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Intracellular Degradation of Histidine-Rich Glycoprotein Mutants: Tokushima-1 and 2 Mutants Are Degraded by Different Proteolytic Systems[1]

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We reported the first case of a congenital histidine-rich glycoprotein deficiency (HRG Tokushima) in which substitution of Gly85 with Glu (G85E) in the first cystatin domain resulted in intracellular degradation and a low plasma level of HRG [Shigekiyo, T. *et al.* (1998) *Blood* 91, 128–133]. Recently, we identified the gene mutation of a second case of HRG deficiency as a Cys223 to Arg (C223R) mutation in the second cystatin domain. To investigate the molecular and cellular bases of these deficiencies, we expressed these HRG mutants in baby hamster kidney (BHK) cells. Pulse-chase experiments in the absence and presence of various proteinase inhibitors revealed that, while wild-type HRG was completely secreted during 4-h chase periods, both the G85E and C223R mutants were only partially secreted and primarily degraded within the cells. The intracellular degradation of the C223R mutant was almost completely inhibited in the presence of a proteasome inhibitor, lactacystin, carbobenzoxy-leucyl-leucyl-leucinal or *N*-acetyl-leucyl-leucyl-norleucinal, resulting in increased secretion of the C223R mutant, and thus implicating the proteasome system in this degradation process. In contrast, the sum of the amounts of the G85E mutant inside and outside the cells decreased during the chase periods even in the presence of the proteasome inhibitor, carbobenzoxy-leucyl-leucyl-leucinal or *N*-acetyl-leucyl-leucyl-norleucinal, although proteasome-specific inhibitor lactacystin and one of the cysteine protease inhibitors, E-64-d, prevented the intracellular degradation. These results suggested that intracellular degradation of G85E HRG occurred to some extent through a hitherto unknown mechanism. Similar studies involving recombinant mutants in which Gly85 or Cys223 was replaced with several other amino acids revealed that proteins with mutations leading to the destruction of the predicted β-sheet structure of the cystatin domains were eliminated by the intracellular quality control system.

Key words: deficiency, histidine-rich glycoprotein, intracellular degradation, proteasome.

Human histidine-rich glycoprotein (HRG) is a single-chain plasma protein with a molecular weight of 67,000 (*1*). It consists of 507 amino acid residues including 66 histidines and 65 prolines (*2*), and consists of six domains, *i.e.*, from the N-terminal, cystatin-domains 1 and 2, a proline-rich domain 1, a histidine-rich domain, a proline-rich domain 2, and a C-terminal domain (*2, 3*). HRG interacts with several plasma proteins including plasminogen (*4*), fibrinogen (*5*), IgG (*6*), and several components of the complement system (*7*). Interaction of HRG with plasminogen inhibits the binding of plasminogen to fibrin, leading to down-regulation of fibrinolysis. Binding of HRG to fibrinogen prolongs the prothrombin time suggesting an anticoagulant effect for HRG, but fibrin clots formed in the presence of HRG are more sensitive to plasmin cleavage than those formed in its absence (*8*). On the other hand, HRG binds to heparin and diminishes the anticoagulant effects of heparin-dependent protease inhibitors such as antithrombin (*9, 10*), heparin cofactor II (*11*), and protein C inhibitor (*12*). Thus, HRG is thought to act as a modulator of coagulation and fibrinolysis depending upon the physiological conditions. HRG is one of the acute phase negative proteins, and its plasma level is low in pregnant women (*13*) and newborns (*14*). In the past decade, a relationship between high HRG levels and thrombosis has been proposed, but an unequivocal connection has not been established yet (*15–17*). In 1993, the

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Abbreviations: HRG, histidine-rich glycoprotein; LLL, carbobenzoxy-leucyl-leucyl-leucinal; LLN, *N*-acetyl-leucyl-leucyl-norleucinal; TPP II, tripeptidyl peptidase II.

first case of a congenital HRG deficiency (HRG Tokushima) was reported by Shigekiyo *et al.* (*18*). The proband had a history of thrombosis and five members of the family were affected. Shigekiyo *et al.* identified the cause of this HRG deficiency to be a single mutation of G6215 to A in exon 3 of the HRG gene (*19, 20*), resulting in the replacement of Gly-85 with Glu. A second HRG deficiency family was reported by Souto *et al.* in 1996 (*21*), but the molecular basis of this deficiency remains unclear. Recently, Shigekiyo *et al.* identified a third family with a congenital deficiency of HRG (HRG Tokushima 2), and determined that the deficiency was associated with the point mutation of T11438 to C in exon 6 of the HRG gene, resulting in a Cys-223 to Arg mutation (*22*).

