Final Report for Project #98-0097
"Participation of Proteasomal Degradation in Heritable Plasma Alpha1-antitrypsin Deficiency"

SUMMARY

The combination of pulse-chase radiolabeling, immunoprecipitation, velocity sedimentation, and selective oligosaccharide processing inhibition, has revealed the mechanism by which alpha1-antitrypsin (AAT) quality control is orchestrated in the secretory pathway of hepatoma cells, a model of the hepatocyte. Cycles of binding to calnexin initially facilitates the productive folding of newly synthesized AAT. We have demonstrated that in the absence of correct folding persistent rounds of binding to calnexin allows asparagine-linked oligosaccharides to be processed by endoplasmic reticulum (ER) mannosidase I (ERMI). Importantly, the removal of a single mannose unit from multiple asparagine-linked oligosaccharides, by ERMI, abrogates the dissociation misfolded AAT from calnexin, leading to its selective degradation by the cytosolic proteasome. As such, the modification couples the "retention" and "degradation" phases of AAT's intracellular disposal by functioning as a clock, or molecular switch, that directs molecular capture. Also, we have identified a previously unknown functional hierarchy among the different members of the quality control machinery, with ERManII occupying the highest position.

Recent Abstracts and Presentations:

Cabral, C. M. , Liu, Y., Choudhury, P., and Sifers, R. N. (1998) ER mannosidases control the fate of antitrypsin variant PI Z and are a target for secretion-rescue therapy. Mol. Biol. Cell 9, 460a, Annual meeting for the American Society for Cell Biology, Dec. 12-16.

Sifers, R. N. (1998) Participation of ER mannosidases in alpha1-antitrypsin quality control provides a target for secretion-rescue therapy. Mol. Biol. Cell 9, 460a, Annual meeting for the American Society for Cell Biology, Dec. 12-16.

Choudhury, P., Liu, Y., Cabral, C., and Sifers, R. N. (1998) Proteasome-mediated degradation of misfolded alpha1-antitrypsin involves physical interaction with calnexin and the sec61 complex. Mol. Biol. Cell 9, 220a, Annual meeting for the American Society for Cell Biology, Dec. 12-16.

Sifers, R. N. (1999) Mannose-trimming functions as a clock in glycoprotein folding and quality control. The Keystone Symposium on Protein Folding, Modification, and Transport in the Early Secretory Pathway, April 10-16.

Sifers, R. N. (1999) Mannosidases are master regulators of alpha1-antitrypsin quality control. Annual Meeting for the American Society of Human Genetics, Oct. 19-23 (scheduled).

Recent Publication:

Liu, Y., Choudhury, P., Cabral, C.M., and Sifers, R.N. (1999). Oligosaccharide modification in the early secretory pathway directs the selection of a misfolded glycoprotein for degradation by the proteasome. **J. Biol. Chem.** 274: 5861-5867. (A reprint is enclosed.)

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Oligosaccharide Modification in the Early Secretory Pathway Directs the Selection of a Misfolded Glycoprotein for Degradation by the Proteasome*

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The role of conformation-based quality control in the early secretory pathway is to eliminate misfolded polypeptides and unassembled multimeric protein complexes from the endoplasmic reticulum, ensuring the deployment of only functional molecules to distal sites. The intracellular fate of terminally misfolded human α_1-antitrypsin was examined in hepatoma cells to identify the functional role of asparagine-linked oligosaccharide modification in the selection of glycoproteins for degradation by the cytosolic proteasome. Proteasomal degradation required physical interaction with the molecular chaperone calnexin. Altered sedimentation of intracellular complexes following treatment with the specific proteasome inhibitor lactacystin, and in combination with mannosidase inhibition, revealed that the removal of mannose from attached oligosaccharides abrogates the release of misfolded α_1-antitrypsin from calnexin prior to proteasomal degradation. Intracellular turnover was arrested with kifunensine, implicating the participation of endoplasmic reticulum mannosidase I in the disposal process. Accelerated degradation occurred in a mannosidase-independent manner and was arrested by lactacystin, in response to the posttranslational inhibition of glucosidase II, demonstrating that the attenuated removal of glucose from attached oligosaccharides functions as the underlying rate-limiting step in the proteasome-mediated pathway. A model is proposed in which the removal of mannose from multiple attached oligosaccharides directs calnexin in the selection of misfolded α_1-antitrypsin for degradation by the proteasome.

The endoplasmic reticulum (ER)[1] functions as the intracellular site where nascent polypeptides enter the central vacuolar system (1) and fold into their correct functional conformation (2), which is dictated by the primary amino acid sequence (3). Quality control machinery resident to that compartment facilitates the selective elimination of incompletely folded proteins to ensure that only functional molecules are deployed to distal sites (4, 5). As such, the role of conformation-based quality control is fundamental to normal cell physiology.

Although initially unexpected, it is now recognized that the 26 S proteasome, a constituent of the cytosol (6), is responsible for the degradation of many ER-situated proteins (7–10). Indeed, the cytoplasmic delivery of proteasomal substrates has been reported (11–14). As yet, however, the molecular basis by which proteins of the ER are selected for proteasomal degradation remains unclear, although the molecular chaperone calnexin, which binds monoglucosylated oligosaccharides (15), has been implicated as a possible participant in the sorting process (16, 17).

