Grp78, Grp94, and Grp170 interact with α1-antitrypsin mutants that are retained in the endoplasmic reticulum

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Submitted 26 May 2004; accepted in final form 19 April 2005

Schmidt, Bela Z., and David H. Perlmutter. Grp78, Grp94, and Grp170 interact with α1-antitrypsin mutants that are retained in the endoplasmic reticulum. Am J Physiol Gastrointest Liver Physiol 289: G444–G445, 2005. First published April 25, 2005; doi:10.1152/ajpgi.00237.2004.—In α1-antitrypsin (α1-AT) deficiency, a mutant form of α1-AT polymerizes in the endoplasmic reticulum (ER) of liver cells resulting in chronic hepatitis and hepatocellular carcinoma by a gain of toxic function mechanism. Although some aspects of the cellular response to mutant α1-AT Z have been partially characterized, including the involvement of several proteasomal and nonproteasomal mechanisms for disposal, other parts of the cellular response pathways, particularly the chaperones with which it interacts and the signal transduction pathways that are activated, are still not completely elucidated. The α1-AT Z molecule is known to interact with calnexin, but, according to one study, it does not interact with Grp78. To carry out a systematic search for the chaperones with which α1-AT Z interacts in the ER, we used chemical cross-linking of several different genetically engineered cell systems. Mutant α1-AT Z was cross-linked with Grp78, Grp94, calnexin, Grp170, UDP-glucose glycoprotein:glucosyltransferase, and two unknown proteins of ~110–130 kDa. Sequential immunoprecipitation/immunoblot analysis and coimmunoprecipitation techniques demonstrated each of these interactions without chemical cross-linking. The same chaperones were found to interact with two nonpolymerogenic α1-AT mutants that are retained in the ER, indicating that these interactions are not specific for the α1-AT Z mutant. Moreover, sucrose density gradient centrifugation studies suggest that ~85% of α1-AT Z exists in heterogeneous soluble complexes with multiple chaperones and ~15% in extremely large polymers/aggregates devoid of chaperones. Agents that perturb the synthesis and/or activity of ER chaperones such as tunicamycin and calcium ionophore A23187, have different effects on the solubility and degradation of α1-AT Z as well as on its residual secretion.

α1-antitrypsin deficiency; molecular chaperones; endoplasmic reticulum quality control; endoplasmic reticulum retention

α1-ANTITRYPSIN (α1-AT) deficiency is the most common genetic cause of liver disease in children and emphysema/destructive lung disease in adults. The lung disease is caused by a gain of toxic function mechanism, which somehow results from the retention of a mutant α1-AT molecule in the endoplasmic reticulum (ER) of liver cells. The lung disease involves a loss of function mechanism in which the lack of α1-AT secretion from the liver into the blood and, in turn, transfer to the lungs permits uninhibited proteolytic damage to the connective tissue matrix of the lung.

A prospective nationwide screening study in Sweden that began 30 years ago has indicated that only a subgroup (~10%) of individuals with the classic homozygous PIZZ form of α1-AT deficiency develop clinically significant liver disease during childhood (42, 43). Our studies of genetically engineered skin fibroblast cell lines from PIZZ individuals with and without liver disease have indicated that there is a lag in the ER degradation of mutant α1-AT Z in the subgroup that is “susceptible” to liver disease and, therein, that differences in ER degradation/quality control are important determinants of the liver disease phenotype in this deficiency (49). Studies done by a number of laboratories (37, 46) have indicated that the ER degradation pathway for mutant α1-AT Z involves several mechanisms including ubiquitin-dependent and -independent proteasomal mechanisms as well as nonproteasomal mechanisms. Autophagy and a tyrosine phosphatase-dependent reaction have been implicated as potential nonproteasomal mechanisms (4, 45).

Examination of the structure of α1-AT and other serpins by Carrell and Lomas has shown that the mutant α1-AT Z is prone to polymerization by a loop-sheet insertion mechanism (8, 27, 28). Moreover, by showing that the secretion defect of mutant α1-AT Z is partially reversed by the introduction of additional mutations in the α1-AT Z molecule that prevent its polymerization, they have provided evidence that polymerization plays a role in the ER retention of α1-AT Z. Recently, we have shown that a naturally occurring mutation of α1-AT, called α1-AT Saar, which causes truncation of the COOH terminus of α1-AT, is retained in the ER even though it does not polymerize. In fact, when the Saar mutation is introduced into the α1-AT Z molecule, the resulting α1-AT Z + Saar mutant is retained in the ER to the same, or perhaps even to a greater, extent as α1-AT Z itself, even though this double mutant no longer has polymerogenic properties (25). These results imply that there are different mechanisms for ER retention of polymerogenic and nonpolymerogenic mutants of α1-AT or that polymerization is an effect, rather than the cause, of ER retention.

A growing body of literature has characterized the role of ER chaperones in determining the fate of glycoproteins. The ER chaperones facilitate the folding of wild-type proteins that is necessary for translocation out of the ER to the appropriate destination. They also play a role in the quality control mechanism, inhibiting secretion of incompletely folded or misfolded proteins by retaining them in the ER and/or by targeting them for degradation. Grp78 (BiP) is the most well-studied ER chaperone. It is a nonglycosylated protein of the ER lumen that belongs to the heat shock protein (Hsp)70 family (17). It binds
transiently to newly synthesized proteins, more extensively to misfolded proteins, and plays a role in the gating of the translocon pore. Grp78 also plays a crucial role as a sensor of unfolded proteins in the ER and triggering the unfolded protein response (UPR), caused by the accumulation of unfolded proteins in the ER. The involvement of Grp78 in maintaining the solubility of misfolded proteins has been demonstrated in many cases (29, 39).

Grp94 is a resident ER member of the Hsp90 protein family (1, 36). Similarly to Grp78, it is also induced under many conditions, leading to the accumulation of unfolded proteins in the ER. Compared with Grp78 and calnexin, Grp94 interacts with only a restricted set of proteins and seems to bind to advanced folding intermediates and incompletely assembled oligomers. Grp170 (Orp150) is an ER resident glycoprotein with a COOH-terminal ER retention signal (15). The physiological role of Grp170 is poorly understood. It has been shown to interact with both Grp78 and Grp94 and secretory proteins, and it may be cooperatively involved in the folding of proteins.

Calnexin (p88 or IP90) is a type I transmembrane protein with ER retention signal in its COOH terminus (9, 22, 23, 38, 48). Calnexin binds monoglucosylated high-mannose oligosaccharides produced by the partial deglucosylation of the Glc3Man9GlcNAc2 core or by the reglucosylating action of UDP-glucose:glycoprotein glucosyltransferase (UGGT) on Man9-αGlcNAc2 oligosaccharides (34). In addition to retaining misfolded proteins, calnexin also increases the folding efficiency of glycoproteins, preventing premature oligomerization and suppressing the formation of nonnative disulfide bridges (22). UGGT (47) adds a single glucose unit to Man9-αGlcNAc2 oligosaccharides covalently attached to partially folded proteins (7). It is the only component of the “calnexin cycle” identified so far that has been shown to be able to specifically recognize unfolded proteins. Chaperones have also been shown to interact with each other to form chaperone networks in the ER (16, 31, 44). These networks are thought to facilitate the transient sequential interactions involved in folding of cargo.

