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Cellular and Functional Characterization of Three Recombinant Antithrombin Mutants That Caused Pleiotropic Effect-Type Deficiency[1]

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Inherited antithrombin deficiency is associated with a predisposition for familial venous thromboembolic disease. Pleiotropic effect-type mutants of antithrombin that have an amino acid replacement in a distal hinge region including strands 1C, 4B, and 5B of the polypeptide chain are known to exhibit impaired interactions with both thrombin and heparin, coupled with a secretion defect. To examine the mechanism of pleiotropic effect-type antithrombin deficiency, we expressed three mutants, Oslo (Ala404→Thr), Kyoto (Arg406→Met), and Utah (Pro407→Leu), in baby hamster kidney (BHK) cells, and compared their secretion rates, affinities for heparin and abilities to form thrombin-antithrombin (TAT) complexes with those of wild-type (Wt) antithrombin. Pulse-chase experiments showed that the Oslo- and Kyoto-mutants were secreted at rates similar to Wt antithrombin. In contrast, the Utah-mutant underwent partial intracellular degradation. The intracellular degradation of the Utah-mutant was not inhibited by lysosomotropic inhibitors, but by proteasome inhibitors such as carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (LLL) and lactacystin, indicating that a part of the Utah-mutant was degraded by proteasome through quality control in the endoplasmic reticulum (ER). Crossed immunoelectrophoresis in the presence of heparin showed that only the Oslo-mutant lacks heparin-binding ability. Incubation with thrombin showed that the Kyoto- and Utah-mutants, but not the Oslo-mutant, formed a weak but detectable TAT complex. Furthermore, heparin enhanced the TAT complex formation by the Kyoto- and Utah-mutants, suggesting heparin cofactor activities of these mutants. These results show that each of the Oslo-, Kyoto-, and Utah-mutants exhibits different properties as to secretion, intracellular degradation and functional activity, although they are grouped as pleiotropic effect-type mutants.

Key words: antithrombin, intracellular degradation, proteasome, quality control, secretion defect.

Antithrombin, one of the major proteinase inhibitors in mammalian plasma, inhibits the coagulation proteases, thrombin, factors VIIa, IXa, Xa, XIa, and XIIa, and plasma kallikrein. The inhibition of a protease by antithrombin is associated with the concomitant formation of a stable 1:1 molar complex, the rate of which is enhanced as much as 1,000-fold in the presence of heparin, a sulfated polysaccharide (*1, 2*). Human antithrombin is a single chain glycoprotein (58 kDa) consisting of 432 amino acid residues with three disulfide bridges (*3, 4*). Most of the circulating antithrombin in humans is post-translationally modified to contain four Asn-linked oligosaccharides at positions 96, 135, 155, and 192, which accounts for 15% of its molecular weight. Antithrombin has two functional domains: the NH_2-terminal heparin-binding domain and the COOH-terminal protease-binding domain including the reactive-site Arg393-Ser394 (P1-P1′) bond (*1-4*).

Inherited antithrombin deficiency is frequently associated with a predisposition for familial venous thromboembolic disease (*5*). Typically, the deficiency is classified into two types (*6*): the type I ("classical") deficiency is characterized by reduced levels of both the circulating antigen and its activity, whereas type II deficiency is characterized by the production of a variant protein, of which the antigen level is essentially normal, but the functional level is reduced to about 50% of normal in the case of heterozygous abnormality. Most type I deficiencies are caused by a small insertion or deletion of bases in the antithrombin gene, resulting in a frameshift mutation and usually premature stop signal formation. Previously, we expressed two type I deficiency mutants, ⊿Glu (deletion of Glu313) and P→stop (Pro429→stop codon), in baby hamster kidney (BHK) cells and showed that type I deficiency (low antigen level) of

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Abbreviations: BHK, baby hamster kidney; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; LLL, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; LLM, *Nα*-acetyl-L-leucyl-L-leucyl-L-methioninal; LLnL, *Nα*-acetyl-L-leucyl-L-leucyl-L-norleucinal; LLnV, carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal; PNGase F, peptide: *N*-glycosidase F; TAT, thrombin-antithrombin; Wt, wild-type.

antithrombin is caused by intracellular degradation of mutant proteins by proteasome through the quality control mechanism in the endoplasmic reticulum (ER) (*7*).

In contrast to type I deficiency, type II deficiency is caused by a single base mutation resulting in a single amino acid replacement. Type II deficiency is further classified into three subclasses as follows: HBS, functional abnormalities limited to the heparin binding site; RS, functional abnormalities limited to the reactive site; and PE, pleiotropic effect-type (*6*). Pleiotropic effect-type deficiency is characteristic of mutations in the COOH-terminal region to the reactive site, resulting in impaired interactions with both thrombin and heparin, coupled with a reduced plasma concentration of the mutant molecule (*6, 8*). As shown in Fig. 1, eleven distinct mutations and 17 cases in total are currently listed in this category (*6*). These mutations, located on strand 1C or in the region leading into strands 4B and 5B, are thought to reflect a conformational linkage between the reactive site and the heparin-binding region of the molecule (*8*). However, the cellular basis and functional characterization of pleiotropic effect-type deficiency of antithrombin remain to be investigated.

To examine the molecular and cellular mechanisms of pleiotropic effect-type antithrombin deficiency, we expressed three typical pleiotropic effect-type mutants, Oslo (Ala404→Thr) (*8-11*), Kyoto (Arg406→Met) (*6, 12*), and Utah (Pro407→Leu) (*8, 13-15*), in BHK cells, and compared their secretion rates, intracellular localizations, and abilities of heparin-binding and thrombin-antithrombin (TAT) complex formation with those of wild-type (Wt) antithrombin. Using these antithrombin mutants, we have characterized the cellular basis and functional properties of each type of the pleiotropic effect-type antithrombin deficiency.

