Intracellular Disposal of Incompletely Folded Human $\alpha_1$-Antitrypsin Involves Release from Calnexin and Post-translational Trimming of Asparagine-linked Oligosaccharides*

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Protection of lung elastin fibers from proteolytic destruction is compromised by inefficient secretion of incompletely folded allelic variants of human $\alpha_1$-antitrypsin from hepatocytes. Pulse-chase radiolabeling with [35S]methionine and sucrose gradient sedimentation and immunoprecipitation techniques were employed to investigate quality control of human $\alpha_1$-antitrypsin secretion from stably transfected mouse hepatoma cells. The secretion-incompetent variant null(Hong Kong) (Sifers, R. N., Brakeburn-Macatee, S., Kidd, V. J., Muenesch, H., and Woo, S. L. C. (1988) J. Biol. Chem. 263, 7330–7335) cannot fold into a functional conformation and was quantitatively associated with the molecular chaperone calnexin following biosynthesis. Assembly with calnexin required cotranslational trimming of glucose from asparagine-linked oligosaccharides. Intracellular disposal of pulse-radiolabeled molecules coincided with their release from calnexin. Released monomers and intracellular disposal were nonexistent in cells chased with cycloheximide, an inhibitor of protein synthesis. Post-translational trimming of asparagine-linked oligosaccharides and intracellular disposal were abrogated by 1-deoxymannojirimycin, an inhibitor of $\alpha$-mannosidase activity, without affecting the monomer population. The data are consistent with a recently proposed quality control model (Hammond, C., Braakman, I., and Helenius, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 913–917) in which intracellular disposal requires dissociation from calnexin and post-translational trimming of mannose from asparagine-linked oligosaccharides.

Conformational maturation of nascent polypeptides is facilitated by transient physical interaction with molecular chaperones. The general consensus is that rounds of binding prevent misfolding and subsequent entrance of partially folded intermediates into nonproductive folding pathways (1). In eukaryotic cells, nascent polypeptides destined to traverse compartments of the secretory pathway are translocated into the lumen of the endoplasmic reticulum (ER) during biosynthesis. Incompletely folded proteins often exhibit a persistent physical association with one or more molecular chaperones and are retained in the ER prior to intracellular disposal (for a review, see Ref. 3). This mechanism has been termed "quality control" (4) and apparently functions to ensure transport of only correctly folded proteins beyond the ER.

Calnexin (also designated p88 or IP90), a calcium-binding molecular chaperone of the ER membrane (5), forms a transient noncovalent association with several newly synthesized proteins in the ER (6–14). An important feature of calnexin is that it exhibits an affinity for monoglucosylated oligosaccharides (15), which are intermediates formed during cotranslational trimming by ER $\alpha$-glucosidases (16). Importantly, it can also be generated by post-translational reglucosylation of glycans, an event catalyzed by the ER resident protein UDP-glucose:glycoprotein glucosyltransferase (17–19). Interaction with calnexin can be prevented by drugs that either inhibit asparagine-linked glycosylation or arrest cotranslational trimming of attached glucose residues (6, 7, 20–22) and is virtually nonexistent in mutant cell lines deficient in ER $\alpha$-glucosidase I or II (23).

Protein folding and quality control machinery may participate in the molecular pathogenesis of plasma $\alpha_1$-antitrypsin (AAT) deficiency (24, 25). Human AAT is a monomeric glycoprotein of 394 amino acids (26, 27) and is secreted from liver hepatocytes (28). It is a member of the serine proteinase inhibitor superfamily (29) and protects lung elastin fibers from proteolytic destruction by inhibiting the activity of elastase released from activated neutrophils (30). Several allelic variants of the inhibitor exist (31), and many exhibit a distinct mutation predicted to preclude conformational maturation of the encoded polypeptide following biosynthesis (32). Defective intracellular transport of the aberrantly folded protein through compartments of the secretory pathway can diminish circulating levels of the inhibitor (25). Proteolytic destruction of lung elastin is associated with severe plasma AAT deficiency and is implicated in the pathogenesis of chronic obstructive lung disease (33).