There are relatively limited data on the interaction of mutant α1-AT Z with ER chaperones. This mutant has been shown to interact with calnexin in cell lines and in cell-free microsomal systems (5, 37, 49). There are conflicting reports about Grp78. Early reports suggested that α1-AT Z did not interact with Grp78 in mouse hepatoma cell lines (nor did the α1-AT Hong Kong mutant) or in the liver of a transgenic mouse model (18). However, recent studies in a human embryonic kidney cell line suggest an association with Grp78 but not with Grp94 (5).

Interactions with calnexin and UGGT have been demonstrated with the α1-AT Hong Kong mutant that is prematurely truncated at its COOH terminus (10, 21), but because there are significant differences in the properties of α1-AT Hong Kong compared with α1-AT Z, the interactions with α1-AT Hong Kong are not necessarily reflective of what happens with α1-AT Z.

Thus, in this study, we used a chemical cross-linking approach to provide a systematic unbiased analysis of the ER chaperones associated with mutant α1-AT Z. We also examined the possibility that nonpolymerogenic mutants α1-AT Saar and α1-AT Z + Saar interact differently with ER chaperones. Because chaperone interactions have been shown to be very sensitive to detergents, we chose a cross-linking approach to increase the probability of detecting all interactions. The interactions found by cross-linking were subsequently verified without cross-linking.

MATERIALS AND METHODS

Materials. Dithiobis(succinimidylpropionate) (DSP) and the biocinonic acid (BCA) protein assay were purchased from Pierce (Rockford, IL). Tunicamycin, protein G-bearing formalin-fixed Streptococcus sp. cells, and grade VI apyrase were from Sigma (St. Louis, MO). Reagents for analytical gel electrophoresis were from Bio-Rad (Hercules, CA). Goat anti-human α1-AT was purchased from DAKO (Carpinteria, CA). Rabbit anti-Grp170 serum was a kind gift of Dr. John Subjeck (Roswell Park Cancer Institute, Buffalo, NY). Rabbit anti-UDP-glucose glycoprotein:glucosyltransferase serum was generously provided by Dr. Armando Parodi (Institute for Biotechnological Research, University of San Martin, Buenos Aires, Argentina). Mouse anti-calnexin was obtained from Affinity Bioreagents (Golden, CO). Goat anti-Grp78 and anti-Grp94 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antiserum raised against Grp78, Grp94, the COOH-terminal sequence of canine calnexin, and glucosidase II were purchased from Stressgen (Victoria, British Columbia, Canada). Antibody to ER mannosidase II was kindly provided by Dr. Joyce Bischoff (Children’s Hospital Boston, Harvard Medical School, Boston, MA). Secondary antibodies used in immunoblotting experiments (peroxidase-labeled donkey anti-goat IgG and donkey anti-rabbit IgG) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Peroxidase-labeled streptavidin was from Pierce. Radioactive molecular mass markers were from Amersham Biosciences (Piscataway, NJ), and the prestained protein standard used for immunoblotting was purchased from Invitrogen (Carlsbad, CA). Cell culture reagents were from Invitrogen and Mediatech (Herndon, VA).

Cell lines. Fibroblast cell lines that were transduced with amphotropic recombinant retroviral particles bearing α1-AT Z cDNA and have stable constitutive expression of α1-AT Z (CI712B) have been described previously (49). Chinese hamster ovary (ChoK1) cell lines expressing α1-AT, α1-AT Saar, α1-AT Z, or α1-AT Z + Saar were established as described previously (25). The HepaTO/Z cell line, with inducible expression of α1-AT Z, was established from the murine hepatoma Hepa 1–6. Hepa 1–6 was first engineered for expression of tetracycline-controlled transactivator (TtTa) using the pTet-Off plasmid (BD Biosciences, Palo Alto, CA) and geneticin-resistant colonies were isolated and screened by transfection with the pTREd2eGFp reporting plasmid (BD Biosciences). The clone with the highest expression level in the absence of doxycycline and lowest background in the presence of 1 μg/ml doxycycline was selected for further use (HepaTO). The α1-AT Z sequence was inserted into the pTRE2Hyg plasmid (BD Biosciences) and was used to transfect HepaTO cells. Hygromycin-resistant clones with highest expression of α1-AT Z in the absence of doxycycline and lowest background in the presence of 1 μg/ml doxycycline were propagated, frozen in aliquots, and used in further experiments. These cell lines are appropriate model systems because pulse-chase studies have shown that the mutants are retained in the ER (data not shown) just as they are in the liver of deficient individuals (25, 49).

Metabolic labeling, immunoprecipitation, sequential immunoprecipitation, analytical gel electrophoresis, and immunoblotting. Cell lines were subjected to pulse-chase radiolabeling as described previously (37). For the pulse period, the cells were incubated at 37°C in 100–500 μCi/ml Tran35S-label (MP Biomedicals, Irvine, CA) in Dulbecco’s modified Eagle’s medium lacking methionine. In pulse-chase experiments, the cells were then rinsed vigorously and incubated in their regular culture medium with excess unlabeled methionine for the indicated time intervals as the chase period. At the end of each chase period, the extracellular medium was harvested and the cells were lysed in 0.1 M PBS, 1% Triton X-100, 0.5% sodium.
deoxycholate, and 10 mM EDTA supplemented with the Complete protease inhibitor cocktail (Roche, Indianapolis, IN) and 2 mM PMSF. The radiolabeled cell lysates were subjected to clarification and immunoprecipitation, and immunoprecipitates were analyzed by SDS-PAGE/fluorography exactly as described previously (46). In experiments when sequential immunoprecipitation was performed, the immunoprecipitated material was released from protein G by boiling in 50 μl gel loading buffer for 5 min. The supernatant was then transferred to new tubes, and the volume was made up to 1 ml with 0.1 M PBS, 1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, and 1% bovine serum albumin and then processed for another round of immunoprecipitation. Aliquots of the radiolabeled cell lysates were also subjected to trichloroacetic acid precipitation and scintillation counting to ensure that there was equivalent incorporation between cells under comparison. In experiments when nonlabeled cells were used, equal amounts of total cellular protein were used as determined by the BCA assay. For coimmunoprecipitations, cells were lysed either in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl containing 0.5% Nonidet P-40 (NP-40), and protease inhibitors as indicated above or in non-Chaps cell extract buffer (Cell Signaling Technology, Beverly, MA), supplemented with 50 U/ml apyrase or 20 mM CaCl₂, as indicated. Subsequent washes were done in the same buffer. For immunoblotting, proteins were transferred to a supported nitrocellulose membrane (Schleicher & Schuell BioScience, Keene, NH). The membrane was blocked in Dulbecco’s modified PBS, 1% bovine serum albumin, 1% nonfat dry milk powder, and 0.05% Tween 20. The same buffer was used for incubation with the primary antibody, and the secondary antibody was applied in 0.1 M PBS and 0.05% Tween 20. All washes were done with 0.1 M PBS and 0.05% Tween 20. Blots were developed with the Supersignal chemiluminescent substrate (Pierce), and antibodies were removed from membranes with Restore (Pierce) before they were reused. Autoradiographs and immunoblots were quantitated using ImageJ, a public domain Java image-processing program (downloaded from the National Institutes of Health, Bethesda, MD). Mean (SD) and Student’s t-test calculations were done with Excel 2000 (Microsoft, Redmond, WA).

