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Processing by Endoplasmic Reticulum Mannosidases Partitions a Secretion-impaired Glycoprotein into Distinct Disposal Pathways*

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In the early secretory pathway, a distinct set of processing enzymes and family of lectins facilitate the folding and quality control of newly synthesized glycoproteins. In this regard, we recently identified a mechanism in which processing by endoplasmic reticulum mannosidase I, which attenuates the removal of glucose from asparagine-linked oligosaccharides, sorts terminally misfolded α_1-antitrypsin for proteasome-mediated degradation in response to its abrogated physical dissociation from calnexin (Liu, Y., Choudhury, P., Cabral, C., and Sifers, R. N. (1999) *J. Biol. Chem*. 274, 5861–5867). In the present study, we examined the quality control of genetic variant PI Z, which undergoes inappropriate polymerization following biosynthesis. Here we show that in stably transfected hepatoma cells the additional processing of asparagine-linked oligosaccharides by endoplasmic reticulum mannosidase II partitions variant PI Z away from the conventional disposal mechanism in response to an arrested posttranslational interaction with calnexin. Intracellular disposal is accomplished by a nonproteasomal system that functions independently of cytosolic components but is sensitive to tyrosine phosphatase inhibition. The functional role of ER mannosidase II in glycoprotein quality control is discussed.

In the endoplasmic reticulum (ER),[1] an assortment of molecular chaperones and folding enzymes facilitate the conformational maturation of newly synthesized polypeptides destined for deployment to the cell surface as biologically active proteins (1). In recent years, a picture has emerged that describes how asparagine-linked glycosylation, in combination with several independently acting enzymes, facilitates glycoprotein folding (for reviews, see Refs. 2 and 3). In a widely accepted model (4), the partial deglucosylation of asparagine-linked $Glc_3Man_9GlcNAc_2$ induces cotranslational physical interaction between glycoproteins and members of a small family of lectins, each of which recognizes the monoglucosylated glycan as ligand (2). Dissociation of the complex coincides with the removal of glucose by glucosidase II (5). In the absence of conformational maturation, UDP-glucose:glycoprotein glucosyltransferase (UGTR) functions as a folding sensor (6) that recognizes structural determinants common to nonnative glycoprotein structure (7, 8). Reglucosylation of asparagine-linked oligosaccharides induces the reassembly of folding intermediates with calnexin (4). As such, reversible glucosylation hinders premature exit from the ER (2, 4, 9) until correctly folded molecules that are no longer substrates for UGTR are released from the lectin-mediated retention cycle (4).

As a rule, failure to attain conformational maturation following biosynthesis results in the selective elimination of misfolded polypeptides and unassembled protein complexes by a relatively stringent mechanism of conformation-based quality control (10, 11). Molecular characterization of primary and secondary disposal systems, plus the identification of the full repertoire of quality control machinery, is currently under intense investigation (for a review, see Ref. 11). Because the efficiency of modification by UGTR and glucosidase II is sensitive to the number of mannose units within the asparagine-linked oligosaccharide (6, 13), it is possible that oligosaccharide processing represents the meeting point between protein folding and quality control pathways. For this reason, recent work has focused toward elucidating the potential role of processing mannosidases in glycoprotein quality control (9, 14, 15).

In addition to its fundamental importance in normal cell physiology (16), the process of quality control in the early secretory pathway has been implicated as a key factor in the molecular pathogenesis associated with several human disorders (17, 18). To this end, a major physiologic role for the monomeric secretory glycoprotein α_1-antitrypsin (AAT) is to prevent the destruction of lung elastin. The hindered secretion and disposal of allelic variants from liver hepatocytes, the predominant site of biosynthesis (19), can lead to plasma AAT deficiency. A severe deficiency of the plasma protein is known to function as a heritable risk factor for the development of chronic obstructive lung disease (for reviews, see Refs. 20 and 21). Gene expression studies performed in stably transfected murine hepatoma cells have allowed for the characterization of AAT quality control mechanisms in a physiologically relevant model system (9, 22–25). The truncation of carboxyl-terminal amino acids in genetic variant PI QO Hong Kong (null(Hong Kong)) (22) precludes conformational maturation following biosynthesis, resulting in its lectin-mediated intracellular retention prior to disposal (9). Recently, we (26) proposed a model of quality control in which the removal of a single terminal α1,2-linked mannose unit from multiple asparagine-linked oligosaccharides abrogates the physical dissociation of null(Hong Kong) from the ER lectin calnexin (27), leading to its selective degradation by the cytosolic proteasome (28). In this process of "molecular capture," the attenuated removal of glucose from asparagine-linked oligosaccharides functions as the underlying mechanism by which the misfolded glycoprotein is selected for degradation.

In the present study, stably transfected hepatoma cells were

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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.

used for the molecular characterization of the conformation-based quality control of variant PI Z, the most common severe deficiency variant of human AAT (29). A single amino acid substitution at the base of the reactive center loop (12, 30) favors inappropriate polymerization (31) of a late folding intermediate (32), hindering its secretion from liver hepatocytes. The structural anomaly has been detected by velocity sedimentation (24), fluorescence-based and ultrastructural analyses (31), and transverse urea gradient gels following *in vitro* refolding (32). Enhanced secretion of recombinant PI Z bearing site-directed mutations predicted to impede loop-sheet polymerization (33) has confirmed that the structural anomaly plays a predominant role in the intracellular transport defect. The intracellular accumulation of an insoluble fraction of PI Z polymers is responsible, in part, for a heritable form of liver cirrhosis (21), and a hindered rate of disposal has been linked to this phenotype (34). Thus, the molecular characterization of PI Z quality control may lead to the development of therapeutic interventions to alleviate the severity of the lung and liver disease. Here we demonstrate that the selective elimination of variant PI Z is accomplished by a nonproteasomal mechanism that is cytosol-independent but sensitive to general inhibitors of tyrosine phosphatase activity. The results of glycosidase inhibitor studies plus coimmunoprecipitation analyses indicate that the combined processing of asparagine-linked oligosaccharides by ER mannosidases I and II (35) partitions variant PI Z away from the conventional proteasome-mediated disposal mechanism by preventing posttranslational assembly with calnexin. These data implicate ER mannosidase II as a component of glycoprotein quality control and identify a natural molecular strategy to partition variant PI Z away from the conventional proteasome-mediated disposal pathway.