Intracellular retention of most "null" AAT variants results from mutations that cause premature truncation of the polypeptide at its carboxyl terminus (34), a phenomenon predicted to prevent formation of specific secondary structural features (32, 35). In this study, conformation-based quality control of human AAT secretion was investigated in mouse hepatoma cells stably expressing the nonfunctional allelic variant QO Hong Kong (null(Hong Kong)), which is incapable of folding into the appropriate native structure. Null(Hong Kong) exhibits a Ca$^{2+}$-sensitive physical interaction with the molecular chaperone calnexin during intracellular retention (36) and requires release from the molecular chaperone as well as post-translational trimming of its asparagine-linked oligosaccharides for normal disposal. Predicted roles for each

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1 The abbreviations used are: ER, endoplasmic reticulum; AAT, $\alpha_1$-antitrypsin; PAGE, polyacrylamide gel electrophoresis.
of these events in the quality control of human AAT secretion are discussed.

MATERIALS AND METHODS

**Chemicals and Reagents**—Protein G-agarose, castanospermine, and 1-deoxymannojirimycin were purchased from Calbiochem. Importantly, 1-deoxymannojirimycin purchased from this source did not inhibit protein synthesis. Tunicamycin (homolog A1), cycloheximide, and all salts and buffers were purchased from Sigma. Easy Tag \[^{35}S\]Smethionine was purchased from DuPont NEN. All media used in tissue culture were purchased from Life Technologies, Inc. Fetal bovine serum was procured from Summit Biotechnology.

**Antisera**—An immunoglobulin fraction of goat anti-human AAT was purchased from Organon Teknika-Cappel. Rabbit polyclonal antisera against a synthetic polypeptide homologous to amino acids 487–505 in the cytoplasmic tail of canine calnexin was a gift from Drs. John J. M. Bergeron (McGill University) and Ari Helenius (Yale University).

**Cultured Cells**—The cell line H1A/N13 was previously generated by stable transfection of the mouse hepatoma cell line Hepa 1a with the cDNA encoding the human null (Hong Kong) AAT variant (37). Cells were maintained as monolayers in Dulbecco’s modified Eagle’s/Waymouth medium (3:1) containing 15% fetal bovine serum, 1

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identified as the human AAT monomer.

**Cotranslational Assembly with Calnexin**—Sucrose gradient sedimentation and coprecipitation techniques were used to characterize the time course of assembly between calnexin and newly synthesized null(Hong Kong). The entire population of newly synthesized molecules radiolabeled during a 15-min incubation with [35S]methionine sedimented at 6.8 S (Fig. 2A). In a double immunoprecipitation analysis, radiolabeled null(Hong Kong) was detected in a calnexin immunoprecipitate generated from the 6.8 S species (Fig. 3A, lane 2). Because less than quantitative coprecipitation may occur by this methodology, an experiment was performed in which the null(Hong Kong)-calnexin interaction was disrupted by chelation of calcium ions (36) prior to sucrose gradient sedimentation. This shifted sedimentation of the entire population of radiolabeled molecules to 4.5 S (Fig. 2A) and coincided with the disappearance of radiolabeled null(Hong Kong) in the calnexin immunoprecipitate (Fig. 3A, lane 4). Importantly, sedimentation of the 6.8 S complex was unaffected in response to an identical incubation without EGTA (data not shown), suggesting that dissociation was specific.

**Cotranslational Trimming of Glucose from Asparagine-linked Oligosaccharides Is Required for Assembly with Calnexin**—Ware et al. (15) have reported that calnexin exhibits an affinity for monoglucosylated oligosaccharides, and for many glycoproteins, interaction with calnexin is prevented by compounds that inhibit asparagine-linked glycosylation or that arrest cotranslational trimming of attached glucose residues (6, 7, 20–22). Since covalent addition of the oligosaccharide moiety Glc₃-Man₃-GlcNAc₂ occurs cotranslationally (16), the necessity of oligosaccharides in generating the null(Hong Kong)-calnexin association was tested by preincubating H1A/N13 cells with tunicamycin prior to pulse radiolabeling (Fig. 4). Inhibition of asparagine-linked glycosylation was confirmed by increased electrophoretic mobility of pulse-radiolabeled molecules (Fig. 4A, panel TUN, lane 1). Assembly with calnexin was not detected (Table I). Since the three terminal glucose residues are subjected to cotranslational hydrolysis (16), cells were preincubated with castanospermine, an inhibitor of ER α-glucosidases I and II (16), to maintain the oligosaccharide in the original
null(Hong Kong) (Fig. 4), confirmed by retarded electrophoretic mobility of pulse-radiolabeled molecules had resulted from their association with calnexin (Fig. 3), physical association with calnexin was not detected in the insoluble portion of the cell lysate (data not shown), which would be expected if loss of immunoprecipitable molecules had resulted from their interaction with calnexin, coincided with the onset of intracellular disposal. Approximately 70% of the radiolabeled molecules continued to sediment at 6.8 S after 2 h of chase (Fig. 2B). Double immunoprecipitation analysis demonstrated their physical interaction with calnexin (Fig. 3B, lane 4). Furthermore, the 6.8 S species was quantitatively shifted to 4.5 S after 2 h of chase (Fig. 2B), which coincided with loss of interaction with calnexin (Fig. 3B, lane 6). Importantly, ~30% of the radiolabeled molecules sedimented at 4.5 S after the 2-h chase (Fig. 2B), and no physical interaction with calnexin was detected by double immunoprecipitation (Fig. 3B, lane 2). Since the entire population of pulse-radiolabeled molecules was associated with calnexin immediately following biosynthesis (Fig. 2A), detection of the 4.5 S species was consistent with the idea that this population had been released during the chase.

