Proteasome degradation of protein C and plasmin inhibitor mutants

Miwako Nishio1,Takatoshi Koyama1, Masako Nakahara2, Nagisa Egawa1, Shinsaku Hirosawa1
1Laboratory Molecular Genetics of Hematology, Graduate School of Health Sciences, Tokyo Medical and Dental University, Tokyo, Japan; 2School of Allied Health Sciences, Kitasato University, Sagamihara, Kanagawa, Japan

Summary
Protein C (PC) deficiency and plasmin inhibitor (PI) deficiency are inherited thrombotic and haemorrhagic disorders. We investigated the intracellular degradation of mutant proteins, using naturally occurring PC and PI mutants that lead to congenital deficiencies. To examine the necessity of N-linked glycosylation for the proteasomal degradation of PC and PI, PC178 and PC331 mutants treated with tunicamycin and N-glycosylation-lacking mutants, PC92Stop and PI-America were pulse chased. The analysis revealed that the speed of degradation of the tunicamycin-treated PC mutants, PC92Stop and PI-America lacking glycosylation, was slower than that of N-glycosylated mutants. Immunoprecipitation and immunoblot analysis showed that PC178 and PC331 mutants were associated with molecular chaperones, Bip, GRP94, and calreticulin. PI-America was associated with only Bip. Although degradation of mutants was mediated by proteasomes, no association with ubiquitin was detected. Cotransfection of endoplasmic reticulum (ER) degradation enhancing α-mannosidase-like protein (EDEM) accelerated the degradation of N-glycosylated PC. In the absence of autophagy using Atg5-deficient cell lines, the degradation of the PC331 mutant was mildly accelerated but that of PC178, PI-America and PI-Okinawa mutants was not influenced. While the degradation of the PC and PI mutants was facilitated by N-glycosylation moieties, they were ubiquitin-independently degraded by proteasomes, irrespective of the presence or absence of N-glycosylation. Molecular chaperone binding was influenced by the presence of N-glycosylation moieties. When the misfolded or truncated mutant proteins are functionally active, proteasome inhibitors such as bortezomib may have therapeutic potential for treatment of protein deficiencies.

Keywords
Endoplasmic reticulum-associated degradation, N-linked glycosylation, protein C, plasmin inhibitor, proteasome inhibitor

Introduction
Regulation of coagulation and fibrinolysis are important for maintenance of homeostasis in blood. In many inherited disorders, protein deficiency is one of the major etiologies. Congenital deficiency of proteins in coagulation and fibrinolysis leads to thrombotic or haemorrhagic tendency.

Protein C (PC) is a plasma serine protease precursor that acts as an anticoagulant and plays an important physiological role in haemostasis (1). It is a vitamin K-dependent glycoprotein synthesized in the liver, along with prothrombin, factor VII (FVII), FIX and FX. This protein is activated by thrombin-thrombomodulin complex on endothelial cells. Activated PC inactivates activated coagulation factors V and VIII (FVa and FVIIIa) and becomes an inhibitor of clotting factors that initiates the inactivation of the clotting cascade. PC deficiency is an autosomally inherited disorder associated with a high risk of recurrent venous thrombosis (2, 3).

Plasma plasmin inhibitor (PI) is an important physiological inhibitor of plasmin-mediated fibrinolysis to stabilize haemostatic plugs (4). PI inhibits fibrinolysis by inhibition of plasmin proteolytic activity, and interference with binding of plasminogen to fibrin, and its factor XIII-catalyzed covalent binding to fibrin. In patients with PI deficiency, haemostatic plugs are dissolved prematurely before the restoration of injured vessels, resulting in a tendency for severe haemorrhage (5).

Congenital protein deficiency is caused by impaired protein activity, which results from a broad spectrum of mutations within the protein gene. While gene inversion is a frequent cause of haemophilia A, a defect in the expression of messenger RNA or its rapid degradation seems to be a minor contributor in the mechanism of protein deficiencies (6). In most cases of protein...
deficiencies, mutant proteins may be degraded in the cells that normally produce the proteins.

