Inhibition of Intracellular Degradation Increases Secretion of a Mutant Form of α1-Antitrypsin Associated with Profound Deficiency

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Abstract

The mutant Z form of α1-antitrypsin (α1AT) is responsible for > 95% of all individuals with α1AT deficiency, an important inherited cause of emphysema and liver disease. Since secreted Z α1AT is a functional antiprotease, we hypothesized that interrupting catabolism of retained Z α1AT might increase its transport out of cells, causing an increase in extracellular protease protection. Both the protein translation inhibitor cycloheximide and the specific inhibitor of proteasome function, lactacystin, prevented intracellular degradation of Z α1AT. Moreover, this inhibition of degradation was associated with partial restoration of Z α1AT vesicular transport. This effect was observed in a model system of transfected CHO cells as well as in human alveolar macrophages synthesizing Z α1AT. This study supports the hypothesis that altering the intracellular fate of a mutant protein may be an option in the treatment of diseases associated with misfolded but potentially functional proteins. (*J. Clin. Invest.* 1998. 101:2693–2701.) Key words: α1-antitrypsin • degradation • proteasome • cirrhosis • emphysema

Introduction

α1-Antitrypsin (α1AT)[1] deficiency, an autosomal recessively inherited disease, affects up to 1 in 2,500 Caucasians. The classic disorder is associated with early onset of emphysema and premature death. Less frequently, α1AT deficiency causes severe liver disease, particularly in children. Currently liver transplant is the only effective therapy for these children (1, 2). Individuals with intermediate α1AT levels appear entirely normal. α1AT, a glycoprotein secreted in abundance by hepatocytes and mononuclear phagocytes, serves as the major serum antiprotease responsible for critical protease–antiprotease balance in the lung and elsewhere (1, 2).

Although there are more than 30 variants that cause α1AT deficiency, the Z form exists in > 95% of all α1AT-deficient individuals (1–3). The Z protein differs from the normal M variant in the replacement of a glutamic acid by lysine near the base of the reactive site loop; this replacement disturbs a salt bridge between two strands in β sheet A of α1AT (1, 4–8). The amino acid substitution also leads to a delay in protein folding and subsequent linkage of the Z α1AT molecules via loop–sheet polymerization (2, 7–9). The intracellular fate of Z α1AT has been extensively studied by several laboratories (5, 7, 10–14). Although ~ 15% of Z α1AT is secreted and has half the function of normal M α1AT, the vast majority remains in the RER and is degraded before entering the Golgi apparatus (1, 2, 5, 6, 12–17). Intracellular degradation of Z α1AT in a transfected rat hepatocyte system is nonlysosomal, energy-dependent, and sensitive to inhibitors of protein translation (12, 15). Recently, inhibitors of multicatalytic proteasomes have been implicated in the catabolism of intracellular Z α1AT in transfected human fibroblasts (18). Several types of α1AT-secreting cells have been used to determine the molecular basis of α1AT variants, including transfected or infected mouse hepatoma cells, COS1 cells, NIH 3T3 cells, human fibroblasts, alveolar macrophages, and recently, CHO cells (5, 10, 12, 13, 16, 18–20). CHO cells do not synthesize α1AT mRNA, and have been used extensively in studies of glycoprotein transport (10, 19). In this study, we demonstrate increased secretion of functional Z α1AT after inhibition of its intracellular degradation in a model CHO cell system and human alveolar macrophages expressing the Z α1AT gene.

Methods

Plasmid construction and in vitro model system. To determine the molecular mechanisms associated with the profound reduction of secreted Z α1AT, we used CHO cells (CCL61; American Type Culture Collection, Rockville, MD) transfected with eukaryotic expression vectors pRc/RSV (Invitrogen, San Diego, CA) containing the normal M or Z gene. Oligonucleotide site-directed mutagenesis and PCR were used to generate the Z mutation using the normal α1AT cDNA as a template. Intron II of the α1AT genomic sequence was introduced into its native position to increase gene expression. Transfected cells were selected using G418 (GIBCO BRL, Gaithersburg, MD), and G418-resistant colonies were pooled to establish a polyclonal population of transfected cells. For all experiments, the transfected cells were at 80–90% confluence (19, 20).

Metabolic labeling studies. Nearly confluent monolayers of CHO cells expressing Z or M α1AT in 100-mm–diameter culture dishes were incubated for 30 min with [^{35}S]methionine (200–500 μCi/ml of medium; ICN Pharmaceuticals Inc., Irvine, CA), and were then chased for 0–12 h by incubation in 3 ml of Ham's F-12 (Biofluids, Inc., Rockville, MD) with 10% FCS containing a fivefold excess of unlabeled methionine. Subconfluent culture plates with similar numbers of cells were labeled with the same amount of [^{35}S]methionine, and the radiolabeled plates of cells were randomly chosen for either treatment or no treatment with cycloheximide. To determine rates and mechanisms of intracellular degradation, the following compounds were included in the chase medium: cycloheximide (50 μg/ml), an inhibitor of protein synthesis, or the metabolic poisons: 40 mM NaN_3, 10 mM NaF, 5 mM 2,4-dinitrophenol, and 10 mM 2-deoxy-D-glucose.

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1. *Abbreviation used in this paper:* α1AT, α1-antitrypsin.

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Brefeldin A (2 μg/ml), an inhibitor of normal RER-to-Golgi transport, or inhibitors of lysosomal function, 50 mM NH_4Cl, 0.1 mM chloroquine, 0.1 mg/ml leupeptin, or 10^{-5} M colchicine (Sigma Chemical Co., St. Louis, MO), were present for the entire labeling and chase period (12, 21). Alveolar macrophages obtained from bronchoalveolar lavage of individuals with PI*Z α1AT deficiency evaluated at the National Institutes of Health Clinical Center as part of an institutional review board approved protocol. Alveolar macrophages were incubated for 20 h in 100-mm–diameter culture dishes (7.5×10^6 cells) in RPMI 1640 medium (Biofluids, Inc.) with 10% FCS, pulse-radiolabeled with [^{35}S]methionine (500 μCi/ml of medium) for 30 min at 37°C, and chased for up to 6 h with or without 50 μg/ml cycloheximide. Alveolar macrophages and CHO cells were treated with 10 μM lactacystin for 2 h before pulse-radiolabeling studies, and were maintained at the same concentration throughout the pulse and chase periods (22–24). Cells were harvested in a total volume 1.25 ml of lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 10 mM EDTA, and 1 mM PMSF in 1× PBS, pH 7.6; Sigma Chemical Co.), frozen in dry ice, and centrifuged to precipitate cell debris. α1AT was immunoprecipitated from cell lysate or medium using rabbit anti–human α1AT antibodies (Accurate Chemical and Scientific Co., Westbury, NY; or DAKO Corp., Carpinteria, CA) bound to protein A Sepharose CL-4B (Pharmacia Biotech AB, Uppsala, Sweden). Immunocomplexes were washed, suspended in 50 μl of sample buffer (63 mM Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.0025% bromophenol blue), heated at 95°C for 5 min, and analyzed using SDS Tris-glycine 4–12% PAGE (Novex, San Diego, CA; 5, 19, 20). Radiolabeled α1AT was detected by autoradiography and quantified using a densitometer (Molecular Dynamics, Sunnyvale, CA). Immunoprecipitates from lysates after 1 h of chase were treated with 2 mU endoglycosidase-H (Oxford Glyco Sciences, Inc., Wakefield, MA) at 37°C for 20 h. Similar immunoprecipitates were analyzed without endoglycosidase-H treatment (19, 20).