MATERIALS AND METHODS

Materials—Full-length human antithrombin cDNA was prepared as described (*7*). Human thrombin was prepared by activation of prothrombin with *Echis carinatus* venom (Sigma Chemical, St. Louis, MO). Rabbit anti-human antithrombin antiserum was prepared as described previously (*16*) and the antiserum was affinity-purified on a human antithrombin-Sepharose column. Peptide:*N*-glycosidase F (PNGase F) was obtained from New England Biolabs (Beverly, MA). Chloroquine, *Nα*-acetyl-L-leucyl-L-leucyl-L-methioninal (LLM), *Nα*-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL), and chymostatin were purchased from Sigma Chemical. Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (LLL), and carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal (LLnV) were obtained from Peptide Institute (Osaka). Lactacystin prepared from *Streptomyces* sp. OM-6519 as described in Ref. *17* was a generous gift from Dr. Satoshi Omura (The Kitasato Institute). Geneticine disulfate (G418) was purchased from Wako Pure Chemicals (Osaka). Other materials were of the highest grade commercially available.

Construction of the Expression Vector, Transfection into Cells, and Pulse-Chase Analysis—Site-directed mutagenesis of antithrombin was performed using the Sculptor *in vitro* mutagenesis system (Amersham) and 17-mer mutagenic primers, as described previously (*7*). The primers used were as follows: Toyama (*18*) and Rouen II (*19*), 5′-CCAGACAC(A/T)CCGGTTGG-3′ [complementary sequence to nucleotide Nos. 227-243 of human antithrombin cDNA (*3*), the mutagenic bases were incorporated as a mixture at the site shown underlined and in parentheses]; Oslo, 5′-CCTGTTGGTCCTGAAAG-3′ (complementary sequence to Nos. 1298-1314, the mutagenic T is underlined); Kyoto, 5′-GGAAAGGCATGTTGGCC-3′ (complementary sequence to Nos. 1305-1321, the mutagenic A is underlined); and Utah, 5′-CCAGGAAAAGCCTGTTG-3′ (complementary sequence of Nos. 1308-1324, the mutagenic A is underlined). The mutations were verified by sequence analyses. Then, cDNAs for Wt and the six antithrombin mutants were separately ligated into the *Eco*RI site of the pcD2-SR*α* expression vector as described previously (*20*). These expression vectors were purified by CsCl gradient ultracentrifugation. BHK cells, obtained from the Japan Research Cell Bank (Tokyo), were maintained in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum/antibiotic-antimycotic liquid (Gibco BRL Life Technology). Seven micrograms of expression vector was transfected into 2×10^5 cells by the calcium phosphate co-precipitation method (*20*). To obtain a stable transfectant, cells were selected by the addition of 400 μg/ml G418. The resistant cells fully grew in 2-3 weeks. A pool of stable BHK cells was used in the pulse-chase experiments and colonies of stable BHK cells which expressed the highest amounts of antithrombin among clones selected with the pulse-chase method were used for the production of recombinant mutants of antithrombin. To determine the rates of secretion of recombinant antithrombin mutants, pulse-chase experiments were performed as described previously (*7, 21*).

PNGase F Digestion—For PNGase F digestion, immunoprecipitated samples were dissolved according to the instruction manual and incubated with 500 units of PNGase F at 37°C for 1 h.

Production of Recombinant Antithrombins—To obtain recombinant Wt and mutant antithrombins, stably-transfected BHK clones producing the highest amounts of the antithrombin mutants were cultured for three days in DMEM/10% fetal calf serum in eight Triple-flasks (500 cm^2, Nunc). After washing with phosphate-buffered saline, the medium was changed to DMEM/Ham's F-12 (1:1) without phenol red (Sigma) supplemented with insulin-transferrin-selenium-X (Gibco BRL), followed by further culture for a week. The serum-free culture medium (1,200 ml each) thus obtained was centrifuged at 8,000 rpm for 30 min, and the resultant supernatant was concentrated to 20 ml with a Diaflo membrane (Amicon). The concentrated sample was used for the crossed immunoelectrophoresis analysis.

Crossed Immunoelectrophoresis—Crossed immunoelectrophoresis in the presence of heparin in the first direction was performed as described (*22*). Briefly, 10 μl of normal plasma, or recombinant Wt or mutant antithrombin was electrophoresed on a 1% agarose gel containing 20 U/ml of heparin until free Bromophenol Blue migrated to the anodal terminal. Then, the gel was cut into slabs, transferred to a second gel containing 2% anti-human antithrombin antiserum, and electrophoresed at right angles to the first run at 10 V/cm for 4 h. Immunoprecipitin lines were visualized by staining with Coomassie Brilliant Blue.

TAT Complex Formation—To examine the functional

activity of recombinant antithrombin mutants, the ability of a mutant to form a TAT complex was studied using ^{35}S-labeled antithrombin and cold human α-thrombin. Stable BHK cells (1×10^6 cells) expressing an antithrombin mutant were labeled for 1 h with 100 μCi/ml of EXPRE-^{35}S^{35}S, and then chased for 4 h with 1 ml of DMEM/Ham's F-12 (1:1) without serum and phenol red (Sigma). The Utah-mutant was expressed for 8 h. The supernatant containing an ^{35}S-labeled antithrombin mutant was mixed with 1 μg/ml α-thrombin in the absence or presence of 0.1 U/ml heparin. Following incubation at 37°C for a preselected time, samples were heat-inactivated, immunoprecipitated and then subjected to SDS-PAGE in the presence of 2-mercaptoethanol.

RESULTS

Comparison of the Secretion Rates of Wt and Mutant Antithrombins—Pleiotropic effect-type antithrombin deficiency is a novel category of antithrombin deficiency characterized by impaired interactions with both thrombin and heparin, coupled with a reduced plasma concentration (*6, 8*). At present, eleven distinct mutations have been identified in this category (Fig. 1) (*6*). These mutations, located on strands 1C to 5B, are thought to reflect a conformational linkage between the reactive site and the heparin-binding region of the molecule (*8*). To investigate the cellular and molecular bases for pleiotropic effect-type antithrombin deficiency, we constructed expression vectors containing cDNA for typical pleiotropic effect-type antithrombin mutants; Oslo (Ala404→Thr), Kyoto (Arg406→Met), and Utah (Pro407→Leu). Although, *in vivo*, antithrombin is expressed in liver cells, the expression vectors were transfected into BHK cells for the following reasons: (i) it is difficult to distinguish a transfected mutant from the endogenously expressed molecule, as liver cell lines, such as HepG2 cells, secrete antithrombin endogenously (*23*), and (ii) BHK cells have been well characterized as to the expression of recombinant antithrombin (*24*). A pool of stably-transfected BHK cells was used in the pulse-chase experiments to average the transfection efficiency of many resistant clones.