The molecular pathogenesis of several human diseases, including cystic fibrosis, familial hypercholesterolemia, and a heritable form of pulmonary emphysema, are caused, in part, by the participation of conformation-based quality control factors (for reviews, see Refs. 18 and 19). The latter disorder is caused by the impaired secretion of misfolded genetic variants of human α_1-antitrypsin (AAT) from liver hepatocytes. Human AAT is a monomeric glycoprotein that is a member of the serine proteinase inhibitor superfamily that protects lung elastin fibers from elastolytic destruction. Defective intracellular transport of the aberrantly folded glycoprotein diminishes circulating levels of the inhibitor, resulting in the elastolytic destruction of lung elastin (reviewed in Ref. 20).

Defective secretion of AAT has been investigated by us (21–26) and others (10, 17, 27, 28) as a model to dissect the involvement of quality control machinery in the molecular pathogenesis of human disease. As observed for other hepatic secretory glycoproteins (29), rounds of binding to calnexin are predicted to facilitate the folding of the newly synthesized AAT into its functional conformation (25). Our analyses have shown that prolonged physical association with calnexin accompanies intracellular retention of the terminally misfolded glycoprotein (25) and that the removal of mannose from asparagine-linked oligosaccharides is essential for subsequent degradation (25).

The focus of the present study was to investigate the intracellular degradation of misfolded AAT as a model to elucidate the role of intracellular mannosidase activity in glycoprotein quality control, a phenomenon recently observed by us (25) and others (30–33). Results from the present investigation are consistent with a model in which modification by ER mannosidase I mediates the proteasomal degradation of terminally misfolded AAT by abrogating its dissociation from calnexin in response to the attenuated deglucosylation of asparagine-linked oligosaccharides. This finding provides evidence that ER-situated mannosidase activity occupies a previously unrecognized elevated hierarchical position among known members of the glycoprotein folding and quality control machinery and extends the role of calnexin beyond that of a simple molecular chaperone.

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[1] The abbreviations used are: ER, endoplasmic reticulum; AAT, α_1-antitrypsin; PAGE, polyacrylamide gel electrophoresis; UGTR, UDP-glucose:glycoprotein glucosyltransferase.

MATERIALS AND METHODS

Reagents and Antisera—All salts, buffers, and protease inhibitors were purchased from Sigma, except for lactacystin, which was synthesized by Dr. E. J. Corey (Harvard University). Endoglycosidase H, jack bean mannosidase, and all oligosaccharide processing inhibitors were purchased from Boehringer Mannheim, with the exception of castanospermine (CalBiochem) and kifunensine (Toronto Research Chemicals, Inc). All tissue culture media were purchased from ICN Biochemicals and Life Technologies, Inc. Fetal bovine serum was procured from Summit Biotechnology. Polyclonal rabbit antiserum against a synthetic peptide homologous to the cytoplasmic tail of canine calnexin was purchased from StressGen, and an IgG fraction of polyclonal goat anti-human AAT was procured from Organon Teknika-Cappel.

Metabolic Radiolabeling and Immunoprecipitation—Mouse hepatoma cells were stably transfected with DNA encoding human AAT in which 33 amino acids are absent from the carboxyl terminus of the 394-amino acid polypeptide (cell line H1A/N13) (21). Unless stated otherwise, incubation of cells with inhibitors was performed for 60 min in regular growth medium (25) prior to a 15-min pulse in methionine-free medium containing [^{35}S]methionine (NEN Life Science Products) (23). Cell lysis was performed with buffered Nonidet P-40 (25), and protein immunoprecipitation was accomplished by a 2-h incubation of the soluble cell lysate with an excess of specific antiserum immobilized to protein G-agarose, as described (25). Following immunoprecipitation, radiolabeled proteins were resolved by SDS-PAGE and detected by fluorographic enhancement of vacuum-dried gels. Increased electrophoretic mobility of radiolabeled AAT, resulting from oligosaccharide modification, was resolved in 20-cm gels (25).

Velocity Sedimentation—Calnexin-associated AAT was separated from released monomers by velocity sedimentation of Nonidet P-40 cell lysates in linear 5–20% sucrose gradients as described previously (24, 25). Physical interaction with calnexin was confirmed by sequential coimmunoprecipitation of radiolabeled molecules with antiserum against the molecular chaperone as described previously (25). The relative amount of AAT in each sedimenting species was assessed by scintillation spectrophotometry of immunoprecipitated protein excised from the gel.

Detection of Asparagine-linked Glucosylated Oligosaccharides—Prior to treatment, calnexin was dissociated from immunoprecipitated AAT by incubation with 5 mM EDTA at 37 °C (24). Digestions were performed for 16 h at 37 °C with 0.03 μg of jack bean mannosidase in 50 μl of 10 mM citrate, pH 5.0, followed by an additional 8-h incubation upon the addition of another aliquot of enzyme. Resolution of AAT glycoforms was accomplished by SDS-PAGE and fluorography. Electrophoretic AAT standards in which all three attached oligosaccharides contain glucose was generated by preincubating cells with 0.2 μg/ml castanospermine prior to pulse-radiolabeling and immunoprecipitation. The standard in which one of the attached oligosaccharides contain glucose was generated by chasing pulse-radiolabeled cells in medium containing metabolic poisons to disrupt the physical interaction with calnexin (26), resulting in the hydrolysis of glucose residues prior to immunoprecipitation.