Intracellular Disposal Coincides with Appearance of the 4.5 S Species—Since intracellular disposal of pulse-radiolabeled null(Hong Kong) is preceded by a 45-min lag (40), we asked whether appearance of released monomers coincided with the onset of disposal. The radiolabeled 4.5 S species was not detected until 60 min of chase (Fig. 5). At this time point, 20% of the radiolabeled molecules sedimented at 4.5 S (Fig. 5). Approximately 80% sedimented at 6.8 S, and only these exhibited a physical association with calnexin as judged by double immunoprecipitation analysis and altered sedimentation in response to EGTA (data not shown). Significantly, appearance of the 4.5 S species coincided with the first detectable loss of null(Hong Kong). The ratio of calnexin-associated (6.8 S) and monomeric (4.5 S) molecules remained relatively constant during the next 2 h, during which the majority of molecules were removed from the cell. It should be noted that radiolabeled null(Hong Kong) was not detected in the insoluble portion of the cell lysate (data not shown), which would be expected if loss of immunoprecipitable molecules had resulted from their insolubility.

Inhibition of Protein Synthesis Prevents Appearance of the 4.5 S Species and Arrests Intracellular Disposal—Cycloheximide is an inhibitor of protein synthesis and somehow blocks intracellular disposal of several transport-impaired proteins in the secretory pathway (40, 41). Disposal of pulse-radiolabeled null(Hong Kong) was nonexistent during a 3-h chase in medium containing 20 μg/ml cycloheximide (Fig. 6A). Furthermore, the gradual increase in the electrophoretic mobility of variant null(Hong Kong) that normally accompanies its intracellular retention (40) was absent (Fig. 6A, asterisk). Purumycin, another inhibitor of protein biosynthesis, had these same effects (data not shown). Significantly, arrested disposal coincided with our inability to detect the radiolabeled 4.5 S species by sucrose gradient sedimentation (Fig. 6B). Cycloheximide did not reverse the intracellular stability of null(Hong Kong) in cells preincubated with either tunicamycin or castanospermine (Fig. 6B), consistent with the idea that the mechanism of inhibiting disposal required association with calnexin.

I-Deoxymannojirimycin Arrests Disposal without Affecting Appearance of the 4.5 S Species—Deglycosylation with endoglycosidase H and subsequent fractionation by SDS-PAGE indicated that the gradual increase in the electrophoretic mobility of variant null(Hong Kong) during intracellular retention had resulted from post-translational modification of asparagine-linked oligosaccharides (data not shown). This conclusion was supported by the ability of I-deoxymannojirimycin, a mannoside analog capable of inhibiting the activity of several ER and Golgi α-mannosidases (42–45), to prevent this post-translational anomaly (Fig. 4A, panel DM). Preincubation with this mannoside analog did not prevent nascent null(Hong Kong) from interacting with calnexin (Table I). However, intracellular disposal of pulse-radiolabeled molecules was completely arrested by this

| Preincubation | Inhibits | Predicted oligosaccharide structure | NHK associated with calnexin (%) |
|--------------|---------|----------------------------------|---------------------------------|
| Control      | None    | N-linked glycosylation           | 100                             |
| Tunicamycin  | None    | α-Glucosidases I and II          | 100                             |
| Castanospermine | None | α-Mannosidase                     | 0                               |
| 1-Deoxymannojirimycin | None | Glc3-Man9-GlcNAc2                 | 100                             |

a. Monolayers were incubated with various inhibitors for 60 min prior to pulse radiolabeling.

b. NHK, null(Hong Kong).

c. Values were determined by sucrose gradient sedimentation and double immunoprecipitation.