In this report, we present the results of detailed studies on the cellular basis of the deficiency in the cases of HRG Tokushima 1 (G85E mutant) and Tokushima 2 (C223R mutant). We also present the results for several other mutants in which Gly85 or Cys223 was replaced with various amino acids and analyzed with respect to their intracellular fates.

MATERIALS AND METHODS

DNA—The cDNA for human HRG was isolated from a human liver cDNA library as described (*20*), and spans from nucleotide 118 to 2067 of the reported sequence (*2*). Expression vectors ZMB3 and ZMB4 (*23*) were kindly supplied by Dr. D. Foster (ZymoGenetics, Seattle, WA). Mutations in the HRG cDNA were introduced by two successive rounds of PCR as described previously (*19*). Briefly, the first round of PCR was performed using a mutated sense primer and the 3′-end antisense primer that has a *Bam*HI recognition site, TTGGATCCCTCTTCTCAGGG, where the underlined bases were changed. The oligonucleotide primers used for the mutation of Gly85 were GTGATCGAACAATGTAAGGT (Gly85 to Glu), GTGATCKYACAATGTAAGGT (Gly85 to Ala, Val, Leu, or Ser), GTGATCGATCAATGTAAGGT (Gly85 to Asp), and ATAGTGATCARACAATGTAA (Gly85 to Arg or Lys). The primers used for the mutation of Cys 223 were ATAAACCGTGAAGTCTTCGAC (Cys223 to Arg) and ATAAACKHCGAAGTCTTCGA (Cys223 to Xaa). The second round of PCR was carried out using the 5′-end universal primer M4, CGCCAGGGTTTTCCCAGTCACGAC, and the first round PCR products as primers. The product was initially digested with *Eco*RI and *Hin*cII, ligated into pUC19, and then sequenced to verify that the desired mutation was introduced without any undesired substitutions using either a Shimadzu DSQ-1 or DSQ-1000L DNA sequencer. The *Eco*RI/*Hin*cII fragment was then ligated with the original unmutated *Hin*cII/*Bam*HI fragment to reconstitute the entire HRG cDNA on the pUC19 plasmid, cleaved with *Eco*RI and *Bam*HI, and finally inserted into expression vectors.

Transfection of BHK Cells and Selection of Stably-Transfected HRG-Expressing Clones—Baby hamster kidney (BHK) cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum under a 5% CO_2 atmosphere. The constructed expression vectors were transfected into BHK cells by the calcium phosphate method (*24*). Stably-transfected cells were selected by culturing in the presence of 400 μg/ml geneticin (Gibco BRL) for cells transfected with ZMB3 vectors, whereas cells transfected with ZMB4 vectors were successively selected with 0.5, 1, 5 μM amethopterin, and then subjected to a filter immunoassay (*25*).

Pulse-Chase Experiments—In order to examine the secretion of the wild-type or mutant HRG, pulse-chase experiments were carried out as previously described (*19*). Approximately 5×10^5 HRG-expressing cells were cultured overnight under standard conditions, and subsequently starved of methionine and cysteine for 30 min prior to pulse-labeling with 50 μCi of EXPRE^{35}S^{35}S (NEN Life Science Products). After labeling for 1 h, the cells were washed successively with phosphate buffer saline and culture media containing 2 mM each of methionine and cysteine, and finally chased in the same media for defined periods. At selected times, culture media and cells were harvested. The collected culture media were then treated with 40 mM Tris-HCl buffer, pH 7.4, containing 0.4% SDS, 0.4% Nonidet P-40, 10 mM methionine and 2 mM EDTA, and immunoprecipitated with affinity-purified rabbit anti–human HRG IgG. The precipitates were recovered by the addition of ZYSORBIN (ZYMED Laboratories, San Francisco, CA, USA) and analyzed by SDS–polyacrylamide gel electrophoresis. Cells were washed with PBS, and then lysed with 10 mM Tris-HCl buffer, pH 7.4, containing 0.1% SDS, 1% Nonidet P-40, 0.15 M NaCl, 10 mM Met, 2 mM EDTA, 0.1 mM PMSF, and 1 μg/ml each of leupeptin and aprotinin. HRG was immunoprecipitated and analyzed by SDS–polyacrylamide gel electrophoresis as described above. The radioactivity of the HRG preparation was measured with a Fuji BAS 2000 Bio-Imaging Analyzer.

