Mannose Trimming Targets Mutant α₂-Plasmin Inhibitor for Degradation by the Proteasome*

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Dong Hui Chung‡, Kazuteru Ohashi‡, Makoto Watanabe§, Nobuyuki Miyasaka‡, and Shinsaku Hiroswa‡¶

From the ¶First Department of Internal Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8519, Japan and the §Department of Biochemistry, Oita Medical School, 1-1, Idaigaoka, Hazama-cho, Oita-gun, Oita-ken 880-8692, Japan

We have previously characterized the molecular and cellular mechanisms of α₂-plasmin inhibitor (α₂PI) deficiency. The mutant α₂PI-Nara and α₂PI-Okinawa proteins were found to be retained and degraded in cells stably expressing these mutant forms of α₂PI. Degradation of the two mutant α₂PI proteins, mediated by proteasomes, occurred after a lag time of 1.5 h during which glucose trimming took place. The mutant α₂PI proteins were not ubiquitinated. Inhibition of mannosidase activity blocked the degradation of the mutant α₂PI proteins without resulting in any changes in their binding to calnexin. Inhibition of glucose removal completely blocked the interaction between the α₂PI proteins and the molecular chaperone calnexin. Under these conditions, mannose residues were removed from the oligosaccharides even when glucose residues were not processed. With mannose removal, the glucose-untrimmed mutant forms of α₂PI, which failed to bind to calnexin, were degraded by proteasomes. The initiation of mannose trimming was a prerequisite for their degradation. Our findings show that modification of oligosaccharides of the mutant forms of α₂PI determines their recognition by the degradation apparatus and that mannose trimming is important for targeting the mutant α₂PI proteins for the degradation pathway.

α₂-Plasmin inhibitor (α₂PI) is a plasma glycoprotein with an estimated four glycosylation sites and a molecular mass of 67 kDa, which contains about 11% carbohydrate (1). α₂PI belongs to the serine protease inhibitor superfamily. It is able to inhibit several different serine proteases, but its main function is to inhibit plasma-mediated fibrinolysis (2–4). The physiological importance of α₂PI was established by the discovery of individuals with congenital α₂PI deficiency in whom hemostatic plugs are dissolved prematurely before the restoration of injured vessels, resulting in a severe hemorrhagic tendency (5, 6). Genetic abnormalities of α₂PI have been well characterized at the molecular level in two Japanese familial cases affected with the deficiency (7–9). One familial case of congenital α₂PI deficiency, designated as α₂PI-Nara, involves a frameshift mutation that results in the substitution of 178 amino acid residues for 12 carboxyl-terminal amino acid residues of the wild-type α₂PI (7). The other case, designated as α₂PI-Okinawa, involves a trinucleotide deletion that gives rise to the deletion of Glu-137 (9, 10). The mutant molecules of α₂PI-Nara and α₂PI-Okinawa, which were expressed transiently in COS-7 cells, were retained for a prolonged period as endoglycosidase H (Endo H)-sensitive forms, and in each instance only a small proportion of the expressed proteins was secreted.

Retention within the endoplasmic reticulum (ER) has been reported for several other naturally occurring or genetically engineered mutant proteins that do not fold correctly. It has been shown that newly made secretory or membrane proteins do not move from the rough ER to the Golgi apparatus unless they fold into a native or near-native conformation (11). Misfolded proteins do not accumulate in the ER and are eventually removed from the ER by proteolysis. This rapid and selective degradation of proteins that are unable to reach the Golgi apparatus has been described as ER or pre-Golgi degradation. The process called ER degradation was generally assumed to occur inside the ER or a pre-Golgi compartment (12, 13). However, the proteases present in the ER have not yet been identified. Several recent reports have suggested a role for the proteasome in the ER degradation of some membrane or luminal proteins (14, 15). For example, misfolded cystic fibrosis transmembrane conductance regulator molecules are rapidly degraded in a process that requires both ubiquitin and that is blocked by lactacystin, a specific proteasome inhibitor (16, 17). Degradation of mutant α₁-antitrypsin (18), yeast carboxypeptidase Y (19), and major histocompatibility class I heavy chains have also recently been shown to require proteasome activity (20–22).

