The Endoplasmic Reticulum Degradation Pathway for Mutant Secretory Proteins α1-Antitrypsin Z and S Is Distinct from That for an Unassembled Membrane Protein*

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We have theorized that a subset of PIZZ α1-antitrypsin (α1-AT)-deficient individuals is more susceptible to liver injury by virtue of second inherited trait(s) or environmental factor(s), which exaggerate the accumulation of mutant α1-AT Z within the endoplasmic reticulum (ER) of liver cells. Using a complementation approach in which cell lines from PIZZ individuals with liver disease (“susceptible” hosts) and from PIZZ individuals without liver disease (“protected” hosts) are transduced with the mutant α1-AT Z gene, we have recently shown that there is a delay in ER degradation of mutant α1-AT Z protein that is only present in cell lines from susceptible hosts and correlates with the liver disease phenotype. In the present study we examined the specificity of this ER degradation pathway to determine if it is responsible for degrading other misfolded mutants of α1-AT and/or for unassembled membrane proteins. The S mutant of α1-AT and H2a subunit of the asialoglycoprotein receptor (ASGPR H2a) were expressed in skin fibroblast cell lines from susceptible and protected hosts. The results showed in both susceptible and protected hosts that α1-AT S was associated with a delay in secretion as compared with wild type α1-AT. The α1-AT S mutant was retained in ER, albeit to a lesser extent than the α1-AT Z mutant. There was, however, a significant increase in retention of α1-AT S in the ER of susceptible as compared with protected host cells. The same host cell lines were transduced to express an unassembled membrane protein, ASGPR H2a. There was no difference in the kinetics of ER degradation of ASGPR H2a in susceptible as compared with protected hosts. Taken together, the results show that α1-AT S is associated with a defect in biogenesis, intracellular retention, which is similar to but milder than α1-AT Z. Like α1-AT Z, α1-AT S is degraded by a pathway in the ER, which is relatively inefficient in PIZZ individuals with the liver disease phenotype. However, this pathway appears to be different from that previously described for a model unassembled membrane protein.

Homozygous PIZZ α1-AT deficiency affects approximately 1 in 1800 live births and is the most common genetic cause of liver disease in children (reviewed in Ref. 1). It is also associated with chronic adult onset liver disease and hepatocellular carcinoma and the premature development of pulmonary emphysema in adults (2, 3). The α1-AT Z molecule is characterized by a single nucleotide substitution, which results in substitution of lysine for glutamate 342. An abnormally folded mutant α1-AT Z protein is retained in the ER of liver cells, and there is a reduction in plasma concentrations of α1-AT to 10–15% of normal concentrations.

Liver injury in PIZZ α1-AT deficiency is thought to result from the toxic effects of the abnormal α1-AT molecule that is retained within the endoplasmic reticulum of hepatocytes (4, 5). However, prospective studies have shown that only 10–15% of individuals with this same ZZ genotype develop liver disease (6, 7). We have theorized that a subset of PIZZ α1-AT deficient individuals is more susceptible to liver injury by virtue of second inherited trait(s) or environmental factor(s), which exaggerate the accumulation of mutant α1-AT Z within the ER of liver cells. Using a complementation approach in which skin fibroblast cell lines from PIZZ individuals with liver disease (“susceptible” hosts) and from PIZZ individuals without liver disease (“protected” hosts) are transduced with the mutant α1-AT Z gene, we have recently shown that there is a delay in ER degradation of mutant α1-AT Z that is only present in cell lines from susceptible hosts and correlates with the liver disease phenotype (8).

There is now a considerable body of evidence that secretory proteins and membrane proteins which are abnormally folded because of mutations or unassembled because they are expressed in excess of other subunits with which they ordinarily interact, are degraded early in the pathway of biogenesis (reviewed in Ref. 9). In fact, many of these are degraded in the ER by a nonlysosomal proteolytic system. It is not yet clear, however, whether ER degradation is mediated by a single proteolytic pathway, several distinct proteolytic pathways, or several pathways that have both overlapping common and nonoverlapping steps. It is also not clear whether there is segregation of secretory and membrane proteins, mutant and unassembled proteins.