Enzymes and Chemicals—Restriction endonucleases were purchased from either New England Biolabs (Beverly, MA, USA), Boehringer Mannheim (Mannheim, Germany), or Takara Shuzo (Kyoto). The thermostable DNA polymerase used for PCR was ExTaq from Takara. Oligonucleotide primers were obtained from Tabai Espec Oligo Service (Ibaraki). Reagents for cell culture were from Gibco BRL Life Technologies (Rockville, MD, USA). Protease inhibitors, E-64-d [L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl-L-leucine(3-methylbutyl)amide] and carbobenzoxy-leucyl-leucyl-leucinal (LLL, MG132), were purchased from Peptide Institute (Osaka), and *N*-acetyl-leucyl-leucyl-norleucinal (LLN, MG101, or calpain inhibitor-I) was from Boehringer. All other reagents and chemicals used in these experiments were of the highest grade commercially available and obtained from Wako Pure Chemicals Industries (Osaka) or Nacalai Tesque (Kyoto).

RESULTS

Intracellular Degradation of G85E and C223R Mutants—To examine the intracellular degradation of the mutant HRG preparations, we initially performed site-directed mutagenesis by PCR and constructed ZMB-HRG expression vectors. These constructs were transfected into BHK cells and then stably expressing cells were selected as described under "MATERIALS AND METHODS". The secretion of newly synthesized HRG was analyzed by means of pulse-chase experiments. As shown in Fig. 1, wild-type HRG was completely secreted during 4 h chase periods, while the total radioactivity for the G85E and C223R mutants decreased to 20 and 40%, respectively, after a 4 h chase. The secreted G85E and C223R mutants represented only 7 and

30%, respectively, of the initially radiolabeled proteins. Thus, the majority of the G85E and C223R mutants were degraded within the cells.

Effects of Proteasome Inhibitors on the Degradation of G85E and C223R Mutants—The amounts of the labeled proteins inside or outside the cells were determined after a 4 h chase in the presence of various inhibitors for either lysosomal proteolysis, thiol proteases, proteasomes or intracellular translocation (Fig. 2). Inhibitors for lysosomal proteolysis, chloroquine or ammonium chloride, had little effect on the secretion of wild-type HRG or degradation of the C223R mutant, while secretion of the G85E mutant decreased on chloroquine treatment but increased on ammonium chloride treatment (Fig. 2). E-64-d, an inhibitor of thiol proteases, also had little effect on the secretion of wild-type HRG. However, it strongly inhibited the degradation and facilitated the secretion of the G85E mutant. E-64-d also significantly increased the intracellular pool of the C223R mutant (Fig. 2). When protein transport from the endoplasmic reticulum to the Golgi was inhibited by brefeldin A, secretion of wild-type HRG was completely blocked, leading to its accumulation within the cells. Degradation of each mutant was not inhibited by this reagent, and the majority of the labeled proteins disappeared (Fig. 2). Interestingly, three proteasome inhibitors had different effects on the three recombinant HRG preparations. Neither lactacystin, *N*-acetyl-leucyl-leucyl-norleucinal (LLN), nor carbobenzoxy-leucyl-leucyl-leucinal (LLL) had any effect on the secretion of wild-type HRG (Fig. 2). The degradation of the G85E mutant was only inhibited by lactacystin, while degradation of the C223R mutant was completely inhibited by all of these proteasome inhibitors (Fig. 2).

Effect of Amino Acid Replacement at Position 85 on Intracellular Degradation of Mutant Proteins—To examine the effects of mutation at position 85 on the quality control of HRG, Gly85 was replaced with several other amino acids. When Gly85 was replaced with Ala, Val or Ser, the resulting mutants were secreted as well as wild-type HRG. However, the Asp- or Lys-mutant was intracellularly degraded similar to the G85E mutant, while the Arg-mutant was partially secreted (Fig. 3).