Endoplasmic reticulum (ER) has been reported to have quality control mechanisms. When newly synthesized proteins enter the ER, they are glycosylated with a core oligosaccharide consisting of three glucose, nine mannoses, and two N-acetylglucosamines (Glc₃Man₉GlcNAc₂) to a specific sequence, Asn-X-Ser/Thr, by the oligosaccharyltransferase complex. Immediately after synthesis, the terminal glucose units are trimmed by glucosidases I and II localized in the ER (7). Another ER-resident enzyme, UDP-glucose: glycoprotein glucosyltransferase (UGT), is capable of adding a glucose residue to the glucose-terminated high mannose oligosaccharides present on incompletely folded or assembled protein (8), favoring their binding to calnexin (CNX) or calreticulin (CRT). It is known that several proteins are retained in the ER in association with molecular chaperones (9). Two of these ER chaperones are retained in the folding-promoting ER environment. ER mannosidases I and II remove the terminal mannoses. It is thought that oligosaccharides lacking the terminal mannoses are poorer substrates for glucosidase II and UDP-glucose, thereby bringing the CNX cycle to an end. Hence, it is possible that the different kinetics of the ER sugar-processing enzymes provide a molecular mechanism regulating retention and dislocation/degradation.

When improperly folded or incompletely assembled proteins fail to restore their functional states, they are degraded by an ER-associated degradation (ERAD) system, which involves transfer of mutant proteins from the ER to the cytoplasm followed by degradation by the proteasomes. Evidence for a role of mannose trimming in diverting misfolded glycoproteins to proteasomal degradation has been found in mammalian cells. Recently, it was reported that endoplasmic reticulum degradation enhancing α-mannosidase like protein (EDEM) is a Man₅GlcNAc₂ (Man8)-binding lectin and accelerates the degradation of misfolded glycoproteins (10). In addition to the proteasomal system, recent studies have revealed another pathway of clearance, namely autophagy. While autophagy is known to be a bulk protein degradation pathway (11), it acts on an alternative ERAD pathway for the degradation of several mutant molecules (12, 13).

We have previously reported that glycosylated PC mutants (Arg178Gln and Cys331Arg) and PI mutants (PL-Nara and PL-Okinawa) are similarly degraded by proteasomes after mannose trimming irrespective of the different structures of the molecules (14, 15). However, a selective mechanism that directs the misfolded proteins to the degradation by proteasomes is not yet clear. In this study, we investigated the common degradation process of naturally occurring mutants that leads to congenital deficiency, using N-glycosylation-lacking PC and PI mutants, EDEM-overexpressed cells, and autophagy-deleted cells.

**Materials and methods**

**Chemicals and reagents**

Rabbit polyclonal anti-human PC antibody (3.7 mg/ml) was purchased from Dako (Glostrup, Denmark). Goat polyclonal anti-human PI antibody was from Biopool (Umeda, Sweden). Anti-Bip, anti-GRP94, anti-CNX, anti-CRT and anti-ubiquitin polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Proteasome inhibitors, MG132, MG101 and N-α-acetyl-leucyl-leucyl-norleucinal (ALLN), and the serine/cysteine protease inhibitors, N-α-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) and leupeptin, and a lysosomal inhibitor, chloroquine, were purchased from Sigma Chemicals (St. Louis, MO, USA). Brefeldin, a specific blocker of anterograde transport from ER to the Golgi was also from Sigma. Lactacystin (LCT) and bortezomib, proteasome inhibitors, were obtained from Kyowa Medex (Tokyo, Japan) and Millenium Pharmaceuticals (Cambridge, MA, USA), respectively. Doxycycline hydrochloride (Dox), used to regulate autophagic ability, was purchased from Sigma. [³⁵S] Met/Cys (>1000 Ci/mmol) was obtained from Amersham Biosciences (Piscataway, NJ, USA). All other chemicals were reagent-grade products and were purchased from Wako Pure Chemicals (Osaka, Japan) unless otherwise indicated.