RESULTS

Calnexin Is an Active Participant in the Proteasome-mediated Disposal of Misfolded AAT—In the absence of COOH-terminal amino acids, human AAT is unable to progress to the correctly folded conformation (34). In pulse-chase studies using [^{35}S]methionine, the terminally misfolded molecules were completely eliminated from stably transfected mouse hepatoma cells within 4 h of chase ($t_{1/2}$ = 2 h) (Fig. 1*a*). Although unaffected by the lysosomotrophic amine chloroquine, intracellular disposal was inhibited >85% upon incubation of cells with the membrane-permeable peptide aldehydes *N*-acetyl-leu-leu-norleucinal and *N*-acetyl-leu-leu-methioninal (Fig. 1*a*), both of which are nonspecific inhibitors of proteasomal degradation (35). Nearly complete inhibition occurred upon incubation with lactacystin, a specific irreversible covalent inhibitor of the proteasome (36), confirming the participation of the multicatalytic proteolytic complex in the disposal process.

Incubation of cells with the glucosidase inhibitor castanospermine prior to pulse-radiolabeling with [^{35}S]methionine was performed to maintain asparagine-linked oligosaccharides in the original $Glc_3Man_9GlcNAc_2$ structure, preventing posttranslational assembly between the newly synthesized glycoprotein and calnexin (25). Under these conditions, intracellular turnover was unaffected by each of the three proteasome inhibitors (Fig. 1*a*). Because no insoluble radioactive AAT was detected under the latter conditions (not shown), these findings indicate that misfolded molecules are substrates for degradation by an alternative proteolytic system under conditions that prevent physical interaction with calnexin.

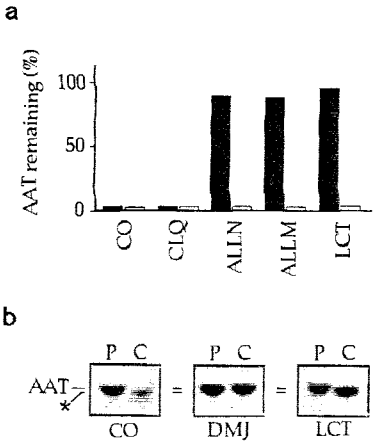

FIG. 1. **Effect of proteasomal inhibitors on the disposal of misfolded AAT and modification of asparagine-linked oligosaccharides.** *a*, the percentage of radiolabeled misfolded AAT molecules remaining in cells after a 4-h chase in media containing either no additions (*CO*), 50 μM chloroquine (*CLQ*), 100 μM *N*-acetyl-leu-leu-norleucinal (*ALLN*), 100 μM *N*-acetyl-leu-leu-methioninal (*ALLM*), or 25 μM lactacystin (*LCT*) (*solid bars*). Cells were treated as above, except that media, including that used for preincubation, were supplemented with 0.2 mg/ml castanospermine (*open bars*). *b*, electrophoretic mobility of immunoprecipitated misfolded AAT in SDS-PAGE following a 15-min pulse (*P*) and subsequent 3-h chase (*C*) in cells treated with either no additions (*CO*), 25 μM lactacytin (*LCT*), or 1 mM 1-deoxymannojirimycin (*DMJ*). Enhanced electrophoretic mobility is shown by an *asterisk*.

Endogenous Mannosidase Activity Abrogates the Dissociation of Misfolded AAT from Calnexin Prior to Proteolysis—Simultaneous detection of calnexin-associated (6.8 S) and released AAT monomers (4.5 S) by velocity sedimentation (25) has provided evidence for the partitioning of the unfolded glycoprotein between the chaperone-associated and unbound state. A sedimentation coefficient of 6.8 S was exhibited by the entire population of misfolded AAT molecules following a 4-h chase with lactacystin (Fig. 2*a*, *LCT*), and the observation was duplicated in two additional experiments. This anomaly coincided with the quantitative coimmunoprecipitation of radiolabeled AAT with antiserum against calnexin (Fig. 2*b*). An identical manipulation did not hinder the secretion of wild-type AAT (not shown), indicating that the ability of lactacystin treatment to prevent the release of the glycoprotein from calnexin was restricted to the terminally misfolded molecule. Because the detection of 4.5 S AAT monomers requires posttranslational release from calnexin (25), it became apparent that the abrogated dissociation of the 6.8 S complex precedes proteasomal degradation, possibly as a normal step in the disposal process.

Enhanced electrophoretic mobility of radiolabeled molecules in SDS-PAGE (Fig. 1*b*, *CO*), which results from the removal of mannose from attached oligosaccharides (24, 25), was arrested, as was intracellular disposal, by incubating cells with 1-deoxymannojirimycin (Fig. 1*b*, *DMJ*), an inhibitor of ER mannosidases (37). However, of the two events, only intracellular disposal was arrested with lactacystin (Fig. 1*b*, *LCT*), confirming that the removal of mannose from asparagine-linked oligosaccharides precedes proteolysis. The importance of this finding was revealed in the next set of experiments, in which a popu-