Several ER-resident proteins that function as molecular chaperones have a role in ER quality control and contribute to the retention of misfolded proteins (23). Calnexin (CNX) and calreticulin preferentially interact with the nascent one-glycosylated forms of N-linked oligosaccharides after glucose trimming and are thought to select proteins for degradation or secretion pathways. Their substrate-binding function is linked to a number of diseases with an ER-storage phenotype, such as cystic fibrosis, familial hypercholesterolemia, and α₁-antitrypsin deficiency (24, 25). In these disease states, they serve as a part of the machinery that retains the misfolded glycoproteins in the ER. However, the selective mechanism that directs the misfolded proteins to the degradation pathway is not clear. Recent studies have indicated that the misfolded glycoproteins
show prolonged association with CNX; removal of mannose from N-linked oligosaccharides resulted in misfolded glycoproteins being led to a subsequent degradation pathway (26–28).

In this study, we demonstrate that mutant α2PI proteins are degraded by proteasomes. We also examine whether oligosaccharide processing and association with CNX play a role in ER quality control in the case of the mutant α2PI proteins.

**MATERIALS AND METHODS**

**Chemicals and Reagents**—Goat polyclonal anti-human α2PI antibody was purchased from Biopool (Umeå, Sweden). Anti-calnexin-α, anti-calreticulin-β, and anti-ubiquitin polyclonal antibodies were purchased from StressGen (Victoria, Canada). Anti-p53 monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [35S]Methionine (1000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. All other chemicals were reagent-grade products and were purchased from Wako Pure Chemicals (Osaka, Japan) unless otherwise indicated.

**Construction of Expression Vectors and Transfection**—The plasmid pSV2PI, a wild-type expression construct, carries the normal α2PI cDNA under the control of the SV40 promoter. The α2PI-Nara expression vector, pSV2PN, and the α2PI-Okinawa expression vector, pSV2PO, were constructed by replacing the normal α2PI cDNA with the cDNA fragments carrying the mutations. Chinese hamster ovary (CHO) cells were maintained in F-12 Nutrient Mixture (Life Technologies, Inc.), and HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan). Both media were supplemented with 10% fetal calf serum. For stable expression in CHO cells, both the expression vector DNA and pSVneo, which carries the G418 gene conveying neomycin resistance, were introduced into the cells by the LipofectAMINE-mediated transfection procedure, and neomycin was employed for selection.

**Immunochromatographic Analysis of [35S]Methionine-labeled Recombinant α2PI**—The CHO cells transfected with pSV2PI, pSV2PN, or pSV2PO were pulse-labeled for 15 min with [35S]methionine and chased as described previously (7). CST, DMM, and LCT were added to the medium before the pulse-chase analysis. The inhibitors TLCK, TPCK, ALLN, ALLM, chloroquine, and leupeptin were added to the medium after pulse labeling. The α2PI proteins radiolabeled in the course of synthesis were immunoprecipitated using an excess of the antibody and protein G from cell lysates or conditioned medium and were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [10% acrylamide gel] and Endo H digestion and was performed as described previously (9). A nonstringent buffer, HBS containing 0.2M NaCl and 50 mM HEPES, pH 7.5, with 2% CHAPS and 1 mM N-ethylmaleimide, and CHAPS were purchased from Sigma. Endo H was obtained from Roche Molecular Biochemicals. LipofectAMINE was obtained from Life Technologies, Inc. Lactacytin (LCT) was kindly provided by Dr. Satoshi Omura (Kitasato Institute, Tokyo, Japan) (16).