Chemical cross-linking. A fresh 20 mM stock solution of DSP, a cell membrane-permeable cross-linker cleavable by reducing agents, was prepared every time in DMSO. Cells were washed three times with Dulbecco’s modified PBS, and cross-linking was done on ice using 2 mM DSP in Dulbecco’s modified PBS for 30 min. Then, the cross-linking solution was aspirated, and the cells were lysed in the same buffer as described above supplemented with 20 mM glycine and 20 mM N-ethyl-maleimide. In the case of the HepaTO/Z cells, cross-linking was done at room temperature for 20 min, followed by a 5-min incubation in 20 mM glycine in Dulbecco’s modified PBS before lysis in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl containing 0.5% NP-40, 20 mM glycine, 20 mM N-ethyl-maleimide, and protease inhibitors.

Preparation of soluble and insoluble fractions from cell lysates. Cell lysates were prepared in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 0.5% Triton X-100, 0.5% sodium deoxycholate, and 2 mM N-ethyl-maleimide supplemented with protease inhibitors as above and homogenized by 12 passages through a 26-gauge needle on ice (14). Insoluble material was recovered by centrifugation at 16,000 g for 20 min. Pellets were washed once and solubilized in 50 μl of 50 mM Tris-HCl (pH 6.8), 5% SDS, and 10% glycerol with 1 min of sonication and then 10 min of boiling. The volume of the resolubilized pellet was then made up to 1 ml with 0.1 M PBS, 1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, and 1% bovine serum albumin and then processed for immunoprecipitation exactly as the other samples. This protocol has been shown to provide specific separation of the α₁-AT Z mutant into soluble and insoluble fractions (25).

Equilibrium centrifugation on sucrose gradients. Cells grown to confluence were lysed in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 0.5% Triton X-100, 0.5% sodium deoxy-

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Fig. 1. Cross-linking of mutant α₁-antitrypsin (α₁-AT) Z in human fibroblasts. Human fibroblasts expressing α₁-AT Z (CJZ12B cells) were subjected to radiolabeling for 24 h with 100 μCi/ml [35S]methionine followed by chemical cross-linking using 2 mM dithiobis(succimidyl)propionate (DSP), a cell membrane-permeable cross-linker cleavable by reducing agents. A radiolabeling period of 24 h was used to get sufficient incorporation into the fibroblast cell line, but identical patterns could be seen with a 4-h pulse (data not shown). At the end of the cross-linking period, cell lysates were prepared and subjected to immunoprecipitation, the immunoprecipitated material was separated on 10% SDS-PAGE under reducing conditions, and the results were analyzed using fluorography. Equal aliquots of lysates of control (−2 mM DSP) and cross-linked (+2 mM DSP) cells were immunoprecipitated with either antiserum to α₁-AT or with nonimmune serum (ni. se) as marked on the right. The approximate molecular mass of the major bands is marked on the right in kDa.

RESULTS

Cross-linking of α₁-AT Z in a human fibroblast cell line. First, we used chemical cross-linking to determine other proteins that interact with mutant α₁-AT Z in a human fibroblast cell line engineered for stable expression of α₁-AT Z, the CJZ12B cell line. Previous studies (49) have shown that this cell line is a good model of the classic form of α₁-AT deficiency with retention of the mutant protein in the ER. The results (Fig. 1) show that the ~52-kDa α₁-AT polypeptide coprecipitates polypeptides of ~50, ~94, and ~170 kDa. The presence of ~80- and ~150-kDa bands could be discerned after a longer exposure of the film (data not shown). These polypeptides were specific as shown by their absence when...
nonimmune serum was used instead of anti-human α₁-AT. A lesser amount of the ~80-kDa polypeptide was coprecipitated in the absence of cross-linking. The relatively diffuse ~55-kDa polypeptide represents a biosynthetic intermediate of α₁-AT Z with more extensive glycosylation, as shown by previous endoglycosidase H- and N-glycosidase F studies (30, 49).

Cross-linking of α₁-AT mutants in ChoK1 cells. To exclude the possibility that these results are peculiar to the human fibroblast cell line and also to establish a model system with higher incorporation of biosynthetic radiolabeling, we subjected a ChoK1 cell line engineered for stable expression of α₁-AT Z to chemical cross-linking. We also compared the ChoK1 cell line expressing α₁-AT Z with ChoK1 cell lines that expressed mutant α₁-AT Saar and α₁-AT Z + Saar. The α₁-AT Saar mutant is truncated for the carboxyl-terminal 19 amino acids. The α₁-AT Z + Saar mutant has the substitution that characterizes α₁-AT Z (Glu342 to Lys342) and the 19-amino acid carboxyl-terminal truncation. Our previous studies have shown that the α₁-AT Saar and α₁-AT Z + Saar mutants are retained in the ER, although they do not form insoluble aggregates, in contrast to α₁-AT Z (25). We reasoned that a comparison of these mutants to α₁-AT Z might reveal differences in interaction with chaperones that are specific for mutant α₁-AT Z. The results show that there are no substantial differences between these mutants (Fig. 2). Both α₁-AT Saar and α₁-AT Z + Saar migrate slightly faster than α₁-AT Z. Polypeptides of ~80, ~88, ~94, ~110, ~130, ~150, and ~170 kDa coimmunoprecipitate in each case. Wild-type α₁-AT (α₁-AT M) comigrates with α₁-AT Z but has significantly more of the fully glycosylated ~55-kDa intermediate than α₁-AT Z or the other mutants but much less, if any, of the coprecipitating polypeptides. We presume this is because the interactions with wild-type α₁-AT are too transient to be detected, but it is also possible that interaction of chaperones with the α₁-AT mutants reflect the degradative pathways that are invoked specifically by mutant but not by wild-type α₁-AT. The interaction of all α₁-AT mutants with the ~80-kDa polypeptide was apparent in the absence of cross-linking.

We also examined the possibility that α₁-AT Z interacts with other chaperones after an extensive chase period. ChoK1 Z cells were subjected to pulse labeling for 6 h and then to a chase period of 18 h. Cross-linking of these cells showed that the same ~80-, ~88-, and ~94-kDa polypeptides interacted with residual undegraded α₁-AT Z (data not shown).

