATZ mRNAs were generated by in vitro transcription and used to program a rabbit reticulocyte lysate system supplemented with canine pancreatic microsomal membranes. After 1 h in the translation reaction mixture supplemented with [³⁵S]Met, the microsomal vesicles were pelleted by centrifugation and resuspended in fresh proteolysis-primed lysate containing ATP, an ATP regenerating system in the absence of [³⁵S]Met, but using the conditions which have been described for many studies of the ubiquitin-dependent proteosomal system (13). After several different time intervals, aliquots were homogenized and subjected to SDS-PAGE/fluorography analysis (Fig. 2a). The results show that a 52-kDa radiolabeled polypeptide is generated from both α₁-ATM and α₁-ATZ mRNAs. This polypeptide corresponds to α₁-AT that has acquired high-mannose carbohydrate side chains. In the case of α₁-ATM, there are two distinct cleavages during the 16-h chase period, but no significant disappearance of total radiolabeled α₁-AT. The mechanism for the two cleavages is not known, but presumably involves luminal endoproteolytic activity. There was also no significant disappearance over 16 h for several different polypeptides that were translocated into the lumen in the cell-free microsomal translocation system using the exact same "pulse-chase" protocol but programming it with mRNA from normal adult human liver (data not shown). For α₁-ATZ, there is very little of the two cleavages, but there is progressive disappearance of the α₁-AT molecule beginning within 1 h and complete disappearance between 2 and 4 h of the chase period. There is some variability from experiment to experiment in the pattern of disappearance of α₁-ATZ during the first three time points, but it is always completely degraded by 4 h of the chase period. The t½ for degradation of α₁-ATZ in five experiments shown in Figs. 2 and 3 is 1.12 ± 0.58 h. Taken together, the data indicate that the α₁-ATZ molecule is degraded in microsomal vesicles in a highly
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Specific manner. Moreover, the data show that α₁-ATZ degradation can be characterized in the cell-free system. For instance, we could examine the effect of ATP on α₁-ATZ degradation in this system. When chased in the absence of ATP (Fig. 2b) or in the presence of ATP (Fig. 2c), there is minimal, if any, degradation of α₁-ATZ during the chase period. In addition, when chased in the absence of proteolysis-primed lysate, there is no degradation of α₁-ATZ (data not shown). Thus, degradation of α₁-ATZ is dependent on ATP and on the constituents of the proteolysis-primed lysate.

Next, we examined the effect of lactacytin in the cell-free system (Fig. 2d). The results show that lactacytin markedly inhibits degradation of α₁-ATZ in this system. To ensure that lactacytin was in fact inhibiting proteasomal activity, we added ¹²⁵I-lysosome, a known substrate for the proteasome (15), to the proteolysis-primed lysate in the absence or presence of 20 μM lactacytin. The results showed that there was time-dependent appearance of trichloroacetic acid-soluble radioactivity in the absence of lactacytin, but markedly inhibited in the presence of lactacytin (data not shown). Taken together, these data provide evidence that ER degradation of α₁-ATZ has the characteristics of proteolysis mediated by the 26 S proteasome and provide evidence for the novel use of a cell-free microsomal translocation system to characterize the kinetics of degradation of secretory proteins.

Although it is relatively easy to conceptualize how a transmembrane protein like CFTRΔF508 might be accessible on the cytoplasmic aspect of the ER membrane for ubiquitination and degradation by the 26 S proteasome, it is more difficult to conceptualize how this might occur for a luminal polypeptide. Several results militate against the possibility that α₁-ATZ is somehow exposed on the external surface of the microsomes in the cell-free system. First, the α₁-ATZ being degraded in Fig. 2 is glycosylated. This is proven by its susceptibility to Endo H as shown in Fig. 2a. After Endo H digestion, α₁-ATZ migrates to approximately 46 kDa, a mobility that corresponds to the nascent α₁-ATZ polypeptide after its signal peptide has been cleaved. Second, the 52-kDa α₁-ATZ polypeptide that is being degraded in the cell-free system is protected from proteasome K (Fig. 3b) added at the end of the pulse period (left) or at the end of the 2-h chase period (right). In each case, proteasome K by itself has no effect, even though it can completely degrade α₁-ATZ if it is added together with Triton X-100 that homogenizes the microsomal membranes. In order to exclude the possibility that a portion of the newly synthesized and glycosylated α₁-ATZ is pumped out of the microsomal vesicles, we separately analyzed the supernatant and pellet vesicles at the end of each chase time point (Fig. 3c). There was no evidence that the 52-kDa α₁-ATZ polypeptide ever reached the supernatant in this experiment, in experiments in which ATP is and lactacytin were used to inhibit degradation (data not shown), or in experiments in which ATP was added to stimulate any possible transport activity (data not shown).

