Intracellular Association between UDP-glucose:Glycoprotein Glucosyltransferase and an Incompletely Folded Variant of $\alpha_1$-Antitrypsin*

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Genetic variants of human $\alpha_1$-antitrypsin unable to fold into the native structural conformation are poorly secreted from hepatocytes. The molecular chaperone calnexin coimmunoprecipitates with secretion-incompetent variant null(Hong Kong) retained in stably transfected mouse hepatoma cells (Le, A., Steiner, J. L., Ferrell, G. A., Shaker, J. F., and Sifers, R. N. (1994) J. Biol. Chem. 269, 7514–7519). Mobilization of intracellular Ca$^{2+}$ stores with metabolic poisons diminished interaction with calnexin and coincided with coimmunoprecipitation of a 150-kDa protein (p150). Mobilization of endoplasmic reticulum luminal Ca$^{2+}$ with thapsigargin, an inhibitor of the microsomal Ca$^{2+}$ ATPase, gave a similar result. Coimmunoprecipitation of p150 was specifically disrupted in response to incubation of the cell lysate with exogenous CaCl$_2$. Finally, in ECL Western blotting, p150 was recognized by polyclonal antiserum against UDP-glucose:glycoprotein glucosyltransferase that likely functions in glycoprotein folding and quality control (Sousa, M. C., Ferrero-Garcia, M. A., and Parodi, A. J. (1992) Biochemistry 31, 97–105). The data are consistent with a model in which perturbation of endoplasmic reticulum Ca$^{2+}$ results in a stable physical association between unfolded human $\alpha_1$-antitrypsin and UDP-glucose:glycoprotein glucosyltransferase.

In eukaryotes, proteins destined for secretion are translated as nascent polypeptides into the lumen of the endoplasmic reticulum (ER) (for a review, see Ref. 1). Folding into the native conformation, a structure dictated by the primary amino acid sequence (2), is facilitated through transient interaction with one or more molecular chaperones (3). Conformational fidelity of folded structures is monitored by a poorly understood quality control system (4) which prevents transport of incompletely folded and unassembled proteins beyond the ER (5).

Cotranslational addition of Glc$_3$Man$_9$GlcNAc$_2$ to specific asparagine residues and hydrolysis of attached glucose units can accompany translocation of the nascent polypeptide (6). Reglucosylation of high mannose-type glycans has been detected in microsomal preparations from mammals, plants, fungi, yeast, and protozoa (7, 8), and it is catalyzed by the ER resident protein UDP-glucose:glycoprotein glucosyltransferase (UGTR) (8–10). Importantly, only high mannose-type oligosaccharides attached to unfolded proteins function as acceptors in the glucose transfer reaction (11–13). Results from a cell-free system indicate that the unfolded polypeptide and asparagine-linked GlcNAc are responsible for eliciting glucose transfer (13).

Several nascent proteins (14–18) form transient associations with calnexin (also designated p88 or IP90), a calcium-binding protein of the ER membrane (19). Since calnexin functions as a molecular chaperone for glycoproteins (17, 20) and interacts with monoglucosylated oligosaccharides (21), Hammond et al. (15) proposed that reglucosylation by UGTR may function to initiate assembly between unfolded glycoproteins and the molecular chaperone. In support of this idea, Labriola et al. (22) have reported that delivery of a nascent acid hydrolase to lysosomes of Trypanosoma cruzi is delayed by inhibition of ER $\alpha$-glucosidase activity and is a predictable response if attached monoglucosylated oligosaccharides are interacting with calnexin.

Recent evidence suggests that protein folding and quality control machinery may participate in the molecular pathogenesis of several human diseases caused by defective intracellular transport of an aberrantly folded protein through the secretory pathway (23–25). Human $\alpha_1$-antitrypsin (AAT) is a 394-amino acid protein (26, 27) glycosylated at three specific asparagine residues (28). It is folded into a highly ordered tertiary structure containing three $\beta$-sheets, nine $\alpha$ helices, and three internal salt bridges (29). The human AAT structural gene is highly polymorphic (30), and several alleles exhibit a distinct mutation predicted to preclude conformational maturation of the encoded polypeptide following biosynthesis (31). Secretion of AAT from hepatocytes (32, 33) is impaired in response to incomplete folding of the polypeptide (34, 35).