To determine the identity of the coprecipitating polypeptides, we first subjected the ChoK1 cell lines to immunoprecipitation with anti-α₁-AT followed by immunoblot analysis with anti-Gpr78. In Figure 3A, this approach shows that the ~80-kDa polypeptide is Gpr78 present in roughly equivalent amounts for the α₁-AT mutants. To provide further evidence that Gpr78 interacts with mutant α₁-AT Z, we subjected unlabeled ChoK1 Z cells lysed in the presence or absence of apyrase to immunoprecipitation with anti-Gpr78 followed by immunoblot analysis with anti-α₁-AT (Fig. 3B). The results show that a small amount of α₁-AT Z coprecipitates with Gpr78, and it is significantly increased in the presence of apyrase. ATP binding allows Gpr78 to release its substrate (24); therefore, ATP depletion with apyrase has been previously used to enhance detection of complexes of Gpr78 with its substrates (26, 30). Compared with the amount of α₁-AT that is detected by immunoprecipitation with anti-α₁-AT followed by immunoblot analysis with anti-α₁-AT, densitometric analysis indicates that 15% (SD 3) [means (SD), n = 4] of the steady-state α₁-AT Z pool is complexed with Gpr78 when lysates were depleted of ATP, whereas only 8% (SD 2) was in complex with Gpr78 without ATP depletion (n = 4, P < 0.05; Fig. 3B). The specificity of the interaction with Gpr78 is further verified by comparing ChoK1 cells that express mutant α₁-AT Z to ChoK1 cells that express wild-type α₁-AT M (Fig. 3C). When first immunoprecipitated with anti-Gpr78, α₁-AT is detected by immunoblot in cells expressing α₁-AT Z to a much greater extent than in those expressing wild-type α₁-AT.

We suspected that the ~88-kDa polypeptide cross-linked with the α₁-AT mutants was calnexin. To definitively determine that calnexin interacted with α₁-AT mutants, we used the immunoprecipitation/immunoblot strategy. ChoK1 cells expressing mutant α₁-AT Z were lysed in the absence or presence of 20 mM CaCl₂ and then were immunoprecipitated with antibody to α₁-AT, complement factor B as a control, and

![Fig. 2. Cross-linking of α₁-AT Z in ChoK1 cells. ChoK1 cells stably transfected with mutants α₁-AT M, α₁-AT Saar, α₁-AT Z, or α₁-AT Z + Saar (as indicated on top of gel) were subjected to radiolabeling for 6 h with 500 μCi/ml [35S]methionine followed by chemical cross-linking (+2 mM DSP) or mock treated (−2 mM DSP) as indicated. At the end of the cross-linking period, cell lysates were prepared and subjected to immunoprecipitation with antiserum to α₁-AT. The immunoprecipitated material was separated on 6% SDS-PAGE under reducing conditions, and the results were analyzed using fluorography. The relative molecular mass of the major bands cross-linked to or coimmunoprecipitated with α₁-AT Z is marked with arrows on the right. The positions of the ~55- and ~52-kDa forms of α₁-AT M and Z as well as that of the ~50-kDa α₁-AT Saar and α₁-AT Z + Saar are marked on the right with arrows as well. Note that ~80-kDa polypeptide coimmunoprecipitated with α₁-AT Saar, α₁-AT Z, and α₁-AT Z + Saar without cross-linking.](http://ajpgi.physiology.org/doi/10.1152/ajpgi.00334.2017)
calnexin coprecipitates α1-AT, and that increases when the lysis is done in the presence of calcium (Fig. 4). The interaction is specific, as shown by its absence using antibody to factor B. Quantitative analysis indicates that 46% (SD 8) of the total cellular α1-AT Z is in complex with calnexin in lysates supplemented with calcium, whereas only 26% (SD 7) of the α1-AT Z was bound to calnexin without calcium supplementation (n = 4, P < 0.05; Fig. 4).

We also suspected that the ∼94-kDa polypeptide in the cross-linking experiment was Grp94. To address this possibility, we subjected all of the ChoK1 cell lines to cross-linking. Lysates were immunoprecipitated with anti-α1-AT followed by immunoblot analysis for Grp94 (Fig. 5A). The results show that Grp94 is indeed cross-linked to α1-AT in ChoK1 cell lines expressing α1-AT M, α1-AT Saar, and α1-AT Z + Saar in roughly equivalent amounts. A significantly lesser amount of Grp94 is complexed with wild-type α1-AT M. To show that this interaction occurs in the absence of cross-linking, we first used a different type of sequential immunoprecipitation protocol. ChoK1 cells expressing α1-AT Z were metabolically labeled to steady state, and then lysates were prepared and subjected to a first round of immunoprecipitation with antibody to α1-AT or a control, complement C1-INH inhibitor. The immunoprecipitated material was released and subjected to a second round of immunoprecipitation with anti-Grp94 antibody (Fig. 5B). The results show a 94-kDa polypeptide only when antibody to α1-AT was used in the first round. With the use of similar immunoprecipitation/imunoblot analysis as in Figs. 3B and 4, we found that 30% (SD 6) (n = 3) of the total calnexin, and the immunoprecipitates were analyzed by immunoblot for α1-AT.

It has been shown that depletion of ER Ca2+ stores by thapsigargin decreases the association of calnexin with its substrates (6, 10, 12); therefore, an excess amount of Ca2+ was included in the lysis buffer to maintain the association of calnexin and α1-AT Z. The results show that antibody to

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**Fig. 3.** A: the ∼80-kDa protein cross-linked to α1-AT in ChoK1 cells is Grp78. ChoK1 M, ChoK1 Saar, ChoK1 Z, and ChoK1 Z + Saar cells (indicated on top of gel) were mock treated (−2 mM DSP) or subjected to cross-linking (+2 mM DSP) as indicated. The cell lysates were immunoprecipitated with antibody to α1-AT, and the immunoprecipitated proteins were blotted to nitrocellulose membranes. The membrane was developed using goat anti-human Grp78 and peroxidase-conjugated donkey anti-goat IgG. The position of Grp78 is marked on the right with arrows. B: Grp78 coimmunoprecipitates with α1-AT Z. ChoK1 Z cells were lysed in the presence or absence of 25 U/ml apyrase, and equal aliquots of cell lysates were subjected to immunoprecipitation with goat anti-Grp78, as indicated. The material resulting from the immunoprecipitation was separated on 10% SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane, and the membrane was developed using biotinylated antibody against α1-AT and streptavidin-conjugated peroxidase. The position of α1-AT is marked on the right with an arrow. The bottom shows quantitative results of several experiments. Results are presented as %values of the amount of α1-AT immunoprecipitated with anti-α1-AT; α1-AT Z 8% (SD 2; n = 4) was found in complex with Grp78, and this figure rose significantly to 15% (SD 3; n = 4) when lysates were depleted of ATP with apyrase (P < 0.05). C: Grp78 coimmunoprecipitates with α1-AT Z but not with wild-type α1-AT M. ChoK1 M or ChoK1 Z cells (as indicated on top) were lysed, and the cell lysates were immunoprecipitated with antibody to α1-AT or anti-Grp78, as indicated. The immunoprecipitated proteins were blotted to nitrocellulose membranes, and the membrane was developed using biotinylated antibody against α1-AT and streptavidin-conjugated peroxidase. The position of α1-AT is marked on the right with an arrow.