Effect of Amino Acid Replacement at Position 223 on Intracellular Degradation of Mutant Proteins—To examine the effects of mutation at position 223 on the quality control of HRG, Cys223 was replaced with several other amino acids. Mutation of Cys223 to Ala or Ser had little effect on

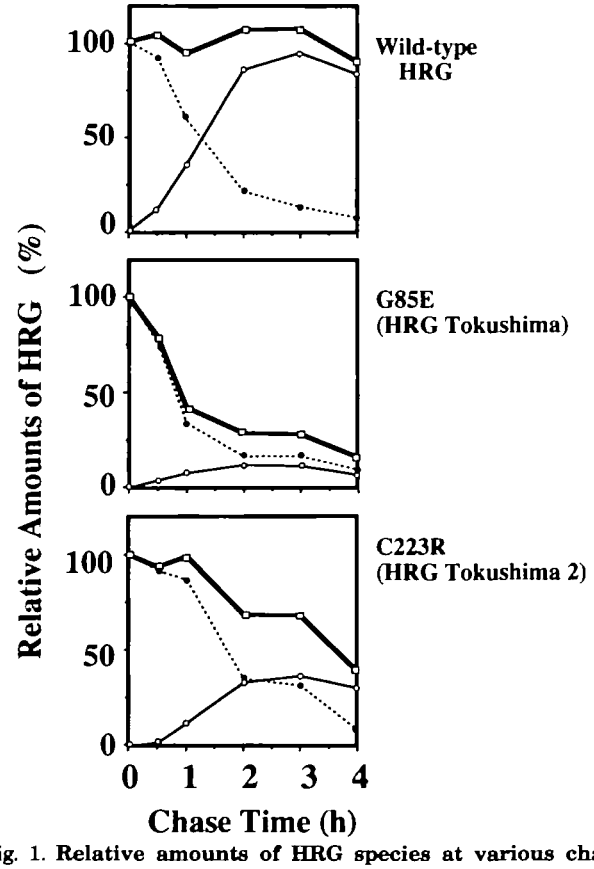

Fig. 1. **Relative amounts of HRG species at various chase times.** Intracellular HRG, secreted HRG and total HRG are depicted as dotted, solid and thick lines, respectively. The amount of intracellular HRG at the end of the labeling (chase 0 h) was taken as 100%.

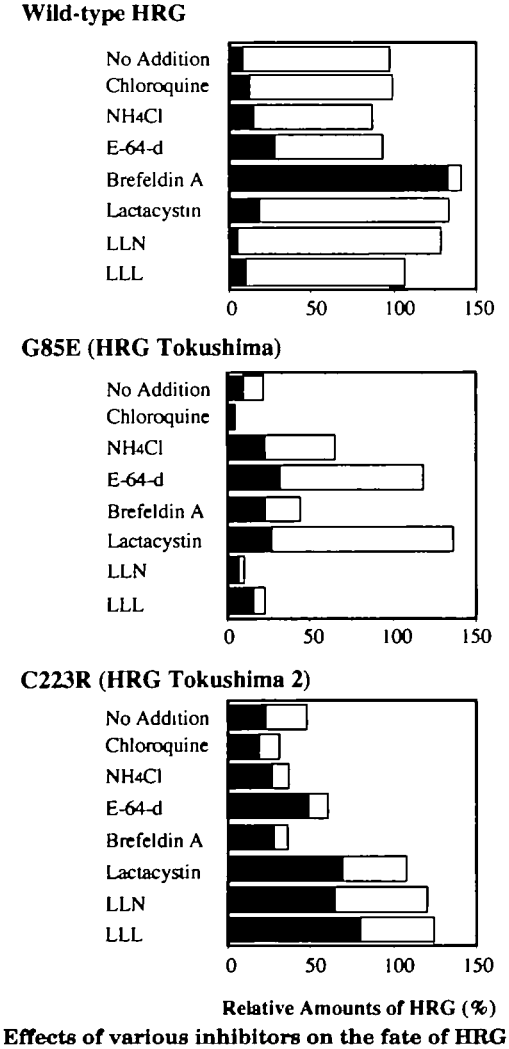

Fig. 2. **Effects of various inhibitors on the fate of HRG during a 4 h chase.** The amounts of intracellular and secreted HRG are presented as solid and open bars, respectively. The values are expressed relative to the amount of intracellular HRG at chase 0 time. The concentrations of inhibitors were as follows: chloroquine, 100 μM; ammonium chloride, 30 mM; E-64-d, 50 μM; brefeldin A, 3 μg/ml; lactacystin, 10 μM; LLN, 50 μM; LLL, 25 μM.

