Intracellular degradation of secretion defect-type mutants of antithrombin is inhibited by proteasomal inhibitors

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Abstract To examine the cellular basis for secretion defect-type antithrombin deficiency, we expressed two mutants, P→stop (Pro^{429} to stop codon) and ΔGlu (deletion of Glu^{313}). Pulse-chase experiments using stably transfected BHK cells showed that little (<5%) of P→stop mutant as well as ΔGlu mutant was secreted and the total amount of radioactivity was significantly reduced, suggesting an intracellular degradation. The degradation was not inhibited by brefeldin A, indicating it occurring in a preGolgi apparatus. However, the degradation was strongly inhibited by proteasomal inhibitors, such as carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (LLL), carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal (LLnV) and lactacystin. By endoglycosidase H digestion and immunofluorescence staining, these mutants were shown to localize in the endoplasmic reticulum (ER). These results suggest that the secretion defect-type mutants of antithrombin are degraded by proteasome through the ER-associated quality control mechanism in the cells.

Key words: Antithrombin; Endoplasmic reticulum-associated degradation; Quality control; Lactacystin; Proteasome

1. Introduction

Antithrombin, one of the major serine proteinase inhibitors in mammalian plasma, inhibits most of the coagulation proteases. The inhibition of each protease by antithrombin is concomitant with the formation of a stable 1:1 molar complex and is enhanced as much as 1000-fold in the presence of heparin, a sulfated polysaccharide [1,2]. Human antithrombin is a 58 kDa glycoprotein consisting of 432 amino acid residues and three disulfide bridges [3]. Most of the circulating antithrombin is post-translationally modified resulting in four Asn-linked oligosaccharides at positions 96, 135, 155, and 192. Antithrombin has two functional domains; one is the NH_2-terminal heparin-binding region and the other is the reactive-site region of Arg^{393}–Ser^{394} located in the COOH-terminal region [1–3].

Inherited antithrombin deficiency is associated with a predisposition to familial venous thromboembolic disease [4]. The deficiency has been classified into two types [5]: type I ('classical') deficiency is characterized by a reduced level of both the circulating antigen and its activity, and type II deficiency is by production of variant protein, in which the antigen is approximately normal but functional levels are reduced to 50% of that of normal. At present, a total of 128 distinct mutations have been compiled in the antithrombin deficiency database, 93 cases (73%) of which belong to type I deficiency [6]. However, cellular mechanism for the type I antithrombin deficiency has not been understood well.

To examine the secretion defect resulting in antithrombin deficiency, we expressed two mutants of type I deficiency, ΔGlu (deletion of Glu^{313}) and P→stop (Pro^{429}→stop codon). The proband of ΔGlu mutant showed 47% for antigen and 57% for functional activity of antithrombin [7]. P→stop mutant was studied from the result of the study on α_1-proteinase inhibitor that COOH-terminal truncation to Pro^{391} (Pro^{429} in antithrombin) of α_1-proteinase inhibitor led to a secretion defect [8]. In this communication, we show that the type I deficiency (secretion defect) of antithrombin is caused by an intracellular degradation of mutant proteins by proteasome through the quality control mechanism in the cells.

2. Materials and methods

2.1. Materials

Full-length human antithrombin cDNA was prepared from a human liver cDNA library (Clonetech) and its nucleotide sequence [3] confirmed. Rabbit anti-human antithrombin antiserum was prepared as described previously [9], and rabbit anti-bovine PDI antiserum was kindly provided by Prof. Masakazu Kikuchi (Ritsumeikan University, Kyoto). FITC-labeled sheep anti-rabbit IgG (H+L) and FITC-labeled wheat germ lectin were obtained from The Binding Site (Birmingham, UK) and Sigma Chemicals (St. Louis, MO), respectively. Brefeldin A and LLnL were purchased from Sigma Chemicals. LLL and LLnV were obtained from Peptide Institute Inc. (Osaka). Lactacystin was prepared from Streptomyces sp. OM-6519 as described [10].