**Construction of expression vectors**

Normal PC cDNA with 5'- and 3'-untranslated and coding regions was used to construct an expression vector, pCIPC, which was transcribed under the control of the cytomegalovirus enhancer and promoter (Promega). The XhoI–XbaI fragment was released from the pcIPC vector and was subcloned into a pBlueScript vector to introduce two mutations, Arg178 (CGG) to Gln (CAG) and Cys331 (TGC) to Arg (CGC) using a Chameleon Double-Stranded Site-Directed Mutagenesis Kit (Stratagene Ltd, Cambridge, UK), resulting in the mutant expression vectors, pCIPC178 and pCIPC331 (14, 16). Induction of their mutations was confirmed by DNA sequencing. Another mutation, Glu92 (GAG) to Stop (TAG) (17), was introduced in the pCIPC vector using a Gene Tailor Site-Directed Mutagenesis System (Invitrogen, Paisley, UK), resulting in the mutant expression vector, pCIPC92.

On the other hand, a 2048 bp fragment of normal PI cDNA including 5'- and 3'-untranslated end coding regions was used to construct an expression vector pCIP1, which was also transcribed under the control of the cytomegalovirus (CMV) enhancer and promoter (Promega, Southampton, UK). The XhoI–XhoI fragment was released from the pCIP vector and was subcloned into a pBluescript vector to introduce the single thymine deletion at nucleotide position 332 (del332T) (18). The XhoI–XhoI fragment of the normal pCIP vector was replaced with the corresponding fragment with the mutation, resulting in the mutant PI-America expression vector, pCIPAM. Induction of the single deletion was confirmed by DNA sequencing. PI-Okinawa expression vector, pSV₂PO, was constructed as previously described (15, 19).

Expression vector of mouse EDEM was regulated by CMV promoter (10). The expression vector of human Myc-ubiquitin, pRGG4-Myc-ubiquitin, was kindly provided by Dr S. Miyake, Department of Molecular Oncology, Tokyo Medical and Dental University (20).

**Transient or stable expression of recombinant PC and PI**

In the presence of 10 ng/ml doxycycline (Dox), autophagy-related gene Atg5 expression is completely suppressed in a mouse embryonic fibroblast cell line, m5–7 cells and these cells...
are autophagy-defective (21). Chinese hamster ovary (CHO) cells and m5–7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). CHO cells were transfected with pCIPC92, pCIPC178, pCIPC331, pCIPAM, or pSV2PO. For stable expression in CHO cells, the cells were cotransfected with each expression vector DNA and pSV2neo (10:1) by LipofectAMINE reagent (Invitrogen), and neomycin was employed for selection. Stable cell lines of recombinant PC were cultured in a medium containing 10 µg/ml vitamin K1. Molecular profiles of PC and PI mutants used in this study are summarized in Table 1.

### Immunoprecipitation
Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed on ice with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemicals) for 30 minutes (min). The lysates were centrifuged at 15,000 rpm for 30 min at 4°C. Supernatants and culture media were immunoprecipitated with rotation for 16 hours (h) at 4°C using an excess of the polyclonal antibody (0.2% v/v), 10 µl of protein A-Sepharose (Santa Cruz), and 0.2% SDS to remove nonspecifically bound proteins. The Sepharose beads were washed four times with lysis buffer and were boiled in 2× SDS sample buffer (100 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, and 0.2% bromophenol blue) containing 10% 2-mercaptoethanol for 5 min.

### Pulse-chase experiment
CHO cells transfected with pCIPC92, pCIPC178, pCIPC331, pCIPAM, and pSV2PO were pulse-labeled for 30 min with [35S] Met/Cys and chased as previously described (14). Tunicamycin, which prevented the transfer of the glycosyl core to the protein, was added to the medium 16 h before pulse-labeling. Proteolysis inhibitors were added to medium at the onset of the chase period. Proteins that were radio labeled in the course of synthesis were immunoprecipitated from cell lysates or conditioned medium, and were then analyzed by SDS-PAGE, followed by autoradiography (BAS 2000, Fujifilm, Tokyo, Japan), and quantified using Scion Image (Microsoft, Washington, D.C., USA).

### Immunoblotting
CHO cells expressing either pCIPC178 or pCIPC331 were preincubated with MG132 (10 µM) for 16 h. The cells were lysed in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM PMSF and 0.5 mM dithiothreitol), and the mutant PC proteins were immunoprecipitated with anti-PC antibody. The immunoprecipitates were subjected to SDS-PAGE, and western blot analysis was performed with detection using anti-ubiquitin antibody.