MATERIALS AND METHODS

Inhibitors and Salts—Glycosidase inhibitors were purchased from Toronto Research Chemicals, Inc. and Roche Molecular Biochemicals. All phosphatase inhibitors and proteasome inhibitors, except for lactacystin, were purchased from Calbiochem. Lactacystin was purchased from the E. J. Corey laboratory (Harvard Medical School). All routine buffers and salts were procured from Sigma.

Metabolic Radiolabeling of Stably Transfected Murine Hepatoma Cells—Previously established mouse hepatoma cells stably transfected with human genomic DNA encoding human AAT variants (cell lines H1A/M-15, H1A/N13, and H1A/Z8) were used in these analyses (22–24). Stably transfected clones that exhibited the highest rate of AAT biosynthesis relative to endogenous murine albumin were chosen to mimic the authentic *in vivo* conditions of hepatocytes. Monolayers of semiconfluent cells were pulse-radiolabeled for 15 min at 37 °C in methionine-free medium (ICN Pharmaceuticals, Inc.) containing [^{35}S]methionine (9) (NEN Life Science Products) and then chased in methionine-free medium for up to 3 h. Cells were lysed with buffered Nonidet P-40 detergent (9) either immediately following the pulse or at the specified time point during the chase. Experiments involving kifunensine, swainsonine, 1-deoxymannojirimycin, castanospermine, and lactacystin included a 1-h preincubation period prior to pulse radiolabeling. Unless stated otherwise, all other inhibitors were added to cells at the onset of the chase period. Pervanadate was generated from sodium orthovanadate prior to use, as described previously (36). Steady-state radiolabeling of cells with [^{35}S]methionine was performed as described previously (37).

Immunoprecipitation and Quantitation—Proteins were immunoprecipitated by a 2-h incubation at 4 °C with an excess of specific polyclonal antiserum against human AAT (ICN Pharmaceuticals, Inc.) or murine albumin (Bethyl Laboratories) immobilized to protein G-agarose (Calbiochem) (25). Radiolabeled proteins were resolved by SDS-PAGE and detected by fluorographic enhancement of vacuum-dried gels. Quantitation of radiolabel was performed by scintillation counting of excised gel slices (9, 25). For the detection of insoluble radiolabeled PI Z in pulse-chase experiments, the insoluble pellet resulting from the centrifugation (10,000 × *g*, 10 min) of the Nonidet P-40 cell lysate was agitated with 1% lithium dodecylsulfate for 10 min. (37), prior to immunoprecipitation of the released protein and detection by fluorography.

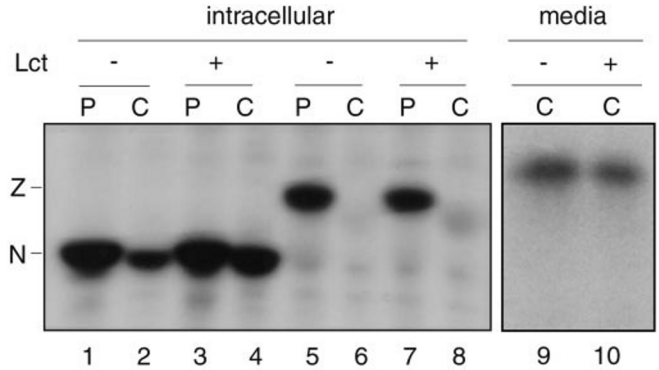

FIG. 1. **Transport-incompetent PI Z is eliminated by a nonproteasomal mechanism.** Fluorographic detection following SDS-PAGE of variants null(Hong Kong) (*N*) and PI Z (*Z*) immunoprecipitated from cell lysates. Following a 15-min pulse with [^{35}S]methionine, cells were lysed immediately (*P*) or chased (*C*) for 3 h in the absence (−) or presence (+) of 0.025 mM lactacystin (*Lct*). In the *right panel*, an overexposed gel shows radiolabeled secreted PI Z in the medium following the chase (*lanes 9* and *10*).

Selective Permeabilization of the Plasma Membrane—Selective permeabilization of the plasma membrane was performed at isotonic conditions as described for human hepatoma cells (38). Briefly, confluent monolayers of pulse-radiolabeled cells in semiconfluent 100-mm diameter dishes were washed at 25 °C with CSK buffer (0.3 M sucrose, 0.1 M KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 10 mM PIPES, pH 6.8) and then incubated for 5 min at that same temperature with 3 ml of CSK buffer containing 0.050 mg/ml digitonin (Roche Molecular Biochemicals). The optimal concentration of digitonin needed to efficiently permeabilize >98% of cells was determined by Trypan blue exclusion (39) and by the loss of the immunoreactive 20 S proteasome α-subunit. Digitonized cells were washed three times at 25 °C with CSK buffer prior to the initiation of a 3-h incubation at 37 °C in CSK buffer. Following each experiment, AAT was immunoprecipitated from the incubation buffer and the Nonidet P-40 lysate derived from the permeabilized cells, as described for intact cells.

Velocity Sedimentation—Aliquots of the buffered Nonidet P-40 cell lysate or media were subjected to velocity sedimentation in linear 5–20% sucrose gradients in a manner that was identical to that described previously (9, 24, 25). Variant PI Z was immunoprecipitated from individual gradient fractions, as described above, and then resolved by SDS-PAGE, detected by fluorography, and quantitated by scintillation counting of excised gel pieces.

Enhanced Chemiluminescent (ECL) Western Blotting—After the electrophoretic transfer of protein from SDS-PAGE gels onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech), ECL Western blotting was performed under previously described conditions (9, 39) with a 1:1000 dilution of a polyclonal rabbit antiserum against a synthetic peptide homologous to the cytoplasmic tail of canine calnexin (StressGen) or a 1:5000 dilution of rabbit antiserum against the α-subunit of the 20 S proteasome (Calbiochem). Incubation with conjugated secondary antibodies, subsequent washings, and the detection of immunospecific bands were performed in a manner identical to that reported previously (9, 39). Relative band intensities were quantified by standard densitometric analysis.