Viability of cultured cells was determined by trypan blue exclusion and/or by counting the number of detached cells. Throughout the metabolic studies, CHO cell and alveolar macrophage death did not exceed 1% of total cell number.

To determine if lactacystin and cycloheximide caused cytotoxic-mediated leakage of normally cytosolic proteins, the 29-kD intracellular resident protein neomycin phosphotransferase, the gene product that confers G418 resistance in the transfected CHO cell line, was used as a marker for extracellular leakage of cytosolic protein content. CHO cells expressing Z α1AT were incubated in the presence of 50 μg/ml cycloheximide or 10 μM lactacystin for 0–10 h under conditions identical to those used to characterize the intra- and extracellular fate of α1AT. Cell lysates and medium were immunoprecipitated with antibody to the 29-kD intracellular resident protein neomycin phosphotransferase (5 Prime→3 Prime Inc., Boulder, CO) and separated according to apparent molecular mass by a 4–12% SDS-PAGE. No radiolabeled neomycin phosphotransferase was detected in medium of transfected CHO cells under conditions used to characterize the intracellular fate of Z α1AT, indicating that cytotoxicity and intracellular release of cytosolic proteins did not contribute to the total amount of extracellular α1AT. In addition, in all experiments involving transfected CHO cells, no intracellular high-mannose 50-kD α1AT was detected in the medium.

Quantitative assessment of intra- and extracellular α1AT. To evaluate the effect of cycloheximide on α1AT intracellular transport in CHO cells, cells were lysed in a total volume 1.25 ml of lysis buffer, and α1AT was isolated by immunoprecipitating 0.5 ml of lysate (40% of total). The amount of α1AT secreted by CHO cells was determined by immunoprecipitating 0.5 ml medium from a total volume of 3 ml (17% of total). The amount of intra- and extracellular α1AT from alveolar macrophages treated with either lactacystin or cylcoheximide was determined by immunoprecipitating 1.25 ml (100% of total) lysate and 3 ml medium (100% of total). Since transfected CHO cells synthesize five to tenfold more α1AT than do alveolar macrophages, the amount of cell lysate and medium from CHO cells used to evaluate the effect of lactacystin was reduced to 0.25 ml and 0.6 ml, respectively (20% of total) to assure that all bands on the autoradiograms were in a similar range. The total intracellular and extracellular α1AT was estimated by multiplying the band densities by the appropriate ratio of medium or lysate total volume. Experiments that quantified the effects of cycloheximide and lactacystin on alveolar macrophages and CHO cells were performed in triplicate or more.

Functional assessment of secreted α1AT. To compare the function of Z and M α1AT, the samples of medium (0.5 ml) after 4 h of chase with or without treatment with either 50 μg/ml cycloheximide or 10 μM lactacystin, were concentrated (Microcon 30; Amicon, Inc., Beverly, MA) to 50 μl, and were incubated with an excess amount of human neutrophil elastase (0.45 ml of 100 nM neutrophil elastase solution; Athens Research and Technology, Inc., Athens, GA) in 0.1 M Hepes (pH 7.5), 0.5 M NaCl, and 0.1% Brij 35 for 2 h at 37°C. Complex formation was accomplished in slight excess of neutrophil elastase compared with α1AT. To generate this complex, we performed a pilot titration experiment using a fixed amount of labeled α1AT with increasing amounts of neutrophil elastase. Using this approach, we were able to titrate formation of complexes without causing nonspecific degradation of the complex by excess neutrophil elastase. Complexes were immediately immunoprecipitated by anti-α1AT, washed, suspended in 50 μl of SDS sample buffer (63 mM Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.0025% bromophenol blue), heated at 95°C for 5 min, and subjected to 4–20% SDS-PAGE.

Immunofluorescence. Transfected cells grown on coverslips were incubated with or without 50 μg/ml cycloheximide or 10 μM lactacystin, fixed with 2% paraformaldehyde, and treated serially with appropriate dilutions of primary antibodies (rabbit anti–human α1AT [Dako Corp.] and mouse anti-BiP [StressGen Biotechnologies Corp., Victoria, British Columbia], or anti-p58 and β-COP [Sigma Chemical Co.]) and secondary antibodies ([FITC-conjugated goat anti–rabbit and rhodamine-conjugated goat–anti-mouse; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA]) with 0.05% saponin and 1% BSA in PBS. Cells were washed, mounted on glass slides, and photographed using a fluorescence microscope (Nikon, Inc., Melville, NY) with a 60× objective, or with a TCS 4D laser scanning confocal microscope (Leica, Heidelberg, Germany) with a 100× objective.

Statistical analysis. Statistical analysis was performed using a paired two-tailed *t* test. Intra- and extracellular α1AT concentrations were expressed as the mean increase over baseline±SEM. The determination of half-lives of intracellular Z and M α1AT were performed in triplicate or more and are expressed as the mean±SEM.

Results

Vesicular transport of M and Z α1AT. Using a transfected CHO cell system, we compared the amount of intra- and extracellular α1AT in M- vs. Z-transfected cells under various conditions. Z α1AT accumulated in the intracellular compartment in an endoglycosidase H–sensitive, high-mannose oligosaccharide 50-kD form, and was secreted in amounts 4.3–6.7-fold less than those of M α1AT (Fig. 1, *A* and *B*). Since M, and to a lesser extent Z α1AT are secreted, brefeldin A, a drug that disrupts vesicular transport between the RER and Golgi, was used to evaluate the intracellular half-lives of M and Z α1AT. Using the best-fit logarithmic curve ($r^2 > 0.95$) on a log-linear plot, the intracellular half-life of Z α1AT in brefeldin A–treated cells (9.8±0.6 h) was significantly less than that of M α1AT (24±2.8 h; $P = 0.005$; see Fig. 1 *C*).