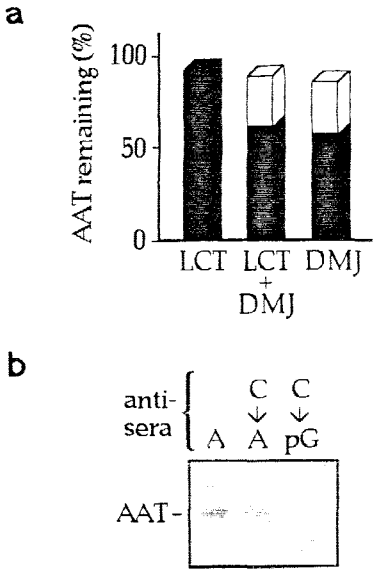

FIG. 2. **Differential sedimentation of misfolded AAT in sucrose gradients following inhibition of disposal.** *a*, relative ratio of radiolabeled molecules sedimenting at either 6.8 S (*closed bars*) or 4.5 S (*open bars*) following a 4-h chase in cells incubated with medium containing either 25 μM lactacystin (*LCT*), 1 mM 1-deoxymannojirimycin (*DMJ*), or a combination of the two inhibitors (*LCT + DMJ*). Each *bar* represents the average result of three determinations. *b*, equal aliquots of the 6.8 S species resulting from lactacystin treatment were subjected to coimmunoprecipitation analyses. Shown are immunoprecipitation of misfolded AAT (*A*), immunoprecipitation of misfolded AAT following its release from a calnexin immunoprecipitate (*C* → *A*), and incubation with protein G-agarose following the release of misfolded AAT from a calnexin immunoprecipitate (*C* → *pG*), as a negative control.

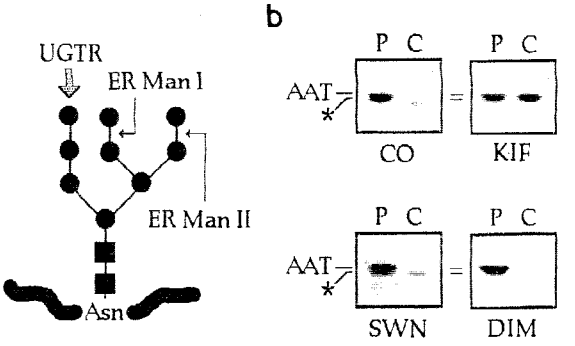

FIG. 3. **Effect of ER mannosidase inhibitors on misfolded AAT disposal.** *a*, the branched $Man_9GlcNAc_2$ structure is shown that contains nine mannose units (*closed circles*) and two GlcNAc units (*closed squares*). Shown are the sites of hydrolysis by ER mannosidases (*ER Man*) I and II and the site at which glucose is added by *UGTR* (*shaded arrow*). *b*, electrophoretic mobility of immunoprecipitated misfolded AAT in SDS-PAGE following a 15-min pulse (*P*) and subsequent 3-h chase (*C*) in cells incubated with medium containing either no additions (*CO*), 100 μM kifunensine (*KIF*), 100 μM swainsonine (*SWN*), or 1 mM 1,4-dideoxy-1,4-imino-D-mannitol (*DIM*). Enhanced electrophoretic mobility of misfolded AAT is shown by an *asterisk*.

lation of released misfolded monomers, in addition to the 6.8 S complex, was detected following coincubation of cells with both lactacystin and the mannosidase inhibitor (Fig. 2*a*, *LCT+DMJ*). Under these conditions, the relative ratio of the 6.8 and 4.5 S species was identical to that observed when disposal had been arrested with 1-deoxymannojirimycin alone (Fig. 2*a*, *DMJ*). These findings demonstrate that intracellular mannosidase activity is responsible for abrogating the dissociation of misfolded AAT from calnexin prior to proteolysis.

The Activity of ER Mannosidase I Is Required for Disposal—In the absence of sufficient material to characterize the mannose content of attached oligosaccharides, we examined the effect of processing inhibitors on AAT disposal as a means to identify the mannosidase activity essential for degradation. Because the oligosaccharide-dependent size shift has been localized to the ER (24), we examined the involvement of two processing α-mannosidases, each of which can initiate the removal of mannose from asparagine-linked oligosaccharides in higher eukaryotes (38) and is inhibited with 1-deoxymannojirimycin (39). Of the two enzymes, ER mannosidase I catalyzes the removal of the outermost mannose unit from the middle branch of $Man_9GlcNAc_2$ (Fig. 3*a*), and is inhibited by the plant alkaloid kifunensine (39). A separate mannose unit is excised by the kifunensine-resistant ER mannosidase II (40), which is sensitive to both swainsonine and 1,4-dideoxy-1,4-imino-D-mannitol (38). Incubation of cells with kifunensine arrested the intracellular disposal of misfolded AAT (Fig. 3*b*, *KIF*), whereas no demonstrable effect was exerted by either of the ER mannosidase II inhibitors (Fig. 3*b*, *SWN*, *DIM*), indicating that trimming of attached glycans to $Man_8GlcNAc_2$, by ER mannosidase I, is responsible for mediating the onset of misfolded AAT degradation by the proteasome in these cells.