RESULTS

Proteasome-independent Disposal of Variant PI Z—Variants null(Hong Kong) and PI Z are retained in the ER of stably transfected hepatoma cells by distinct mechanisms (9, 24), but each is subjected to intracellular disposal (23). Intracellular turnover was examined in pulse-chase studies following a 15-min pulse with [^{35}S]methionine. After 3 h of chase, 30% of pulse-radiolabeled null(Hong Kong) remained undegraded in the cell lysate ($t_{½}$ = 120 min) (Fig. 1, compare *lanes 1* and *2*). Under identical conditions, the intracellular population of radiolabeled PI Z was not detected ($t_{½}$ = 90 min) (Fig. 1, compare *lanes 5* and *6*), and only a fraction (~15%) was secreted into the medium during this period (compare *lanes 1* and *9*), as previously reported (23). No fraction of either radiolabeled variant was detected in the membrane pellet following cell lysis with

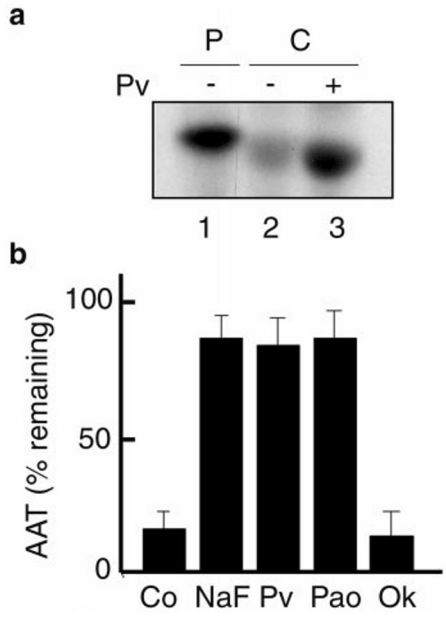

FIG. 2. **PI Z disposal is arrested in response to tyrosine phosphatase inhibition.** *a*, fluorographic detection following SDS-PAGE of variant PI Z immunoprecipitated from cell lysates. Following a 15-min pulse with [^{35}S]methionine, cells were lysed immediately (*P*) or chased (*C*) for 3 h in the absence (−) or presence (+) of sodium pervanadate (*Pv*) prior to immunoprecipitation. *b*, quantitation of the percentage of pulse-radiolabeled PI Z remaining in cells following a 3-h chase with specific inhibitors. The mean value (plus 1 S.D.) from a total of four individual experiments is shown. Pulse-chase experiments were performed as above but in the presence of no supplements (*Co*), 5 mM sodium fluoride (*NaF*), 0.5 mM pervanadate (*Pv*), 0.05 mM phenylarsine oxide (*Pao*), or 0.001 mM okadaic acid (*Ok*).

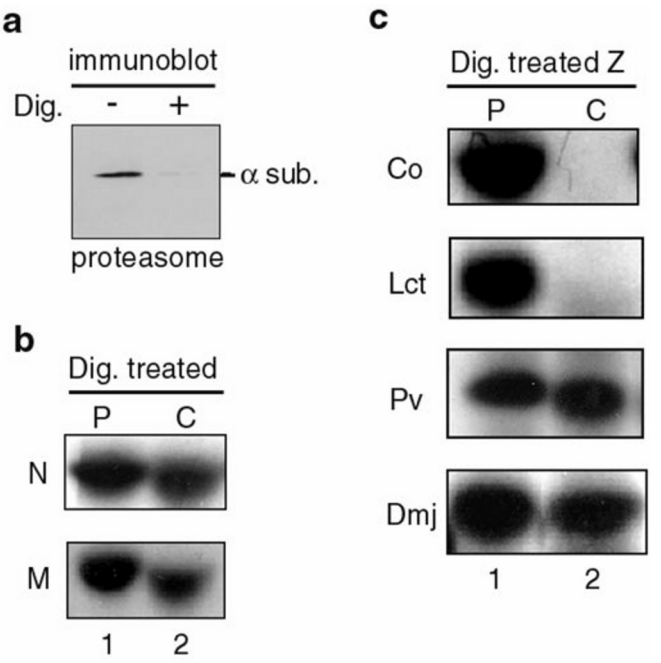

FIG. 3. **Nonproteasomal elimination of variant PI Z in permeabilized cells.** *a*, immunoblot showing the loss of the proteasome α-subunit (*α sub.*) following selective permeabilization of the plasma membrane with digitonin (see "Materials and Methods"). *b*, fluorographic detection following SDS-PAGE of variant null(Hong Kong) (*N*) and the correctly folded wild type variant PI M (*M*) immunoprecipitated from permeabilized cells. Following a 15-min pulse with [^{35}S]methionine, cells were permeabilized with digitonin and then lysed immediately (*P*) or chased (*C*) for 3 h in isotonic incubation buffer prior to lysis and immunoprecipitation. *c*, same as in *b*, except that variant PI Z was immunoprecipitated from permeabilized cells following a 3-h incubation with no supplements (*Co*) or in the presence of 0.025 mM lactacystin (*Lct*), 0.5 mM pervanadate (*Pv*), or 1 mM 1-deoxymannojirimycin (*Dmj*). The results are representative of at least three independent experiments.

Nonidet P-40; nor was the loss altered in response to the use of alternative polyclonal antisera for immunoprecipitation (data not shown). These findings indicate that the intracellular turnover of both variants was the result of intracellular degradation rather than a reflection of protein insolubility or hindered antibody recognition.

Lactacystin, a specific irreversible covalent inhibitor of the multicatalytic proteasome (40), arrested null(Hong Kong) turnover such that 95% of the pulse-radiolabeled molecules were detected in cells following a 3-h chase (Fig. 1, compare *lanes 3* and *4*). In contrast, <5% of pulse-radiolabeled PI Z was detected in cells under identical conditions (Fig. 1, compare *lanes 7* and *8*), and this was not the result of accelerated secretion (compare *lanes 9* and *10*). In a separate set of experiments, not lactacystin, MG132, or *N*-acetyl-Leu-Leu-norleucinal, all inhibitors of proteasomal activity (28, 40, 41), hindered PI Z turnover by more than 5% (data not shown). Overall, these findings indicate that variants null(Hong Kong) and PI Z are eliminated in hepatoma cells by distinct disposal pathways, the latter being somewhat more efficient.