2.2. Construction of the expression vector and transfection to cells

Site-directed mutagenesis of antithrombin mutants was performed with the Sculptor in vitro mutagenesis system (Amersham) and 17-mer mutagenic primers. Primers used were as follows: ΔGlu, 5′-GCATCATCTCCAATTCATCCAG-3′ (complementary sequence of Nos. 1019–1042 of antithrombin [3] with deletion of codon (GGA) for Glu^{313}) and P→stop, 5′-TTACTTAACACACATTAGTTGGCTACTCT-3′ (complementary sequence of Nos. 1369–1395, mutagenic TTA is underlined). Mutations were verified by sequence analyses. The cDNAs for wild-type (Wt) and mutant antithrombin were ligated into the *Eco*RI site of pcD2-SRα expression vector [11], and these expression vectors were purified by CsCl gradient ultracentrifugation. Seven milligram of expression vector were transfected into 2×10^5 BHK cells by the calcium phosphate coprecipitation method [11]. To obtain stable transfectants, cells were selected by the addition of 400 mg/ml G418 (Wako Pure Chemicals, Osaka).

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Abbreviations: endo H, endoglycosidase H; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; LLnL, *N*α-acetyl-L-leucyl-L-leucyl-L-norleucinal; LLL, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; LLnV, carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal; PDI, protein disulfide isomerase; Wt, wild-type

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2.3. Pulse-chase, immunoprecipitation, and gel electrophoresis

To examine the secretion rate and intracellular localization of recombinant antithrombin mutants, pulse-chase analysis and endo H digestion were performed as described previously [12,13].

2.4. Immunofluorescence

To examine the subcellular localization of recombinant antithrombin in transfected cells, immunofluorescence analysis was performed as described previously [12,13]. The rabbit anti-human antithrombin antibody was stained with FITC-labeled sheep anti-rabbit antibody. For organelle markers of the ER and Golgi apparatus, rabbit anti-bovine PDI antiserum and FITC-labeled wheat germ lectin, respectively, were employed.

3. Results

3.1. Identification of intracellular degradation of antithrombin mutants

To examine the secretion efficiency of antithrombin mutants, pulse-chase experiments were performed first. Fig. 1 shows the SDS-PAGE of Wt antithrombin, P→stop and ΔGlu mutants. In Wt-expressing cells, the majority of the pulse-labeled radioactivity was recovered from the medium after a 2 h chase, and the total amount of radioactivity was maintained during the chase period (Fig. 1A). In contrast, little (< 5%) of P→stop mutant was secreted into the medium during the chase period and a significant reduction in the total amount of radioactivity was observed (Fig. 1B). Pulse-chase analysis of ΔGlu mutant-expressing cells showed a similar but slightly better secretion pattern when compared with that of P→stop mutant cells (Fig. 1C). The time for 50% disappearance of the total amount of radioactivity in P→stop and ΔGlu mutants cells were estimated to be 5.7 and 3.3 h, respectively. These results suggest that P→stop and ΔGlu mutants of antithrombin are degraded intracellularly.

To further examine the intracellular degradation of these antithrombin mutants, we performed pulse-chase experiments in the presence of a transport inhibitor, brefeldin A, and protease inhibitors (Fig. 2). In these experiments, antithrombin in cell extracts and in the conditioned medium was immunoprecipitated, followed by SDS-PAGE analysis and detected by autoradiography (Fig. 2A). By taking the amount of radioactivity in cell extracts derived from 1 h pulse-labeled cells as 100%, 71% and 8% of the radioactivity were detected in the cell extracts and medium, respectively, of 8 h chased Wt cells. In contrast, in P→stop mutant cells, 21% and 2%, and in ΔGlu mutant cells, 6% and 9% of the radioactivity were detected in the 8 h chased cell extracts and medium, respectively. Therefore, in P→stop and ΔGlu mutants cells, there were 77% and 85%, respectively, disappearance of the pulse-labeled radioactivity during the 8 h chase period (Fig. 2B). In the presence of brefeldin A, almost all the pulse-labeled Wt was recovered from the cell extracts of 8 h chased cells. In contrast, in P→stop and ΔGlu mutants cells, about 67% of the total amount of radioactivity disappeared during an 8 h chase period in the presence of 3 μg/ml brefeldin A. Brefeldin A is an inhibitor of intracellular transport by redistributing Golgi proteins into the ER and preventing further movement through the secretory pathway [14]. These results suggest that both P→stop and ΔGlu mutants were selectively degraded and brefeldin A had no apparent effects on the degradation (Fig. 2B).