First, to compare the secretion rates of Wt and mutant antithrombins, pulse-chase experiments were performed. The pulse-chase patterns, and the results of quantitative analyses of Wt antithrombin and the three pleiotropic effect-type mutants are shown in Fig. 2. For Wt antithrombin, a single band corresponding to an apparent molecular mass of 54 kDa was detected for cell extracts, which disappeared gradually with the chase. Concomitant with the decrease in antithrombin in cell extracts, a single chain form of antithrombin with an apparent molecular mass of 60 kDa increased in the medium (Fig. 2A). For the Oslo- and Kyoto-mutants, as well as Wt antithrombin, the majority of the pulse-labeled radioactivity was recovered in the medium after a 2 h chase, and the total radioactivity remained unchanged (Fig. 2B). Two heparin-binding defect mutants, Toyama (Arg47→Cys) (*18*) and Rouen II (Arg47→Ser) (*19*), exhibited the same secretion kinetics as those of Wt, Oslo, and Kyoto (data not shown). In contrast, only ~25% of the pulse-labeled Utah-mutant was secreted into medium in an 8 h chase, and a 50% decrease in the total amount of radioactivity was observed. Thus, partial intracellular degradation of the Utah-mutant was suggested.

Cellular Basis for the Secretion Defect of the Utah-Mutant—In addition to a partial secretion defect, the Utah-mutant showed aberrant migration on SDS-PAGE analysis. As shown in Fig. 3A, intracellular and secreted Wt antithrombins showed apparent molecular masses of 54 and 60 kDa, respectively, under reducing conditions. The Oslo- and Kyoto-mutants showed the same mobility as that of Wt antithrombin (data not shown). In contrast, the apparent molecular masses of the intracellular and secreted forms of the Utah-mutant were estimated to be 48 and 58 kDa, respectively (Fig. 3A). Under nonreducing conditions, the relative migration of Wt and the Utah-mutant was unchanged. To eliminate the effect of carbohydrate chains on the difference in molecular masses between Wt and the Utah-mutant, PNGase F digestion was performed (Fig. 3B). Then, the intracellular and secreted Wt, and the Oslo- and Kyoto-mutants each exhibited a molecular mass of 48 kDa, whereas the molecular masses of the intracellular and secreted forms of the Utah-mutant were each estimated to be 42 kDa. Endoglycosidase H and *N*-glycanase digestions of intracellular and secreted antithrombins, respectively, gave the same results as PNGase F digestion (data not shown). The aberrant migration of the Utah-mutant on SDS-PAGE may suggest abnormal folding, which eventually caused partial intracellular degradation of the Utah-mutant.

To further investigate the partial intracellular degradation of the Utah-mutant, we performed pulse-chase experiments in the presence of various inhibitors (Fig. 4). For Wt, all the pulse-labeled antithrombin was detected in the medium after a 12 h chase, while the total amount of the Utah-mutant decreased to 33% during this chase period (lane 1 *versus* 2). The lysosomotropic agents, chloroquine (lane 3) and NH_4Cl (lane 4), both of which accumulate in lysosomes, elevate the intravesicular pH by virtue of their weak basic properties, and thereby reduce the activities of lysosomal proteases (*25*), showed no inhibitory effect on either the secretion of Wt antithrombin or the intracellular degradation of the Utah-mutant. Moreover, inhibitors of endosomal and lysosomal proteases, chymostatin (lane 5) and leupeptin (data not shown), also showed no effects on the degradation of the Utah-mutant. These results provide suggestive evidence that lysosomes or endosomes are not responsible for the degradation of the intracellular Utah-mutant.

Previously, we showed that proteasome inhibitors inhibited the degradation of secretion defect-type antithrombin mutants, ΔGlu and P→stop, in stable BHK cells (*7*). To examine a possible role of proteasome in the intracellular degradation of the Utah-mutant, we employed several proteasome inhibitors (lanes 6-10). The results showed that LLL (lane 6), LLnV (lane 7), and LLnL (lane 9), all known as potent proteasome inhibitors (*26*), strongly inhibited the intracellular degradation of the Utah-mutant, 60-77% of the radioactivity being retained within cells after a 12 h chase. The order of inhibitory potency of LLL ~LLnV > LLnL > LLM agreed well with the inhibitory spectrum of these compounds on proteasome (*26*). Lactacystin, a specific inhibitor for proteasome through modification of the active threonine residue of β type proteasome subunits (*27*), also strongly inhibited the intracellular degradation of the Utah-mutant (lane 8). For Wt, the

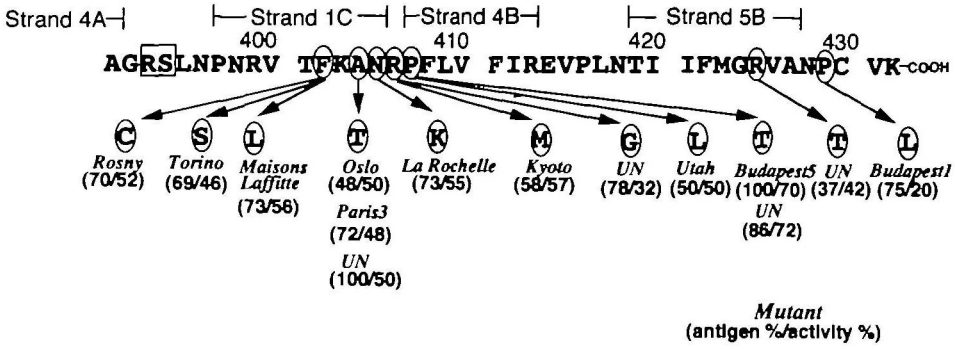

Fig. 1. **Amino acid sequence of the COOH-terminus of human antithrombin and mutation sites in pleiotropic effect-type antithrombin deficiency.** The reactive site (Arg393-Ser394) is boxed. The mutation sites, replaced amino acids, trivial names of mutants, and antigen/activity percentages are shown. *UN*, unnamed mutant.