PI Z Disposal Is Sensitive to Tyrosine Phosphatase Inhibitors—To initiate the biochemical description of the nonproteasomal disposal system, we explored the reported inhibitory effect of the metabolic poison sodium fluoride on PI Z disposal (24), since it fails to inhibit the intracellular turnover of variant null(Hong Kong) (39). Since combinations of metabolic poisons are required to deplete intracellular ATP (43), we extended our investigation to test the hypothesis that the effect of sodium fluoride on PI Z disposal actually involves its reported role as an inhibitor of intracellular phosphatases (44). Consistent with this notion, 80% of radiolabeled PI Z remained undegraded in cells following a 3-h chase in the presence of sodium pervanadate (Fig. 2*a*, *lane 3*), a membrane-permeable inhibitor of protein-tyrosine phosphatase activity (45), as compared with 20% under control conditions (*lane 2*). Pervanadate treatment had no demonstrable effect on null(Hong Kong) disposal under an identical set of conditions (data not shown). Importantly, PI Z turnover was inhibited to a similar extent as with sodium fluoride when pulse-radiolabeled cells were incubated with phenylarsine oxide (Fig. 2*b*), an additional general tyrosine phosphatase inhibitor (46). In contrast, incubation with okadaic acid, a general inhibitor of serine/threonine phosphatase activities (47), had no demonstrable inhibitory effect on PI Z disposal (Fig. 2*b*). Incubation with pervanadate arrested PI Z disposal without hindering the enhanced electrophoretic mobility of radiolabeled molecules in SDS-PAGE (Fig. 2*a*), which reflects the removal of mannose from asparagine-linked oligosaccharides during intracellular retention (24). This latter observation was common to all the tyrosine phosphatase inhibitors (data not shown), indicating that reversible tyrosine phosphorylation plays an important role at a relatively late step in the nonproteasomal disposal of variant PI Z.

Pervanadate-sensitive Disposal Proceeds in the Absence of Cytosolic Components—Selective permeabilization of the plasma membrane was performed after pulse radiolabeling with [^{35}S]methionine (see "Materials and Methods") as an alternate method to confirm that PI Z disposal occurs independently of the cytosolic proteasome. Quantitation after ECL Western blotting demonstrated that selective permeabilization of the plasma membrane had removed >95% of the immunoreactive proteasomal α-subunit (Fig. 3*a*). Under these conditions, only a negligible loss (~20%) of pulse-radiolabeled variant null(Hong Kong) (Fig. 3*b*, *N*) or wild type AAT (Fig. 3*b*, *M*)

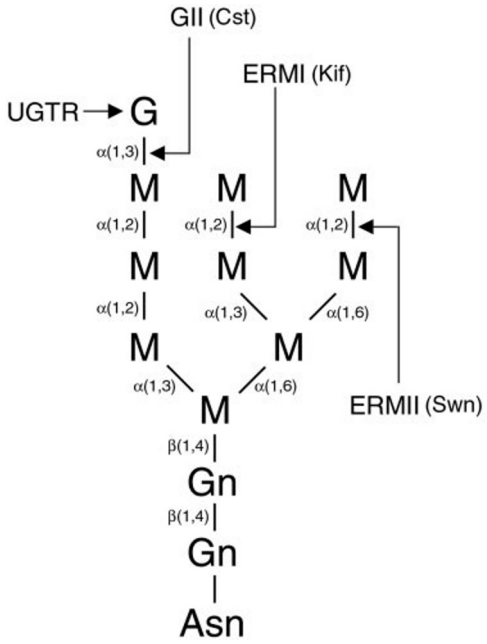

FIG. 4. **Asparagine-linked oligosaccharide modifications in the ER.** The Asn-linked $Man_9GlcNAc_2$ precursor and sugar linkages are depicted. The site where UGTR transfers glucose (*G*) to a distinct terminal α1,2-linked mannose (*M*) unit is shown. The *arrows* show the specific site of action for glucosidase II (*GII*), ER mannosidase I (*ERMI*), and ER mannosidase II (*ERMII*). An inhibitor of each enzyme is shown within the adjacent *parentheses*. In addition to interacting with the unfolded polypeptide, UGTR recognizes the innermost GlcNAc (*Gn*) residue.