**Fig. 4.** Calnexin coimmunoprecipitates with α1-AT Z. ChoK1 Z cells were lysed in the presence or absence of 20 mM CaCl2, and equal aliquots of cell lysate were subjected to immunoprecipitation with goat anti-α1-AT, rabbit anti-complement factor B, or rabbit anti-calnexin. The immunoprecipitated proteins were blotted to nitrocellulose membranes, and the membrane was developed using biotinylated antibody against α1-AT and streptavidin-conjugated peroxidase. The position of α1-AT is marked on the right with an arrow. The bottom shows a quantitation of the α1-AT coimmunoprecipitated with calnexin in the absence or presence of calcium in several experiments; data are expressed as %values of the amount of α1-AT immunoprecipitated with anti-α1-AT. α1-AT Z [26% (SD 7); n = 4] was found in complex with calnexin, and this figure rose significantly to 46% (SD 8; n = 4) when lysates were supplemented with calcium (P < 0.05).

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Fig. 5. A: the ∼94-kDa protein cross-linked to α1-AT in ChoK1 cells is Grp94. ChoK1 M, ChoK1 Saar, ChoK1 Z, and ChoK1 Z + Saar cells (indicated on top of gel) were mock treated (−2 mM DSP) or subjected to cross-linking (+2 mM DSP) as indicated. The cell lysates were immunoprecipitated with antibody to α1-AT, and the immunoprecipitated proteins were blotted to nitrocellulose membranes. The membrane was developed using goat anti-human Grp94 and peroxidase-conjugated donkey anti-goat IgG. The position of Grp94 is marked on the right with an arrow. B: Grp94 coimmunoprecipitates with α1-AT Z. ChoK1 Z cells were radiolabeled for 26 h with 100 μCi/ml [35S]methionine, lysed, and equal aliquots of cell lysate were subjected to immunoprecipitation with the following antibodies: goat anti-C1 inhibitor and goat anti-α1-AT, as indicated on top of the lanes (1st IP). The immunoprecipitated material was released and subjected to a second round of immunoprecipitation with goat anti-Grp94 (2nd IP). The material resulting from this second immunoprecipitation was separated on 8% SDS-PAGE under reducing conditions, and the results were analyzed using fluorography. The position of Grp94 is marked on the right with an arrow. C: significant portion of α1-AT Z is in complex with Grp94. ChoK1 Z cells were immunoprecipitated with goat anti-α1-AT or goat anti-Grp94; the immunoprecipitates were blotted to nitrocellulose membranes, and the membrane was developed using biotinylated antibody against α1-AT and streptavidin-conjugated peroxidase. The position of α1-AT is marked on the right with an arrow.

intracellular α1-AT Z pool was complexed with Grp94 (Fig. 5C).

Next, we examined the possibility that the ∼150- and ∼170-kDa polypeptides cross-linked to α1-AT Z were Grp170 and UGGT. Grp170 is an ∼150-kDa ER protein, structurally related to Hsp70, and has chaperone activity, but its specific ligands in mammalian cells are still not definitely characterized (15). UGGT is an ∼170-kDa protein that adds glucose to deglucosylated unfolded proteins in the ER (7, 16). Sequential immunoprecipitation with antibody to Grp170 and then with anti-α1-AT also proved that the 52-kDa form of α1-AT Z is specifically associated with Grp170 in the absence of cross-linking (Fig. 6A). Using immunoprecipitation/immunoblot analysis, we estimated that ∼16% (SD 6) (n = 3) of the total intracellular α1-AT Z pool was complexed with Grp170 (data not shown). In Fig. 6B, sequential immunoprecipitation with antisem to UGGT or nonimmune serum and then with anti-α1-AT shows that anti-UGGT specifically recognized (coprecipitated with) the 52-kDa form of α1-AT Z and that this interaction can be detected in the absence of cross-linking. Immunoprecipitation/immunoblot indicated that ∼12% of the total intracellular α1-AT Z pool was complexed with UGGT (data not shown).

We do not know the identity of the ∼110- and ∼130-kDa bands that coprecipitate in cross-linking experiments in each of the cell lines. These bands were not recognized by antibodies to glucosidase II or ER mannosidase II (data not shown).

Identification of chaperones in soluble complexes with α1-AT mutants under non-denaturing conditions. To determine whether α1-AT Z interacts with Grp78 and Grp94 under non-denaturing conditions and to determine the relative molecular mass of the putative complexes, we subjected transfected ChoK1 Z cells to sucrose density gradient centrifugation under non-denaturing conditions. A previous study of cells transfected with α1-AT Z analyzed with this technique showed that α1-AT Z existed within the ER in soluble complexes of ∼150-kDa size and did not seem to form larger aggregates but was degraded from this soluble pool (20). However, the narrow sucrose gradient (5–20%) used in that study did not allow the detection of much larger complexes than the ones reported. Although the investigators used mild cell lysis conditions, known not to dissolve loop-sheet polymers of α1-AT Z, it was not reported how much α1-AT Z could be recovered from the pellet at the bottom of the sucrose gradient. Here, we used a wider gradient (5–60%) to allow detection of larger complexes.

When fractions from the sucrose density gradient of lysate of ChoK1 Z cells were immunoblotted for α1-AT, the results showed that the bulk (85%) of α1-AT Z was found in fractions 2–11, corresponding to sizes from under 66 kDa to over 443 kDa (Fig. 7A). Approximately 4% of α1-AT Z was found in fractions 18–21 and ∼11% was recovered from the pellet at the bottom of the gradient.