To determine the specificity of the protease responsible for the intracellular degradation of P→stop mutant, various

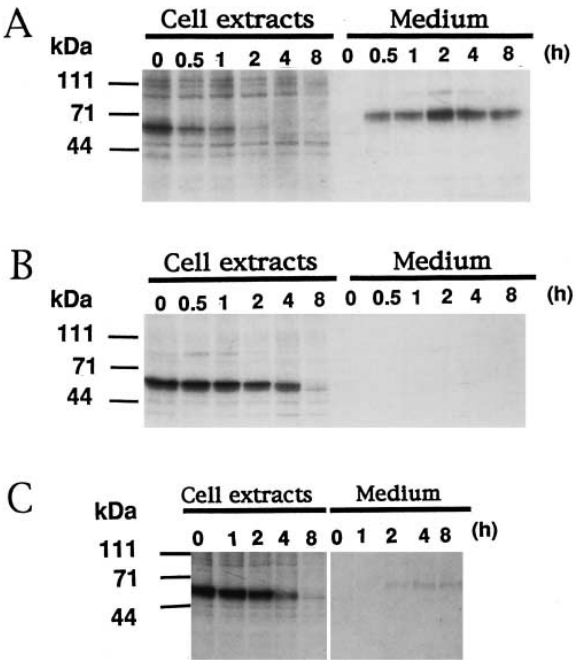

Fig. 1. Pulse-chase analysis of Wt antithrombin and P→stop and ΔGlu mutants in stably transfected BHK cells. A: Stable BHK cells were pulse-labeled for 1 h with 100 μCi/ml EXPRE^{35}S^{35}S (a mixture of [^{35}S]Met and [^{35}S]Cys, NEN) and chased for 0, 0.5, 1, 2, 4, and 8 h. Labeled antithrombin from cell extracts and from the medium were immunoprecipitated and analyzed on 12.5% SDS-PAGE. B: In the kinetic analyses, the amount of radioactivity in the pulse-labeled cell extracts, quantitated by Bio-Imaging Analyzer (Fuji Film), is taken as 100%, and relative radioactivities of intracellular (●) and secreted fractions (○) are shown. Sum of the radioactivities of both fractions at each time is shown by open squares (□).

types of membrane-permeable protease inhibitors were examined. LLL and LLnV known as potent synthetic proteasomal inhibitors [15], strongly inhibited the intracellular degradation of both P→stop and ΔGlu mutants, and almost all the pulse-labeled radioactivity was maintained intracellularly after the 8 h chase (Fig. 2B). The order of the potency of inhibitors, LLnV–LLL > LLnL, agreed well with the inhibitor spectra for proteasome [15]. Lactacystin, known as a specific inhibitor for proteasome through modifying the active threonine residue of β-type proteasome subunits [16], also strongly inhibited the intracellular degradation of these mutants, suggesting proteasome is responsible for the intracellular degradation of both P→stop and ΔGlu mutants. These results suggest that both mutants are degraded principally through the same cellular mechanism. In Wt antithrombin-transfected cells, the total amount of radioactivity was unchanged during the chase period in the presence of these proteasomal inhibitors (Fig. 2B), indicating these inhibitors had no inhibitory effect on the secretion of Wt antithrombin. Leupeptin and E64(d) known to inhibit the activities of ER proteases, ER60 and ER72, in vitro [17,18], had no effects on the secretion of Wt antithrombin nor the degradation of two antithrombin mutants. Also other protease inhibitors against aminopeptidase (amastatin), cysteine proteases (antipain and E64), elastase (elastatinal) and metal proteases (phosphoramidon) showed no effect (data not shown). Furthermore, lysosomotropic agents, chloroquine and NH_4Cl, which accumulate in lysosomes and elevate the intravesicular pH by virtue of their weak basic prop-