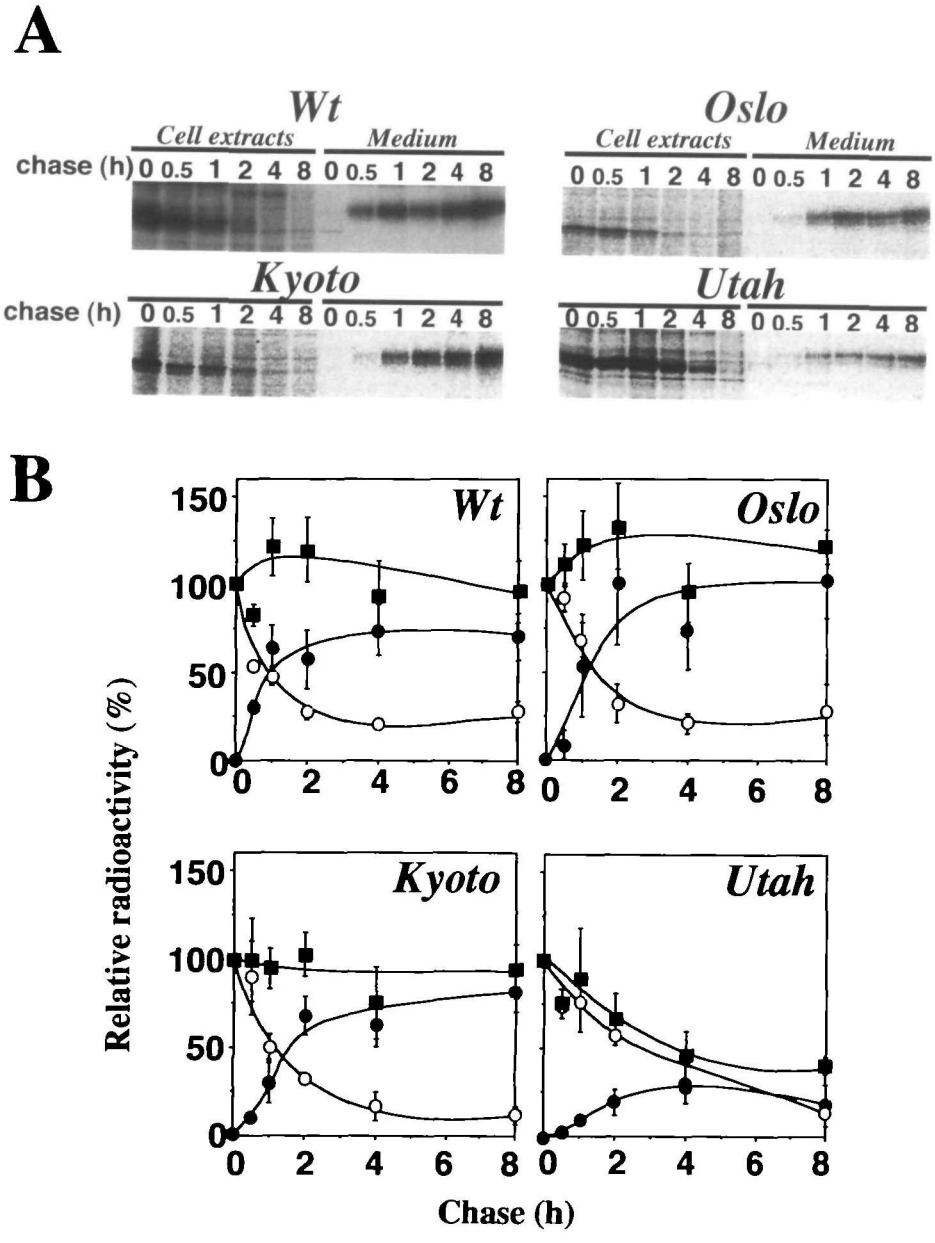

Fig. 2. **Pulse-chase analyses of pleiotropic effect-type antithrombin mutants in stably-transfected BHK cells.** A: Stable BHK cells were pulse-labeled for 1 h with 100 μCi/ml [^{35}S]-Met and [^{35}S]Cys, and then chased for 0, 0.5, 1, 2, 4, and 8 h. Labeled antithrombins from cell extracts and medium were immunoprecipitated and analyzed by 12.5% SDS-PAGE. B: On kinetic analyses, the amount of radioactivity in the pulse-labeled cell extracts was taken as 100%, and the relative radioactivities of the intracellular and secreted fractions are shown by open and closed circles, respectively. The sum of the radioactivities of the two fractions at each time is shown by the closed square. The mean ± SD values for three independent experiments are also shown.

amount of secreted antithrombin was maintained during the chase period in the presence of these inhibitors, indicating no disturbance of the secretion pathway by the proteasome inhibitors. It should be noted that, although proteasome inhibitors inhibited the intracellular degradation of the Utah-mutant, the mutant being retained intracellularly, none of these inhibitors enhanced the secretion of the mutant.

Heparin-Binding Ability of Mutant Antithrombins—Next, to examine the heparin-binding ability of the pleiotropic effect-type mutants, we performed crossed immunoelectrophoresis in the presence of heparin. The crossed immunoelectrophoresis patterns of human plasma and recombinant Wt antithrombins that migrated to the anode terminal, indicate heparin-binding ability (Fig. 5, A and B). The recombinant Kyoto- and Utah-mutants showed similar migration distances to that of Wt (Fig. 5, C and E). In contrast, the recombinant Oslo-mutant migrated less anodally (Fig. 5D), its mobility being similar to those of the heparin-binding defect-type mutants, recombinant Toyama and Rouen II (Fig. 5, F and G). These data suggest that among pleiotropic effect-type mutants, the Kyoto- and Utah-mutants possess heparin-binding ability, whereas the Oslo-mutant possesses little, if any, heparin-binding ability.