With these considerations in mind, in the current study we examined the specificity of the pathway that is responsible for ER degradation of mutant α1-AT Z. Another mutant α1-AT molecule, α1-AT S, and an unassembled membrane protein, ASGPR H2a, were expressed in prototype protected cell lines (competent for ER degradation) and susceptible cell lines (resistant to ER degradation) in the ER of liver cells. This article must therefore be hereby marked "costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: α1-AT, α1-antitrypsin; ER, endoplasmic reticulum; ASGPR, asialoglycoprotein; RSV, Rous sarcoma virus; LTR, long terminal repeat; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; endo H, endoglycosidase H; CFTR, cystic fibrosis transmembrane conductance regulator.
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Site-directed Mutagenesis, Plasmid Constructs, and Generation of Amphotrophic Recombinant Retroviral Particles—Based on the informative results in our previous study (8), we used amphotrophic recombinant retroviral particles to transduce the designated gene into skin fibroblast cell lines from protected and susceptible hosts. In each case, the gene of interest was initially subcloned downstream of the Rous sarcoma virus long terminal repeat in plasmid pMON1375B. The RSV LTR directs levels of transcription in human skin fibroblast cell lines that are moderate enough to facilitate detection of the encoded gene product but not high enough to overwhelm the post-transcriptional, translational, and post-translational processing pathways in a way that would confound interpretation of comparisons. The designated gene, together with its promoter, was then subcloned into the recombinant retroviral plasmid pN2.

α-1-AT M and Z cDNAs were constructed and sequenced as described previously (8, 12). The α-1-AT S cDNA was constructed by polymerase chain reaction-based mutagenesis using the overlap extension technique and the wild type α-1-AT M cDNA as a template (13). The oligonucleotides used in the polymerase chain reaction were constructed such that the outside 5′-sense flanking oligonucleotide primer contained a new XbaI restriction site, and the outside 3′-flanking primer contained a new PstI restriction. The inside oligonucleotide primers were designed to mutate base 867 from A to T and, therefore, generate the S mutant of α-1-AT. The resulting polymerase chain reaction product was isolated by standard techniques. The RSV LTR was then digested from the BamHI sites of vector pMON 1375B and ligated into the BamHI site of a PEG424-based shuttle vector. Next, the α-1-AT S cDNA was ligated into the XbaI and PstI sites of the shuttle vector downstream from the RSV LTR. The RSV LTR-α-1-AT cDNA insert was then isolated and ligated into the Xhol site of retrovector pN2 (14).

Orientation was checked by endonuclease digestion analysis. DNA sequence analysis of the α-1-AT S gene within the pN2 vector confirmed the presence of the mutation specific for α-1-AT S and the absence of other inadvertent mutations.

Packaging cell lines (15) Psi2 and PA317 were used for production of amphotrophic recombinant retroviral particles, as described (8).

The retroviral vector pRA2 containing the ASGPR H2a cDNA was obtained as a generous gift from Dr. Michael Shia (Boston University). This plasmid contains ASGPR H2a cDNA cloned downstream of the murine leukemia virus LTR in the retroviral vector pDOL (11). It was already shown to direct moderate levels of transcription of ASGPR in murine fibroblast cell lines. This vector was used to produce amphotrophic recombinant retroviral particles in the PA317 packaging cell line exactly as described for the α-1-AT constructs above.

Human Skin Fibroblast Cell Lines—Punch skin biopsies from the forearm of PIZZ individuals were explanted to allow for proliferation of skin fibroblasts. Before they were in the second or third passage, each of the fibroblast cell lines was transduced with amphotrophic recombi-

RESULTS

Biogenesis of α-1-AT S in Skin Fibroblast Cell Lines from Protected and Susceptible Hosts—First, a prototype protected cell line and a prototype susceptible cell line were transduced with the α-1-AT Z gene to re-establish the validity of the study. These cell lines were subjected to pulse-chase radiolabeling, and the results were analyzed by immunoprecipitation followed by SDS-PAGE/fluorography (Fig. 1). In each case, α-1-AT Z is synthesized as a ~52-kDa partially glycosylated polypeptide as shown at time 0 of the chase period. This polypeptide is retained in the intracellular contents over 1 h of the chase period. In the protected host cell line, it begins to disappear by