**RESULTS**

**Mutant α2PI Proteins Are Degraded in the Pre-Golgi Area**—We examined the secretory process for normal and mutant α2PI proteins in stably transfected CHO cell lines. Most of the wild-type α2PI molecules with a molecular mass of 64 kDa disappeared from the cells within 2 h and were secreted into the culture medium with a molecular mass of 67 kDa (Fig. 1A). The secreted wild-type protein had complex oligosaccharides, which were resistant to Endo H digestion. The α2PI molecules in the cell extract were Endo H-sensitive. In contrast, the α2PI-Nara and α2PI-Okinawa molecules were not detected in the culture medium even after a 6-h chase period, and they were retained as Endo H-susceptible forms within the cells (Fig. 1B). Because Endo H removes N-linked oligosaccharides from polypeptides during transit through the ER without any effect on the complex oligosaccharides already transferred to the medial stacks of the Golgi apparatus, the mutant molecules were retained in the ER. As observed in the case of the cells that transiently expressed these molecules, the retained mutant molecules did not accumulate but eventually disappeared. We tried to isolate misfolded proteins from the cells, but no insoluble fraction was detected. The mutant α2PI-Nara molecules were degraded in the pre-Golgi area. The time course of degradation of the mutant α2PI-Nara and α2PI-Okinawa molecules is shown in Fig. 1C. The degradation of α2PI-Nara started 1.5 h after protein labeling. The half-life of the mutant molecules was about 4 h. The degradation of α2PI-Okinawa showed a similar pattern.

**The Precursor Forms of α2PI-Nara and α2PI-Okinawa Are Decreased in Molecular Size in the ER**—The retained α2PI-Nara and α2PI-Okinawa each showed a change in molecular size during the pulse-labeling and chase periods (Fig. 1B). The deglycosylated polypeptides treated with Endo H showed identical mobility. These findings indicate that the decrease in size of the retained proteins was the result of modification of the oligosaccharide moieties and not the result of a change in the radiolabeled polypeptides.

We determined whether the decrease in size of the retained mutant α2PI molecules was a result of the hydrolysis of either glucose or mannose residues. The mobility of immunoprecipitated α2PI-Nara molecules preincubated with CST was retarded compared with that of the molecules without CST treatment. Digestion of the α2PI proteins with Endo H produced molecules with the same mobility (Fig. 2a). This finding indicates that glucose residues were co-translationally hydrolyzed from oligosaccharides of the α2PI-Nara proteins. CST decreased the amount of expression of the proteins. The inhibition of glucose trimming might impair effective translation and translocation of the proteins. Treatment with dMM inhibited the reduction of the molecular size of α2PI-Nara. The molecular size of α2PI-Nara treated with dMM was smaller than that of the molecules at 0 h that were co-translationally hydrolyzed (Fig. 2b). The post-translational removal of glucose residues preceded mannose trimming. These findings indicate that the decrease in size of the retained α2PI-Nara molecules was the result of post-translational hydrolysis of glucose and mannose residues from oligosaccharides of the mutant protein in the ER. Because the retained α2PI-Nara molecules were localized to the ER, it is evident that mannose processing was attributable to dMM-inhibitable mannosidase present in the ER (30–32). Detailed analysis by SDS-PAGE showed that the mobility of molecules treated with dMM corresponded to that observed after a 1.5-h chase period (Fig. 2c). The initiation of mannose trimming occurred after the 1.5-h chase period during which glucose...
trimming had occurred. The oligosaccharides of α₂PI-Okinawa proteins were also found to undergo similar post-translational hydrolysis in the cells (data not shown).

Degradation of the Mutant Proteins Is Proteasome-mediated—To examine whether specific proteases are involved in degradation of the mutant proteins, pulse-chase experiments were performed in the presence of inhibitors of several proteases and the proteasome. The lysosomal protease inhibitors used in this experiment had no effect on the total amount of radioactivity in the cell extracts after the 6-h chase period. The proteasome inhibitors ALLN and LCT markedly inhibited the degradation of the α₂PI-Nara proteins, but the other inhibitors had no effect (Fig. 3A). These proteins were not secreted from the cells even in the presence of the protease inhibitors or proteasome inhibitors. The mutant molecules that accumulated in cells treated with ALLN or LCT for 6 h had the same mobility as observed after a 6-h chase period in the absence of inhibitors, indicating the accumulation of mannose-trimmed forms (Fig. 3B). On the other hand, neither ALLN nor LCT had any effect on the secretion of wild-type α₂PI stably expressed in CHO cells. The intracellular degradation of α₂PI-Okinawa was also inhibited by ALLN and LCT (data not shown). These results indicate that the degradation of the mutant α₂PI proteins was mediated by proteasomes.