The metabolic inhibitors NaN_3 and NaF, or 2,4-dinitrophenol and 2-deoxy-D-glucose, used in combination to block the energy-dependent components of intracellular degradation, reduced degradation of Z α1AT by 50–60%. Inhibitors of lyso-

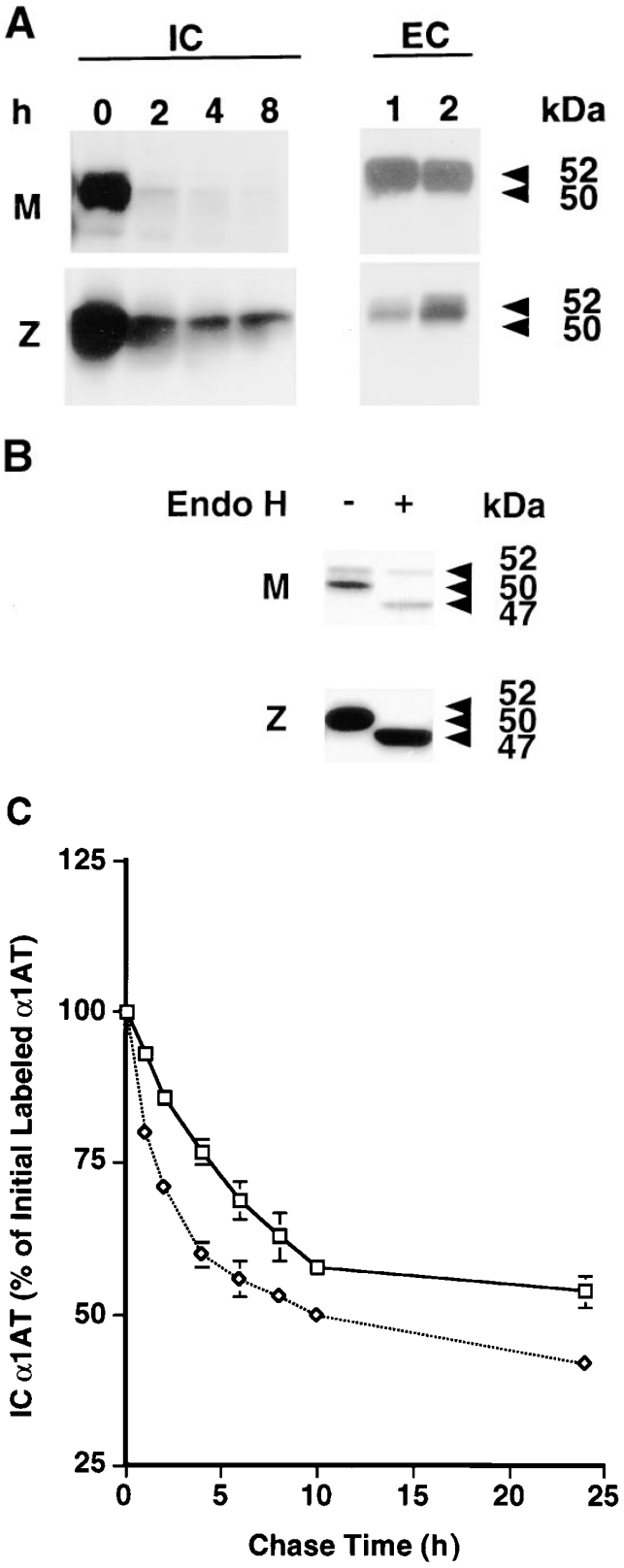


Figure 1. Vesicular transport of normal (*M*) and Z α1AT in CHO cells. (*A*) SDS-PAGE of radiolabeled intracellular and extracellular (secreted) M and Z α1AT. (*Top*) Intracellular (*IC*) and extracellular (*EC*) α1AT. Each lane represents chase time (*h*) from 0–8 h for IC and 1–2 h for EC. (*Left*) M and Z represent M and Z α1AT. (*Right*) The apparent molecular masses (kD) of the fully glycosylated 52-kD and high mannose 50-kD forms of α1AT. (*B*) SDS-PAGE of radiolabeled intracellular M and Z α1AT digested with endoglycosidase-H. (*Top*) Intracellular α1AT with (+) or without (−) endoglycosidase-H (*EndoH*). (*Left*) M and Z represent M and Z α1AT. (*Right*) The molecular masses (kD) of the fully glycosylated 52-kD, high-mannose 50-kD, and endoglycosidase-H–cleaved 47-kD forms of α1AT after 1 h of chase. (*C*) Intracellular half-lives of M and Z α1AT. Ordinate,% total radiolabeled intracellular (*IC*) α1AT after brefeldin A treatment. Abscissa, chase time in hours (*h*). M α1AT is the solid line (□), and Z α1AT is dotted line (◇). Positive and negative error bars are the SEM.

somal function, including NH_4Cl, chloroquine, leupeptin, and colchicine, did not affect the rate of Z α1AT degradation of Z α1AT (data not shown). Because of its long intracellular half-life, the effect of metabolic and lysosomal protease inhibitors on M α1AT could not be determined before significant cell death occurred.

Cycloheximide increases secretion of Z and M α1AT. Cycloheximide treatment of Z α1AT-transfected CHO cells resulted in a transient increased accumulation of intracellular Z α1AT followed by a 2.4±0.28-fold increase in its secretion by 8 h ($P = 0.018$; Fig. 2, *A* and *B*). Comparison of the total intra- and extracellular α1AT at time points after synthesis of the radiolabeled α1AT was completed demonstrated that > 80% of the newly synthesized α1AT is eventually exported to the extracellular compartment in the presence of cycloheximide. In the absence of cycloheximide, ~ 25% of the synthesized Z α1AT is exported extracellularly (Fig. 2, *C* and *D*). This effect was not limited to cycloheximide, since Z α1AT-expressing CHO cells treated with puromycin demonstrated similar results (data not shown). While cycloheximide did not measurably increase intracellular M α1AT, its secretion was ~ 34± 9.3% more than that from untreated cells ($P = 0.012$; Fig. 2, *E* and *F*). The rapid extracellular transport of M α1AT (~ 90 min) in both cycloheximide-treated and -untreated cells (Fig. 2 *F*) contrasts with the delay in reaching maximal secretion of Z α1AT in both cycloheximide-treated and -untreated cells (8 h and 4 h, respectively; Fig. 2 *B*). Interestingly, the intracellular half-life of Z α1AT after pretreatment with 2 μg/ml brefeldin A plus 50 μg/ml cycloheximide was similar to brefeldin A alone (9.8 and 10 h, respectively).