AAT is a member of the serine proteinase inhibitor superfamily (36). Since elastase released by activated neutrophils is rendered inactive by the inhibitor (37), diminished circulating levels can result in proteolytic destruction of lung elastin, a phenomenon implicated in the pathogenesis of chronic obstructive lung disease (38). “Loop-sheet” polymerization is apparently responsible for accumulation of a subset of human AAT variants in the ER of hepatocytes (31). However, impaired secretion of the majority of variants does not include detectable intracellular accumulation. Most “null” alleles encode a polypeptide truncated at the carboxyl terminus (39), which is a phenomenon predicted to preclude formation of specific secondary structural features (31) and can prevent loop-sheet polymerization. Variant QO Hong Kong (null[Hong Kong]) cannot attain conformational maturation following biosynthesis, forms a physical interaction with molecular chaperone calnexin.

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¶ The abbreviations used are: ER, endoplasmic reticulum; AAT, $\alpha_1$-antitrypsin; PAGE, polyacrylamide gel electrophoresis; UGTR, UDP-glucose:glycoprotein glucosyltransferase.
Briefly, H1A/N13 cells were cultured overnight on coverslips and then chased from Life Technologies, Inc. Fetal bovine serum was procured from Summit Biotechnology.

**Cultured Cells**—The cell line H1A/N13 was previously generated by stable transfection of the mouse hepatoma cell line Hepa 1a with subcloned DNA encoding the human null(Hong Kong) AAT variant (40). Cells were maintained as monolayers in Dulbecco’s modified Eagle's Waymouth medium (3:1) containing 15% fetal bovine serum, 1 M NaCl, 1 M NaF, and a mixture of protease inhibitors as described previously (24). Insoluble material was removed by centrifugation at 10,000 × g for 10 min. Cellular toxicity was measured by trypan blue dye exclusion. The hemocytometer was used to detect cells stained during a 5-min incubation with dye. Greater than 98% remained viable in response to all reported manipulations.

**Immunoprecipitation**—Detection of proteins coprecipitating with variant null(Hong Kong) was performed as described previously (24). Briefly, soluble lysates were incubated for 120 min at 4 °C with an IgG fraction of goat anti-human AAT (Organon Teknika-Cappel) pre-immobilized to Protein G-agarose (24). Immunocomplexes were washed with cold phosphate-buffered saline and scraped with a spatula in cell lysis buffer (0.1 M Tris-HCl, pH 7.4, containing 0.5% Nonidet P-40, 0.15 M NaCl) and a mixture of protease inhibitors as described previously (24). Insoluble material was removed by centrifugation at 10,000 × g for 10 min. Cellular toxicity was measured by trypan blue dye exclusion. The hemocytometer was used to detect cells stained during a 5-min incubation with dye. Greater than 98% remained viable in response to all reported manipulations.

**Immunoblotting**—Immunoprecipitated proteins were fractionated by SDS-PAGE and electrophoretically transferred to Hybond nitrocellulose (Amersham Corp.) as described previously (24) except that 0.1% SDS (0.1%) was included in the transfer buffer. Enhanced chemiluminescence (ECL) Western blotting (Amersham Corp.) was performed according to the manufacturer’s instructions, except that SuperSignal (Pierce) was used as the detection reagent. Calnexin was detected by incubation of blots with a 1:1000 dilution of rabbit polyclonal antiserum against rat UDP-glucose:glycoprotein glucosyltransferase (43). Protein G-agarose (Pierce) was used as the detection reagent. Calnexin was detected by chemiluminescence (ECL) Western blotting (Amersham Corp.) was performed using the Howtek Scanmaster 3 quantitative methods scanner was used for quantitation of band intensities corresponding to radiolabeled proteins detected in fluorograms.