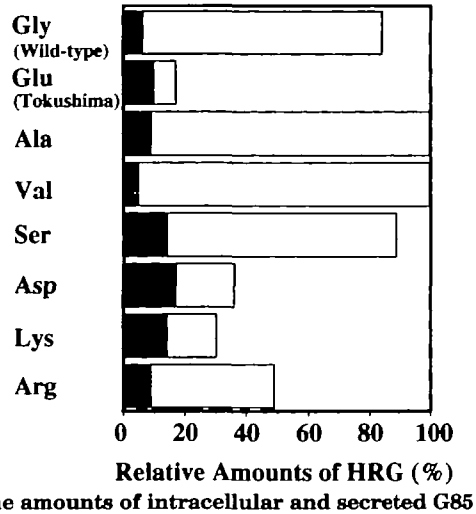

Fig. 3. **The amounts of intracellular and secreted G85 mutant HRGs after a 4 h chase.** Intracellular and secreted HRG are represented by solid and open bars, respectively.

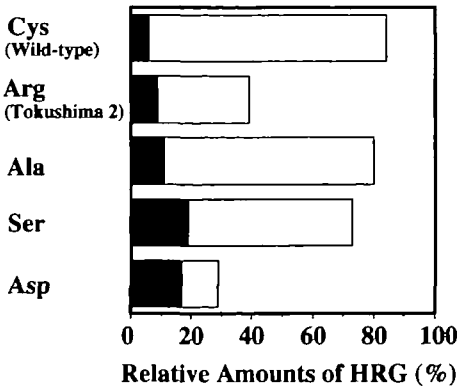

Fig. 4. **The amounts of intracellular and secreted C223 mutant HRGs after a 4 h chase.** Intracellular and secreted HRG are depicted as solid and open bars, respectively.

the secretion of these mutants, while mutation to Asp as well as to Arg resulted in a secretion-defect mutant (Fig. 4).

DISCUSSION

Recently, we reported that the basis of a congenital histidine-rich glycoprotein deficiency (HRG Tokushima) was a G to A mutation resulting in the replacement of Gly85 with Glu. Pulse-chase experiments suggested that the Glu85 mutant was intracellularly degraded, and consequently the plasma level of HRG was reduced (*19*). In our second case (HRG Tokushima 2), a T to C mutation resulting in the replacement of Cys223 with Arg was identified. We have expressed the Arg223 mutant in BHK cells and showed that the recombinant mutant was degraded within the cells. As shown in Fig. 1, the secretion of the C223R mutant (30%) is slightly higher than that of the G85E mutant (7%). The plasma HRG levels of probands of HRG Tokushima and Tokushima 2 were 20% and 50%, respectively, and possibly may reflect this secretion efficiency, although we cannot fully explain the value of 20% for HRG Tokushima in the heterozygous patient. To characterize the nature of the intracellular degradation of these mutants, we performed pulse-chase experiments in the absence and presence of various proteinase inhibitors. The effects of inhibitors of lysosomal proteolysis on the intracellular degradation of these mutants were not clear, but it was suggested that lysosomal proteinases do not participate, at least, in the degradation of the C223R mutant. Also, brefeldin A had no effect on the degradation, suggesting the degradation process takes place before these proteins are transported into the Golgi apparatus, probably in the ER or cytoplasm after retrotranslocation from the ER, as suggested by recent reports (*26*, *27*). In most cases, the intracellular degradation of mutant proteins has been reported to be mediated by the proteasome system since the degradation is inhibited by proteasome specific inhibitors, especially by lactacystin (*27–29*). Lactacystin, LLL, and LLN inhibited the degradation of the C223R mutant indicating that the proteasome is responsible for the intracellular degradation of the C223R mutant. On the other hand, the degradation of the G85E mutant was only inhibited by lactacystin, *i.e.* not by LLL or LLN. Therefore, the degradation of the G85E mutant may be catalyzed by the novel proteolytic system. Although lactacystin has been reported to be a quite specific inhibitor of the proteasome (*30*), a recent report stated that the giant protease tripeptidyl peptidase II (TPPII) is also inhibited by lactacystin (*31*). The degradation of the G85E mutant is sensitive to E-64-d and lactacystin, but insensitive to LLL and LLN, which is similar to the inhibitor sensitivity of TPPII. So, we suggest that one of the possible candidates responsible for this unusual degradation is TPPII, which is inhibited by lactacystin but not by LLN (*31*). TPPII is known as a serine protease, and human erythrocyte TPPII was not inhibited by a cysteine protease inhibitor, E-64, although it was inhibited by some thiol-reactive compounds like *p*-chloromercuribenzoate and *N*-ethyl-maleimide (*32*, *33*). So, it remains to be clarified whether or not TPPII from BHK cells is inhibited by E-64-d. The involvement of a cysteine protease in the quality control is also proposed for the pre-Golgi, nonlysosomal degradation of the Gly97Cys mutant of coagulation factor VII (*34*).