Fig. 6. A: Grp170 coimmunoprecipitates with α1-AT Z. ChoK1 Z cells were radiolabeled for 26 h with 100 μCi/ml [35S]methionine and lysed, and equal aliquots of cell lysate were subjected to immunoprecipitation with rabbit nonimmune serum or rabbit anti-Grp170, as indicated on top of gel (1st IP). The immunoprecipitated material was released and subjected to a second round of immunoprecipitation with goat anti-α1-AT (2nd IP). The material resulting from this second immunoprecipitation was separated on 10% SDS-PAGE under reducing conditions, and the results were analyzed using fluorography. The position of α1-AT is marked on the right with an arrow. B: UGGT coimmunoprecipitates with α1-AT Z. ChoK1 Z cells were radiolabeled for 6.5 h with 200 μCi/ml [35S]methionine and lysed, and equal aliquots of cell lysate were subjected to immunoprecipitation with rabbit nonimmune serum or rabbit anti-UGGT, as indicated on top of gel (1st IP). The immunoprecipitated material was released and subjected to a second round of immunoprecipitation with goat anti-α1-AT (2nd IP). The material resulting from this second immunoprecipitation was separated on 10% SDS-PAGE under reducing conditions, and the results were analyzed using fluorography. The position of α1-AT is marked on the right with an arrow.
Fig. 7. α₁-AT Z and α₁-AT Z + Saar form soluble complexes of the same size with chaperones. A: homogenized lysates of control (−2 mM DSP) or cross-linked (+2 mM DSP) ChoK1 Z and ChoK1 Z + Saar cells were subjected to separation on a 5–60% sucrose gradient. Twenty-three fractions were taken starting from the top of the gradient, and the pellet (P) was also recovered from the bottom of the tube. One-half of each fraction was subjected to trichloroacetic acid (TCA) precipitation, and the other half was immunoprecipitated with antibody to α₁-AT and then Grp78 was detected from the immunoprecipitated material by immunoblotting. Sucrose gradient profile of α₁-AT Z (α) is compared with that of α₁-AT Z + Saar (β). C: same as B, except α₁-AT immunoblotting results of cross-linked cells were used. Cross-linked α₁-AT Z (α) is compared with cross-linked α₁-AT Z + Saar (β). D: one-half of each sucrose gradient fraction of cross-linked ChoK1 Z or ChoK1 Z + Saar cells was immunoprecipitated with antibody to α₁-AT and then Grp78 was detected from the immunoprecipitated material by immunoblotting. Sucrose gradient profile of Grp78 cross-linked to α₁-AT Z (α) is compared with that of α₁-AT Z + Saar (β). E: ChoK1 Z cells were lysed in the presence of apyrase and separated on a sucrose gradient, and one-half of each fraction was subjected to TCA precipitation, and the other half was immunoprecipitated with antibody to α₁-AT; then both TCA precipitates and immunoprecipitates were immunoblotted for Grp78. Sucrose density gradient profiles of Grp78 coimmunoprecipitating with α₁-AT Z (β) is compared with that of total Grp78 (κ). F: soluble and insoluble fractions of ChoK1 Z cell lysate were prepared as described in MATERIALS AND METHODS, and the soluble fraction was analyzed as in A, top. Total cell lysate of ChoK1 Z cells was supplemented with SDS to a final concentration of 2% and then analyzed as in A, bottom. Bars on top represent the approximate position of the size markers: bovine serum albumin, 66 kDa; β-amylase, 200 kDa; and apoferritin, 443 kDa.

When fractions from the gradient loaded with ChoK1 Z + Saar lysate were analyzed in the same way, all of the α₁-AT Z was found in fractions 2–11. None was found in the pellet or in the very large complexes of fractions 18–21, distinguishing it from α₁-AT Z. Interestingly, quantification of the signals for α₁-AT Z and α₁-AT Z + Saar showed almost identical sucrose gradient profiles in the range of 5–30% sucrose (Fig. 7B).

Because it might better preserve interactions with chaperones, we also subjected cells to cross-linking and then analyzed the lysates on sucrose density gradients (Fig. 7A). The results showed a partial shift of α₁-AT Z to higher fractions within the 2–12 range. The results were identical for α₁-AT Z + Saar. The bulk of protein was detected in fractions 3–10. Quantitative results (Fig. 7C) showed the near identity of sucrose density gradient profile of α₁-AT Z and that of α₁-AT Z + Saar with the shift toward complexes of 200–443 kDa in cross-linked cells (compare Fig. 7, C with B). This is probably due to stabilization of complexes of larger size by cross-linking.

As expected, α₁-AT Z was also present in the pellet at the bottom of the tube, although this insoluble α₁-AT Z represented a much smaller proportion of the total α₁-AT Z signal compared with that in the noncross-linked cells (compare top and top middle of Fig. 7A). The difference between the bottom ends of gradients of lysates from cross-linked and noncross-linked ChoK1 Z cells, namely, the reduction of insoluble α₁-AT Z and the disappearance of the very large soluble complexes (detected in fractions 18–21 of the non-cross-linked cells), is most likely due to the sequestration of α₁-AT Z by the debris in lysates of cross-linked cells during the short, low-speed centrifugation done to clear lysates before application to the sucrose gradient. In every case, cell lysates of cross-linked cells produced a markedly increased pellet (data not shown).

On the basis of the results presented in Figs. 2–6, we suspected that the high-molecular-weight forms of the mutants in fractions 5–12 contained chaperones in complex with α₁-AT
Z or α1-AT Z+ Saar. We examined this possibility for Grp78 by subjecting sucrose gradient fractions to immunoprecipitation with antibody to α1-AT and then immunoblotting for Grp78. The results showed that Grp78 was indeed present in large complexes with α1-AT Z as well as with α1-AT Z + Saar, ranging in size from under 200 kDa to over 443 kDa (Fig. 7D). Grp78 was not present in fractions 18–21 or in the pellet, indicating that the extremely high-molecular-weight forms of α1-AT Z are not associated with chaperones. Repeating the immunoblottting for Grp94 showed that the very same fractions that contain Grp78 also contain Grp94 (data not shown). These results, showing complexes of α1-AT mutants with Grp78 and Grp94 of ~200–600 kDa, are very similar to what has been seen for incompletely folded immunoglobulin heavy chains present in chaperone-containing complexes of heterogeneous size ranging from ~140 to over ~700 kDa (31).

The failure to detect Grp78 and Grp94 in complex with the extremely high-molecular-weight forms of α1-AT Z could not be attributed to insensitivity of immunoblotting. A similar conclusion could be drawn from experiments in which the soluble and insoluble fractions of lysates of ChoK1 Z cells subjected to radiolabeling and cross-linking were analyzed by SDS-PAGE and autoradiography. The results showed that, although polypeptides cross-linked to α1-AT Z could readily be detected in the soluble fraction, none was seen in the insoluble fraction (data not shown).

To show that the complexes containing Grp78 and α1-AT Z exist in the absence of cross-linking, ChoK1 Z cells were lysed in the presence of apurase (as in Fig. 3B) and subjected to separation on a sucrose gradient (Fig. 7E). Fractions were either TCA precipitated or immunoprecipitated for α1-AT Z, and Grp78 was detected from both the TCA precipitates and from the immunoprecipitates by immunoblotting. The results showed a shift in the sucrose gradient profile of Grp78 that coimmunoprecipitates with α1-AT Z compared with total Grp78 (Fig. 7E). Probably due to the instability of these complexes in the absence of cross-linking, a smaller portion of Grp78 was detected in complexes of the size seen with cross-linking.

We suspected that the extremely high-molecular-weight forms of α1-AT Z in fractions 18–21 and in the pellet, that are not associated with chaperones Grp78 and Grp94, represent relatively insoluble polymers/aggregates. To examine this possibility, we compared by sucrose density gradient analysis whole lysate to the soluble fraction of ChoK1 Z cells (Fig. 7F). The soluble fraction was prepared by a previously described homogenization/centrifugation protocol (25). This protocol has been shown to result in a pellet that contains ~17% of the total cellular α1-AT Z in ChoK1 Z cells but none of the total cellular α1-AT Z + Saar in ChoK1 Z + Saar cells (25). The results in Fig. 7F show that the extremely high-molecular-weight forms of α1-AT Z in fractions 18–21 and in the pellet disappear when the soluble fraction is analyzed this way, providing evidence that the extremely high-molecular-weight material is relatively insoluble α1-AT Z polymers/aggregates (compare Fig. 7, F, top, with A, top). Supplemeting total cell lysate with SDS that is known to dissolve loop-sheet polymers of α1-AT Z (2, 18) also caused the disappearance of the extremely high-molecular weight forms of α1-AT Z in fractions 18–21 and in the pellet (compare Fig. 7, F, bottom, with A, top).