Mutant Proteins Are Not Ubiquitinated—If the proteasome actually degrades the mutant proteins, mutant α₂PI should be ubiquitinated before degradation. We examined whether the mutant proteins were conjugated with ubiquitin. Since ubiquitination of p53 has been reported (33), we used p53 as a positive control. Although ubiquitinated p53 was detected, the mutant proteins were not ubiquitinated even in the presence of the inhibitors (Fig. 3C).

CST Accelerates and dMM Inhibits the Degradation of the Mutant Proteins—As shown in Fig. 2b, the blocking of mannose trimming by the mannosidase inhibitor dMM changed the mobility on SDS-PAGE and also inhibited the degradation of the mutant proteins. In contrast, blocking of glucose trimming by CST seemed to have no such inhibitory effect on their degradation. Therefore, we examined the effect of oligosaccharide trimming on their degradation (Fig. 4, A and B). The degradation of the mutant proteins was rather accelerated as a result of CST treatment. On the other hand, dMM treatment markedly inhibited the degradation of the mutant proteins. The lag time of degradation seen in the case of the other inhibitors was not observed in the case of CST treatment. On the other hand, dMM treatment markedly inhibited the degradation of the mutant α₂PI proteins was comparable with that seen in the case of LCT. Exposure of the cells to a combination of dMM and LCT did not result in an additive effect (data not shown). Similar results were obtained in the case of α₂PI-Okinawa (data not shown).

dMM Also Inhibits Degradation of the Mutant Proteins Treated with CST—We examined the effect of the mannosidase inhibitor dMM on the degradation of the molecules when glucose trimming had been blocked by CST. Pre-incubation with
CST blocked co-translational glucose trimming and accelerated the degradation of the mutant α2PI proteins (Fig. 5). Comparing the molecular sizes of the mutant proteins at 0 and 6 h, the mobility changed during the chase period. dMM blocked both their decrease in size and their degradation. LCT markedly inhibited the degradation of the molecules, but not the size reduction. When the cells were incubated with CST only after radiolabeling, post-translational glucose trimming was blocked, and the molecules were degraded. dMM and LCT also inhibited their degradation. These results suggest that the processing of mannose residues in the molecules occurred even without the removal of glucose. Also, mannose trimming was important for the accelerated degradation of the mutant α2PI-Nara molecules in cells treated with CST.

**Wild-type and Mutant α2PI Proteins Associated with CNX—** Because CNX has been shown to bind monoglucosylated N-linked oligosaccharides of nascent proteins in the ER, it has been speculated that the repeated binding of glucose to aglucoylated oligosaccharides of proteins might play a role in the selection of properly folded proteins for secretion and in the retention of unfolded proteins in the ER. To examine the role of the interaction between CNX and the α2PI proteins, pulse-labeled cells were lysed, and these proteins were co-immunoprecipitated by anti-CNX antibody under nonstringent conditions. The intensity of each band was quantified by a Fuji BAS 2000 Bio-imaging analyzer. The ratio of the intensity of the band of α2PI protein immunoprecipitated by anti-CNX antibody to that of the band of α2PI protein immunoprecipitated by anti-α2PI antibody at each time point was calculated and expressed as a percentage. About 10% of the wild-type α2PI molecules were associated with CNX for a short duration of 30 min (Fig. 6A) and then secreted from the cells. In the case of α2PI-Nara, about 20% of the mutant proteins interacted with CNX for a longer duration of 90 min. Therefore, CNX dissociated from the mutant α2PI-Nara despite the fact that the proteins were retained within the cells. α2PI-Okinawa also interacted with CNX. In the case of the hepatoma cell line HepG2, the cells are known to produce α2PI, which remains associated with CNX for a short duration. Despite the increase in retained mutant proteins, the mannosidase inhibitor dMM had no effect on the association of the mutant α2PI proteins with CNX (data not shown). On the other hand, treatment of the stable cell lines, or HepG2 cells, with CST blocked the interaction of the wild-type and mutant α2PI proteins with CNX (Fig. 6B). Binding of another ER chaperone, calreticulin, which is a soluble homologue of CNX, and binding of Bip with the wild-type or mutant α2PI molecules were not detected.