FIG. 2. Kinetics of secretion of α-1-AT Z in fibroblast cell lines from a protected versus a susceptible host. Separate monolayers were subjected to pulse-chase radiolabeling. At the end of each time interval of the chase period, as indicated at the top, extracellular fluid (EC) was harvested, and cell lysates (IC) were prepared. Each sample was subjected to immunoprecipitation and immunoprecipitates analyzed by SDS-PAGE/fluorography. The relative electrophoretic mobility of the 52-kDa α-1-AT Z polypeptide is indicated at the left margin as S2. There were no significant differences between the protected and susceptible cell line in total trichloroacetic acid-precipitable radioactivity or in α-1-AT-specific radioactivity at time 0 of the chase period.

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2 h and has almost completely disappeared by 3 h of the chase period as contrasted to the susceptible host cell line, in which this polypeptide is still present in substantial amounts in the cell lysates by 5 h of the chase period. There is negligible mature α1-AT secreted by these 2 cell lines. The wild type M α1-AT is ordinarily synthesized as a 52-kDa polypeptide, converted into a 55-kDa fully glycosylated form within 30 min, and secreted into the extracellular medium with a half-time of approximately 40–50 min (8). These results, indeed, provide confirmation of our previous studies showing that α1-AT is subject to intracellular degradation, less efficiently in cells from susceptible than in those from protected hosts. There was no evidence for expression of α1-AT in the parent untransduced cell line (data not shown) and no difference in the fate of other endogenous fibroblast proteins in the transduced cell lines (data not shown).

We could now take the same prototype protected and susceptible cell lines and subclone them for transduction of the α1-AT S gene. Stable transduced cell lines were selected in G418 and subjected to the same pulse-chase radiolabeling protocol (Fig. 2). In each case, the α1-AT S protein is also synthesized as a 52-kDa partially glycosylated polypeptide as shown at time 0 (intracellularly). It is also retained over the first hour of the chase period. In the protected host, it is converted to the 55-kDa intermediate and rapidly secreted by 2 h of the chase period. Analysis of three separate experiments in this particular cell line by densitometry of fluorograms demonstrated a half-time for appearance in the extracellular medium of 120 min (data not shown). These results indicate that the defect in biogenesis of α1-AT S is similar to, but milder than, that of α1-AT Z. The kinetics of secretion of α1-AT S (t_{1/2} = 120 min) are delayed when compared with wild type α1-AT M (t_{1/2} = 40–45 min), but a significantly greater proportion of newly synthesized α1-AT S is secreted when compared with α1-AT Z (compare Fig. 2 with Fig. 1).

In the susceptible host, the kinetics of secretion of the 55-kDa α1-AT were similar to that in the protected host (Fig. 2, bottom panel). Analysis of three separate experiments in this cell line by densitometry of fluorograms demonstrated a half-time for appearance in the extracellular medium that was also 120 min (data not shown). However, a significant proportion of the newly synthesized α1-AT was retained within the cells at 3 h of the chase period and was still apparent by 5 h of the chase period. These results indicate that there is a lag in intracellular degradation of retained α1-AT S in the susceptible host. Because there is a lesser degree of intracellular retention for α1-AT S than for α1-AT Z (compare Fig. 2 with Fig. 1), the difference in rate of intracellular degradation is apparently less severe.

To ensure that this difference was significant, two types of controls were done. First, we examined the rate of disappearance of endogenous secretory proteins from the same two cell lines. In fact, the same pulse-chase experiment was subjected to sequential immunoprecipitation for α2-macroglobulin. There was no difference between the protected and susceptible cell lines in rate of disappearance of α2-macroglobulin. There was also no difference between these two cell lines in rate of disappearance of complement protein factor B (data not shown) or of unidentified radiolabeled polypeptides seen in SDS-PAGE/fluorography of total cell lysates (data not shown).