Taken together, these results confirm previous results indicating that α1-AT mutants are retained in the ER in complex with Grp78 and Grp94, but, in this case, it is shown using conditions that permit biochemical/immunological analysis of cellular constituents but are relatively gentle and non-denaturing. Second, ~15% of total cellular α1-AT Z in ChoK1 Z cells can be found in extremely large, relatively insoluble polymers/aggregates that are not associated with chaperones. Because this is not seen for α1-AT in ChoK1 Z + Saar and ChoK1 M cells, it is unlikely to be randomly aggregated protein. Third, the results suggest that most (~85%) of the α1-AT Z in the cell is in a monomeric form in heterogeneous complexes with multiple chaperones. This is because the sucrose gradient profile of the α1-AT Z mutants is so similar to that of the α1-AT Z + Saar mutant in the range of ~66 kDa to over 443 kDa. However, it is not possible to exclude the existence of oligomers in complex with a lesser number of chaperones or with lesser proportion of large chaperones. This also means that it is not possible to conclude that chaperones only interact with monomers of α1-AT Z.

There are two other important implications of these results. First, the similar disposition of most of α1-AT Z and α1-AT Z + Saar provides further evidence for the concept that polymerization is an effect rather than the cause of α1-AT Z retention within the cells. Second, the presence within the cells of heterogeneous complexes that contain multiple chaperones and α1-AT Z monomers and oligomers makes it very difficult to apply the lessons learned from studies on the polymerization of purified α1-AT Z ex vivo to what goes on in the secretory pathway in vivo.

Grp78, calnexin, Grp94, and Grp170 interact with α1-AT Z in cells of hepatocytic origin. To determine whether α1-AT Z interacts with the same chaperones in cells of hepatocytic origin, we carried out cross-linking experiments in the HepaTO/Z cell line (Fig. 8). The results show that UGGT, Grp170, Grp94, calnexin, and Grp78 polypeptides are detected by immunoblotting of cell lysates from HepaTO/Z cells after immunoprecipitation with antibody to α1-AT Z.

Fate of mutant α1-AT Z in ChoK1 Z cells when chaperones are perturbed. Next, we examined the fate of α1-AT Z when the ChoK1 cell line was subjected to treatment with tunicamycin. Tunicamycin inhibits N-glycosylation and induces synthesis of Grp78, Grp94, and Grp170 as part of the unfolded protein response. First, we subjected the cells to chemical cross-linking (Fig. 9A). The results show that the unglycosylated form of α1-AT Z (α1-AT Z*) cross-linked increased amounts of Grp78, unglycosylated Grp94 (Grp94*), and unglycosylated Grp170 (Grp170*).

Second, we subjected ChoK1 Z cells to pulse-chase analysis in the absence or presence of tunicamycin (Fig. 9B). In the presence of tunicamycin, the faster-migrating unglycosylated α1-AT Z protein disappeared more slowly from the cells and secretion was completely abrogated. Examination of the insoluble fraction of lysates from the same experiment revealed that tunicamycin treatment caused accelerated partitioning of α1-AT Z into the insoluble fraction and an increase in the amount of insoluble α1-AT Z detected (Fig. 9C). After tunicamycin treatment, α1-AT Z begins to accumulate in the insoluble fraction within 1 h compared with 2 h in control. In other experiments, it could be detected in the insoluble fraction within 30 min of chase (data not shown). The studies shown in
cross-linking of polypeptide Grp94 to α1-AT Z but no change in Grp170 and UGGT. A similar increase in binding of Grp78 and Grp94 to thyroglobulin after depletion of ER Ca2⁺ stores by thapsigargin treatment was reported by Di Jeso et al. (12). Pulse-chase analysis performed after same treatment showed that A23187 mediates a delay in the disappearance of α1-AT Z

Fig. 7 indicate that the insoluble fraction includes extremely large polymers/aggregates of α1-AT Z not associated with Grp78.

Next, we examined the effect of calcium ionophore A23187 on the fate of mutant α1-AT Z. In addition to its effect on calcium, A23187 is known to induce increased synthesis of Grp78 and Grp94 (13). We treated ChoK1 Z cells with calcium ionophore A23187 for 6 h and then subjected them to cross-linking (Fig. 10A). The results show a marked increase in association of Grp78 with α1-AT Z and a modest increase in cross-linking of polypeptide Grp94 to α1-AT Z but no change in Grp170 and UGGT. A similar increase in binding of Grp78 and Grp94 to thyroglobulin after depletion of ER Ca2⁺ stores by thapsigargin treatment was reported by Di Jeso et al. (12). Pulse-chase analysis performed after same treatment showed that A23187 mediates a delay in the disappearance of α1-AT Z
from the intracellular compartment (Fig. 10B). The amount of α₁-AT Z detected in the extracellular media was also slightly decreased compared with control, but the secreted α₁-AT Z migrated more rapidly. The more rapid migration of secreted glycoproteins caused by A23187 has been observed before and is attributed to inhibition of the addition of sialic acid to complex oligosaccharide side chains (11).

Next, we examined α₁-AT Z in the insoluble fraction of lysates from the same pulse-chase experiment and found that calcium ionophore A23187 had a very different effect from tunicamycin. There was a significant decrease in the amount of insoluble α₁-AT Z in cells treated with calcium ionophore A23187 (Fig. 10C).

These results indicate that agents that perturb ER chaperones have distinct effects on the fate of mutant α₁-AT Z. Tunicamycin mediates an increase in the association of α₁-AT Z with Grp78, Grp94, and Grp170, an increase in the partitioning of α₁-AT Z into the insoluble state, and an increase in intracellular retention due to both delayed degradation and abrogation of residual secretion. The calcium ionophore mediates a more modest increase in association of α₁-AT Z with Grp78 and Grp94. In the presence of the calcium ionophore, there is a delay in intracellular degradation and less insoluble α₁-AT Z.

DISCUSSION

The results of this study indicate that mutant α₁-AT Z interacts with a complex array of chaperones in the ER. In addition to calnexin, α₁-AT Z interacts with Grp78, Grp94, Grp170, and UGGT in several different cell lines. These interactions were initially detected by chemical cross-linking as a method that would detect interacting proteins in an unbiased and relatively sensitive manner. On the basis of relative electrophoretic mobility, it was possible to surmise the identity of the putative interacting proteins and then by using several types of communoprecipitating techniques, in both cross-linked- and noncross-linked cells, to more definitively establish the proteins that interact with mutant α₁-AT Z. We estimated the fraction of the total α₁-AT Z pool that is complexed with the individual chaperones under steady-state conditions: 15% was complexed with Grp78, 46% with calnexin, 30% with Grp94, 16% with Grp170, and 12% with UGGT. The

Fig. 10. The effects of calcium ionophore A23187 on α₁-AT Z processing. A: ChoK1 cells stably transfected with α₁-AT Z were subjected to radiolabeling for 6 h with 250 μCi/ml [³⁵S]methionine in the presence of vehicle as control or calcium ionophore A23187 (1 μM) as indicated on top of gel, followed by chemical cross-linking. At the end of the cross-linking period, cell lysates were prepared and subjected to immunoprecipitation with anti-α₁-AT serum. The immunoprecipitated material was separated on 6% SDS-PAGE under reducing conditions, and the results were analyzed using fluorography. The position of α₁-AT Z, Grp78, Grp94, and Grp170, and UGGT is marked on the right with arrow.