**DISCUSSION**

In this study, we have shown that both α2PI-Nara and α2PI-Okinawa mutant proteins are degraded by proteasomes without ubiquitination. We also have demonstrated that inhibition of mannose trimming blocked the degradation of the mutant α2PI proteins with or without removal of glucose. Also, we characterized the difference in association of the wild-type and mutant α2PI proteins with CNX after translation.

ER-associated degradation is well characterized for several transmembrane and luminal proteins (34). This degradation of newly synthesized proteins in the ER is referred to as quality control and is distinct from lysosomal degradation. It has been recognized that the degradation of mutant proteins is mediated by the proteasome in the cytoplasm. ER forms of the multimeric–spanning cystic fibrosis conductance regulator have been shown to be degraded by proteasomes, with the generation of ubiquitinated intermediates (17). Misfolded forms of this complex protein are recognized by enzymes of the ubiquitin-conjugating system and then targeted for destruction. Our study shows that mutant α2PI proteins also are degraded by the proteasome, but these mutants could not be detected in a ubiquitinated form. The proteasome has been shown to degrade ubiquitinated forms of proteins. However, several authors have reported that the proteasome is involved in degradation of proteins without ubiquitination. Ornithine decarboxylase, the best characterized substrate for regulated proteolysis, is degraded by a 26S proteasome in a ubiquitin-independent fashion (35). Ornithine decarboxylase proteolysis depends on another targeting system for proteasomal degradation. It is unknown whether the mutant α2PI molecules are really degraded by the proteasome in a ubiquitin-independent manner. There are several possibilities to be considered in explaining the absence of ubiquitinated forms of the α2PI mutants. First, the ubiquitination of these proteins may vary depending on the cell lines used. Recent studies have shown that T-cell receptor-α subunit chains do not require ubiquitination for their degradation by proteasomes in non-T cells (36), whereas ubiquitinated T-cell receptor-α is detected easily when the proteasome function is inhibited in a T-cell line (27). Second, unlike other short-lived proteins, the half-life of the mutant α2PI is about 4 h, which is a relatively slow degradation process. The steady-state level of ubiquitinated forms may be low compared with the total number of molecules, probably depending on the activity of the cellular de-ubiquitinating enzymes, in which case the ubiquitinated forms would be scarcely detected. Another possible explanation is that there may be another ER-resident protease sensitive to LCT and ALLN.

The quality control of mutant α2PI proteins is associated with oligosaccharide trimming and the assembly with molecular chaperones. It was found that degradation of the mutant α2PI molecules took place after a lag time of 1.5 h in the chase period. The end point of the lag time corresponded to the initiation of mannose trimming. N-linked oligosaccharides are co-translationally added to luminal asparagine residues of proteins as Glc3Man9GlcNAc2 (31). The three terminal glucose residues are rapidly cleaved in the ER, and ER and cis-Golgi mannosidases carry out trimming of these high mannose chains (37). During the glucose trimming, the carbohydrate-binding luminal chaperone CNX preferentially associates with monoglucosylated oligosaccharides and facilitates protein fold-
The cells were lysed, and the mutant proteins were identified by antibodies. After the lag time, degradation of the mutant proteins began with mannose trimming. The initiation of degradation corresponded to the time when the mutant proteins were recognized by both antibodies. To void detection of the antibody heavy chain (50–55 kDa), the membrane was cut at a position corresponding to 60 kDa. Western blot analysis of immunoprecipitates was performed using anti-α2PI antibody, anti-p53 antibody, or anti-ubiquitin antibody for detection. To void detection of the antibody heavy chain (50–55 kDa), the membrane was cut at a position corresponding to 60 kDa. The ubiquitinated forms of p53 (p53-Ub) were recognized by both antibodies.