One possible explanation is that the proteasome attacks the cytoplasmic tail of a transmembrane ER protein to which α₁-ATZ has bound. Because our previous studies had shown that α₁-ATZ interacts with one such protein, calnexin (3), we examined the possibility that α₁-ATZ must interact with calnexin to be degraded by the proteasome. First, we examined the relative electrophoretic mobility of endogenous calnexin in the canine pancreatic microsomal vesicles under the conditions used for the cell free translocation/pulse-chase assay. Several recent studies (16–18) have shown that calnexin is inactive as a chaperone if it is reduced by DTT and that this reduced state can be detected in SDS-PAGE under nonreducing conditions by a shift to a slower electrophoretic mobility. Western blot analysis showed that, under the conditions of our assay, calnexin migrated to an electrophoretic mobility that was consistent with the presence of a high-mannose oligosaccharide.

Degradation of α₁-AATM and α₁-ATZ in a cell-free system. a, α₁-AATM mRNA and α₁-ATZ mRNA were translated for 60 min at 30 °C in a rabbit reticulocyte lysate supplemented with canine pancreatic microsomes. After translation, the microsomal vesicles were harvested by centrifugation. The pellets were resuspended in 50 μl of proteolysis-primed lysate and incubated at 37 °C for several different time intervals. Aliquots were taken at each time point and mixed with SDS-sample buffer. All products were analyzed on SDS-PAGE/fluorography. The relative electrophoretic migration of the 52-kDa α₁-AT polypeptide is indicated at the left margin. b, α₁-ATZ mRNA was subjected to translation as described above, but chased in proteolysis-primed lysate. c, α₁-ATZ mRNA was subjected to the same protocol, but chased in the presence (+ATP) or absence (−ATP) of ATP and an ATP regenerating system. d, rabbit reticulocyte lysate was preincubated in the absence (−) or presence (+) of lactacytin (20 μM) prior to being used for translation. α₁-ATZ mRNA was then subjected to translation as described above, but chased in proteolysis-primed lysate also in the absence (−) or presence (+) of lactacytin (20 μM).

Fig. 2. Degradation of α₁-AATM and α₁-ATZ in a cell-free system. a, α₁-AATM (52 kD) was translated in the absence (−) or presence (+) of lactacytin (66 kD) added at the end of the pulse period (P). After pulse, the vesicles were incubated overnight at 37 °C in the absence (−) or presence (+) of ATP (5 mM) or ATPS (5 mM). b, the 52-kDa α₁-ATZ polypeptide that is being degraded in the cell-free system is protected from proteinase K (35) added at the end of the pulse period (left) or at the end of the 2-h chase period (right). In each case, proteinase K by itself has no effect, even though it can completely degrade α₁-ATZ if it is added together with Triton X-100 that homogenizes the microsomal membranes. In order to exclude the possibility that a portion of the newly synthesized and glycosylated α₁-ATZ is pumped out of the microsomal vesicles, we separately analyzed the supernatant and pellet vesicles at the end of each chase time point (Fig. 3c). There was no evidence that the 52-kDa α₁-ATZ polypeptide ever reached the supernatant in this experiment, in experiments in which ATP is and lactacytin were used to inhibit degradation (data not shown), or in experiments in which ATP was added to stimulate any possible transport activity (data not shown).