In the detection of ubiquitination, as a positive control, Jurkat T cells, a human leukemic T cell line, were used. Jurkat T cells were also preincubated with MG132 (10 µM) for 16 h, lysed with SDS-sample buffer and sonicated on ice. Western blot analysis was performed using the anti-ubiquitin antibody.

### Statistics
Statistical analysis for nonparametric multiple comparisons was performed by Steel test or Steel-Dwass test using the data analy-

---

**Table 1: Molecular profiles of protein C and plasmin inhibitor mutants used in this study.**

<table>
<thead>
<tr>
<th>Nucleotide mutation</th>
<th>Amino acid change</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC178 G6246A (exon 7) CGG→CAG</td>
<td>Arg178Gln</td>
<td>May alter the local conformation and influence the removal of the connecting peptide during processing</td>
<td>(16)</td>
</tr>
<tr>
<td>PC331 T8706C (exon 9) TGC→CGC</td>
<td>Cys331Arg</td>
<td>Lack of N-glycosylation at the fourth glycosylation site (Asn 329)</td>
<td>(16)</td>
</tr>
<tr>
<td>PC92Stop G3217T (exon 5) GAG→TAG</td>
<td>Glu92Stop</td>
<td>Truncated molecule that lacks N-glycosylation</td>
<td>(17)</td>
</tr>
<tr>
<td>PI-America del332T (exon 5)</td>
<td>Frameshift that substitutes a sequence encoding 11 amino acids and a stop codon</td>
<td>Truncated molecule that lacks N-glycosylation</td>
<td>(18)</td>
</tr>
<tr>
<td>PI-Okinawa del GAA (exon 7)</td>
<td>Absence of Glu137</td>
<td>The significant alteration in the hydrophobicity may cause a structural change by disrupting the normal hydrogen bonding network</td>
<td>(19)</td>
</tr>
</tbody>
</table>
Results

Pulse-chase analysis of PC mutants treated with or without tunicamycin
To examine the relevance of the N-linked glycosylation to the degradation, stable transfectants expressing the PC178 mutant (Fig. 1A, B) or PC331 mutant (Fig. 1A, C) were treated with or without tunicamycin and were used in the pulse-chase analysis. Each of the PC molecules from cell extracts was immunoprecipitated with polyclonal anti-PC antibodies. We measured the density of each band at the indicated times using the densitometer. The results were plotted in Figure 1B and C, and the degradation patterns were analyzed. We have already reported that PC178 and PC331 mutants are not secreted into the media even after a 6 h-chase period (14).

After a lag time of 1.5 h, the degradation of mutants was initiated. The degradation of the tunicamycin-treated PC mutants, lacking the addition of N-glycosylation, was slower than that of the untreated PC mutants. The degradation of the N-glycosylated PC mutants was markedly accelerated compared with mutants lacking N-glycosylation (n = 5, p < 0.05).

Pulse-chase analysis of PC92 mutant
To determine whether the N-linked glycosylation is relevant to the degradation of PC mutants, we introduced a nonsense mutation at the codon of Glu92 of the PC expression vector to translate only 91 amino acids corresponding to mature PC protein, resulting in the mutant PC92 with no putative N-glycosylation site. CHO cells transfected with PC92 mutant lacking N-glycosylation were pulse-labeled and chased for the indicated periods (Fig. 1D). The rate of degradation of PC92 mutant lacking N-glycosylation was slower than that of the N-glycosylated PC178 and PC331 mutants (Fig. 1E) (n = 5, p < 0.05). PC92 was not secreted into the culture media (data not shown). On the other hand we have already reported that wild-type PC molecules were progressively secreted into the culture media and disappeared in the cells with half-life of 1.8 h (14).