**Detection of Intracellular Ca²⁺ Stores**—Intracellular Ca²⁺ distribution was examined by scanning laser confocal microscopy using the fluorescent Ca²⁺ probe Fluo-3 (Molecular Probes Inc., Eugene, OR). Briefly, H1A/N13 cells were cultured overnight on coverslips and then treated as described in the appropriate figure legend. Cells were then incubated for 2 min at 37 °C with 2 μM Fluo-3. Coverslips were transferred to an inverted glass slide to ensure adherence and placed on an inverted Nikon Diaphot microscope (Nikon, New York, NY). Cells were scanned for maximum fluorescence via a section series by scanning laser confocal microscopy.

**RESULTS**

**Coprecipitation of a 150-kDa Protein in Response to Treatment of Cells with Metabolic Poisons**—A. H1A/N13 cell monolayers were pulse-radiolabeled for 15 min with [³⁵S]methionine (lane 1) and then chased for 60 min in regular growth medium (lane 2) or in medium supplemented with 10 mM NaF and 40 mM NaN₃ (lane 3). B. non-transfected Hepa 1a cell monolayers were treated identical to that described in panel A. AAT was immunoprecipitated from cell lysates, and radiolabeled proteins were fractionated by SDS-PAGE prior to detection by fluorography. The molecular mass of radiolabeled proteins is shown in kilodaltons (kDa).

**Association with UGTR**

![Figure 1](image-url)
H1A/N13 cells were pulse-radiolabeled for 15 min with [35S]methionine. Cells were chased for 60 min with regular growth medium (lane 1) or medium supplemented with 10 mM NaF and 40 mM NaN₃ (lane 2). In lane 3, cells were treated as in lane 2 followed by an additional 60-min incubation with regular growth medium. In lane 4, cells were incubated for 120 min in medium supplemented with 10 mM NaF and 40 mM NaN₃ (lane 2), 10 mM NaF (lane 3), or 40 mM NaN₃ (lane 4). Variant null(Hong Kong) (NHK) was immunoprecipitated, and radiolabeled proteins were detected by fluorography after being fractionated by SDS-PAGE. The molecular mass of radiolabeled proteins is shown in kilodaltons (kDa).

**Reversible and Cumulative Intracellular Association**—To rule out the possibility that intracellular association with p150 was merely an artifact of cellular toxicity, pulse-radiolabeled cells were incubated for 60 min in medium containing NaF and NaN₃, and then incubated for an additional 60-min period in regular growth medium. Coimmunoprecipitation of p150 was not detectable following the second incubation (Fig. 2A, compare lanes 2 and 3), suggesting that the interaction was reversible. In a parallel experiment, trypan blue was excluded by >98% of cells (not shown), confirming that treatment with metabolic poisons had not resulted in cell death.

Intensity of coimmunoprecipitating p150 increased approximately 5-fold in cells incubated for 120 min with medium containing NaF and NaN₃ (Fig. 2A, lane 4) as compared with those incubated for only 60 min (Fig. 2A, lane 2). Because intracellular disposal was unaffected by the longer incubation period increased coimmunoprecipitation of p150 likely reflected gradual intracellular accumulation of the null(Hong Kong)-p150 complex.

As combinations of metabolic poisons are required for efficient reduction of intracellular ATP levels (44), we tested the ability of NaF and NaN₃ individually to facilitate coprecipitation of p150. Incubation with either compound alone did not result in detectable coimmunoprecipitation of p150, and there was no apparent increase in electrophoretic mobility of radiolabeled molecules (Fig. 2B, lanes 3 and 4). Importantly, coimmunoprecipitation of p150 required incubation with both compounds (Fig. 2B, lane 2). These data are consistent with the idea that detectable interaction between null(Hong Kong) and p150 had resulted from reduction of intracellular ATP levels.

**Exogenous Ca²⁺ Disrupts the Null(Hong Kong)-p150 Complex**—Since coimmunoprecipitation of p150 resulted from incubation of cells with metabolic poisons we asked whether the complex would dissociate in the presence of nucleotide triphosphates. In separate experiments cell lysates containing the accumulated complex were incubated for 10 min at 37 °C with exogenous ATP or GTP prior to immunoprecipitation of null(Hong Kong). Coimmunoprecipitation of p150 was unaffected (not shown). Reduction of intracellular ATP levels can inactivate microsomal Ca²⁺-ATPase, the enzyme responsible for maintaining the high ER luminal Ca²⁺ concentration (45, 46), so we considered the possibility that the formation of the complex might have resulted from mobilization of intracellular Ca²⁺ stores. To test this hypothesis cell lysates containing the accumulated complex were incubated for 10 min at 37 °C in the presence of 1 mM CaCl₂. As shown in Fig. 3, coimmunoprecipitation of p150 was diminished to 36% of that detected in the absence of the divalent cation. In three separate experiments incubation with CaCl₂ diminished co-immunoprecipitation of p150 to a mean value of 40.1 ± 5.3% (not shown).