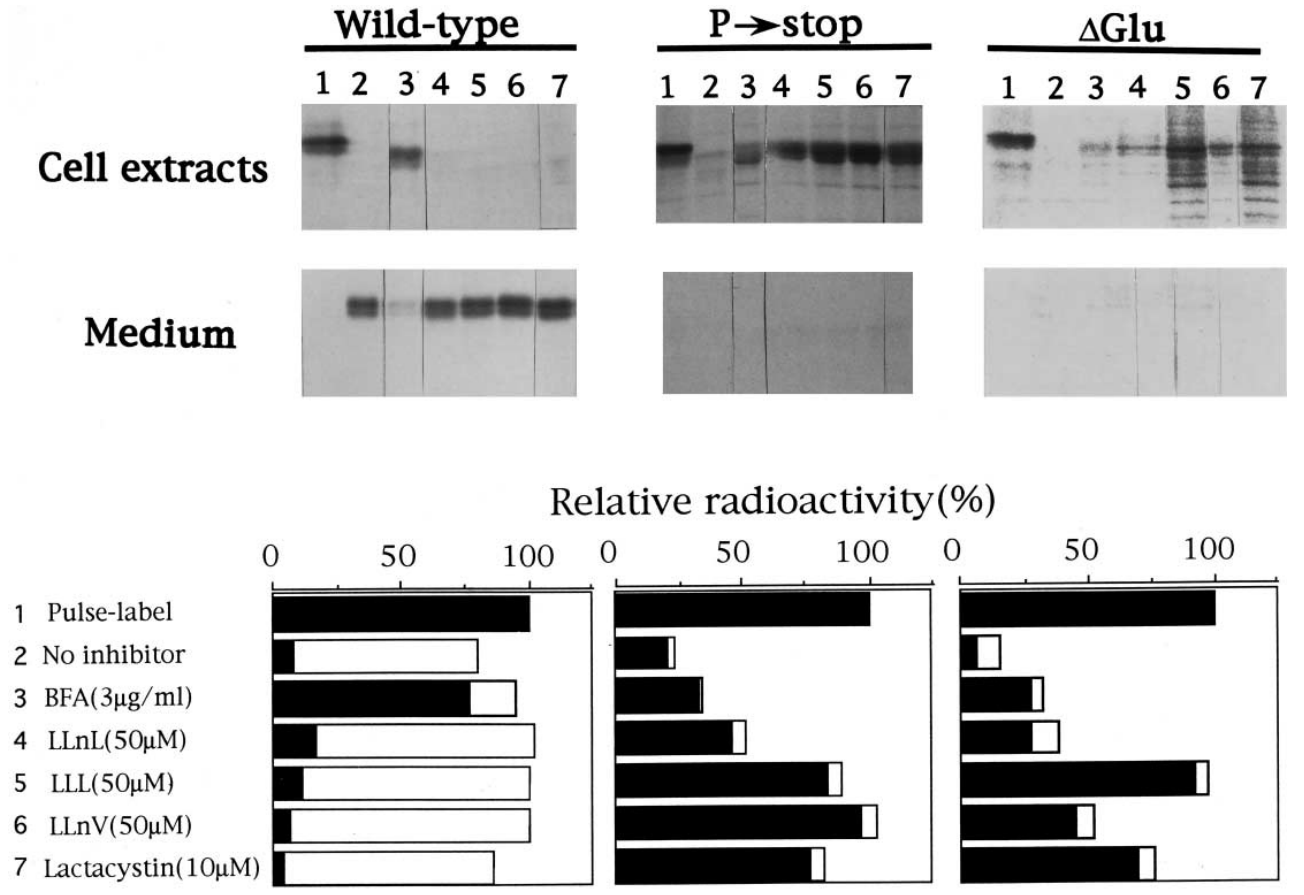

Fig. 2. Effects of brefeldin A and proteasomal inhibitors on the secretion and degradation of Wt antithrombin and P→stop and ΔGlu mutants. A: Stably transfected BHK cells expressing Wt antithrombin, P→stop and ΔGlu mutants were pulse-labeled for 1 h with 50 μCi/ml of EXPRE^{35}S^{35}S and then chased for 8 h in the presence of various inhibitors. Antithrombin in the cell extracts and in the conditioned medium were immunoprecipitated, followed by SDS-PAGE analysis in the presence of 2-mercaptoethanol. Lane 1: Sample from 1 h pulse-labeled cells. Lane 2: Sample from 8 h chased cells without inhibitors. Lanes 3–7: Samples from 8 h chased cells in the presence of inhibitors: lane 3, 3 μg/ml brefeldin A; lane 4, 50 μM LLnL; lane 5, 50 μM LLL; lane 6, 50 μM LLnV; lane 7, 10 μM lactacystin. B: Radioactivities of bands corresponding to antithrombin in (A) were quantitated by Bio-Imaging Analyzer. Taking the radioactivity of pulse-labeled cell extracts as 100%, the relative radioactivities of antithrombin in cell extracts and medium are shown by closed and open bars, respectively.

erties and then reduce the activities of lysosomal proteases [19], had no inhibitory effect on the degradation of antithrombin mutants (data not shown). These results are inconsistent with a lysosomal or an autophagic degradation of the antithrombin mutants.

3.2. Intracellular localization of antithrombin mutants

To examine the intracellular localization of the antithrombin mutants, we used biochemical and morphological techniques. First, ^{35}S-labeled Wt antithrombin and P→stop and ΔGlu mutants were subjected to endo H digestion followed by SDS-PAGE analysis. About 10% of Wt antithrombin were endo H-resistant, while all the P→stop and ΔGlu mutants