Pulse-chase analysis of PI-America
To examine the relevance of N-linked glycosylation in another protein undergoing degradation, the PI mutant lacking N-glycosylation sites (designated PI-America) was transiently expressed in CHO cells (18). The cells were pulse-labeled and chased for the indicated periods (Fig. 1D). The time course of degradation of the mutants PI-America and PI-Okinawa is shown in Figure 1F. The rate of degradation of PI-America was also slower than that of the N-glycosylated PI mutant, PI-Okinawa (n = 5, p < 0.05). PI-America was minimally secreted into the culture media (18). On the other hand we have already reported that wild-type PI molecules were progressively secreted into the culture media and disappeared in the cells with half-life of 2 h (15).

Association of PC mutants with molecular chaperones
Molecular chaperones have been shown to function as foldases, essentially guiding nascent polypeptides along the folding and...
assembly pathways. To examine the role of the interaction between molecular chaperones and PC mutants, CHO cells were transfected with PC178, PC331 or PC92 mutant and were lysed. The cell extracts were immunoprecipitated with anti-PC, Bip, GRP94, CRT or CNX polyclonal antibodies. Western blot analysis of the immunoprecipitates was performed using the anti-PC monoclonal antibody for detection (Fig. 2A).

The N-glycosylated PC mutants interacted with most molecular chaperones, aside from no binding of the PC331 mutant to CNX. The difference in the pattern of the PC178 mutant associated with Bip was observed as two bands. One corresponded to the binding with other molecular chaperones, but the other was larger in molecular size. Binding of PC92 with molecular chaperones was not detected.

**Association of PI-America with Bip**

To examine the role of the interaction between molecular chaperones and PI-America in comparison with the N-glycosylated PC mutants, PI-America was transiently expressed in CHO cells and lysed. PI-America lacking N-glycosylation was associated with Bip only. Binding of GRP94, CRT and CNX with PI-America was not detected (Fig. 2B).

**Proteasome-mediated degradation of PC92 mutant and PI-America**

To examine whether specific proteases are involved in the degradation of the PC92 and PI-America, pulse-chase experiments were performed in the presence of inhibitors for several proteases and proteasomes. The proteasome inhibitors, LCT, MG132, MG101 and ALLN, markedly inhibited the degradation of the PC92 (Fig. 3A, C) and PI-America (Fig. 3B, D). The other inhibitors had no significant effects.

**The effect of bortezomib, a proteasome inhibitor on inhibition**

Bortezomib is the first clinically approved proteasome inhibitor for the treatment of refractory multiple myeloma. To examine the effect of bortezomib, pulse-chase experiments were performed in the presence of various concentrations of bortezomib. The degradation of PC mutants was significantly inhibited by 15.6 µM bortezomib, a clinically relevant concentration (Fig. 3E).

**Ubiquitination-independent degradation of PC mutants**

Ubiquitination is known to play an important role in the regulated destruction of proteins. The ubiquitin-proteasome system works by marking specific substrates with ubiquitins and degrading the marked proteins by proteasomes in an ATP-dependent manner. To find out whether the degradation of PC mutants is due to ubiquitination, CHO cells transfected with PC178 and PC331 mutants were immunoprecipitated with anti-PC antibody and Western blot analysis was performed with anti-ubiquitin antibody. The proteasome inhibitor, MG132 (1 µM), was used to prevent the rapid degradation of PC mutants. Although the ubiquitinated proteins of Jurkat T cells were recognized by the anti-ubiquitin polyclonal antibody (22), ubiquitination of PC mutants was not detected even in the presence of the proteasome inhibitor MG132 (Fig. 4A). Ubiquitination was not detected even when the myc-tagged ubiquitin was cotransfected (20). Association between the mutants and an ubiquitin ligase Skp1 was not detected, either (data not shown).

**The effect of EDEM overexpression on the degradation of PC mutants**

EDEM is an enzymatic inactive mannosidase homolog and most likely binds to Man8-misfolded glycoproteins and accelerates its degradation. To examine the function of EDEM on the degradation of PC mutants, PC178 (Fig. 4B, C) and PC331 mutants (Fig. 4B, D) were cotransfected with or without EDEM and used in pulse-chase analysis. Overexpression of EDEM remarkably accelerated the degradation of PC mutants and decreased its half-life (n = 5, p<0.05).