B: ChoK1 Z cells were preincubated with calcium ionophore A23187 (1 μM) for 6 h and then subjected to radiolabeling for 30 min with 250 μCi/ml [³⁵S]methionine in the presence of calcium ionophore A23187. At the end of the labeling period, cells were washed and incubated in culture medium containing calcium ionophore A23187 and excess amount of unlabeled methionine for the time periods indicated on top of each lane. Control cells received vehicle only. Cell lysates and extracellular media were immunoprecipitated for α₁-AT, and results were analyzed with 10% SDS-PAGE/fluorography. Bottom: shows quantitative data from at least 2 experiments. The amount of intracellular or extracellular α₁-AT detected at each time point is expressed as a % value of the intracellular α₁-AT detected at the end of the pulse period. C: insoluble fractions prepared from cell lysates in B were resolubilized and immunoprecipitated for α₁-AT, and the results were analyzed with 10% SDS-PAGE/fluorography. Bottom: amount of insoluble α₁-AT detected at each time point expressed as a % value of the intracellular α₁-AT detected at the end of the pulse period. Each time point represents data from at least 2 experiments.
sum of these fractions therefore accounts for 19% more than the total cellular pool of \( \alpha_1 \)-AT Z. This may reflect experimental error. Certainly, the many technical factors involved in sequential immunoprecipitation/immunoblot analysis could result in errors of this magnitude. However, a much more likely explanation is that this reflects that \( \alpha_1 \)-AT Z is in ternary complex with multiple chaperones. There is ample evidence for this in recent studies of ER chaperones including interactions between Grp170 and Grp94 as well as Grp170 and Grp78 (31, 40). Moreover, the results of coimmunoprecipitation studies from sucrose density gradient fractions here (Fig. 7) provide even stronger evidence for the extent of heterogeneous complexes of \( \alpha_1 \)-AT Z with multiple chaperones.

The interaction of \( \alpha_1 \)-AT Z with Grp78 is particularly important. It was demonstrated here in human fibroblasts as well as in the Chok1 cell lines. It was demonstrated by several different techniques including sequential immunoprecipitation/immunoblot analysis without cross-linking, and the interaction was enhanced by ATP depletion with apyrase. We have also demonstrated this interaction in the Hepato/Z cell line (Fig. 8) as well as in genetically engineered HeLa cell lines (data not shown). Finally, the interaction of \( \alpha_1 \)-AT Z with Grp78 was demonstrated under non-denaturing conditions with evidence for their presence together in large soluble complexes from under \( \sim \)200 kDa to over \( \sim \)443 kDa. The functional significance of the interaction of \( \alpha_1 \)-AT Z with Grp78 in mammalian cells is unknown, but Grp78 has been shown to play a role in the degradation \( \alpha_1 \)-AT Z in yeast (4).

It was somewhat surprising to us that the nonpolymerogenic mutants, \( \alpha_1 \)-AT Saar and \( \alpha_1 \)-AT Z + Saar, had interactions with ER chaperones that were identical to those of \( \alpha_1 \)-AT Z. These data indicate that the interactions of \( \alpha_1 \)-AT Z with Grp78, calnexin, Grp94, Grp170, and UGGT are not a function of its polymerogenic properties. Because they provide further evidence for the similar fate of these mutants in the ER, these data also cast further doubt on the idea that the polymerogenic properties are the cause, as opposed to the effect, of ER retention of the \( \alpha_1 \)-AT Z mutant.

Recent studies have shown that the mannose binding protein ER degradation-enhancing \( \alpha \)-mannosidase-like protein (EDEM) binds substrates of the ER degradation pathway and collaborates with calnexin in the disposal of these substrates (19, 32). In fact, Oda et al. (33) have shown that the \( \alpha_1 \)-AT Hong Kong mutant binds to EDEM. We were unable to detect a polypeptide that would correspond to EDEM in association with \( \alpha_1 \)-AT Z, \( \alpha_1 \)-AT Saar, or \( \alpha_1 \)-AT Z + Saar in the cross-linking experiments described here. However, it is not possible to completely exclude the possibility that this negative result is due to the specific conditions used. We do know that the \( \alpha_1 \)-AT Hong Kong mutant is much more rapidly degraded than \( \alpha_1 \)-AT Z, \( \alpha_1 \)-AT Saar, and \( \alpha_1 \)-AT Z + Saar, and its ER degradation pathway differs from that of \( \alpha_1 \)-AT Z, \( \alpha_1 \)-AT Saar, and \( \alpha_1 \)-AT Z + Saar in a number of other ways.

The results of this study also show quite different effects on the fate of \( \alpha_1 \)-AT Z when the synthesis or functional activity of chaperones is perturbed pharmacologically. The effect of tunicamycin led to an increase in the association of \( \alpha_1 \)-AT Z with Grp78, unglycosylated Grp94, and unglycosylated Grp170, probably reflecting the increase in synthesis of these chaperones as a result of the UPR. There was also an increase in the partitioning of \( \alpha_1 \)-AT Z into the insoluble fraction, but it is not possible to determine whether this is due to the effect of the UPR directly, of underglycosylation, of the increase in association with the glucose regulated proteins, or due to the increased time that \( \alpha_1 \)-AT Z resides in the ER as a result of the action of tunicamycin. A23187 also induces the UPR, but, under the conditions used here, there was an increase in association of mutant \( \alpha_1 \)-AT Z with Grp78 and Grp94 and a decrease in the accumulation of insoluble \( \alpha_1 \)-AT Z, although degradation is delayed. The fact that we detected less insoluble \( \alpha_1 \)-AT Z in the presence of A23187 may reflect the capacity of Grp78 to suppress aggregation of substrates (41). However, again, because the two drugs have multiple effects, only some of which are overlapping, it is possible that other effects of tunicamycin and A23187 account for these differences. Nevertheless, these data do provide further evidence that the processes of polymerization, aggregation, degradation, and secretion of mutant proteins such as \( \alpha_1 \)-AT Z are not necessarily interrelated.

ACKNOWLEDGEMENTS

Dr. Li Lin established the ChoK1 cell lines used in this study. The advice and encouragement of Drs. Jeff Brodsky and John Subjeck are greatly appreciated. The authors are grateful to Jennifer Goecckeler and Craig Scott for help with the sucrose gradient experiments.

GRANTS

This work was supported in part by grants from the National Institutes of Health and the Alpha-1 Foundation.

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