Although some proteasome population has been proved to be tightly associated with the ER membranes (*35*), both proteasome and TPPII are mainly cytosolic enzymes, and the degradation processes are believed to occur in the cytosol. However, the retrotranslocation of the target proteins from the ER to the cytosol and the degradation processes are so tightly coupled that no accumulation of the target proteins in the cytosol has been proved yet. The location of the HRG mutants not degraded on the addition of protease inhibitors was not studied in this experiment, but presumably they are localized in the ER compartment.

The amino acid sequences around Gly85 are well conserved among HRG from different species and Gly85 is common among all species so far sequenced, as indicated in the previous report (*19*). Therefore, Gly was thought to be essential at this locus for proper folding. To determine whether or not Gly85 can be replaced by any other amino acid residues, and also to determine whether or not any particular kind of amino acid residue confers the susceptibility of the mutant protein to intracellular degradation, we expressed several mutants in BHK cells by replacing Gly85 with other amino acid residues. Our results showed that

Fig. 5. **Predicted secondary structure of the cystatin domains in HRG together with the determined secondary structure of chicken cystatin.** Line 1 is the amino acid sequence of chicken cystatin. The disulfide bonds are indicated by thin lines above the sequence. The α-helices and β-strands of chicken cystatin are indicated by dotted and thick lines, respectively, below the sequence (*37*). Lines 2 and 3 represent the amino acid sequences of cystatin domains 1 (residues 1–112) and 2 (residues 113–227) of human HRG, respectively, together with the predicted secondary structures. Weakly suggested β-strand regions are presented as thin lines. Gly85 and Cys223 are boxed.

the replacement of Gly85 with a neutral amino acid, Ala, Val, or Ser, had no remarkable effect on the quality control system and these mutants were secreted as well as wild-type HRG. In contrast, replacement of Gly85 with a charged amino acid residue, Asp, Lys, or Arg, resulted in reduced secretion and intracellular degradation of the mutant, as in the case of HRG Tokushima (Gly85Glu). These results indicate that position 85 of HRG can be occupied by amino acid residues with a small side chain without having serious effects on the secretion. However, occupation of this locus by an amino acid residue with a charged side chain caused the conformational change of the mutants and resulted in the secretion defect followed by intracellular degradation. Among them, the Arg mutant was relatively well secreted. Similar results were also obtained for the mutants of Cys223. Although Cys223 is involved in the disulfide bond formation with Cys200 (unpublished results), replacement of Cys223 with Ala or Ser did not affect the secretion efficiency of the mutant. On the other hand, charged side chains of Arg and Asp had a profound effect on the proper folding of the polypeptide and led to intracellular degradation of the mutant by the quality control system. The secondary structure of the cystatin domains of HRG can be predicted by the Chou and Fasman method (*36*), which is illustrated in Fig. 5. This predicted structure of HRG correlates well with the crystal structure of chicken egg cystatin (*37*). In this model, both mutation sites are located in the β-sheet structure. The amino acid residues at the putative positions in the well secreted mutants are neutral and known to be favorable for formation of the β-sheet structure, while those of secretion-defect mutants are charged amino acid residues that are unfavorable for the formation of the β-sheet structure. A relatively higher tendency of Arg to preserve the β-sheet structure than the other three charged amino acids (Lys, Asp, and Glu) may be responsible for the slightly higher level of secretion of the mutant. Besides those in this study, among many secretion-defect mutants caused by a single amino acid replacement, not a few have been reported to have occurred in the putative β-sheet region and the mutation had a destructive effect on the β-sheet structure. Such examples are the Gly424 to Arg substitution in sheet 5B of antithrombin (*38*), the *Cpe*^{fat} mutation substituting Ser202 with Pro in mouse carboxypeptidase E (*39*), and the cog Tg mutation replacing Leu2263 with Pro in thyroglobulin (*40*). Overall, we conclude that these mutations which destroy the formation of the core β-sheet structure of the polypeptide are one of the major molecular bases of the secretion defect of the mutant, and intracellular degradation of these mutants is mediated not only by the proteasome but by some other protease(s) like TPPII.

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