Second, we examined the kinetics of disappearance of α1-AT S in the same two cell lines in multiple experiments as well as in cell lines from several other protected and susceptible hosts (Fig. 4). The results show that there is a progressive disappearance of α1-AT S in the protected hosts over 3–4 h. α1-AT-specific radioactivity has completely disappeared by 3 h in one protected host and by 4 h in two other protected hosts. In the susceptible hosts there is a decrease in the rate of disappearance of α1-AT S, which first becomes apparent by 2 h of the chase period. The difference is most apparent at 4–5 h, where approximately 15–20% of the initial α1-AT S is still present in the susceptible host but absent in the protected host. The difference is still lesser in magnitude than that for α1-AT Z (Fig. 1 and Ref. 6) probably because a greater proportion of the initially synthesized α1-AT has successfully traversed the secretory pathway and has reached the extracellular fluid in the case of α1-AT S. Densitometric analysis of α1-AT S in the extracellular fluid in all of these experiments did not reveal any significant differences between protected and susceptible cell lines in amount or rate of appearance. The half-time for appearance of α1-AT in the extracellular medium was again 120 min (data not shown). This suggests that a lag in, or decrease in rate of, intracellular degradation is the major determinant of the apparent retention of α1-AT S in susceptible hosts as compared with protected hosts in Fig. 4.

Intracellular Localization of α1-AT S in Cell Lines from Protected and Susceptible Hosts—To determine whether the 52-kDa α1-AT S is retained in ER, we used biochemical and morphological techniques to determine its intracellular localization. For the biochemical technique we subjected samples from pulse-chase experiments to endo H digestion (Fig. 5). The results show that the 52-kDa α1-AT S polypeptide is sensitive to endo H digestion throughout the chase period of 5 h. It is converted to a 46-kDa polypeptide, which comigrates with the product of endo H digestion of HepG2 cell lysates (right panel). The small amount of 55-kDa α1-AT S that is detected in the cells and the larger amount that is secreted into the medium are resistant to endo H, just as in HepG2 (right panel). These results indicate that the 52-kDa α1-AT S molecule is retained in a pre-Golgi compartment. The smaller amount of 52-kDa...
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**Fig. 4.** Densitometric analysis of the kinetics of secretion of α1-AT S in fibroblast cell lines from protected and susceptible hosts. Fibroblasts from two susceptible hosts and three protected hosts were transduced with Nα-1-AT S. Each of the transduced cell lines was subjected to pulse-chase radiolabeling. Fluorograms with approximately equivalent amounts of radiolabeled α1-AT at time 0 of the chase period were subjected to densitometric scanning. The result for each time point was plotted as percent α1-AT-specific radioactivity remaining as compared with 0. For each cell line, at least one experiment that met the above criteria is shown. For two of the cell lines, three experiments are shown as mean ± S.D.

**Fig. 5.** Effect of endo H on α1-AT S that has accumulated in fibroblast cell lines from the susceptible host. Fibroblasts and HepG2 cells were subjected to pulse-chase radiolabeling for the time intervals shown at the top, and the resulting cell lysates (IC) and extracellular fluid (EC) were subjected to mock digestion (−) or endo H digestion (+) after immunoprecipitation. The relative electrophoretic migrations of 55- and 52-kDa α1-AT S polypeptides and the 46-kDa product of endo H digestion are indicated at both margins as 55, 52, and 46*.

α1-AT S that is retained in the protected host cell line is also sensitive to endo H digestion (data not shown).

For the morphological technique, we subjected the cell lines to immunofluorescence analysis (Fig. 6). There was no significant staining when the secondary antibody was omitted (data not shown), when primary antibody was omitted (panel 1), or when untransfected fibroblasts from the susceptible host were stained with both primary and secondary antibody (panel 2). In the susceptible cell line expressing α1-AT S, antibody to α1-AT stained intensely in a reticular pattern especially prominent in the perinuclear region (panel 3). This pattern of staining was identical to that of antibody to calnexin, a known ER resident protein (panel 5), but clearly distinct from that of anti-58K, a known Golgi protein that has a more localized, polar perinuclear staining pattern (panel 7), and anti-cathepsin D, a known lysososomal protein that has a punctate staining in the peripheral cytoplasm (panel 8) in the same cell line. Staining with anti-α1-AT showed an identical pattern in fibroblasts from the same susceptible host that expresses α1-AT Z (panel 4) as those that express α1-AT S (panel 3), that is, a reticular pattern especially prominent in the perinuclear region. Taken together with the endo H results, these results indicate that the 52-kDa α1-AT S polypeptide is retained in ER of susceptible host fibroblasts in a manner very similar to α1-AT Z. The small amount of α1-AT S that is retained in the protected host fibroblasts also has an “ER” pattern of staining (panel 6).