**The degradation of PC and PI mutants in the absence of autophagy; m5–7 cells**

Autophagy is a bulk protein degradation pathway. To investigate whether PC mutants is also degraded by autophagy, we carried out pulse-chase analysis in m5–7 cells transiently transfected with PC and PI mutants (Fig. 5A, D). Cells were treated with Dox (10 ng/ml) for four days to suppress autophagy. In the absence of autophagy, there was a mild acceleration in the degradation of PC331 mutant (Fig. 5C) but the degradation of PC178, PI-America and PI-Okinawa was not influenced (Fig. 5B, E, F).
Discussion

In the present study, we have shown that the PC178, PC331, PC92, and PI-America mutants enter into the degradation pathway without secretion and are degraded in an ubiquitin-independent manner by proteasomes, irrespective of the presence or absence of N-glycosylation moieties. Also, we have demonstrated that the binding of molecular chaperones with PC and PI mutants was influenced by the presence of N-glycosylation moieties and that EDEM accelerated the degradation of PC mutants. Therefore, N-glycosylation moieties seem to be relevant for the efficient degradation of mutant proteins. Autophagy does not appear to contribute to the degradation of PC and PI mutants.

We have already reported that mannos trimming of N-linked glycosylation moieties triggers the degradation of PC and PI mutants by proteasomes (14, 15). PC proteins have four potential sites for N-glycosylation. All four glycosylation sites of PC178 mutant are added by N-linked oligosaccharides, but the PC331 mutant can neither add N-linked oligosaccharides at Asn329 nor form a disulfide bond with Cys345 in the catalysis domain of PC heavy chain (14, 16). With the results of tunicamycin-treated PC178 and PC331 mutants and those of N-glycosylation-lacking mutants, PC92 and PI-America, we found that the degradation of PC and PI mutants lacking N-linked oligosaccharides was slower than that of N-glycosylated mutants. While the overall speed of degradation was not strictly consistent quantitatively depending on the lot or passage of cells, the results of degradation were qualitatively consistent. Virgilio et al. have previously demonstrated that the half-lives of nonglycosylated glycoprotein ribophorin I was prolonged after tunicamycin pretreatment (23). Tunicamycin is known to inhibit glycosylation and synthesis of proteins, and also trigger the unfolded protein response (UPR) (24), which is responsible for intracellular recycling of unfolded proteins and induces molecular chaperones (25). Taking into account that the degradation of PC92 and PI-America was retarded, the efficient degradation of PC178 and PC331 mutants seems to be disrupted by inhibition of N-glycosylation rather than by the other effects of tunicamycin.

In general, molecular chaperones play a role in the selection of properly folded proteins for secretion and in the retention of unfolded proteins in the ER. Most molecular chaperones, except CRT and CNX, directly bind to the unfolded peptide domains of proteins. Wild-type, mutant PI-Okinawa and PI-Nara with
N-linked oligosaccharides all interacted with CNX (15). A recent paper reported that β3-Cys549Arg mutant also interacted with CNX and retained in ER (26). Disulfide bond disruption by the Cys549Arg or Cys549Ser mutation in the β3 subunit is predicted to have a deleterious effect on the structure of the protein and seems to cause intracellular degradation resulting in reduced surface expression of β3 and αIIbβ3. Immunofluorescence experiments showed that β3 and αIIbβ3 were not properly transported out of ER. We suppose that these mutants may also be degraded in proteasome. PI-America without N-linked oligosaccharides was associated with only Bip, which is consistent with the fact that Bip binds to the peptide domain. On the other hand, it is controversial whether CRT and CNX bind to the defined oligosaccharides, Glc3Man5GlcNAc2, or the unfolded peptide domains directly (27, 28). The lack of binding of the PC331 mutant to CNX may be due to the absence of N-linked oligosaccharides at 329. That is to say, the binding of molecular chaperones is influenced by the presence of N-linked oligosaccharides. Insufficient binding with several molecular chaperones may retard the degradation process in N-glycosylation-lacking mutants.

Furthermore, we have shown that the induction of EDEM significantly accelerates the degradation of PC mutants. We confirmed that EDEM functions as an effective chaperone of misfolded glycoproteins leading to the proteasomal degradation pathway.