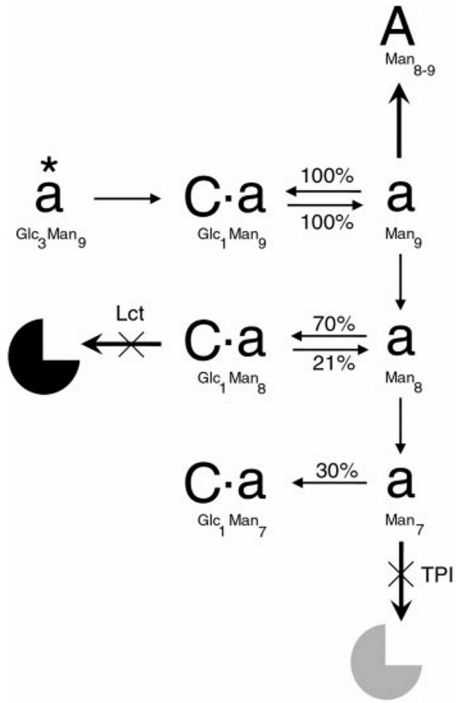

FIG. 5. **Model in which differential mannosidase processing of asparagine-linked oligosaccharides partitions misfolded AAT between distinct disposal pathways.** A model is depicted in which the altered efficiency of reversible oligosaccharide glucosylation enables differential processing by ER-situated mannosidases to regulate the intracellular fate of misfolded AAT. Partial deglucosylation of asparagine-linked oligosaccharides induces physical assembly between newly synthesized (*) unfolded AAT (*a*) and calnexin (*C*) (9). The reversible transfer of Glc to the asparagine-linked Man_9 precursor provides conditions that favor continuous rounds of assembly until conformational maturation is achieved, resulting in deployment of correctly folded AAT (*A*) to the Golgi complex for additional processing prior to secretion. For the incompletely folded molecule, processing by ER mannosidase I (Fig. 5) results in the irreversible transfer of glucose to asparagine-linked Man_8, which abrogates physical dissociation from calnexin, leading to degradation by the cytosolic proteasome (*black three-quarters circle*). The hypothesis to be tested is that the additional processing by ER mannosidase II (Fig. 5) leads to degradation by the alternative mechanism (*gray three-quarters circle*) in response to the low glucose acceptor capacity of asparagine-linked Man_7 (6), which blocks reassembly with calnexin. The *thin vertical arrows* depict the order in which mannose units are removed from the asparagine-linked oligosaccharides. To simplify the model, GlcNAc residues have been omitted. *Numbers above* and *below* the sets of *horizontal arrows* represent the reported efficiencies of reversible oligosaccharide glucosylation at different stages of mannose processing (6, 13). The predicted oligosaccharide structures at specific stages are shown, as are the sites at which lactacystin (*Lct*) and tyrosine phosphatase inhibitors (*TPI*) arrest disposal.

was detected following 3 h of incubation in isotonic buffer. These findings indicated that the manipulation had arrested proteasome-mediated disposal without perturbing the structural integrity of the ER. In contrast, <5% of radiolabeled PI Z remained in the permeabilized cells under identical conditions (Fig. 3*c*, *Co*). Moreover, the loss of radiolabeled protein was unaffected by lactacystin treatment (Fig. 3 *c*, *Lct*). At no period during the course of these experiments was radiolabeled PI Z detected in either the incubation buffer or membrane pellet of the Nonidet P-40 detergent lysate (data not shown). These data confirm that the loss of protein did not reflect its release from the ER or result from insolubility. Importantly, 82% of radiolabeled PI Z remained undegraded in permeabilized cells following a 3-h incubation when pervanadate was included in the incubation buffer (Fig. 3*c*, *Pv*). These data indicate that the selective elimination of PI Z occurs independently of cytosolic factors.

Variant PI Z Partitions into the Proteasome-mediated Disposal Pathway in Response to Selective Glycosidase Inhibition—In a previous report (26), inhibitor studies were utilized to demonstrate how the processing of asparagine-linked oligosaccharides participates in the molecular capture of variant null(Hong Kong) for intracellular disposal. ER mannosidase I is the more abundant of two distinct mannosidases that can initiate the removal of mannose from asparagine-linked oligosaccharides (35). Following removal of the terminal α1,2-linked mannose unit from the middle branch of the $Man_9GlcNAc_2$ precursor (Fig. 4), subsequent reglucosylation by UGTR generates $Glc_1Man_8GlcNAc_2$, which induces the reassembly of glycoprotein substrates with calnexin (48). However, as a relatively poor substrate for glucosidase II (13), dissociation from calnexin is attenuated, which leads to the proteasome-mediated disposal of variant null(Hong Kong) as depicted in Fig. 5. Comparison of the electrophoretic mobility shift in SDS-PAGE (Fig. 1, compare *lanes 1*, *2*, *7*, and *8*) demonstrated that oligosaccharides attached to PI Z were processed to a greater extent than those of null(Hong Kong). Since asparagine-linked $Man_7GlcNAc_2$ is a poor substrate for reglucosylation by UGTR (6), we examined a model in which additional processing by ER mannosidase II is responsible for preventing reassembly of PI Z with calnexin (Fig. 5), resulting in nonproteasomal elimination. Since asparagine-linked $Man_8GlcNAc_2$ plays a pivotal role in the molecular capture process (26), we asked whether PI Z would be degraded by the proteasome during pulse-chase radiolabeling in the presence of kifunensine (35), an inhibitor of ER mannosidase I (50). The hindered electrophoretic mobility of pulse-labeled molecules during intracellular retention (Fig. 6*a*, compare *lanes 2* and *3*) was indicative of limited oligosaccharide processing. Under these conditions, PI Z disposal was diminished ~2.5-fold during a 3-h chase as compared with control (Fig. 6*b*, *Kif*). Importantly, disposal was not completely arrested, even at elevated concentrations of the inhibitor (data

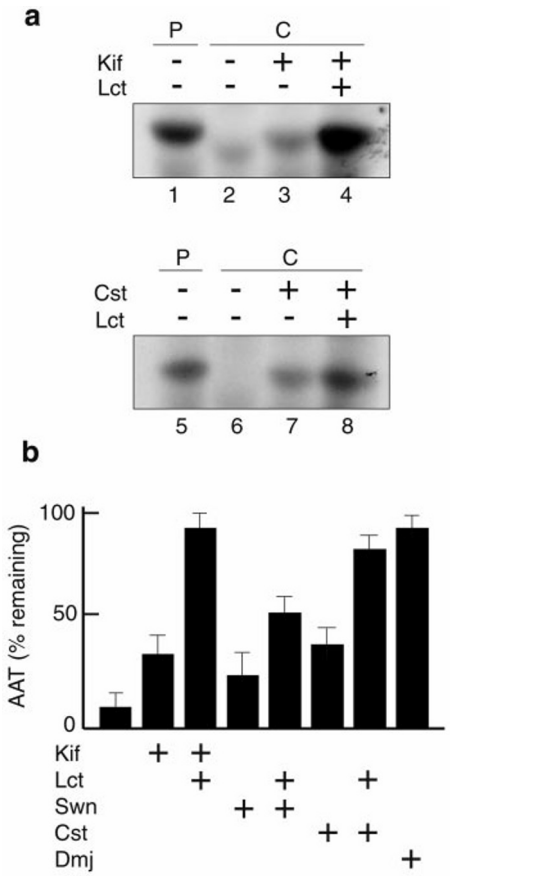

FIG. 6. **Oligosaccharide-processing inhibitors partition variant PI Z into the proteasome-mediated disposal pathway.** *a*, fluorographic detection following SDS-PAGE of variant PI Z immunoprecipitated from cell lysates. Following a 15-min pulse with [^{35}S]methionine, cells were lysed immediately (*P*) or chased (*C*) for 3 h in the absence (−) or presence (+) of 0.1 mM kifunensine (*Kif*), 0.025 mM lactacystin (*Lct*), or 0.2 mg/ml castanospermine (*Cst*) prior to immunoprecipitation. *b*, quantitation of the percentage of pulse-radiolabeled PI Z remaining in cells following a 3-h chase with no supplements (*Co*) or in the presence of the above inhibitors as well as 0.1 mM swainsonine (*Swn*) or 1 mM deoxymannojirimycin (*Dmj*). The mean value (plus 1 S.D.) from a total of four individual experiments is shown for each manipulation.