Degradation of ASGPR H2a Subunit in Skin Fibroblast Cell Lines from Protected and Susceptible Hosts—Next, we examined the possibility that there is overlap in the pathway responsible for ER degradation of α1-AT Z and α1-AT S and that for a model unassembled membrane protein, ASGPR H2a subunit. The same prototype susceptible and protected fibroblast cell lines were transduced with the ASGPR H2a cDNA, and the fate of newly synthesized ASGPR H2a were compared in pulse-chase experiments (Fig. 7). The results show that in the protected host ASGPR H2a is initially synthesized as a 43-kDa polypeptide. After a lag of 0.5 h, the 43-kDa polypeptide begins to disappear coincident with the appearance of a 35-kDa proteolytic fragment. The 43-kDa polypeptide has completely disappeared by 2 h of the chase period, at which time the 35-kDa polypeptide begins to disappear. The 35-kDa polypeptide is almost completely degraded by 3.5 h of the chase period. These results are almost identical to previous results in NIH 3T3 fibroblasts (11, 20). In the susceptible cell line, the kinetics of disappearance of the 43-kDa ASGPR H2a polypeptide, of its conversion to the 35-kDa fragment and of the disappearance of the 35-kDa polypeptide, were similar to that in the protected cell line. The results of these experiments, together with experiments in another pair of prototype susceptible and protected cell lines were subjected to semi-quantitative analysis as shown in Fig. 8. The results show that there is no significant difference in the kinetics of degradation of ASGPR H2a in susceptible as compared with protected cell lines. These results also provide evidence for the specificity of the differences in the kinetics of degradation of α1-AT Z and α1-AT S in the same host cell lines shown in Figs. 1, 2, and 4.

**DISCUSSION**

In this study, we used a unique cell system to examine the specificity of the pathway for ER degradation of mutant α1-AT Z and to determine whether there is a common ER degradation pathway for mutant secretory proteins and membrane proteins. Our previous study had shown that skin fibroblast cell lines from α1-AT-deficient individuals with liver disease (susceptible hosts) are relatively inefficient in ER degradation of α1-AT S as compared with cell lines from PiZZ individuals without liver disease (protected hosts). Prototype susceptible and protected fibroblast cell lines were used here to compare the fate of another mutant secretory protein, α1-AT Z, and a model unassembled membrane protein, ASGPR H2a, which are retained in the ER. The results showed that there is a lag in degradation of α1-AT S in susceptible hosts and that a 52-kDa partially glycoylated intermediate of α1-AT S is retained in the ER in cell lines from these hosts. The lag in ER degradation of α1-AT S in susceptible cells is lesser in magnitude than that for α1-AT Z because a lesser proportion of newly synthesized α1-AT S is retained in the ER, the majority being secreted into the extracellular fluid. In contrast, there is no lag in degradation of the unassembled membrane protein ASGPR H2a in the susceptible cells. The kinetics of an initial endopeptolytic cleavage of ASGPR H2a and the kinetics of disappearance of the major endopeptolytic fragment of ASGPR H2a were identical in cell lines from protected and susceptible hosts.