**Coimmunoprecipitation of p150 Coincides with Mobilization of Intracellular Ca²⁺ Stores**—Changes in intracellular Ca²⁺ distribution was monitored by scanning laser confocal microscopy after incubation with the fluorescent Ca²⁺ probe Fluo-3 (see “Materials and Methods”). Intense staining of intracellular Ca²⁺ stores became very diffuse in response to treatment of cells with metabolic poisons (Fig. 4, compare panels A and B), indicating that coimmunoprecipitation of p150 did coincide with mobilization of intracellular Ca²⁺ stores. Toxicity of Ca²⁺ ionophores A23187 and ionomycin (not shown) prevented their use in our analyses. To ask whether mobilization of ER Ca²⁺ stores was important pulse-radiolabeled cells were incubated for 60 min with medium containing 10 μM thapsigargin, a specific inhibitor of microsomal Ca²⁺ ATPase (45, 47, 48). Coimmunoprecipitating p150 was detected in thapsigargin-treated cells (Fig. 5, lane 2) which was consistent with the notion that reduction of ER luminal Ca²⁺ stores did coincide with intracellular accumulation of the null(Hong Kong)-p150 complex.

**Decreased Association with Molecular Chaperone Calnexin**—Since variant null(Hong Kong) normally exhibits a Ca²⁺-sensitive interaction with molecular chaperone calnexin (24), in the next set of experiments ECL Western blotting was used as a method to quantitate changes in coprecipitation of the molecular chaperone at conditions that favored detectable formation of null(Hong Kong)-p150. Incubation for 60 min with 10 μM thapsigargin diminished coprecipitation of calnexin to only 5.3% of that detected in untreated cells (Fig. 6, compare lanes 3 and 4). Association with calnexin was reduced to 35.4% of normal in response to a 60-min incubation with metabolic poisons NaF and NaN₃ (Fig. 6, lane 5), and this was restored to

**Fig. 2. Association with p150 is reversible and cumulative. A.** H1A/N13 cells were pulse-radiolabeled for 15 min with [35S]methionine. Cells were chased for 60 min with regular growth medium (lane 1) or medium supplemented with 10 mM NaF and 40 mM NaN₃ (lane 2). In lane 3, cells were treated as in lane 2 followed by an additional 60-min incubation with regular growth medium. In lane 4, cells were incubated for 120 min in medium supplemented with 10 mM NaF and 40 mM NaN₃ (lane 2), 10 mM NaF (lane 3), or 40 mM NaN₃ (lane 4). Variant null(Hong Kong) (NHK) was immunoprecipitated, and radiolabeled proteins were detected by fluorography after being fractionated by SDS-PAGE. The molecular mass of radiolabeled proteins is shown in kilodaltons (kDa).

**Fig. 3. Exogenous Ca²⁺ facilitates dissociation of bound p150.** H1A/N13 cells were pulse-radiolabeled for 15 min with [35S]methionine and chased for 2 h in medium supplemented with 10 mM NaF and 40 mM NaN₃. A, cell lysates were incubated for 10 min at 37 °C in the absence (lane 1) or presence of 1 mM CaCl₂ (lane 2). Variant null(Hong Kong) (NHK) was immunoprecipitated, and radiolabeled proteins were fractionated by SDS-PAGE and detected by fluorography. The molecular mass of radiolabeled proteins is shown in kilodaltons (kDa). B, quantification of coimmunoprecipitated p150 described from the experiment shown in panel A following incubation with 1 mM CaCl₂ (Ca⁺⁺) or with no additions (Control). The amount of associated p150 (% UGTR association) was calculated as a percent of the control value, which was 100%.
normal values following an additional 60-min incubation with regular growth medium (Fig. 6, lane 6). The data indicated that detectable binding of p150 to null(Hong Kong) coincided with reduced levels of associated calnexin.