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Fig. 4. a, electrophoretic mobility of endogenous canine pancreatic microsomal calnexin. Canine pancreatic microsomal vesicles were incubated at 22 °C for 30 min in the presence of DTT or GSSG in several different concentrations. The vesicles were then subjected to Western blot analysis with anti-calnexin antibody SPA-880. The relative mobility of the reduced and nonreduced state is indicated at the left margin. b, evidence for binding of α1-ATM to calnexin in the cell-free system. Wild type α1-ATM or α1-ATZ mRNA were subjected to translation/translocation in a reaction mixture that had been preincubated for 10 min in CST, 1 mM. Aliquots were subjected to immunoprecipitation with anti-α1-AT antibody. Immunoprecipitates were run on SDS-PAGE and transferred to nitrocellulose for Western blot analysis with anti-calnexin antibody. Molecular mass markers are indicated at the right margin in kilodaltons. The migration of calnexin and Ig heavy chain is indicated at the left margin. c, effect of castanospermine on the kinetics of degradation of α1-ATZ in the cell-free system. The reaction mixture for translation/translocation was preincubated at 27 °C for 10 min in the absence (−) or presence (+) of CST, 1 mM. One experiment with a short chase is shown on the left and another experiment with a longer chase is shown on the right. The relative mobility of trimmed and untrimmed α1-ATZ is indicated at the left margin. In the experiment on the right, CST induces the formation of one predominant untrimmed molecule and two faster migrating degradation products. d, effect of glyco-acceptor peptide NYT on the kinetics of degradation of α1-ATZ in the cell-free system. The same protocol was used for cell-free translation/translocation in the absence or presence of n-acetyl-NYT-amide, 2 mM. A microsomal vesicle preparation from Promega was used in this particular experiment. The migration of unglycosylated 46-kDa α1-ATZ, and glycosylated 52-kDa α1-ATZ is indicated at the left margin. e, evidence for polyubiquitination of calnexin in the cell-free system. For this experiment, α1-ATM or α1-ATZ mRNA were subjected to translation/translocation for 60 min. The microsomal vesicles were then harvested by centrifugation. The pellets were resuspended in proteolysis primed lysate supplemented with ATP-γS (5 mM), ubiquitin (4 μg/mL), and lactacystin (20 μM). The reaction was terminated after 15 min at 37 °C by homogenizing the vesicles with phosphate-buffered saline, 1% Triton X-100. Aliquots were subjected to immunoprecipitation with anti-ubiquitin antibody. Immunoprecipitates were run on SDS-PAGE and transferred to nitrocellulose for Western blot analysis with anti-calnexin antibody on the left and anti-ubiquitin (right) and anti-calnexin (right) antibodies.

with the nonreduced, active state. Addition of GSSG to a concentration of 10 mM did not change its migration, but addition of DTT to increase the concentration from the 2 mM, used for our assays, to 5 mM resulted in a shift to a slower electrophoretic mobility (Fig. 4a) identical to that recently described by Hebert et al. (16), Ou et al. (17), and Tector and Salter (18). Next, we examined the possibility that α1-ATM forms a stable complex with calnexin in the cell-free system. Microsomes which had translocated α1-ATM or α1-ATZ in the absence or presence of castanospermine were homogenized under nondenaturing conditions (2% CHAPS), subjected to immunoprecipitation with anti-α1-AT antibody, and then immunoprecipitates were subjected to Western blot analysis with anti-calnexin antibody (Fig. 4b). In the presence of castanospermine (CST), an inhibitor of glucosidase I and II, glycoproteins are not converted to the monoglycosylated intermediate that is necessary for interaction with calnexin (19, 20). The results show that calnexin is coprecipitated in microsomes that had translocated α1-ATZ. A band corresponding to coprecipitated calnexin is faint, if present at all, in microsomes which had translocated wild type α1-ATM and in microsomes that had translocated α1-ATZ in the presence of CST.

Next, we examined the effect of CST on the kinetics of degradation of α1-ATZ in the cell-free system (Fig. 4c). The results show that preincubation of the cell-free system with CST alters the electrophoretic mobility of α1-ATZ as expected and inhibits its degradation. The effect of CST is dose-dependent and sustained for 4 h of chase period (Fig. 4c, right panel). We also examined the effect of inhibiting glycosylation with the glycoacceptor peptide NYT on the kinetics of degradation of α1-ATZ in the cell-free system (Fig. 4d). In this experiment, we used a preparation of microsomal vesicles that were less efficient in glycosylation as evidenced by the presence of two α1-ATZ polypeptides, the nascent 46-kDa polypeptide, and the glycosylated 52-kDa polypeptide (left panel). In the absence of NYT, the 52-kDa polypeptide disappears between 1 and 4 h of the chase period, but the 46-kDa nascent polypeptide does not significantly disappear throughout the entire chase period (left panel). In the presence of NYT, there is only a 46-kDa nascent polypeptide that does not undergo degradation during the chase period. These data provide further evidence that glycosylation, which is required for interaction with calnexin, is also required for degradation.

If the proteasome actually degrades the α1-ATZ-calnexin complex, one would predict that this complex is ubiquitinated. In order to examine this prediction, microsomes that had translocated α1-ATM or α1-ATZ were homogenized under denaturing conditions (Triton X-100), subjected to immunoprecipitation with antibody to ubiquitin, and then the immunoprecipitates analyzed by Western blot analysis with anti-
calnexin antibody (Fig. 4e). The results show a ladder of bands migrating slower than native calnexin, only in the microsomes that had translocated α-ATZ, indicating that multiple ubiquitin molecules have been conjugated to calnexin. No ubiquitinated calnexin was detected in the corresponding reaction with α-ATZ. Separate aliquots of the immunoprecipitates were subjected to Western blot analysis with anti-α-AT (right panel), but no polyubiquitinated polypeptides could be detected. These data show that translocation of α-ATZ into the lumen of the microsome and interaction with the resident ER membrane protein calnexin specifically induces the ubiquitination of calnexin and converts a small cohort of endogenous calnexin molecules into substrates for the proteasome. The results also show that ubiquitin is not conjugated to α-ATZ.