Table I

Asparagine-linked glycosylation and trimming of oligosaccharides effect assembly of nascent null(Hong Kong) with calnexin

H1A/N13 cells were preincubated for 1 h with medium containing no additions (control), tunicamycin, castanospermine, or 1-deoxymannojirimycin prior to a 15-min pulse with [35S]methionine. Cell lysates were subjected to sucrose gradient sedimentation and double immunoprecipitation to detect associated calnexin. The predicted oligosaccharide structure resulting from each treatment is shown.

Glc3-Man9-GlcNAc2 structure. Inhibition of glucose was confirmed by retarded electrophoretic mobility of pulse-radiolabeled null(Hong Kong) (Fig. 4A, panel CST, lane 1). Again, assembly with calnexin was prevented (Table I).

Post-translational Appearance of the 4.5 S Species—Variant null(Hong Kong) undergoes intracellular disposal with a half-life of ~2 h (40). However, enhanced instability was observed in cells preincubated with either tunicamycin or castanospermine as compared with control cells (Fig. 4A), suggesting that interaction with calnexin protects from intracellular proteolysis. Since the entire population of pulse-radiolabeled molecules of variant null(Hong Kong) was bound to calnexin, we asked whether appearance of the 4.5 S monomer population, representing release from calnexin, coincided with the onset of intracellular disposal. Approximately 70% of the radiolabeled molecules continued to sediment at 6.8 S after 2 h of chase (Fig. 2B). Double immunoprecipitation analysis demonstrated their physical interaction with calnexin (Fig. 3B, lane 4). Furthermore, the 6.8 S species was quantitatively shifted to 4.5 S after 2 h of chase (Fig. 2B), which coincided with loss of interaction with calnexin (Fig. 3B, lane 6). Importantly, ~30% of the radiolabeled molecules sedimented at 4.5 S after the 2-h chase (Fig. 2B), and no physical interaction with calnexin was detected by double immunoprecipitation (Fig. 3B, lane 2). Since the entire population of pulse-radiolabeled molecules was associated with calnexin immediately following biosynthesis (Fig. 2A), detection of the 4.5 S species was consistent with the idea that this population had been released during the chase.

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**Fig. 5. Intracellular disposal of variant null(Hong Kong) and association with calnexin.** H1A/N13 cells were incubated for 15 min with [35S]methionine, and cells were chased for the designated periods prior to sucrose gradient sedimentation of cell lysates and detection of associated calnexin by double immunoprecipitation analysis (see “Materials and Methods”). Data are shown in the form of a bar graph. The percent of calnexin-associated molecules (6.8 S peak) is shown in black, and the percent of monomers (4.5 S peak) is shown in white.
null(Hong Kong) with calnexin. This indicates that the idea that dissociation from calnexin required protein synthesis. Although our experiments have not addressed the mechanism by which dissociation was inhibited, one possibility is that a short-lived protein is required for normal disruption of the null(Hong Kong)-calnexin complex. Cycloheximide treatment had the added effect of preventing post-translational trimming of oligosaccharides attached to null(Hong Kong). At present, we do not know whether this reflects inaccessibility of glycans for trimming while null(Hong Kong) is bound to calnexin. However, previous experiments have suggested that the null(Hong Kong)-calnexin interaction is stabilized by a peptide-peptide interaction (36). Furthermore, incubation with several combinations of monosaccharides has failed to dissociate the complex after coprecipitation,2 suggesting that the glycan moiety is not involved in stabilizing the interaction after assembly. These data are in agreement with a two-step binding model proposed by Tector and Salter (12) in which the function of the monoglucosylated oligosaccharide is to initiate assembly of glycoproteins with calnexin. However, we cannot disregard the possibility that a lectin-like interaction does exist, but is disrup
tally or immediately following biosynthesis.