Cycloheximide increases secretion of functional Z α1AT. To estimate the functional quantity of extracellular Z α1AT, we compared the amount of SDS-stable complexes formed in the presence of an excess amount of neutrophil elastase, the major α1AT substrate, before and after treatment with cycloheximide. Importantly, secreted Z α1AT from cycloheximide-treated cells formed SDS-stable complexes with neutrophil elastase in proportions similar to those from untreated cells (14% from untreated cells compared with 15% from cycloheximide-treated cells) despite an increase in the total extracellular α1AT (Fig. 3). In addition to complex formation, 4-kD Z and M α1AT C-terminal fragments were identified, and demonstrated neutrophil elastase–mediated cleavage of the P1-P1′ bond (Fig. 3). Comparison of the percentages of complexed M and Z α1AT showed that M α1AT formed twice as many complexes with neutrophil elastase in proportions as Z α1AT (30 and 14%, respectively).

Cycloheximide alters intracellular localization of Z α1AT in CHO cells. Intracellular localization using immunofluores-

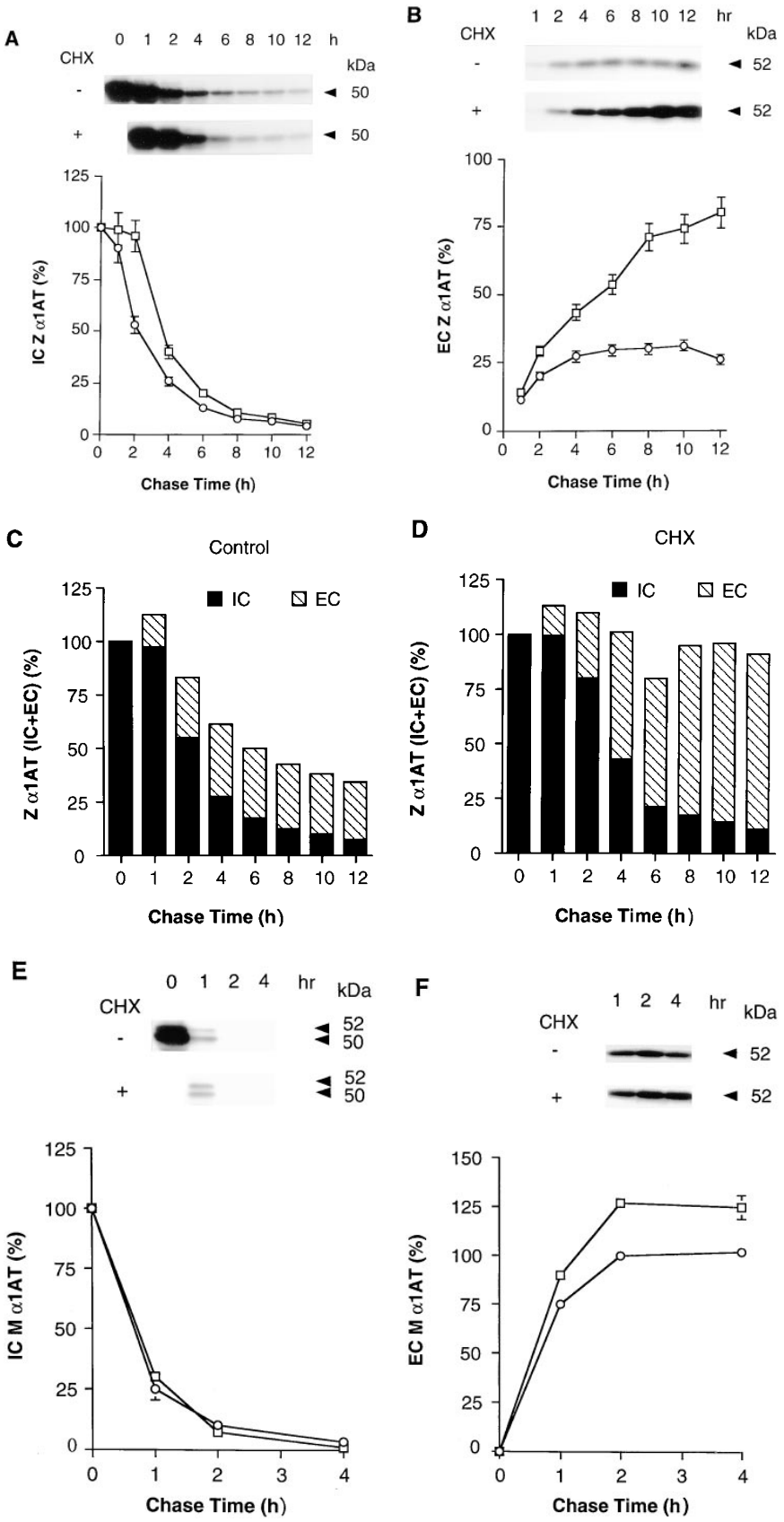


Figure 2. Effect of cycloheximide on vesicular transport of α1AT in CHO cells. (*A*) Intracellular (*IC*); (*B*) extracellular (*EC*); (*C*) total IC and EC; and (*D*) total IC and EC after treatment with cycloheximide Z α1AT at the indicated time of chase were quantified after SDS-PAGE. (*E*) Intracellular (*IC*) and (*F*) extracellular (*EC*) normal M α1AT at the indicated time of chase was quantified after SDS-PAGE. Symbols represent percentage of initial radiolabeled α1AT with (□) and without (○) cycloheximide (*CHX*), 50 μg/ml present during the chase (*bars* represent the SEM for values of three separate experiments). Inserts (*top*) are representative SDS-PAGE used for analysis. (*Right*) Molecular masses (kD) of the fully glycosylated 52-kD and high-mannose 50-kD forms of α1AT.