Numerous studies have emphasized the physiological importance of the ubiquitin-dependent degradation by proteasomes in cytoplasm (29). This type of degradation is referred to as the quality control mechanism for mutant proteins. However, several authors have reported ubiquitin-independent degradation by proteasomes (30). Proteolysis of ornithine decarboxylase and oxidized proteins depends on such a pathway (31). Our previous studies showed that PC178, PC331, PI-Nara and PI-Okinawa mutants were degraded by proteasomes (14, 15), but the ubiquitination could not be detected in these mutants. PC and PI mutants seem to be ubiquitin-independently degraded by proteasomes. Furthermore, both PC92 and PI-America mutants, which lack N-glycosylation, also appeared to be degraded by proteasomes. These findings suggest that PC and PI mutants are degraded by proteasomes, irrespective of the presence or absence of N-linked oligosaccharides. Mutant proteins with misfolding and conformational changes may be supervised and degraded by mediators other than ubiquitin or molecular chaperones associated with N-linked glycosylation.

Here, we report that bortezomib, the first clinically approved proteasome inhibitor for the treatment of refractory multiple myeloma, inhibited the degradation of PC mutants at pharmacological relevant concentrations (32). Bortezomib may also be used for treatment of embolic stroke by promoting eNOS-dependent vascular protection, and by reducing NF-κB-dependent vascular disruption (33). When the misfolded or truncated mutant proteins are functionally active, such proteasome inhibitors may have therapeutic potential for treatment of not only advanced multiple myeloma but also protein deficiency. In such situations, the induction of secretion of undegraded proteins may be necessary.

Autophagy engulfs bulk cytosolic material and organelles within double-membrane vesicles, known as autophagosomes, and then degrades them to liberate nutrients via fusion with lysosome. In addition to ubiquitins/proteasomes, constitutive autophagy plays an important role in protein quality control, as Atg5-deficient mice showed an accumulation of ubiquitinated protein in the brain (34). While autophagy may partly act as an alternative ERAD system in the degradation of several mutant proteins (35, 36), selective degradation of mutant proteins associated with congenital deficiency is not yet clear. In the absence of autophagy, there was a mild acceleration in the degradation of PC331 mutant, but the degradation of PC178, PI-America and PI-Okinawa was not affected. The mechanism of mild acceleration in the degradation of PC331 mutant is not clear, but the PC and PI mutants were preferentially degraded by proteasomes. Thus, autophagy did not seem to participate in selective ERAD of the PC and PI mutants. Most of the known proteins that are degraded by autophagy, as a clearance mechanism, are mutant proteins that have conformational instability with increased propensity to form oligomers and aggregates (37). The PC and PI mutants may be retrotransported to the cytosol without forming insoluble forms such as oligomers and aggregates, and be degraded by proteasomes. If the proteasomal capacity is overloaded or inhibited, soluble misfolded proteins may be degraded by autophagy (37). Since inhibition of proteasomal capacity for four days by proteasome inhibitors induced apoptosis of m5–5 cells in our experimental system, we could not demonstrate such a mechanism.

There seem to be various recognition pathways and degradation pathways for mutant proteins in ERAD. While it seems clear that N-linked oligosaccharides play an important role in ERAD, the functional role connected with various diseases remains to be fully clarified. Thus, understanding the functional roles of N-linked oligosaccharides in ER quality control mechanism may be connected with new targets for the treatment of ER quality control-related diseases. Furthermore, it is also possible that other proteasome regulators and molecular chaperones may be associated with this mechanism.

In summary, our present findings showed that PC and PI mutants were ubiquitin-independently degraded by proteasomes, irrespective of the presence or absence of N-glycosylation moieties. The binding of molecular chaperones influenced by the presence of N-glycosylation moieties may affect the efficient degradation of the mutant proteins. These results may help to elucidate the molecular mechanisms and potential treatments of congenital deficiencies of proteins in a system of coagulation and fibrinolysis.

Acknowledgements

The m5–7 cells were generous gift from N. Mizushima, MD, PhD, Department of Physiology and Cell Biology, Tokyo Medical and Dental University. The expression vector for mouse EDEM was kindly provided by K. Nagata, PhD, Department of Molecular and Cellular Biology, Institute for Frontier Medical Sciences, Kyoto University.
References