TAT Complex Formation of Mutant Antithrombins—To analyze the antithrombin activity of pleiotropic effect-type mutants, we examined the ability of ^{35}S-labeled recombinant Wt and mutant antithrombins to form TAT complexes with cold α-thrombin in the presence and absence of heparin. As shown in Fig. 6A, during 15 min incubation, recombinant Wt antithrombin in the absence of heparin formed SDS-stable TAT complexes of about 100 and 90 kDa, which probably represent the uncleaved and cleaved forms of antithrombin, respectively (lane 2 *versus* 1). The addition of heparin enhanced the TAT formation, suggesting heparin cofactor activity of recombinant Wt antithrombin (lane 3). In contrast, the Oslo-mutant showed no TAT complex formation even in the presence of heparin (lanes 5 and 6). The Kyoto-mutant formed SDS-stable TAT complexes with the same mobility as that of Wt antithrombin, and heparin cofactor activity was also observed (lanes 8 and 9). The Utah-mutant showed much weaker TAT complex formation than Wt and the Kyoto-mutant, presumably the uncleaved form of TAT complex of 92 kDa, under the same conditions (lanes 11 and 12). After the quantification of each TAT complex band, the ratio of TAT complex to total antithrombin in the absence or presence of heparin was calculated (Fig. 6B). Recombinant Wt antithrombin showed progressive TAT complex formation and ~60% of the molecule had formed a complex with thrombin after 120 min in the absence of heparin. In the presence of heparin, a similar degree of TAT complex formation was detected after ~2.5 min, suggesting heparin cofactor activity of recombinant Wt antithrombin as well as plasma antithrombin. Among the pleiotropic effect-type mutants, 20% of the recombinant Kyoto-mutant formed both progressive and heparin-dependent TAT complexes, and the Utah-mutant also formed a very weak but detectable TAT complex. However, only small amounts (<5%) of the Oslo-mutant formed a TAT complex. We also assayed the thrombin inhibitory activities of recombinant Wt antithrombin and the pleiotropic effect-type mutants using a chromogenic substrate, carbobenzoxy-Gly-Pro-Arg-*p*-nitroanilide. Wt as well as plasma antithrombin inhibited ~95% of the thrombin activity during 30 min incubation in the presence of heparin. Under the same conditions, the antithrombin activities of the pleiotropic effect-type mutants were determined to be: Kyoto, 77%; Utah, 33%; and Oslo, 19% (data not shown). Thus, the order of antithrombin activity, Wt > Kyoto > Utah > Oslo, agreed with the ability to form a TAT complex. These data suggest that among the three pleiotropic effect-type mutants, the Kyoto- and Utah-mutants are hypo-functional inhibitors possessing normal heparin-binding ability but weak ability for TAT complex formation, while the Oslo-mutant is a dysfunctional inhibitor.

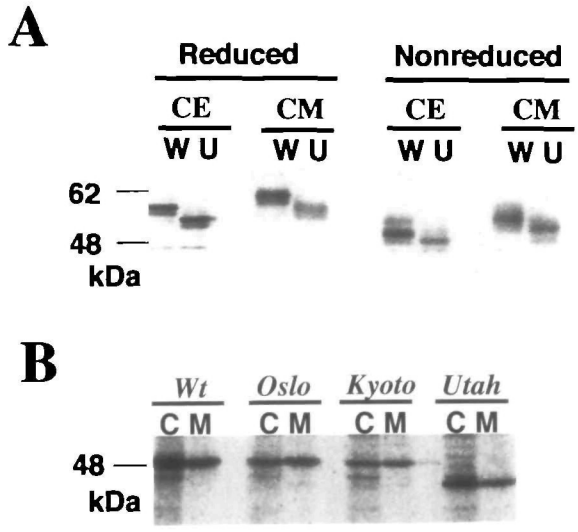

Fig. 3. **Aberrant migration of the Utah-mutant on SDS-PAGE.** A: ^{35}S-labeled Wt (W) and the Utah-mutant (U) from cell extracts (CE) and from the culture medium (CM) of BHK cells were immunoprecipitated and analyzed by 10% SDS-PAGE. The left and right four lanes were electrophoresed in the presence and absence, respectively, of 2-mercaptoethanol. B: ^{35}S-labeled Wt and the pleiotropic effect-type antithrombin mutants of Oslo, Kyoto, and Utah from cell extracts (C) and medium (M) were immunoprecipitated and digested with PNGase-F for 1 h, and then electrophoresed on a 10% SDS-gel.

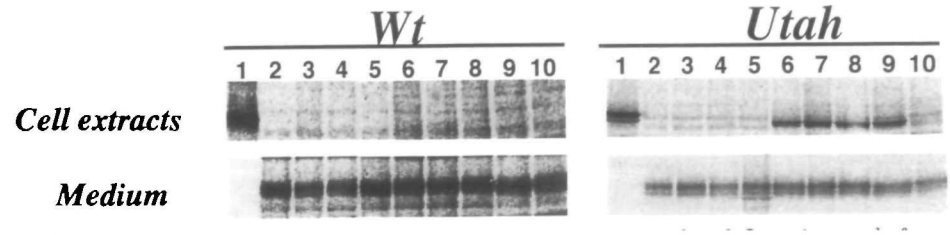

Fig. 4. **Effects of various inhibitors on the secretion and degradation of Wt and the Utah-mutant.** Stably transfected BHK cells expressing Wt antithrombin and the Utah-mutant were pulse-labeled for 30 min with 100 μCi/ml EXPRE^{35}S^{35}S and then chased for 12 h in the presence of various inhibitors. Antithrombin in cell extracts and medium was immunoprecipitated, followed by SDS-PAGE analysis in the presence of 2-mercaptoethanol. Lane 1: sample from 30 min pulse-labeled cells. Lane 2: sample from 12 h-chased cells without an inhibitor. Lanes 3-10: samples from 12 h-chased cells in the presence of inhibitors: lane 3, 100 μM chloroquine; lane 4, 30 mM NH_4Cl; lane 5, 100 μM chymostatin; lane 6, 50 μM LLL; lane 7, 50 μM LLnV; lane 8, 10 μM lactacystin; lane 9, 50 μM LLnL; and lane 10, 50 μM LLM.