Recent studies have shown that mannosidase activity has a role in determining the fate of several proteins including yeast pro/pre-α factor, mutant α2-antitrypsin, and T-cell receptor subunit CD3-δ (26–28). These studies show that the removal of mannose residues from N-linked oligosaccharides plays a role in the selection of mutant proteins to enter the proteasome-dependent degradation pathway. Liu et al. (26, 28) proposed a model in which, after dissociation from CNX, mannose residues of mutant α2-antitrypsin were trimmed and then the mannose-trimmed molecules targeted for disposal by proteasomes became bound to CNX again. Furthermore, Qu et al. (18) have reported that ubiquitination of CNX associated with variant α1-antitrypsin precedes proteasomal degradation of the molecules in a cell-free system. In our study, it was shown that mannose trimming of the mutant α2PI proteins that have dis -
of animal cells, CST was found to prevent the processing of normal glycoproteins and therefore to cause the production of \( N \)-linked glycoproteins of the \( \text{Glc}_3\text{Man}_{7-9}\text{GlcNAc}_2 \) type. In addition, the presence of an inhibitor, dMM (0.5 mM) or LCT (50 \( \mu \)M), was pulse-labeled with \( \text{[35S]} \)methionine for 15 min and chased for the indicated period in the presence of the inhibitor. The molecules that accumulated in the presence of dMM (\( * \)) or LCT (\( \ast \)) are indicated. B, the proteins were quantified on the basis of the intensity of the bands obtained upon SDS-PAGE analysis using the Fuji BAS 2000 system. The change in the degradation of \( \alpha_2\text{PI-Nara} \) molecules upon treatment with the inhibitor, dMM (closed triangles), LCT (open triangles), or CST (closed circles), was plotted along with the results for untreated cells (open circles). The standard deviation is indicated by a bar.

**Fig. 5.** Inhibition of the CST-accelerated degradation of \( \alpha_2\text{PI-Nara} \) by a mannosidase inhibitor. A, CHO cells preincubated with CST (100 \( \mu \)g/ml) were pulse-labeled with \( \text{[35S]} \)methionine for 15 min and then treated with nonradiolabeled methionine for 6 h (the “chase” period) in the presence of the inhibitor. dMM (0.5 mM) or LCT (50 \( \mu \)M) was added to the medium at the start of the chase period. \( M \) indicates the size of the molecules at 0 h in which glucose trimming was blocked. \( G \) indicates the size of the molecules at 0 h in which glucose trimming was added. b, after the 15-min pulse-labeling period, the cells were incubated with CST (100 \( \mu \)g/ml), and dMM (0.5 mM) or LCT (50 \( \mu \)M) was added to the medium at the start of the 6-h chase period. \( g \) indicates the molecules at 0 h in which post-translational glucose trimming was inhibited. \( m \) indicates the molecules after the 6-h chase period in which reduction of the molecular weight occurred.

**Fig. 6.** Association of the wild-type \( \alpha_2\text{PI} \) and mutant \( \alpha_2\text{PI-Nara} \) proteins with CNX. A, in the pulse-chase analysis, these proteins were immunoprecipitated from cell extracts with goat anti-\( \alpha_2\text{PI} \) or rabbit anti-calnexin antibodies after the indicated chase period. The immunoprecipitates were incubated with anti-\( \alpha_2\text{PI} \) antibody again. Aliquots of the immunoprecipitates of molecules released from a \( \alpha_2\text{PI} \) immunoprecipitate (\( \rightarrow \text{A} \)) and from a CNX precipitate (\( \rightarrow \text{CNX} \)) were applied to SDS-PAGE using a 10% acrylamide gel. The proteins were quantified on the basis of the intensity of the bands obtained upon SDS-PAGE analysis using the Fuji BAS 2000 image analyzer. The ratio of the intensity of the band of the \( \alpha_2\text{PI} \) molecules immunoprecipitated by \( \alpha_2\text{PI} \) antibody to that of the \( \alpha_2\text{PI} \) molecules immunoprecipitated by the CNX antibody was calculated as the percentage of \( \alpha_2\text{PI} \) molecules associated with CNX. B, the effect of CST treatment on the binding of the \( \alpha_2\text{PI} \) proteins to CNX was examined in a hepatoma cell line, HepG2, and in cells stably expressing wild-type \( \alpha_2\text{PI} \), \( \alpha_2\text{PI-Nara} \), or \( \alpha_2\text{PI-Okinawa} \). CST was added 1 h before radiolabeling. Immunoprecipitates (IP) recovered from \( \alpha_2\text{PI} \) (A) or CNX (CNX→A) precipitates were analyzed.

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