Recognition of p150 by Antiserum against UDP-glucose:Glycoprotein Glucosyltransferase—ECL Western blotting was employed as a method to confirm the identity of coprecipitating p150. p150 did not cross-react with antiserum against human AAT or calnexin, indicating that it did not consist of an SDS-resistant aggregate of null(Hong Kong) or contain the molecular chaperone, respectively (not shown). In the course of our analyses we tested the hypothesis that p150 might actually be UGTR, which is composed of two identical 150-kDa subunits (49) and has been implicated as a component of glycoprotein quality control (15, 17). The prediction was verified in that coprecipitated p150 exhibited cross-immunoreactivity with a polyclonal antiserum raised against rat UGTR (Fig. 7, lane 1). Furthermore, mobility of p150 in SDS-PAGE was identical to that of immunoreactive murine UGTR detected in the cell lysate (Fig. 7, lane 1), and the signal was absent from a null(Hong Kong) immunoprecipitate generated from untreated cells (Fig. 7, lane 4). It should be noted that the intensity of the band migrating slightly faster than authentic UGTR (Fig. 7, lane 1) is over-represented because of the longer length of exposure needed to detect coprecipitating UGTR.

FIG. 5. p150 coprecipitates in response to thapsigargin treatment. H1A/N13 cell monolayers were pulse-radiolabeled for 15 min with [35S]methionine and chased for 60 min with medium either containing no additions (lane 1) or supplemented with 10 μM thapsigargin (lane 2). Null(Hong Kong) (NHK) was immunoprecipitated from cell lysates, and radiolabeled proteins were fractionated by SDS-PAGE and detected by fluorography. The molecular mass of radiolabeled proteins is shown in kilodaltons (kDa).

FIG. 6. Effect of metabolic poisons and thapsigargin on coprecipitation of calnexin. H1A/N13 cells were incubated with medium containing compounds described below. Null(Hong Kong) was immunoprecipitated from cell lysates and immunocomplexes were fractionated by SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes and coimmunoprecipitating calnexin (CXN) was detected by ECL Western blotting using calnexin-specific antiserum (see "Materials and Methods"). Shown are a crude H1A/N13 cell extract (lane 1), a blank lane (lane 2), cells incubated for 60 min with regular growth medium (lane 3), and after the same incubation in medium supplemented with 10 μM thapsigargin (lane 4). Cells were incubated for 60 min with 10 mM NaF and 40 mM NaN3 (lane 5) or incubated as in lane 5 followed by an additional 60-min incubation with regular growth medium (lane 6). The molecular mass of immunoreactive proteins is shown in kilodaltons (kDa).

DISCUSSION

In the present study, a physical association between immunoreactive UGTR and secretion-incompetent variant null(Hong Kong) was detected in response to incubation of cells with metabolic poisons. The interaction was cumulative and required incubation of cells with both metabolic poisons, sodium fluoride and sodium azide. The exclusion of trypan blue from treated cells and the absence of coprecipitating UGTR following removal of metabolic poisons suggest that formation of the complex did not represent an artifact of cell death. Detection of radiolabeled UGTR in the context of our radiolabeling procedure suggests that it may contain a significant number of methionine residues or exhibit rapid intracellular turnover. Intracellular association between null(Hong Kong) and calnexin was diminished at conditions that induced coprecipitation of UGTR. Because ATP is required to stabilize calnexin-glycoprotein interactions in the ER (43), and reduction of nucleotide levels would be expected to dissociate the null(Hong Kong)-calnexin complex.