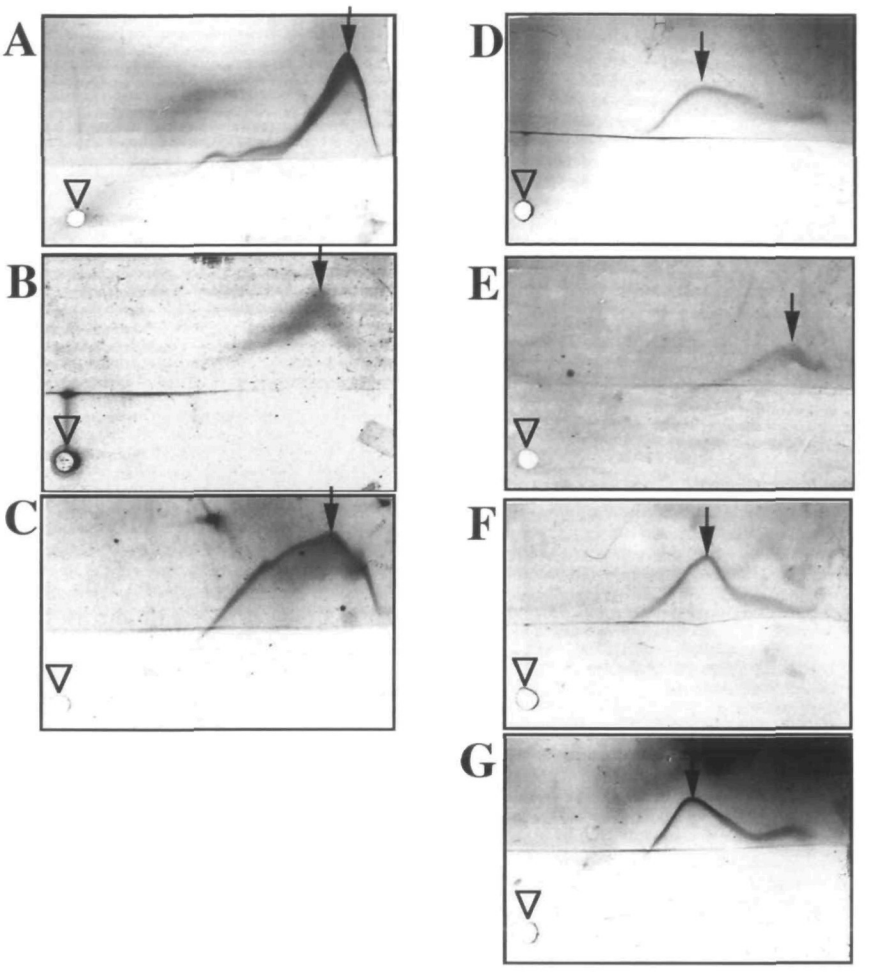

Fig. 5. **Heparin-binding properties of the recombinant antithrombin mutants.** Crossed immunoelectrophoresis was performed as described under "MATERIALS AND METHODS" using 10 μl of normal plasma or recombinant antithrombin. Left, cathode; right, anode. A, normal human plasma; B, Wt; C, Kyoto; D, Oslo; E, Utah; F, Toyama; and G, Rouen II. Arrows indicate the peak positions of the immunoprecipitin lines. ▽, origin.

DISCUSSION

Antithrombin mutants with a single amino acid substitution in a distal hinge region including strands 1C, 4B, and 5B of the polypeptide chain have been reported to have multiple defects of secretion, heparin binding, and thrombin inhibition (*8*). These mutations are classified as "pleiotropic effect-type antithrombin deficiency," and currently eleven distinct mutations are known, as shown in Fig. 1. The antigen and activity levels in patients with pleiotropic effect-type antithrombin deficiency are in the ranges of 37-100% and 20-72%, respectively (*6*). Although Lane *et al.* (*8*) have extensively studied pleiotropic effect-type mutants using patients' plasma, analyses of the secretion mechanism and the functional activity of these mutants have not been reported. Since all patients suffering from pleiotropic effect-type antithrombin deficiency are heterozygotes, they have ~50% normal antithrombin molecules, making it difficult to analyze intracellular events and functional activities of abnormal antithrombin molecules. In this study, we expressed three typical pleiotropic effect-type mutants, Oslo, Kyoto, and Utah, in BHK cells, and examined their secretion mechanisms and functional activities *in vitro*.

The propositus of the Oslo-mutant has been reported to have 48% antigen and 50% activity levels, and was initially classified as type I deficiency (*9*). Subsequently, a small anodal peak due to a mutant antithrombin was detected on crossed immunoelectrophoresis, indicating the presence of abnormal antithrombin in the circulation (*10*). As shown in Fig. 1, two other families having the same mutation as Oslo were reported thereafter (*6*). One designated as Paris 3 showed a 72% antigen level, while the other unnamed patient showed a 100% antigen level. Both families showed ~50% functional activity, suggesting that the mutation of Ala404 to Thr may not always result in a low plasma concentration of antithrombin. In this study, we found that the secretion rate of the Oslo-mutant from BHK cells was indistinguishable from that of Wt antithrombin (Fig. 2), demonstrating that secretion of the Oslo-mutant is normal in BHK cells. The recombinant Oslo-mutant, however, showed little heparin-binding ability on crossed immunoelectrophoresis (Fig. 5), which was similar to that observed for the plasma from the Oslo patient (*10*). In addition, little TAT complex formation was detected in either the presence or absence of heparin (Fig. 6), indicating that the Oslo-mutant is dysfunctional. Watton *et al.* (*28*) investigated the heparin binding affinity of several pleiotropic effect-type mutants, Torino (Phe402→Ser), Maisons Laffitte (Phe402→Leu), Paris 3 (Ala404→Thr), La Rochelle (Asn405→Lys), Budapest 5 (Por407→Thr), and Budapest 1 (Pro429→Leu). Among them, Paris 3, which has the same mutation as Oslo, and La Rochelle showed a