Attenuated Deglucosylation of Attached Oligosaccharides Is a Rate-limiting Step in AAT Disposal—Reversible glucosylation of asparagine-linked oligosaccharides mediates the partitioning of glycoproteins between the calnexin-associated and unbound state (41, 42). Results from separate *in vitro* studies have demonstrated that whereas glucose transfer by UDP-glucose:glycoprotein glucosyltransferase (UGTR) to asparagine-linked $Man_8GlcNAc_2$ remains 70% as efficient as for the $Man_9GlcNAc_2$ precursor (43), subsequent deglucosylation of $Glc_1Man_8GlcNAc_2$ by glucosidase II is diminished to only 21% of that observed for $Glc_1Man_9GlcNAc_2$ substrate (44). Because the deglucosylation of $Glc_1Man_8GlcNAc_2$ is very inefficient, at least for the protein-free oligosaccharide (44), in the next set of experiments we tested the hypothesis that the removal of a single mannose unit from multiple oligosaccharides attached to the unbound monomer prevents complete dissociation of the complex following assembly with calnexin, as depicted in Fig. 4. The accuracy of this prediction was first addressed by asking whether any of the oligosaccharides of calnexin-associated AAT contained glucose following inhibition of proteolysis with lactacystin. Because the removal of mannose by jack bean mannosidase is restricted by terminal glucose units (Fig. 5*a*), differential migration of mannosidase-digested glycoproteins in SDS-PAGE was used as a method to identify the number of oligosaccharides attached to [^{35}S]methionine-radiolabeled AAT that are glucosylated (45). For AAT, a total of four glycoforms can be generated by this methodology in which either 0, 1, 2, or all 3 attached oligosaccharides contain glucose. However, two predominant bands were resolved by SDS-PAGE following lactacystin treatment (Fig. 5*b*, *LCT*). When compared with the migration of known electrophoretic mobility standards (Fig. 5*b*, *Stds*), it was concluded that these species represented populations of calnexin-associated AAT in which either two or all three asparagine-linked oligosaccharides contain glucose (Fig. 5*b*, *LCT*). A slight variation in this pattern has been observed in four additional experiments, including the infrequent detection of a faster migrating band in which only a single glycan contains glucose (not shown). Importantly, the latter species has routinely represented <10% of the total glycoform population, suggesting that it represents a minor species. Finally, radiolabel from [^{14}C]galactose (46) was incorporated as glucose into asparagine-linked oligosaccharides of the misfolded glycoprotein during cycloheximide treatment (not shown), indicating that the sugar units had been added posttranslationally, as would be expected for substrates of UGTR.

Next, we attempted to establish a causal relationship be-

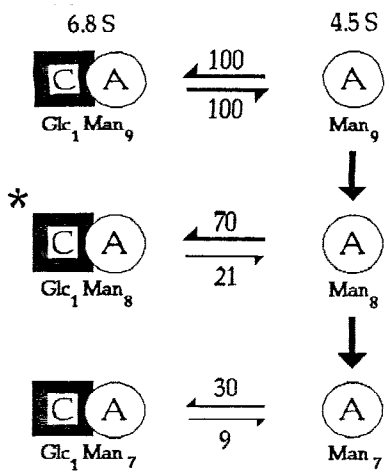

FIG. 4. **Predicted influence of asparagine-linked oligosaccharides on the partitioning of misfolded AAT between the calnexin-associated and unbound state.** *Vertical arrows* represent the order of mannose removal from oligosaccharides attached to the misfolded AAT (*A*) monomer by ER-situated mannosidases. Numbers *above* and *below* the *horizontal arrows* represent the results of separate *in vitro* studies in which the relative percent efficiency of glucosylation by UGTR (43) and deglucosylation by glucosidase II (44), respectively, was determined for each of the depicted oligosaccharide species. Our prediction was that intracellular dissociation from calnexin (*C*) would be abrogated following assembly with the AAT monomer bearing multiple asparagine-linked oligosaccharides with the $Glc_1Man_8GlcNAc_2$ structure (*asterisk*) because it is a poor substrate for deglucosylation. The number of glucose (*Glc*) and mannose (*Man*) units in the oligosaccharides attached to AAT is shown for each step.

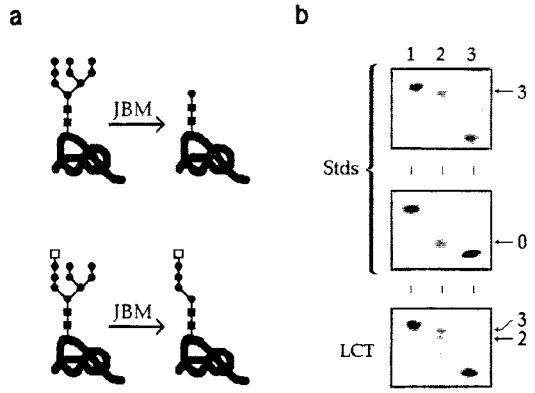

FIG. 5. **Detection and quantitation of glucosylated asparagine-linked oligosaccharides.** *a*, strategy for detecting glycoprotein bearing glucosylated oligosaccharides. A terminal glucose unit (*open square*) hinders the removal of mannose residues (*closed circles*) by jack bean mannosidase (*JBM*). *b*, electrophoretic mobility in SDS-PAGE of radiolabeled misfolded AAT. The differential electrophoretic mobility of standards (*Stds*) is shown in which all three or none of the attached oligosaccharides contain glucose (see under "Materials and Methods"). Following a 4-h chase with lactacystin (*LCT*), radiolabeled misfolded AAT was immunoprecipitated from cells and then was either mock-digested (*lane 1*), digested with jack bean mannosidase (*lane 2*), or digested with endoglycosidase for total deglycosylation (*lane 3*) prior to fractionation by SDS-PAGE and detection by fluorography. For this experiment, all samples were subjected to SDS-PAGE on the same gel, and the predicted number of asparagine-linked oligosaccharides that contain glucose is shown to the *right* of each panel.