were sensitive to endo H (data not shown), suggesting that the intracellular form of these mutants has carbohydrate chains of high mannose-type, localizing in the ER to the *cis*-Golgi compartment. Secondly, we performed immunofluorescence staining of transiently ΔGlu mutant-transfected cells. The stained pattern of ΔGlu mutant (Fig. 3A) was identical with that of PDI, the ER-resident protein (Fig. 3B), but clearly distinct from that of wheat germ lectin, a Golgi marker that has a more localized, polar perinuclear stained pattern (Fig. 3C). P→stop mutant showed similar immunofluorescence with that of ΔGlu mutant (data not shown). These results suggest that secretion defect mutants of antithrombin were predominantly localized in the ER.

4. Discussion

Most of the type I antithrombin deficiencies are caused by a small insertion or deletion of bases in the gene, resulting in a frameshift mutation and usually a premature stop signal [6]. The ΔGlu mutant, however, is caused by an in-frame deletion of trinucleotides (GGA) at position 7644 to 7649 in the antithrombin gene [7], suggesting that the deletion had no effect on the transcriptional or translational step. Since the proband of ΔGlu mutation showed a 50% antigen level compared to normal, the ΔGlu mutant of antithrombin was not thought to be secreted into the circulation. In this study, we observed that the ΔGlu mutant as well as the P→stop mutant of antithrombin showed an impaired secretion resulting in intracellular degradation. This suggests that some type I deficiencies are caused by the intracellular degradation. Moreover, a conservative COOH-terminal Pro residue in serine proteinase inhibitors (serpins) was shown to be critical in the secretion of antithrombin (Fig. 1) as well as α_1-proteinase inhibitor [8],