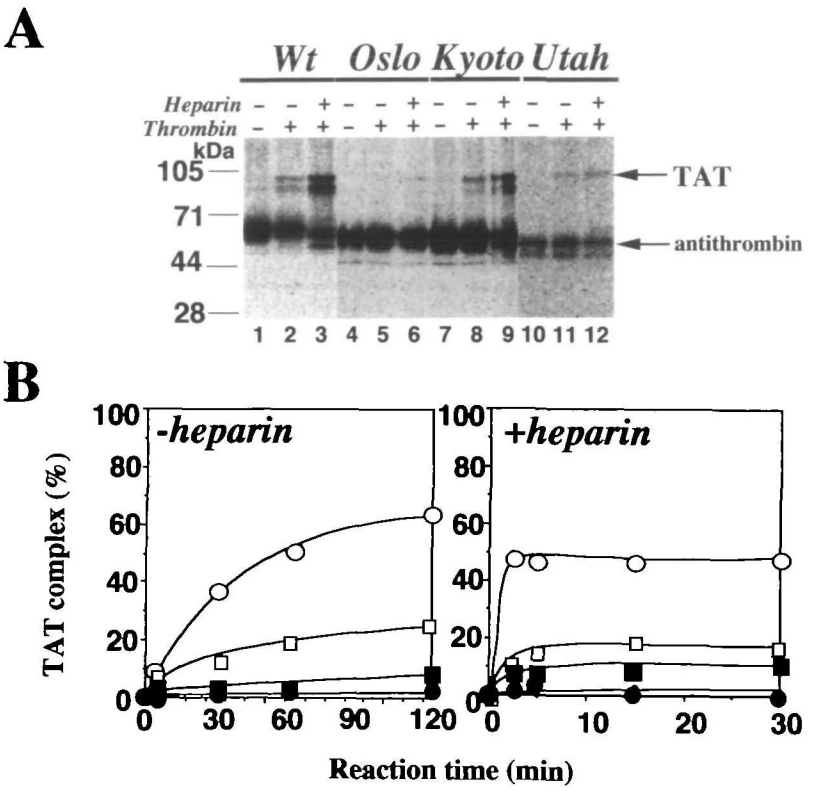

Fig. 6. **Abilities of the recombinant pleiotropic effect-type mutants to form a TAT complex.** A: ^{35}S-labeled Oslo-, Kyoto-, and Utah-mutants were mixed with 1 μg/ml human α-thrombin and then incubated at 37°C for 15 min in either the absence and presence of 0.1 U/ml heparin. Samples were then heat-denatured, immunoprecipitated and subjected to SDS-PAGE analysis in the presence of 2-mercaptoethanol. Arrows indicate the bands of antithrombin and TAT complexes. B: Time course of TAT complex formation. Taking the total radioactivity of antithrombin at each incubation time as 100%, the formation of TAT complexes in the absence (left panel) and presence (right panel) of heparin are shown. Wt, ○; Kyoto, □; Utah, ■; and Oslo, ●.

more than 1,000-fold reduction in heparin-binding affinity, while the others showed a 194-578-fold reduction. Thus, mutations at positions 404 and 405 caused more significant reduction in heparin-binding among pleiotropic effect-type mutants. These mutants also showed reduced pentasaccharide-binding affinity (*29*). Figure 7 shows the spatial distribution of pleiotropic effect-type mutations on antithrombin (*30*). All the seven mutation sites listed in Fig. 1 are located in the neighboring region. Ala404 (P11′ position) of antithrombin is located in the distal hinge between strands 1C and 4B, and a mutation here may disturb the packing at buried sites (*30-32*). One possible reason why the pleiotropic effect-type mutations caused a decrease in heparin-binding ability is that these mutations affect the hydrogen bonding between Glu414 on strand 4B and Arg129, one of the major heparin-binding sites, on the D-helix (Fig. 7) (*30*). It is likely that the mutation of Ala404 to Thr does not cause a secretion defect, but affects both the heparin-binding and thrombin inhibitory abilities of antithrombin.

The proband of the Kyoto-mutant (Arg406→Met) was reported having type Ib deficiency based on the parallel reduction of the antigen and activity levels to 58 and 57%, respectively (*12*). However, isoelectric focusing, followed by immunofixation analysis, revealed that, in addition to normal antithrombin (pI=4.87), the patient's plasma contained abnormal antithrombin (pI=4.70), suggesting the presence of abnormal antithrombin in the circulation (*12*). In this study, we observed that the secretion rate and the heparin-binding ability of the Kyoto-mutant were almost normal (Figs. 2 and 5). However, the Kyoto-mutant showed reduced (about 1/3 of normal) ability of TAT complex formation (Fig. 6), suggesting a low functional molecule. As shown in Fig. 7, Arg406 (P13′ position) of antithrombin is located at the junction site of strands 1C and 4B, and the substitution by Met may reduce the flexibility at the distal hinge (*30-32*), resulting in hypofunctional antithrombin (Fig. 7). Another unnamed mutation at this position (Arg406→Gly) is reported in the database (*6*). Although the details were not given, the antigen and activity levels of the proband for this mutation were 78 and 32%, respectively. Thus, there seems to be a moderate level of secretion of an abnormal molecule that is dysfunctional like the Kyoto-mutant. The reduced level of antithrombin in the plasma of the Kyoto patient may be correlated with the clearance by the serpin-enzyme complex (SEC) receptor on endothelial cells. The minimum sequence in α_1-antitrypsin for binding to the SEC receptor was reported to be Phe370-Val371-Phe372-Leu373-Met374 in strand 4B (*33*), which corresponds to Phe408-Leu409-Val410-Phe411-Ile412 in antithrombin. The mutation of Arg406 may alter the conformation of the ligand motif to a more favorable form for binding to the receptor, resulting in rapid clearance of the Kyoto-mutant from the circulation. However, Maekawa and Tollefsen (*34*) reported that alanine scanning mutations in this region of heparin cofactor II have no effect on the internalization and degradation of thrombin-heparin cofactor II complexes. Thus, it will be controversial to conclude that the reduced antigen level of the Kyoto-mutant is due to rapid clearance through the SEC receptor.