Exogenous ATP had no influence on stability of the accumulated null(Hong Kong)-UGTR interaction, suggesting that lowered intracellular availability of the nucleotide was not solely responsible for intracellular accumulation of the complex. Ability of thapsigargin treatment to result in detectable coprecipitation of p150, plus destabilization of the interaction during a 37 °C incubation with exogenous Ca2+, supported the hypothesis that depletion of ATP by metabolic poisons might have resulted in inactivation of the microsomal Ca2+/ATPase pump,
leading to reduction of ER lumenal Ca\textsuperscript{2+} stores. In support of this concept, depletion of ATP in rat liver microsomes induced calcium release and pump inhibition, and these effects were mimicked by thapsigargin treatment. Considering these data, it is reasonable to conclude that reduction of ER lumenal Ca\textsuperscript{2+}, occurring as an indirect effect of depleting intracellular ATP, likely played a significant role in causing detectable accumulation of the null(Hong Kong)-UGTR complex and is also supported by coprecipitation of UGTR in response to mobilization of the thapsigargin-sensitive Ca\textsuperscript{2+} pool.

The physical interaction between null(Hong Kong) and molecular chaperone calnexin (24, 50) is disrupted by incubation with Ca\textsuperscript{2+} chelators (24). Consistent with this finding was the observation that thapsigargin treatment diminished interaction between calnexin and null(Hong Kong). Furthermore, the treatment diminished interaction with calnexin to a greater extent than did metabolic poisons. Despite this fact, coprecipitation of UGTR in response to mobilization of the thapsigargin-sensitive Ca\textsuperscript{2+} pool.

A recently proposed model (15, 17) suggests that asparagine-linked oligosaccharides of unfolded glycoproteins participate in a cycle of reglucosylation/reglucosylation in which these events are catalyzed by UGTR and α-glucosidase II, respectively. Cycles of transient interaction with UGTR, each resulting in reglucosylation of attached oligosaccharides, is believed to facilitate interaction between unfolded glycoproteins and calnexin and ensure the retention of improperly folded glycoproteins in the ER. We have observed post-translational incorporation of mannose-resistant radiolabel into asparagine-linked oligosaccharides of null(Hong Kong) in H1A/N13 cells incubated with \(^{3}H\)galactose, suggesting that they likely participate as substrates for UGTR. Conceivably, the steady-state concentration of the transiently-formed complex is beyond the detection limits of ECL Western blotting. Conceivably, intracellular accumulation of the null(Hong Kong)-UGTR complex would result if the two components normally interact with one another in a transient fashion but require Ca\textsuperscript{2+} for dissociation. Data generated in the present study lend support to the proposed precursor-product relationship between UGTR association and subsequent assembly with calnexin. However, we cannot exclude the possibility that formation of these null(Hong Kong)-containing complexes, in the context of this study, occurred as mutually exclusive events.

Asparagine-linked oligosaccharides of unfolded glycoproteins with the structure of Man\textsubscript{9,9}GlcNAc\textsubscript{2} function as acceptors in the glucose transfer reaction (11). Since catalysis of glucose transfer by UGTR is a Ca\textsuperscript{2+}-dependent process (49) it has been suggested that hydrolysis of UDP-glucose, which is coupled to glucose transfer, may provide the energy necessary for dissociating bound glycoproteins from UGTR (8). We have been unable to demonstrate transfer of glucose to oligosaccharides of null(Hong Kong) during Ca\textsuperscript{2+}-dependent dissociation of UGTR, but this may reflect poor detection limits associated with our analysis. Also, Ca\textsuperscript{2+}-dependent dissociation of UGTR does not occur following coprecipitation of the complex, even in the presence of UDP-glucose. If dissociation is coupled to glucose transfer then binding of the antibody to null(Hong Kong) may somehow interfere with recognition of oligosaccharides by UGTR.

The concept that UGTR might participate in glycoprotein folding and quality control originally emerged from experiments performed in cell-free systems. However, in a recent report, Fernandez et al. (8) detected a 2–9-fold induction of UGTR mRNA in Schizosaccharomyces pombe at conditions known to perturb glycoprotein folding and induce synthesis of stress proteins. The structural gene was cloned and compared with that in Drosophila melanogaster. A conserved yeast heat shock promoter sequence was detected in the 5′ terminus, providing an explanation of how its expression is induced similar to that of several stress proteins. Our observations demonstrating that intracellular accumulation of the null(Hong Kong)-UGTR complex lend additional support to this hypothesis. Co-immunoprecipitation of intracellular complexes containing AAT furnishes an opportunity to subject the quality control model to experimental analysis, which will provide further insight into the molecular pathogenesis of pulmonary emphysema resulting from severe plasma AAT deficiency.

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