not shown). Intracellular turnover was completely arrested in response to the co-incubation of cells with kifunensine plus the proteasome inhibitor lactacystin (Fig. 6*b, Kif* + *Lct*). A reasonable explanation for these results is that asparagine-linked $Man_8GlcNAc_2$ was generated in the absence of ER mannosidase I activity. To test this hypothesis, pulse-chase radiolabeling was performed in the presence of swainsonine (51, 52), a partial inhibitor of the less abundant kifunensine-resistant ER mannosidase II (Fig. 4). Under these conditions, the rate of PI Z disposal was significantly hindered as compared with control (Fig. 6*b*, *Swn*). Although disposal was not completely arrested in cells coincubated with swainsonine plus lactacystin, ~50% of the radiolabeled molecules remained undegraded following a 3-h chase (Fig. 6*b*, *Swn* + *Lct*), which is the predicted result for the partial inhibitor of ER mannosidase II (51, 52). These findings are consistent with the proposed model (Fig. 5) in which the combined processing by ER mannosidases I and II diverts variant PI Z away from the proteasome.

In the next set of experiments, a posttranslational glucosidase blockade was performed as a method to ask whether hindered oligosaccharide reglucosylation functions as the underlying mechanism by which PI Z is diverted from the proteasome. For this purpose, the glucosidase inhibitor castanospermine (51) was added to cells after a 15-min pulse with [^{35}S]methionine to arrest the posttranslational removal of glucose from attached glycans without hindering cotranslational assembly. A subtle change in the electrophoretic mobility of radiolabeled molecules (Fig. 6*a*, *compare lanes 5*, *7*, and *8*) indicated that mannose units were probably excised from attached glycans during the persistence of attached glucose. Following a 3-h chase under these conditions, approximately 38% of the radiolabeled molecules remained undegraded as compared with only 5% for control (Fig. 6*b*, *Cst*), indicative of a slower rate of disposal. As predicted by the proposed model, PI Z disposal was completely arrested when the posttranslational glucosidase blockade was performed during co-incubation with lactacystin (Fig. 6*b*, *Cst* + *Lct*). Importantly, an identical effect on PI Z disposal was observed when the glucosidase inhibitor 1-deoxynojirimycin (51) was used instead of castanospermine (data not shown). These data support the notion that hindered oligosaccharide reglucosylation is an underlying mechanism by which variant PI Z is diverted away from the proteasome-mediated disposal pathway.

Enhanced Physical Interaction with Calnexin Accompanies the Partitioning of Variant PI Z into the Proteasome-mediated Disposal Pathway—ECL Western blotting was used as a means to determine whether the partitioning of PI Z into the proteasome-mediated disposal pathway corresponds with an enhanced physical association with calnexin. The analysis was performed with unlabeled cells, since the transient cotranslational interaction was beyond the limits of detection by this methodology (Fig. 7*a*, *lane 3*). Lactacystin was included to arrest intracellular disposal to aid in the normalization of the intracellular PI Z concentration. Consistent with the proposed model, associated calnexin was detected in response to treatment with kifunensine plus lactacystin (Fig. 7*a*, *lane 6*). The association was also detected following incubation with castanospermine plus lactacystin (Fig. 7*a*, *lane 7*). Importantly, the complete absence of associated calnexin in response to incubation with the tyrosine phosphatase inhibitor phenylarsine oxide (Fig. 7*a*, *lane 4*) indicated that its detection in the prior experiments had not resulted from an elevated intracellular concentration of variant PI Z. Coimmunoprecipitated calnexin was of an intermediate intensity when cells were coincubated with swainsonine plus lactacystin (data not shown); however, the failure of the inhibitor to totally arrest PI Z disposal precluded the normalization of these data.

In a subsequent set of experiments, we asked whether PI Z would be degraded by the nonproteasomal pathway under conditions that preclude its cotranslational interaction with calnexin. Incubation with castanospermine prior to pulse radiolabeling hindered the migration of newly synthesized molecules in SDS-PAGE, and the results of coimmunoprecipitation confirmed that maintaining asparagine-linked glycans in the initial $Glc_3Man_9GlcNAc_2$ structure arrested cotranslational assembly with calnexin (data not shown). Under these conditions, PI Z was completely degraded within 3 h of chase (Fig. 7*b*, *lane 2*), and this was unaffected by lactacystin treatment (*lane 4*). In contrast, under these conditions, disposal was completely arrested with phenylarsine oxide (Fig. 7*b*, *lane 7*), similar to when the glucosidase inhibitor was omitted from the experiment (*lane 8*). Taken together, these findings confirm our prior conclusion that physical interaction with calnexin is essential for the partitioning of misfolded AAT into the proteasome-mediated disposal pathway.

Simultaneous Inhibition of ER Mannosidases I and II Arrests PI Z Disposal and Enhances the Secretion of Monomers—Results of the previous experiments indicated that molecular capture by calnexin was sufficient to completely arrest the