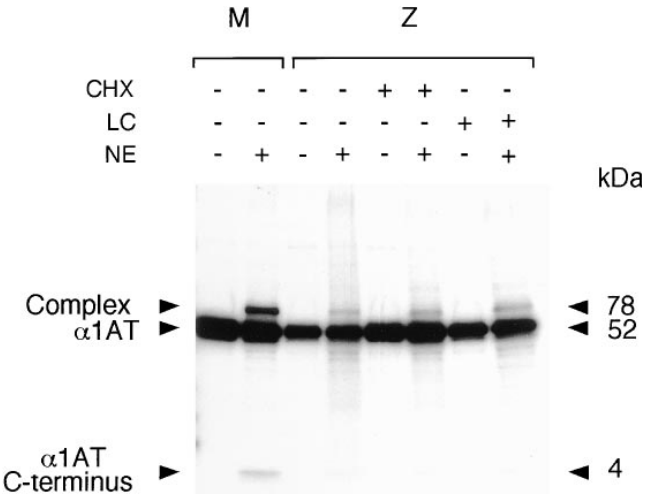
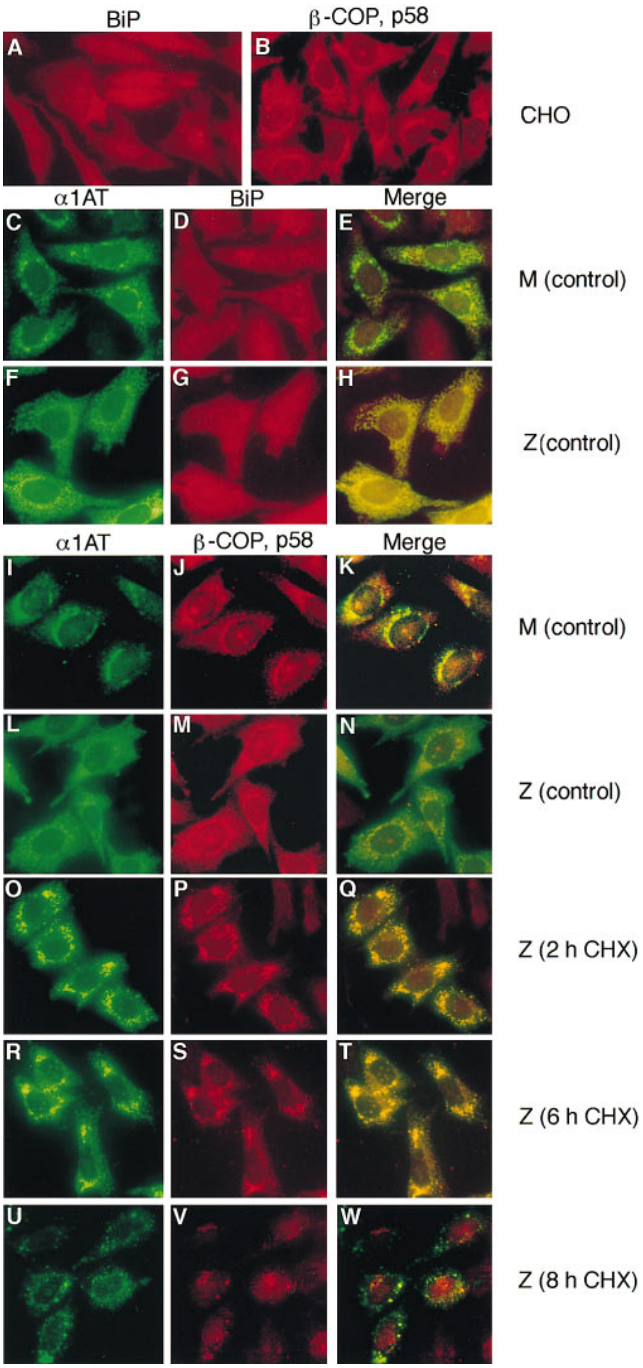


Figure 3. Functional comparison of M and Z α1AT with and without cycloheximide or lactacystin treatment. Autoradiogram of SDS-PAGE of 78-kD SDS-stable complexes of neutrophil elastase with M or Z α1AT secreted by CHO cells. The formation of SDS-stable neutrophil elastase-α1AT complexes indicates that the secreted α1AT is functionally able to inhibit its major target protease. (*Top*) Lanes represent secreted M and Z α1AT with (+) or without (−) treatment with cycloheximide (*CHX*) or lactacystin (*LC*), with (+) or without (−) neutrophil elastase (*NE*). (*Left*) Arrows indicate neutrophil elastase-α1AT complexes (*Complex*), unbound α1AT (*α1AT*), and the α1AT COOH terminus (α1AT C-terminus) after cleavage of the P1-P1′ bond. (*Right*) apparent molecular masses of 78-, 52-, and 4-kD, respectively. Autoradiogram is presented overexposed to demonstrate the 4-kD fragment with a specific activity fivefold less than that of intact α1AT since it contains only 2 of 10 methionines available for radiolabel incorporation.

cence microscopy of CHO cells transfected with α1AT demonstrated a distribution of Z α1AT that was very different from that of M α1AT. Whereas M α1AT was concentrated in a perinuclear area, consistent with a predominantly Golgi localization, Z α1AT was widely dispersed in an RER pattern (Fig. 4, *C–N*). Treatment with cycloheximide appeared to increase the immunofluorescence of Z α1AT briefly (presumably by concentrating in the RER and Golgi), and altered its distribution. Importantly, there was no difference in the patterns of localization of β-COP/p58 or BiP in untransfected CHO cells compared with those expressing α1AT (Fig. 4, *A–N*). Cycloheximide-treated cells demonstrated a dynamic image of Z α1AT vesicular movement, since cycloheximide prevents further translation of α1AT. Two h after cycloheximide treatment, Z α1AT concentrated in an RER-Golgi pattern. At 6 h, Z α1AT had moved into a perinuclear Golgi distribution. By 8 h, there was a significant loss of intracellular immunofluorescence as a result of Z α1AT secretion (Fig. 4, *O–W*). Similarly, treated cells expressing M α1AT lost their immunofluorescence within 90 min of treatment with cycloheximide (data not shown).



FITC staining detected for α1AT in untransfected cells. (*C–N*) Untreated CHO cells expressing M or Z α1AT were double-labeled with FITC-conjugated rabbit anti–human α1AT (*C*, *F*, *I*, and *L*; *green*), and (*D* and *G*) rhodamine-conjugated antibodies to the RER-resident protein BiP (*red*), or Golgi-resident proteins β-COP/p58 (*J* and *M*; *red*). Superimposing the red and green images (*E*, *H*, *K,* and *N*) demonstrates colocalization of α1AT with either BiP or p58/β-COP (yellow). (*O–W*) Z α1AT 2, 6, and 8 h after treatment with cycloheximide, respectively, double-labeled with FITC- conjugated rabbit anti–human α1AT (*O*, *R,* and *U*; *green*) and rhodamine-conjugated antibodies to the Golgi-resident proteins β-COP/p58 (*P*, *S,* and *V*; *red*). Superimposing red and green images demonstrates colocalization of α1AT with p58/β-COP (*Q*, *T,* and *W*; *yellow*). The time-dependent change in the pattern of α1AT fluorescence in *O–W* is consistent with normal vesicular movement of α1AT from the RER and secretion into the extracellular milieu. 60× objective.