The results of these studies also provide further information about the defect in biogenesis of α1-AT S variant. Like α1-AT Z, this variant is associated with a single nucleotide substitution that results in replacement of a glutamate residue at a potential salt bridge. For α1-AT Z, Glu-352 is replaced by Lys; for α1-AT S, Glu-264 is replaced by Val. In the case of α1-AT Z, there is an 85–90% reduction in plasma concentrations of α1-AT, deposition of α1-AT molecules in the ER of liver cells...
that is easily detected by crude morphological techniques, and predisposition to liver injury, at least in a subgroup of the affected individuals. In the case of α1-AT S, there is only 40–50% reduction in plasma α1-AT concentrations, and the variant is not apparently associated with a predisposition to liver injury. The mechanism by which the α1-AT S variant results in reduced plasma concentrations is not entirely understood. In one study of human monocytes and transfected murine fibroblasts, it was attributed to increased intracellular degradation (21). However, relatively little kinetic data were provided in that study. In a more recent study, Brodbeck et al. (22) used Cos cells transiently transfected with α1-AT S cDNA to show, over a relatively short chase period (2 h), that there is a mild degree of increased intracellular retention when compared with Cos transfected with the wild type α1-AT M cDNA (22). In this study using human skin fibroblasts that stably express α1-AT S and longer chase time intervals, there is increased intracellular retention, and the 52-kDa partially glycosylated form of α1-AT S is found in ER. These results indicate that the defect in biogenesis of α1-AT S is similar to, but milder than, that of α1-AT Z. However, from this data we cannot necessarily conclude that the defect in biogenesis completely accounts for the 40–50% reduction in plasma α1-AT concentrations that is associated with the α1-AT S variant. For instance, it is still possible that α1-AT S is more rapidly removed from the circulation in vivo than is wild type α1-AT and that decreased in vivo catabolism also contributes to the reduced plasma levels.

Until recent years, degradation of cellular proteins has been attributed to two major systems, the lysosomal proteases and the cytosolic ubiquitin-dependent proteasome. Several studies in which subunits of multiprotein membrane proteins, truncated membrane proteins, and mutant secretory proteins are expressed in model transfected cell systems have now suggested the presence of a distinct degradation pathway in the ER (9). Examples in which ER degradation has been invoked include asialoglycoprotein receptor subunits (11, 20), the T cell receptor α-subunit (23), carboxyl-terminally truncated forms of ribophorin I (24), CFTR wild type and mutant ∆F508 (25, 26), and mutant α1-AT Z (8, 27). In one case, ER degradation is a mechanism for regulating the expression of a key metabolic enzyme that resides in the ER, 3-hydroxy-2-methyl-coenzyme A reductase (28). In a particularly well characterized example, the H2a subunit of ASGPR is degraded in the ER and that there is an increase in ER degradation of the CFTR mutant F508 (25, 26). A series of more recent studies has now demonstrated that this is, at least in part, attributable to the action of the ubiquitin-dependent proteasome (29, 30). Taken together, these studies have indicated that there are at least two, and perhaps more, pathways for ER degradation. At least one of these pathways is, in fact, not a distinct ER proteolytic system but rather the cytosolic ubiquitin-dependent proteasomal system acting on
the cytoplasmic domains of transmembrane proteins. These studies do not address, and thus do not exclude, the possibility that parts of each ER degradation pathway are distinct and other parts overlapping. In our current study we took advantage of cell lines in which a lag in ER degradation had been previously identified to map parts of the ER degradation pathways that were overlapping. We were especially interested in the comparison of α-1-AT variants Z and S because these two mutant secretory proteins are retained in ER, albeit to different extents. The results showed that the same pathway is responsible for ER degradation of these two mutant proteins. We were also interested in the comparison of α-1-AT variant Z and the unassembled membrane protein ASGPR H2α because previous studies have shown that tosylphenylalanyl chloromethyl ketone inhibited the ER degradation of α-1-AT H2α (20). The results showed that α-1-AT Z and α-1-AT S are degraded by an ER pathway that is at least in part different from that for ASGPR subunits. This does not necessarily mean that ER degradation pathways for mutant secretory proteins are segregated from that, or those, of all unassembled or mutant membrane proteins. Nor do these data exclude the possibility that there are distinct parts of the ER degradation pathways for secretory and membrane proteins that overlap. It will now be possible to use the system described here to identify overlapping steps in the ER degradation pathway for α-1-AT mutants Z and S and other classes of proteins that are retained/degraded in the ER, including other mutant secretory proteins, unassembled secretory proteins, wild type membrane proteins that are resident in ER including HMG CoA reductase and CFTR, mutant membrane proteins such as CFTRΔF508, and viral proteins such as hepatitis B virus surface antigen.

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