Interaction with calnexin was prevented by inhibiting asparagine-linked glycosylation with tunicamycin or by arresting cotranslational trimming of attached glucose residues with castanospermine. This indicates that like many other glycoproteins (11, 47), oligosaccharides may somehow function to facilitate assembly between null(Hong Kong) and calnexin. Intracellular disposal of nascent null(Hong Kong) was accelerated in cells preincubated with either tunicamycin or castanospermine, suggesting that disposal occurs in the absence of the bound molecular chaperone. This idea was consistent with appearance of the 4.5 S species at a time point that coincided with the onset of intracellular disposal. The absence of associated calnexin and sedimentation at 4.5 S suggests that this population of molecules represents the released monomer (36, 39). No changes in the monomer population were observed when cell lysis was performed under less stringent conditions (data not shown), suggesting that the 4.5 S population does not merely reflect weakening of the null(Hong Kong)-calnexin interaction during retention, but represents unassociated molecules. It should be pointed out that an earlier report (36), an error in our fractionation method, overlap between the 4.5 S and 6.8 S species, and incomplete dissociation of complexes in response to incubation with EGTA led to the incorrect conclusion that an additional sedimenting species was detected under steady-state conditions. It is now apparent that the most abundant forms of null(Hong Kong) are the 4.5 S and 6.8 S species. However, we cannot rule out the possibility that additional, more transient species exist.

Intracellular disposal of null(Hong Kong) was arrested during incubation of pulse-radioabeled cells with cycloheximide, and this coincided with complete absence of the 4.5 S monomer population. Cycloheximide had no demonstrable effect on the intracellular stability of null(Hong Kong) in cells preincubated with either tunicamycin or castanospermine, which supported the idea that dissociation from calnexin required protein synthesis. Although our experiments have not addressed the mechanism by which dissociation was inhibited, one possibility is that a short-lived protein is required for normal disruption of the null(Hong Kong)-calnexin complex. Cycloheximide treatment had the added effect of preventing post-translational trimming of oligosaccharides attached to null(Hong Kong). At present, we do not know whether this reflects inaccessibility of glycans for trimming while null(Hong Kong) is bound to calnexin. However, previous experiments have suggested that the null(Hong Kong)-calnexin interaction is stabilized by a peptide-peptide interaction (36). Furthermore, incubation with several combinations of monosaccharides has failed to dissociate the complex after coprecipitation, suggesting that the glycan moiety is not involved in stabilizing the interaction after assembly. These data are in agreement with a two-step binding model proposed by Tector and Salter (12) in which the function of the monoglucosylated oligosaccharide is to initiate assembly of glycoproteins with calnexin. However, we cannot disregard the possibility that a lectin-like interaction does exist, but is disrupted during immunoprecipitation.

Intracellular disposal of null(Hong Kong) was also arrested in cells treated with 1-deoxymannojirimycin, which also prevented demonstrable post-translational trimming of asparagine-linked oligosaccharides. Originally, only one α-mannosidase activity had been assigned to the ER (16). However, in recent years, several α-mannosidases have been identified in the ER, many of which are inhibited by 1-deoxymannojirimycin (42–45). Unlike cycloheximide treatment, in which inhibition

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2 R. N. Sifers, unpublished data.
of disposal coincided with a complete absence of the 4.5 S peak, inhibition with 1-deoxymannojirimycin did not alter the monomer population. Taken together, data from this study suggest that variant null (Hong Kong) is subjected to a quality control pathway identical to that recently proposed by Hammond and Helenius (11). Their model proposes that persistent association between unfolded glycoproteins and calnexin reflects a continuous cycle of binding in which assembly of the complex is facilitated by reglucosylation of asparagine-linked oligosaccharides by the ER resident enzyme UDP-glucose:glycoprotein glucosyltransferase (17–19, 48). Importantly, the model predicts that permanent dissociation from the binding cycle will occur when hydrolysis of mannose residues generates an oligosaccharide unable to participate as a glucose acceptor, thereby leading to disposal of the unfolded glycoprotein. Consistent with this model, inhibition of oligosaccharide trimming by 1-deoxymannojirimycin abrogated intracellular disposal of variant null (Hong Kong) without affecting the percent population of 4.5 S monomers present in the cell. This latter finding would be expected if the protein is degraded only when it can no longer participate in the cycle of binding to calnexin, which would result after extensive post-translational trimming of glycans by α-mannosidase. Further dissection of this quality control pathway will be the subject of future investigations and will enhance our current understanding of how incompletely folded human AAT variants are retained and degraded in hepatocytes.

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