Figure 4. Immunofluorescence photomicrographs showing localization of intracellular α1AT during vesicular transport in CHO cells. (*A*) Nontransfected CHO cells double-labeled with FITC-conjugated anti-α1AT and rhodamine-conjugated antibodies to the RER-resident protein BiP, or (*B*) Golgi-resident proteins β-COP/p58, the RER and Golgi complex staining patterns (*red*), but there was no

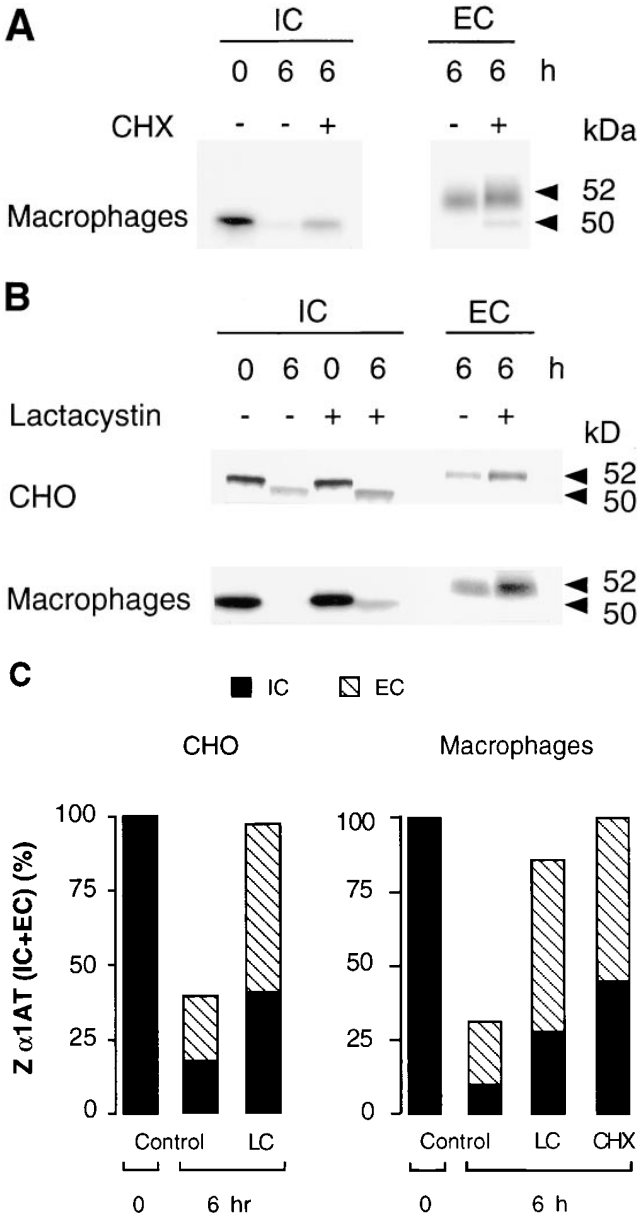


Figure 5. Effect of cycloheximide and the proteasome inhibitor lactacystin on intracellular transport of Z α1AT in human alveolar macrophages and CHO cells. (*A*) Autoradiogram of SDS-PAGE of radiolabeled intracellular (*IC*) and extracellular (*EC*) Z α1AT from human alveolar macrophages (*Macrophages*). Each lane represents chase time of 0 or 6 h for IC and 6 h for EC with (+) or without (−) cycloheximide (*CHX*). Treatment of alveolar macrophages expressing Z α1AT with cycloheximide significantly increases intracellular and extracellular α1AT compared with untreated cells. (*B*) Autoradiogram of SDS-PAGE of radiolabeled IC and EC Z α1AT from CHO cells and human alveolar macrophages with (+) or without (−) exposure to 10 μM lactacystin at 0 and 6 h of chase. On the right, arrows indicate the apparent molecular masses (kD) of the fully glycosylated endoglycosidase-H–resistant 52-kD and endoglycosidase-H–sensitive high-mannose 50-kD forms of α1AT. The 52-kD form of α1AT may be present in both the IC and EC, while the 50-kD form remains in the IC predominantly in the RER. (*C*) Total intracellular (*IC*) and extracellular (*EC*) Z α1AT at 0 and 6 h of chase in CHO and human alveolar macrophages (*Macrophages*) without (*Control*) and with lactacystin (*LC*) or cycloheximide (*CHX*).

Cycloheximide prevents degradation and increases secretion of Z α1AT in alveolar macrophages. Importantly, the effect of cycloheximide on Z α1AT secretion was not limited to α1AT-expressing CHO cells. When treated with cycloheximide, human alveolar macrophages synthesizing Z α1AT demonstrated 4.5±1.0-fold more intracellular Z α1AT ($P = 0.002$), and secreted 1.63±0.13-fold more Z α1AT ($P = 0.04$) than did untreated cells after 6 h of chase (Fig. 5 *A*). The presence of a small amount of the 50-kD Z α1AT form in the medium after 6 h of chase is evidence of cycloheximide-induced alveolar macrophage cytotoxicity (Fig. 5 *A*). Since this form corresponds to the high-mannose RER intracellular form of α1AT, the 50-kD form of α1AT detected in the medium after treatment of alveolar macrophages was not used to determine the extracellular amount of α1AT, since this form was likely not secreted.

Lactacystin increases secretion of Z α1AT in CHO cells and alveolar macrophages. In contrast to the nonspecific and likely indirect effect of cycloheximide, lactacystin specifically irreversibly inhibits intracellular protein degradation mediated by the multicatalytic complex, i.e., proteasome (22, 26–28). Using the proteasome inhibitor lactacystin, degradation of Z α1AT was substantially inhibited in both CHO cells and alveolar macrophages. There were no significant differences at time zero between lactacystin-treated and -untreated cells for either CHO cells or alveolar macrophages ($P = 0.94$ and $P = 0.93$, respectively; Fig. 5 *B*). In CHO cells, 10 μM lactacystin increased intracellular Z α1AT by 1.69±0.33-fold ($P = 0.03$) and secreted Z α1AT by 1.86±0.13-fold ($P = 0.03$) after 6 h of chase (Fig. 5 *B*). Pretreatment with 2 μg/ml brefeldin A prevented lactacystin's inhibitory effect on Z α1AT intracellular degradation (Z α1AT half-lives were ∼ 10 h with or without lactacystin). The additional secreted Z α1AT was functional since it formed SDS-stable complexes with neutrophil elastase in proportions similar to Z α1AT from untreated cells (Fig. 3). Interestingly, 10 μM lactacystin treatment also increased M α1AT secretion by amounts similar to those of cycloheximide (data not shown).