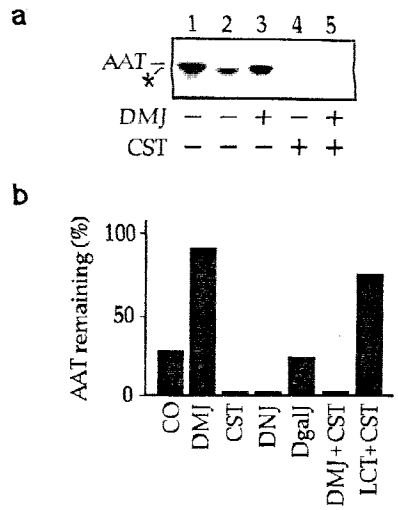

FIG. 6. **Disposal of misfolded AAT is accelerated in response to a posttranslational glucosidase blockade.** *a*, electrophoretic mobility of immunoprecipitated misfolded AAT in SDS-PAGE following a 15-min pulse (*lane 1*) and 3-h chase (*lanes 2–5*) in cells incubated with medium containing combinations of 1 mM 1-deoxymannojirimycin (*DMJ*) and 0.2 mg/ml castanospermine (*CST*). The former compound was included in preincubation and chase medium, whereas the latter was present only during the chase. Enhanced electrophoretic mobility of misfolded AAT is shown by an *asterisk*. *b*, quantitation of the percentage of radiolabeled misfolded AAT molecules remaining in cells following the above treatments and after a 3-h chase in medium containing 1 mM deoxynojirimycin (*DNJ*), 1 mM deoxygalactojirimycin (*DgalJ*), and 25 μM lactacystin plus and 0.2 mg/ml castanospermine (*LCT + CST*) in which the former compound was included in preincubation and chase medium, whereas the latter was present only during the chase.

tween the attenuation of oligosaccharide deglucosylation and proteasomal degradation of misfolded AAT. Because a dynamic interaction with calnexin allows for the liberation of bound glycoprotein substrates in response to the deglucosylation of asparagine-linked oligosaccharides (41), we took advantage of the 2-h half-life of the misfolded protein to test the effect of a posttranslational glucosidase blockade as a means to bypass the predicted role of mannose removal to attenuate oligosaccharide deglucosylation. For this, pulse-radiolabeled cells were chased with medium containing the glucosidase inhibitor castanospermine to directly inhibit the activity of glucosidase II following cotranslational assembly of molecules with calnexin. Disposal of misfolded AAT was accelerated >2-fold under these conditions (Fig. 6*a*, *lane 4*) as compared with control cells (*lane 2*). Accelerated intracellular turnover was also observed when pulse-radiolabeled cells were chased with deoxynojirimycin (Fig. 6*b*, *DNJ*), an alternative glucosidase inhibitor and glucose analog (37). In contrast, incubation with the galactose analog, deoxygalactojirimycin, had no influence on the rate of degradation (Fig. 6*b*, *DgalJ*). A ~1.5-h lag preceded the acceleration of degradation (not shown), possibly reflecting the time required for either inhibitor to attain an appropriate concentration in the ER. It is unlikely that accelerated degradation resulted from the displacement of misfolded AAT by castanospermine because the compound is a transition state analog of the glucosidase reaction, rather than a true structural analog of glucose (37). Coincubation of cells with 1-deoxymannojirimycin during the posttranslational glucosidase blockade had no inhibitory effect on the accelerated rate of disposal (Fig. 6*a*, *lane 5*), as compared with when the mannosidase inhibitor was used alone (*lane 3*). These results were consistent with the notion that the slower means of attenuating oligosaccharide deglucosylation, which normally occurs through the removal of mannose, had been successfully bypassed via the direct inhibition of glucosidase II. Finally, degradation was greatly retarded by incubating cells with the proteasomal inhibitor lactacystin during the posttranslational glucosidase blockade (Fig. 6*b*, *LCT+CST*), indicating that the rapid rate of degradation had reflected an accelerated rate of entrance into the proteasome-mediated disposal pathway. Under none of these conditions was misfolded AAT secreted into the medium or was

the secretion of wild-type AAT impaired (not shown). Together, these findings are consistent with the notion that the attenuated removal of glucose from asparagine-linked oligosaccharides functions as the underlying rate-limiting step in the proteasome-mediated degradation of the misfolded glycoprotein.

DISCUSSION

The complete inhibitory effect of lactacystin on misfolded AAT disposal suggests that its elimination is accomplished almost exclusively by the proteasome, as has been reported for an additional misfolded human AAT variant expressed in transfected yeast (16), transduced human fibroblasts (17), and a cell-free rabbit reticulocyte lysate system supplemented with canine pancreatic microsomal membranes (17). Although the nonproteasomal disposal pathway, which may be of the same type observed for some other mutant ER-situated proteins (27, 32, 35), was not detected in any of these heterologous expression systems (17), our data confirm that calnexin is an active participant in the proteasomal degradation of misfolded AAT, as first reported by Qu *et al.* (17).