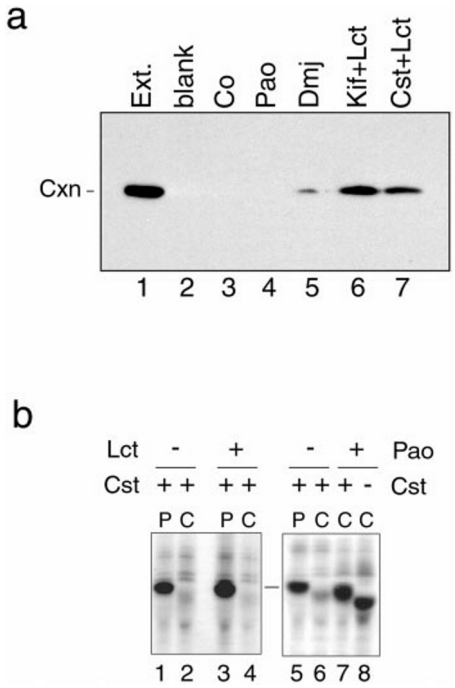

FIG. 7. **Coimmunoprecipitation of calnexin at conditions that predict physical interaction with the lectin.** *a*, following a 90-min incubation with the specified compound, variant PI Z was immunoprecipitated from the Nonidet P-40 cell extract. Coimmunoprecipitated calnexin was detected by ECL Western blotting following SDS-PAGE (see "Materials and Methods"). Quantitation was performed by laser densitometry. *Lane 1*, aliquot of a crude cell extract (*Ext*); *lane 2*, blank; *lane 3*, control (*Co*); *lane 4*, 0.05 mM phenylarsine oxide (*Pao*); *lane 5*, 1 mM deoxymannojirimycin (*Dmj*); *lane 6*, 0.1 mM kifunensine plus 0.025 mM lactacystin (*Kif + Lct*); *lane 7*, 0.2 mg/ml castanospermine plus 0.025 mM lactacystin (*Cst + Lct*). Densitometric analysis indicated that 0.2, 1.6, and 1.0 are the relative intensities of coimmunoprecipitated calnexin in *lanes 5–7* when normalized for the intracellular content of PI Z. *b*, fluorographic detection following SDS-PAGE of variant PI Z immunoprecipitated from cell lysates. In *lanes 1–7*, cells were incubated for 1 h with 0.2 mg/ml castanospermine (*Cst*) prior to a 15-min pulse with [^{35}S]methionine. Cell lysis was performed immediately (*P*) or after a 3-h chase (*C*) in the absence (−) or presence (+) of 0.025 mM lactacystin (*Lct*) or 0.05 mM phenylarsine oxide (*PAO*).

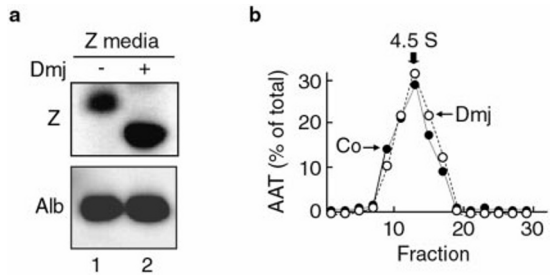

FIG. 8. **Enhanced secretion of PI Z monomers during total mannosidase inhibition.** *a*, fluorographic detection of radiolabeled PI Z (*Z*) and endogenous mouse albumin (*Alb*) immmunoprecipitated from the media of cells following 6.5 h of incubation with [^{35}S]methionine in the absence (−) or presence (+) of 1-deoxymannojirimycin (*Dmj*). *b*, velocity sedimentation of secreted radiolabeled PI Z through 5–20% sucrose gradients (see "Materials and Methods"). The data are representative of three independent experiments.

disposal of PI Z by the nonproteasomal mechanism. Therefore, we asked whether reversible binding to calnexin is equally capable of preventing nonproteasomal disposal. For this, pulse-chase radiolabeling was performed in the presence of 1-deoxymannojirimycin, an inhibitor of both ER mannosidases I and II (35, 51), which is predicted to favor the reversible binding of PI Z to calnexin (Fig. 5). Under these conditions, PI Z disposal was completely arrested in intact cells during a 3-h chase (Fig. 6*b*, *Dmj*). Consistent with a process of reversible binding rather than molecular capture, physical interaction with calnexin was detected but was diminished >6-fold compared with cells that had undergone treatment with either kifunensine or castanospermine (Fig. 7*a*, compare *lane 5* with *lanes 6* and *7*). Although physical interaction with the lectin was not examined in permeabilized cells, the electrophoretic mobility shift of PI Z in SDS-PAGE was blocked by deoxymannojirimycin, and PI Z disposal was arrested (Fig. 3*c*, *panel Dmj*). These data confirm that physical interaction with calnexin is functionally dominant over recognition of variant PI Z by nonproteasomal disposal machinery.

A transient population of PI Z monomers released from secretion-incompetent polymers is predicted to function as the source of the small fraction of molecules that are eventually secreted from cells upon attaining conformational maturation (53). Since a negligible enhancement of PI Z secretion was observed during a 3-h chase with deoxymannojirimycin (data not shown), we chose to address the possibility of secretion rescue in a manner that more fully mimics authentic physiologic conditions in which variant PI Z is continuously synthesized and secreted. Our approach was to compare the amount of PI Z secretion during steady-state radiolabeling conditions (37) in which deoxymannojirimycin was either absent or present in the culture medium. The altered electrophoretic migration of [^{35}S]methionine-radiolabeled molecules immunoprecipitated from the medium of treated cells (Fig. 8*a*, *Z*) reflected the absence of charged sialic acid residues normally added to mannosidase-processed oligosaccharides in the Golgi complex (23). Under both control and experimental conditions, the entire population of secreted radiolabeled PI Z sedimented as a single peak (Fig. 8*b*) identical to that reported for the 4.5 S AAT monomer (9, 25). Importantly, the amount of endogenous mouse albumin in the medium was not enhanced during mannosidase inhibition as compared with control (Fig. 8*a*, *Alb*), arguing against the notion that the manipulation had somehow resulted in a general enhancement of protein secretion. When normalized to the content of secreted albumin, the relative amount of PI Z secreted during deoxymannojirimycin treatment was ~3.2-fold greater than under control conditions (Fig. 8*a*, compare *lanes 1* and *2*). Soluble radiolabeled PI Z persisted in cells during the entire time course of mannosidase inhibition (data not shown), indicating that the majority of molecules were unable to undergo secretion rescue. Importantly, no detectable enhancement in the secretion of variant PI Z occurred in response to the incubation of cells with any of the other inhibitors described in this study (data not shown), suggesting that intracellular retention is not easily overwhelmed in the absence of disposal. Our findings indicate that a fraction of secretion-impaired PI Z is amenable to secretion rescue under conditions that favor reversible binding to calnexin.