In human alveolar macrophages, lactacystin increased intracellular Z α1AT by 2.79±0.33-fold ($P = 0.045$) and secreted Z α1AT by 1.76±0.24-fold ($P = 0.025$) under similar conditions (Fig. 5 *B*). Comparison of the total intra- and extracellular α1AT at time points after synthesis of the radiolabeled α1AT was completed demonstrated that > 60% of the newly synthesized α1AT was exported to the extracellular compartment in the presence of lactacystin after 6 h of chase. In the absence of lactacystin, ∼ 25% of the synthesized Z α1AT is exported extracellularly (Fig. 5 *C*). Alveolar macrophages treated with either lactacystin or cycloheximide demonstrated similar, but not identical, results to transfected CHO cells (Fig. 5 *C*).

Consistent with this observation, studies of intracellular localization using immunofluorescence microscopy demonstrated that lactacystin concentrated Z α1AT and altered its distribution in CHO cells from a predominantly RER to a mixed Golgi–RER pattern (Fig. 6), similar to that in cells treated with cycloheximide for 2 h (compared with Fig. 4, *O–Q*). It was not possible to determine accurately the intracellular location of α1AT in human alveolar macrophages by immunofluorescence microscopy because of their inherent autofluorescence (25).

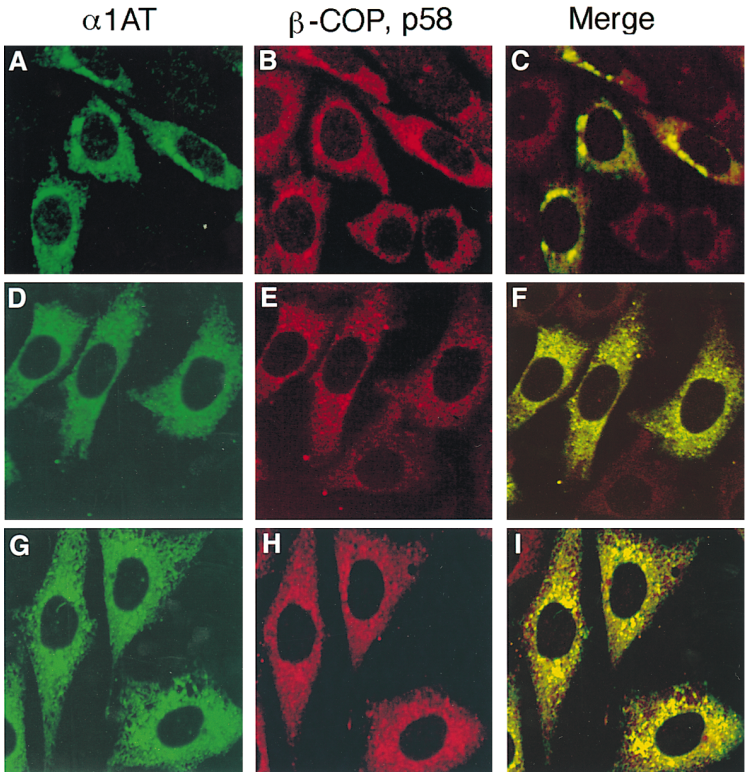


Figure 6. Confocal photomicrographs show immunofluorescence localization of intracellular Z α1AT during vesicular transport in CHO cells with and without treatment with 10 μM lactacystin. (*A*, *D*, and *G*) M and Z α1AT–transfected CHO cells were double-labeled with FITC-conjugated rabbit anti–human α1AT (*green*) and (*B*, *E,* and *H*) rhodamine-conjugated antibodies to the Golgi-resident proteins β-COP/p58 (*red*). (*C*) Colocalization of the green and red labels shows that M α1AT is concentrated in the Golgi (*yellow*). (*F*) Z α1AT untreated (*control*) cells have a diffuse pattern with no concentration in the Golgi (*small amount of yellow*). (*I*) After 6 h of treatment with lactacystin (*lactacystin*), Zα1AT demonstrate a mixed Golgi and presumably, RER pattern (*increased yellow*). 100× objective.

Discussion

The concept of defective protein folding as a basis of some human diseases has gained attention, particularly among laboratories investigating cystic fibrosis and α1AT deficiency (4, 8, 29–33). Much of this interest is based on the fact that our understanding of the mechanisms for processing misfolded proteins has been substantially extended (34, 35). In this study, we used a model CHO cell system and human alveolar macrophages expressing Z α1AT, a variant associated with misfolding and α1AT deficiency, to determine the effect of inhibition of intracellular degradation on its vesicular transport.

Using this model system, we demonstrated that Z α1AT was secreted in amounts four to sixfold less than those of normal M α1AT. This reduction in secreted α1AT correlates well with α1AT levels observed in α1AT-deficient individuals (1). Furthermore, the bulk of Z α1AT was retained in the RER/pre-Golgi compartment and degraded, since most intracellular Z α1AT is endoglycosidase H–sensitive and colocalizes with the RER resident protein, BiP. One potential limitation of the CHO cell model may be that overproduction of a protein that is not normally expressed may alter protein-trafficking characteristics in a manner that does not reflect the natural mechanisms of protein transport. Therefore, observations made in such a model system should be interpreted with caution. In this context, it is critical that the model system share features of protein transport that are similar to those of cells that normally express α1AT (hepatocytes or alveolar macrophages).

Degradation of Z α1AT was prevented by adding the metabolic poisons cycloheximide and lactacystin. The effect of metabolic poisons and cycloheximide on intracellular catabolism of Z α1AT is likely indirect and nonspecific. Lactacystin causes direct inhibition of intracellular degradation of Z α1AT. Z α1AT intracellular degradation was not affected by brefeldin A or inhibitors of lysosomal function. These observations are consistent with previous studies in several in vitro systems (5, 10, 12, 14–18). There was a substantial difference in the rate of intracellular degradation of M and Z α1AT in the presence of brefeldin A (24 vs. 10 h, respectively). Since Z α1AT is known to misfold and forms polymers in the RER, the different intracellular half-lives of M and Z α1AT suggest either that Z α1AT is degraded by another pathway, or that there exists preferential degradation of the misfolded protein (4, 36). The observation that only 50–60% of Z α1AT is degraded by an energy-dependent process provides further evidence that at least one other pathway participates in the degradation of Z α1AT. Since intracellular Z α1AT accumulated very rapidly after adding cycloheximide or puromycin, very short-lived protein(s) seem to play an essential role in Z α1AT degradation.