Although it is well documented that the reversible glucosylation of asparagine-linked oligosaccharides can facilitate glycoprotein folding (41, 42), the role of ER-situated mannosidase activity in regulating the disposal of unfolded glycoproteins has been appreciated only recently (25, 30–32). A molecular explanation for the role of ER-situated mannose trimming in AAT disposal is depicted in Fig. 7, and outlined below, extending the glycoprotein folding model originally proposed by Hammond *et al.* (47). In the absence of conformational maturation, persistent rounds of binding to calnexin, as reflected by the simultaneous detection of the 6.8 and 4.5 S species, functions as a "retention phase," preventing movement to the Golgi complex (Fig. 7). Because glucose transfer by UGTR is restricted to only nonnative glycoproteins (48), it is predicted that conformational maturation results in the permanent release of AAT from the calnexin binding cycle, allowing transport of only functional molecules to the Golgi complex, where further modification of attached glycans can occur (38) prior to secretion (Fig. 7).

Predominance of the 6.8 S species during intracellular retention of the misfolded protein (25) suggests that binding to calnexin is kinetically favored over that of release. In a dynamic, rather than static, interaction with calnexin, as previously suggested by Zapun *et al.* (41), oligosaccharide deglucosylation can precede the complete dissociation and detection of the monomer as the 4.5 S species. In an earlier report (25), we observed that the degradation of misfolded AAT coincided with the appearance of the released 4.5 S monomer. It now seems likely that this observation might reflect a greater level of accessibility of oligosaccharides to ER mannosidases when misfolded AAT exists as the dissociated monomer and can rebind calnexin only after reglucosylation by UGTR. UGTR, which reglucosylates oligosaccharides attached to only nonnative proteins (47), is responsible for selectively recognizing the misfolded AAT monomer and promoting its reassembly with calnexin (Fig. 7). Gradual modification of oligosaccharides attached to the monomer, regardless of the conformational state of the underlying protein, serves as a timer that marks a period during which the polypeptide is provided with multiple attempts to fold properly. Following the removal of a critical number of mannose residues, attenuated deglucosylation by glucosidase II targets the misfolded molecule for degradation by the proteasome (Fig. 7). As such, the removal of mannose functions as a molecular switch to initiate the disposal of misfolded AAT, whereas modification of oligosaccharides attached to correctly folded glycoproteins, even those resident to the ER,

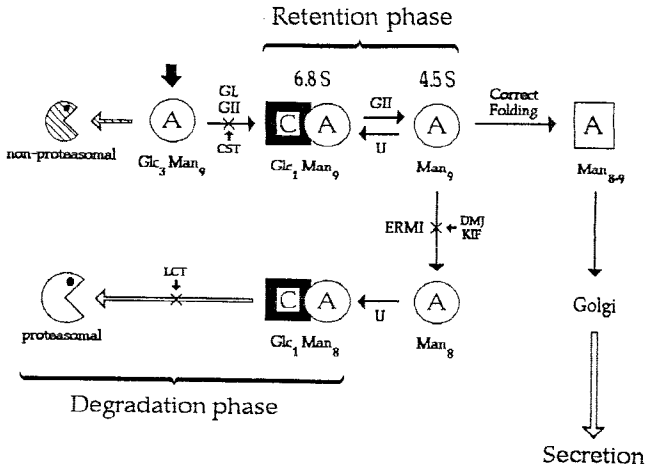

FIG. 7. **Proposed pathway for AAT quality control.** Following cotranslational addition of $Glc_3Man_9GlcNAc_2$ (*closed vertical arrow*) to nascent AAT (*A*), partial deglucosylation by glucosidases I and II (*GI, GII*) generates asparagine-linked $Glc_1Man_9GlcNAc_2$, which promotes cotranslational assembly with calnexin (*C*). The correctly folded glycoprotein (*square*) is no longer recognized by UGTR (*U*), ensuring that only the functional molecule is delivered to the Golgi complex for subsequent secretion. Removal of mannose from glycans attached to correctly folded AAT does occur, but it does not result in degradation because these are not substrates for UGTR. In the absence of bound calnexin, which occurs upon preincubation of cells with the glucosidase inhibitor castanospermine (*CST*), misfolded molecules are degraded by a nonproteasomal pathway, confirming that calnexin is an active participant in proteasomal degradation and identifying the existence of an alternative disposal mechanism. The terminally misfolded glycoprotein (*circle*) enters a retention phase consisting of rounds of binding to calnexin (represented by the *4.5 S* and *6.8 S* species), in which assembly and dissociation are driven by the activities of UGTR and glucosidase II, respectively. Modification by ER mannosidase I (*ER Man I*) allows the glucosylation of oligosaccharides attached to the monomer, but it attenuates subsequent deglucosylation and abrogates dissociation of the 6.8 S complex because $Glc_1Man_8GlcNAc_2$ is a poor substrate for glucosidase II. Abrogated dissociation from calnexin initiates the entrance of misfolded AAT into the degradation phase of the proteasomal pathway in which several steps are arrested by the inhibition of proteolysis during treatment with lactacystin (*LCT*). Inhibition of ER mannosidase I (*ERMI*) with 1-deoxymannojirimycin (*DMJ*) or kifunensine (*KIF*) uncouples the retention and degradation phases. The predicted number of glucose (*Glc*) and mannose (*Man*) units in the oligosaccharides attached to AAT is shown for each step.

should have no effect on their stability because these glycans are not substrates for reglucosylation by UGTR (48). Possibly, the enhanced migration of misfolded AAT in SDS-PAGE may correspond to what Qu *et al.* (17) had considered to represent an endoproteolytic intermediate.