If the proteasome recognizes α-ATZ-polyubiquitinated calnexin as a substrate, one would also predict that an α-ATZ-calnexin-ubiquitin conjugate exists. In order to address this issue (Fig. 4f), we did the same experiment shown in Fig. 4e, but with several critical modifications: the chase after translation/translocation was done in the presence of ATPγS and ubiquitin to trap ubiquitin conjugates; microsomes were homogenized under nondenaturing conditions (2% CHAPS); the aliquots were first immunoprecipitated with anti-α-AT and then subjected to Western blot analysis with anti-ubiquitin (left) and anti-calnexin (right) antibodies. The results show the exact same multi-ubiquitinated ladder as was seen in Fig. 4e. The ladder migrates slower than native calnexin (~90 kDa), is seen in vesicles that have translocated α-ATZ, but not in those which have translocated α-ATM, and is recognized by both anti-ubiquitin and anti-calnexin antibodies. This demonstrates the existence of a complex between α-ATZ and multi-ubiquitinated calnexin. There is also a band at ~60 kDa in each case. We do not know the nature of this band but wonder if it is an α-ATZ-calnexin conjugate.

Several previous studies have shown that calnexin facilitates the folding and assembly of single-chain and multi-chain membrane and secretory proteins (21–24). The current study shows that calnexin also plays a key role in facilitating the degradation of ligands that cannot fold/assemble productively, as exemplified by a naturally occurring mutant secretory protein. Our data cannot, however, exclude the possibility that α-ATZ interacts with other transmembrane proteins of the ER and that such, as yet unidentified, interactions can account for part of its degradation. We do not yet know exactly how the entire α-ATZ-calnexin complex, including the luminal domain of calnexin associated with α-ATZ, is degraded. The proteasome may initiate a process that is completed by other enzymes within the ER membrane or within the ER lumen. Raposo et al. (25) have recently described an electron-dense compartment derived from the ER that may be specialized for ubiquitin-dependent proteasomal degradation. Perhaps, the α-ATZ-calnexin complex is localized to this subcompartment of the ER during its degradation.

A recent study by McCracken and Brodsky (26) has shown that the degradation of uncleaved pro-α-factor in the ER of yeast depends on cytosol, calnexin, and ATP. In this case, the glycosylated form of the polypeptide is not a substrate for ER degradation, and there is evidence that the glycosylated pro-α-factor is transported out of the microsomal vesicles into the cytosol for degradation by an ATP-dependent proteolytic system. We could find no evidence that the glycosylated form of α-ATZ is a substrate for ER degradation (Fig. 4d), and no evidence that the 52-kDa glycosylated α-ATZ polypeptide that is degraded in the cell-free system is ever outside the microsomal vesicles (Fig. 3c). We cannot yet exclude the possibility that the α-ATZ polypeptide is rapidly exchanged across the microsomal membrane, but it is more likely that the difference in the two studies reflect differences in yeast as compared with mammalian microsomal translocation or differences in the substrates pro-α factor and α-ATZ.

The current study also has important implications for the mechanism by which a subgroup of α-ATZ-deficient individuals develop severe liver disease and for potential therapeutic interventions for these individuals. We have shown recently that there is a lag in ER degradation of α-ATZ in these “susceptible” hosts and have predicted that there will be several distinct mechanisms for this lag in ER degradation among the susceptible host population. In one susceptible host, the retained α-ATZ interacts poorly with calnexin (3). In the liver cells of this host, there is likely to be only a very little polyubiquitinated calnexin-α-ATZ complex that can be recognized for proteolysis by the proteasome. In several other susceptible hosts, the retained α-ATZ interacts well with calnexin, but is only slowly degraded. These hosts may have a defect in calnexin that prevents its ubiquitination or may have a defect in ubiquitination or in proteasomal activity. These latter hosts would also be more likely to respond to a pharmacological agent, such as interferon-γ (27), that enhances the activity of the ubiquitin-dependent proteasomal system.

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Footnote 7: H. Teckman, D. Qu, and D. H. Perlmutter, unpublished observation.
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