DISCUSSION

In the present study, comparing and contrasting the intracellular fate of distinct human AAT variants has led to the identification of a previously unrecognized regulatory role for asparagine-linked oligosaccharide processing in glycoprotein quality control. The efficient disposal of variant PI Z in permeabilized cells, resistance to lactacystin, and complete inhibition by general inhibitors of tyrosine phosphatase activity provided several lines of evidence that intracellular degradation is accomplished by a proteasome-independent mechanism. Disposal in permeabilized cells was arrested in response to total mannosidase inhibition, indicating that recognition by nonproteasomal proteolytic machinery occurs downstream of calnexin but within the ER. Although inhibitors of lysosomal degrada-

tion (24) and autophagy had no detectable effect on PI Z turnover, at least in the time course of our experiments,[2] it is premature to disregard the participation of an unconventional lysosomal route that remains intact following cell permeabilization. Certainly, the absence of proteolytic intermediates during intracellular retention (24, 25, 37) suggests that multiple hydrolytic activities probably participate in the disposal process. Finally, it is unlikely that variant PI Z is the only natural substrate for the alternate disposal mechanism, and in this regard, a luminal system has been suggested to participate in the clearance of apolipoprotein B from human hepatoma cells (54).

Acquired sensitivity toward lactacystin plus a detectable physical interaction with calnexin in response to selective mannosidase inhibition provided experimental evidence that the combined processing of asparagine-linked oligosaccharides by ER mannosidases I and II diverts PI Z away from calnexin. Importantly, the formation of asparagine-linked glycans smaller than $Man_7GlcNAc_2$, the predicted intermediates from Golgi-specific processing reactions, should not support physical assembly with calnexin (6). In this regard, it is noteworthy that subcellular fractionation studies have confirmed that transport-impaired molecules of variant PI Z do not enter the *cis*-Golgi compartment during intracellular retention (24). That the posttranslational glucosidase blockade diverted PI Z to the proteasomal disposal pathway in a manner that correlated with the coimmunoprecipitation of calnexin indicates that hindered oligosaccharide reglucosylation plays a significant role in diverting molecules to the nonproteasomal pathway under normal conditions. In support of this interpretation, the posttranslational metabolic incorporation of radiolabeled glucose into the oligosaccharides of variant PI Z is at least 20-fold less efficient than that of null(Hong Kong) under normal conditions.[3]

One explanation for the processing of asparagine-linked oligosaccharides to $Man_7GlcNAc_2$ is that the intracellular accumulation of PI Z polymers may somehow elevate ER mannosidase II activity. In preliminary experiments, the disposal of variant null(Hong Kong) remained sensitive to lactacystin during simultaneous co-expression of PI Z,[4] arguing against the notion that accumulated polymers exert a *trans* effect. Furthermore, neither the biosynthesis nor intracellular accumulation of variant PI Z is sufficient to elicit an unfolded protein response in cultured mouse hepatoma cells or transgenic mice (35). Therefore, although not yet proven, polymerization may induce additional processing by ER mannosidase II simply by enhancing the accessibility of asparagine-linked oligosaccharides not bound to calnexin. In the present study, the apparent hydrolysis of mannose units during the persistence of attached glucose (Fig. 6*a*, compare *lanes 5*, *7*, and *8*) may reflect this situation, and physical interaction with calnexin has been shown to hinder oligosaccharide processing (9, 48). In addition to polymerization, the enhanced processing of asparagine-linked oligosaccharides might function as a mechanism to prevent the posttranslational assembly of PI Z with calnexin. Alternatively, a small population of PI Z monomers may exist in dynamic equilibrium with secretion-impaired polymers (53), functioning as the actual substrate that binds calnexin in the absence of enhanced mannose processing. In this scenario, rapid processing to $Man_7GlcNAc_2$ would occur prior to the release of monomers, the latter of which would serve as the precursor for those molecules secreted from cells in the absence of repolymerization or disposal. Unfortunately, the broad overlapping sedimenting pattern of soluble linear PI Z polymers in sucrose gradients (37) precluded an accurate estimation of both the stoichiometry of the PI Z-calnexin complex as well as the relative intracellular monomer and polymer populations (data not shown). As such, we are currently unable to determine whether the diminished rate of intracellular turnover of PI Z when partitioned into the proteasome-mediated disposal pathway (Fig. 6) reflects a rate-limiting step in which monomers are released from polymers prior to molecular capture *versus* the inefficient retrograde translocation of polymers to the cytoplasm. In either case, it is tempting to speculate that the combined processing of asparagine-linked oligosaccharides by ER mannosidases I and II might function as a general strategy to prevent the potential clogging of the retrograde translocon by which multiple ER-derived proteasomal substrates must pass (55).

Our current findings implicate ER mannosidase II as a participant in glycoprotein quality control, revealing a functional role for this enzyme. As such, it is now understood how all three terminal α1,2-linked mannose units in the conserved $Man_9GlcNAc_2$ structure (Fig. 4) can participate in the facilitation of either glycoprotein folding or degradation. Since differentiation of the secretory pathway may regulate the protein folding and quality control capabilities within specific cell lineages (for a review, see Ref. 11), cell-specific intracellular concentrations of ER mannosidase II (52) might explain why variant PI Z is degraded predominantly by the proteasome in several transfected extrahepatic mammalian cells (56, 57) and in yeast (12), the latter of which exhibits a single ER-situated mannosidase (15). Alternatively, diminished levels of biosynthesis may hinder polymerization, as has been predicted (31). Nevertheless, our present findings plus the recent observation that apolipoprotein B undergoes cell type-specific processing (42) bring into question the physiologic significance of data generated from heterologous expression systems to characterize conformation-based quality control. As such, methods reported to increase PI Z secretion from extrahepatic cells (57) may fail to elevate plasma AAT levels. Of course, the use of mannosidase inhibitors for the same purpose will not be without complications, since Golgi processing enzymes would be affected. However, the partial amenability of the transport-impaired molecule to secretion rescue provides an essential "proof of principle" that may lead to the development of various pharmacological strategies to alleviate distinct heritable pathologies associated with additional protein folding disorders (17, 18). Specific goals for future studies will be to elucidate the functional role of reversible tyrosine phosphorylation in nonproteasomal disposal and to identify the mechanism by which variant PI Z is recognized for elimination by this pathway.

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[3] A. Le, unpublished observation.
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