We conclude that Z α1AT degradation in our model CHO cell system is mediated by both energy-dependent and energy-independent nonserine, nonlysosomal processes, and requires a very short-lived protein component(s). These findings resemble those of Le et al. (12, 15), who used a rat hepatocyte model system.

Recent work by Qu and colleagues has demonstrated that Z α1AT binds to the luminal domain of calnexin, while its cytoplasmic domain becomes ubiquitinated and degraded by proteasomes (18, 37). Since a significant proportion of Z α1AT (40–50%) is also degraded in an energy-independent fashion, ubiquitination-proteasome–mediated proteolysis is not the sole pathway for intracellular Z α1AT degradation (23, 24, 27, 38). Nevertheless, proteasomes clearly mediate the bulk of in-

tracellular Z α1AT degradation since lactacystin prevented Z α1AT degradation in CHO cells and transfected human fibroblasts (18). These findings are consistent with lactacystin's known inhibition of proteasome function, which would reduce intracellular degradation of Z α1AT.

In addition to preventing intracellular degradation, cycloheximide and lactacystin significantly increased secretion of Z α1AT in CHO cells and human alveolar macrophages. Cycloheximide- or lactacystin-treated CHO cells expressing M α1AT secreted 25–43% more α1AT compared with untreated cells, an observation that suggests ongoing degradation of a portion of the M α1AT intracellular pool.

Comparison of the total intra- and extracellular α1AT at time points after radiolabeled α1AT synthesis was completed, demonstrated that $>$ 80% of the newly synthesized α1AT is eventually exported to the extracellular compartment in the presence of cycloheximide. In the absence of cycloheximide, $\sim$ 25% of the synthesized Z α1AT is exported extracellularly. Loss of 55% of the synthesized Z α1AT in the absence of cycloheximide could only be the result of intracellular degradation, since only a small proportion of total labeled α1AT remains in the intracellular compartment at the end of the chase period. Delay in secretion of Z α1AT after inhibition of intracellular degradation is likely due to the increased time required for misfolded Z α1AT to dissociate into the monomeric form and/or to recycle through the vesicular transport system (34, 39).

While direct evidence of cycloheximide's inhibition of intracellular degradation cannot be provided, most of the accumulated Z α1AT exited the cell. This interpretation of the data is supported by the changing pattern of subcellular localization of α1AT immunofluorescence over time in CHO cells treated with cycloheximide. Since cycloheximide prevents further translation of α1AT, time course experiments that follow Z α1AT subcellular localization after cycloheximide treatment present a dynamic picture of its movement from its site of retention in the RER to secretion into the extracellular compartment. In comparison to the nonspecific effect of cycloheximide on Z α1AT intracellular catabolism, treatment with lactacystin specifically interferes with Z α1AT degradation by inhibiting proteasome function (18, 22, 26–28, 37). Therefore, the mixed RER/Golgi pattern of Z α1AT immunofluorescence after treatment with lactacystin represents a new subcellular steady state. This pattern is in contrast with that observed in M α1AT-synthesizing CHO cells that have a predominant Golgi pattern of immunofluorescence, since unlike polymerized Z α1AT, normal unpolymerized M α1AT moves quickly from the RER to the Golgi. By preventing intracellular degradation, lactacystin and cycloheximide allow Z α1AT to become concentrated within the RER. The Z α1AT then slowly enters the vesicular transport system, i.e., moves to the Golgi and beyond. In contrast to specific proteasome inhibitors, cycloheximide likely has no application as a potential therapeutic approach for treating diseases associated with retained mutant protein degradation.

Z α1AT secreted by cells treated with cycloheximide or lactacystin had a similar capacity to form SDS-stable complexes with neutrophil elastase as Z α1AT from untreated cells. The capacity of Z α1AT to form complexes with neutrophil elastase was half that of M α1AT. This finding is consistent with previous studies demonstrating that Z is half as functional as M α1AT (40). It is possible that cycloheximide or lactacystin could alter the specificity of intracellular α1AT transport such that polymers of α1AT were exported. Z α1AT polymers form by reactive site loop incorporation into the β sheet of another molecule. The function of such α1AT polymers would be very low in proportion to the amount of antigenic α1AT detected because their reactive loops would be unavailable for interaction with neutrophil elastase. Release of intracellular polymer cannot account for the appearance of increased α1AT in the medium since the proportion of Zα1AT-neutrophil elastase complexes in treated and untreated cells is similar.

Although cycloheximide and lactacystin almost completely inhibited intracellular catabolism of Z α1AT, pretreatment with brefeldin A abolished their effect on its intracellular degradation. However, in the context that α1AT folding and degradation normally occur in the RER and pre-Golgi compartments, this finding must be interpreted cautiously since brefeldin A is known to disrupt these structures (12, 15, 41). Thus, it is possible that brefeldin A abolishes the effects of cycloheximide and lactacystin on intracellular degradation of Z α1AT only when the compartment contents are mixed, and therefore does not reflect the normal processing of Z α1AT.

In this context, increased secretion of Z α1AT after inhibition of degradation can be explained as follows: (*a*) Misfolded Z α1AT molecules that polymerize by loop-sheet linkage cannot exit the RER because their polymer size prevents their inclusion transport vesicles (7, 8). (*b*) This delay in transport increases the opportunity for a misfolded protein to be either targeted for degradation or retrieved to the vesicular traffic pathway via chaperones such as calnexin (12, 15, 34, 37, 42, 43). (*c*) When proteasome function is inhibited, degradation ceases and only retrieval via vesicular trafficking occurs (26). The observation that inhibition of intracellular degradation of Z α1AT allows apparently normal vesicular trafficking supports the contention of Le et al. that very little Z α1AT exists in an insoluble form within the RER. Therefore, the bulk of intracellular Z α1AT remains available in a soluble form for either degradation or transport to another intracellular compartment (12, 15).

The therapeutic potential of proteasome inhibitors in rescuing misfolded proteins depends upon the functional capacity of the rescued protein and the importance of proteasome function for other processes, such as in regulating cytokines and modulating immune processes involving antigen presentation. (24, 26, 27). In considering the application of this approach to the treatment of α1AT deficiency, it is important to recognize that Z α1AT secreted by alveolar macrophages is likely partially functional. Furthermore, the magnitude of the enhanced secretion that we observed was less than twofold in alveolar macrophages at 6 h compared with approximately threefold in CHO cells; thus, not all types of cell secrete similarly increased amounts of α1AT. Nevertheless, the finding that inhibition of intracellular α1AT degradation increases delivery of α1AT to the extracellular milieu provides a new potential treatment of α1AT deficiency and other diseases associated with misfolded proteins (29, 30).

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