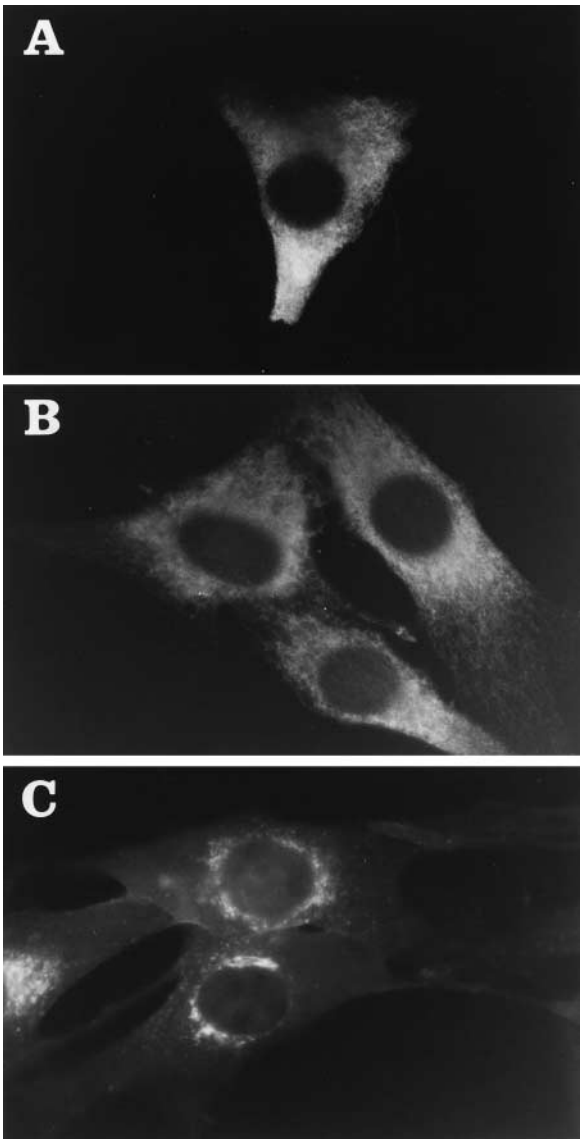

Fig. 3. Immunofluorescence localization of the ΔGlu mutant. BHK cells that transiently expressed the ΔGlu mutant were stained with rabbit anti-human antithrombin IgG, followed by FITC-labeled sheep anti-rabbit IgG antibody (A). For organelle markers of the ER and the Golgi apparatus, PDI stained with rabbit anti-bovine PDI antiserum followed by FITC-labeled sheep anti-rabbit IgG antibody (B) and FITC-labeled wheat germ lectin (C), respectively, are shown.

suggesting that full-length molecules are required for the secretion of serpins and the disruption of a disulfide bridge of antithrombin, Cys^{247}–Cys^{430}, caused the impaired secretion resulting in the intracellular degradation. This intracellular degradation was not inhibited by an ER-Golgi transport inhibitor (brefeldin A), indicating the degradation occurring in the preGolgi apparatus. The endo H digestion and immunofluorescence analysis also suggest that the secretion defect mutants of antithrombin were predominantly localized in the ER (Fig. 3). Thus, the P→stop and ΔGlu mutants were degraded through the quality control mechanism similar to cases of other proteins (see review [20,21]) and under-γ-carboxylated protein C which we observed previously [12].

At present, a cysteine protease(s) inhibited by LLM and LLnL has been implicated in the degradation of HMG-CoA reductase [22], apolipoprotein B [23], and IgM [24], and a serine protease(s) inhibited by dichloroisocoumarin, tosyl-phenylalanyl-chloromethyl ketone, and tosyl-lysyl-chloromethyl ketone is a candidate for IgM light chain breakdown [25]. Very recently, degradations of ΔF508 mutant of cystic fibroblast transmembrane conductance regulator (CFTR) [26,27], MHC class I heavy chain in cytomegarovirus-transfected cells [28] and glycosylphosphatidylinositol (GPI)-linked protein [29] were found to be inhibited by proteasomal inhibitors. In this study, we observed that inhibitors for proteasome, such as LLL, LLnV and lactacystin, showed potent inhibitory effects on the degradation of antithrombin mutants (Fig. 2). These inhibitor spectra strongly suggest that proteasome is responsible for the degradation of antithrombin mutants. We also observed that intracellular degradation of under-γ-carboxylated protein C is also inhibited by these proteasomal inhibitors (Tokunaga et al., submitted), suggesting not only ER-membrane anchoring proteins such as CFTR, MHC class I heavy chain and GPI-linked protein, but typical secretory proteins, such as antithrombin and protein C, are degraded by proteasome through the ER-associated quality control mechanism in the cells.

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