In the present study, inhibitors of ER mannosidase I arrested proteasomal disposal without disturbing the relative ratio of the 4.5 and 6.8 S species, resulting in a futile cycle of binding to calnexin. Thus, it is likely that trimming of attached oligosaccharides to $Man_8GlcNAc_2$, by ER mannosidase I, couples intracellular retention and degradation processes. Although ER mannosidase II apparently is not essential for triggering the proteasome-mediated degradation of misfolded AAT in the mouse hepatoma cell line, we cannot disregard the possibility that it may play a pivotal role in glycoprotein quality control in other cell lines in which the relative level of its activity is elevated (40). Nevertheless, our findings indicate that an oligosaccharide modification that is common to several distinct proteins, rather than a specific amino acid sequence, can function as a signal to "recognize" a misfolded molecule for degradation by the proteasome. By employing this posttranslational modification as the criterion for degradation, it can now be understood how misfolded molecules are preferentially degraded whereas nascent folding intermediates are provided

with ample opportunity to progress to the native conformation, if attainable, prior to oligosaccharide modification.

Although some hydrophobic interaction does exist between misfolded AAT and calnexin (24), several lines of evidence, including (i) the detection of only the 6.8 S complex following lactacystin treatment, (ii) the reversal of this anomaly upon mannosidase inhibition, (iii) the presence of glucose on multiple asparagine-linked glycans of calnexin-associated AAT following lactacystin treatment, and (iv) the accelerated degradation of molecules in response to a simple posttranslational glucosidase blockade, indicate that attenuated oligosaccharide deglucosylation is the molecular event that initiates entrance into the proposed "degradation phase," which sorts misfolded AAT for proteasomal degradation (Fig. 7). Although our study indicates that oligosaccharides of calnexin-associated AAT do contain glucose, these findings do not necessarily reflect an accurate assessment of the number of possible lectin-binding sites present in the molecular chaperone because the persistence of glucose could result from the attenuated deglucosylation of an unbound glycan in response to the removal of mannose. However, it is now apparent that by extending the role of calnexin beyond that of a mere molecular chaperone, ER-situated mannosidase activity occupies a previously unrecognized elevated hierarchical position among the conformation-based glycoprotein quality control machinery.

Abrogated dissociation from calnexin following lactacystin treatment precluded a detailed analysis of the predicted role that calnexin might play in the degradation phase of proteasome-mediated disposal. However, one hypothesis to be considered is that because calnexin is a type I transmembrane protein (49), abrogated dissociation might function to tether misfolded lumenal AAT to the ER membrane, providing a kinetically favorable situation for subsequent physical interaction with retrograde transport machinery, members of which have been implicated as components of the ER translocon (11, 12, 14). Calnexin would serve as a suitable candidate for this purpose because at least one copurifying protein has been shown to cross-link with nascent polypeptides during their translocation into the ER (49). In support of this notion, physical interaction with calnexin has been identified following the inhibition of the degradation of a COOH-terminally truncated form of ribophorin under conditions arresting ubiquitin conjugating activity (14). Furthermore, Qu *et al.* (17) have reported evidence that the induced ubiquination of the cytoplasmic tail of associated calnexin precedes proteasomal degradation of an additional genetic variant of human AAT expressed in a cell-free rabbit reticulocyte system supplemented with canine pancreatic microsomal membranes (17). In the present study, ubiquitin was associated with <10% of calnexin-associated AAT following lactacystin treatment, and none was associated with the bound molecular chaperone (not shown). Also, indirect immunfluorescence microscopy and protease protection analyses have verified that the delivery of misfolded AAT to the cytoplasm is blocked by lactacystin treatment.[2] Because polyubiquination is often required for the cytoplasmic delivery of ER-derived protein substrates (8, 9, 14), removal of the covalent modification by isopeptidases during inhibition of proteolysis might account for the continued ER residence and physical interaction of misfolded AAT with calnexin. Not all proteasomal substrates bear asparagine-linked oligosaccharides, indicating that alternative mechanisms must function in the selection of nonglycosylated ER-situated proteins for degradation by this pathway, none of which apparently recognize terminally misfolded AAT as substrate. Therefore, it is tempting to consider the possibility that a common mechanism for sorting ER-derived proteins to the proteasome is to maintain or promote a constant physical interaction with the translocon.

An essential role for asparagine-linked $Man_8GlcNAc_2$ in initiating the degradation of mutant carboxypeptidase Y in *Saccharomyces cerevisiae* was recently reported by Jakob *et al.* (32). Although the predicted role of the attached glycan in the disposal process in yeast differs from what is proposed to occur in hepatoma cells, data from the two studies agree that the development of systems to eliminate soluble secretory glycoproteins in lower and higher eukaryotes has centered around the conserved $Man_8GlcNAc_2$ structure. Elucidation of the participation of calnexin in proteasomal disposal pathway awaits further experimentation, as does an assessment of how the nonproteasomal pathway might participate in heritable plasma AAT deficiency.

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[2] P. Choudhury, unpublished observations.

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