The Utah-mutant has been well characterized by Bock *et al.* (*8, 13-15*). In clinical tests, the propositus showed parallel reductions of the antigen and activity levels of antithrombin to 50%. However, the presence of abnormal antithrombin in the patient's plasma was observed on

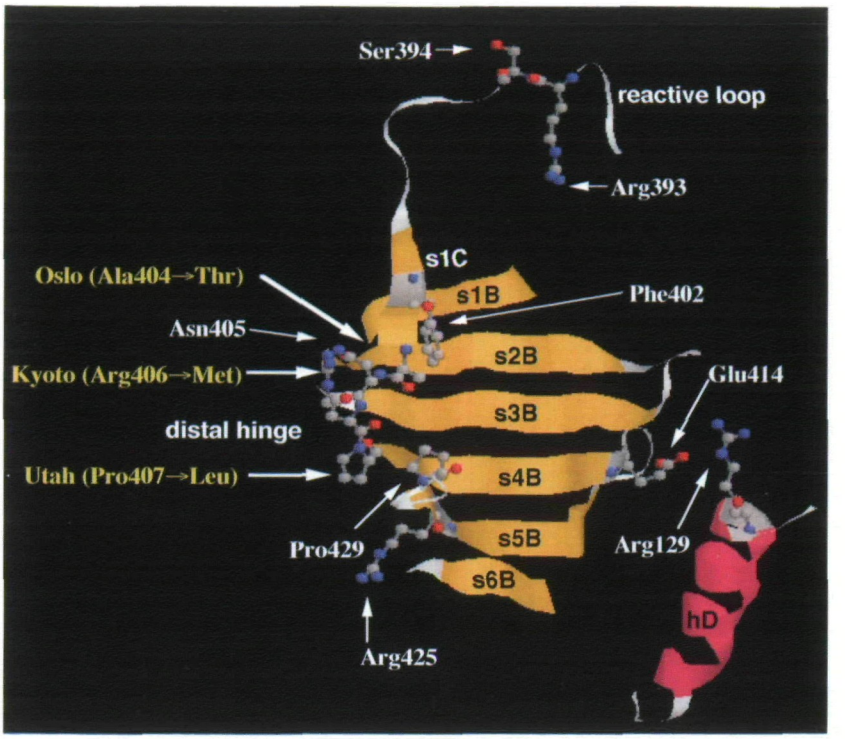

Fig. 7. **Positions of the Oslo-, Kyoto-, and Utah-mutations in the COOH-terminal region of the antithrombin molecule.** The sites of pleiotropic effect-type mutations as well as the reactive site (Arg393-Ser394) and Glu414-Arg129 are indicated by a ball and stick model. Note that all the pleiotropic effect-type mutations occur on a distinct surface of the molecule, ranging from the distal hinge region formed by strand 1C and strands 1C-4B to the C-terminal region of strand 5B. See Refs. *30-32* for detailed explanations of the figure.

crossed immunoelectrophoresis (*14*). In this study, we showed that the secretion rate of the Utah-mutant was significantly lower than those of the Oslo- and Kyoto-mutants as well as Wt antithrombin, and partial (~50%) intracellular degradation was observed for the Utah-mutant (Fig. 2). Sheffield *et al.* (*35*) examined the intracellular event of the Utah-type mutant of rabbit antithrombin expressed in CHO cells, and demonstrated reduced secretion efficiency of the Utah-type mutant on pulse-chase analysis without reference to the partial intracellular degradation of the rabbit Utah-type mutant. Both the intracellular and secreted forms of the Utah-mutant showed lower molecular masses than those of Wt antithrombin (Fig. 3A). Moreover, even after depletion of carbohydrate chains, the Utah-mutant showed faster migration than other antithrombins (Fig. 3B). One possible explanation for this phenomenon is post-translational truncation of the Utah-mutant at the NH_2- and/or COOH-terminus. Since human antithrombin has three disulfide linkages, Cys8-Cys128, Cys21-Cys95, and Cys247-Cys430 (*4*), truncation in the NH_2- and/or COOH-terminal region will easily be reflected by the mobility on SDS-PAGE under reducing and nonreducing conditions. The reducing agent showed little, if any, effect on the mobility of the Utah-mutant. Thus, although we have not determined the NH_2- or COOH-terminal sequence of the recombinant Utah-mutant, we suspect that the Pro407→Leu mutation affects protein folding, resulting in faster migration on SDS-PAGE. Indeed, the plasma of the Utah patient was shown to contain abnormal antithrombin that was coeluted from heparin-Sepharose with normal antithrombin but showed greater electrophoretic mobility than the normal molecule (*14*). Moreover, rabbit antithrombin with the Utah-type mutation secreted from CHO cells showed faster migration than Wt antithrombin (*35*).

In this study, we showed that proteasome inhibitors but not lysosomotropic reagents had inhibitory effects on the intracellular degradation of the Utah-mutant. These data indicated that a part of the Utah-mutant is degraded by proteasome through quality control in the ER. The quality control mechanism in the ER ensures that nascent proteins that fail to fold or to oligomerize correctly are removed from the secretory pathway and degraded intracellularly (for reviews see Refs. *36* and *37*). Initially, this degradation was assumed to be due to through an LLnL-sensitive cysteine protease(s) within the ER. However, Ward *et al.* (*38*) showed that the degradation of the ΔF508 mutant of cystic fibrosis transmembrane conductance regulator (CFTR) was inhibited by proteasome inhibitors. Thus, the mutant CFTR is degraded by proteasome after the attachment of multiubiquitin chains. At present, misfolded ER proteins are proposed to be degraded by proteasome after their retrograde transport from the ER to the cytosol through an ER membrane channel such as Sec61 complex (*36, 37*). Previously, we showed that all the pulse-labeled ΔGlu and P→stop mutants of antithrombin which were classified as type I (secretion defect) deficiency were drastically degraded intracellularly and little (<10%) secretion of the mutants was observed, and the addition of proteasome inhibitors caused the intracellular accumulation of these mutants (*7*). Interestingly, in the case of the Utah-mutant, ~30% of the mutant escaped from quality control and thus was secreted into the medium. Moreover, proteasome inhibitors caused an increase in the remaining amount of the intracellular Utah-mutant without enhancement of its secretion. The folding of the nascent Utah-mutant and its interaction with ER chaperones remain to be investigated.

With respect to the functional properties of the Utah-mutant, Sheffield *et al.* (*35*) reported that the rabbit

Utah-mutant, produced in a cell-free system, exhibited no TAT complex formation ability. However, we found that the recombinant Utah-mutant possessed normal heparin-binding ability (Fig. 5), and reduced, but detectable (~20% of Wt antithrombin) TAT complex formation ability (Fig. 6). This difference may be due to the expression system, since only 10-30% of Wt antithrombin expressed in their system formed a TAT complex (*35*), suggesting the production of heterogeneous antithrombin. The Utah-mutant expressed in BHK cells seems to be properly processed having attached carbohydrate chains which may contribute to the normal heparin-binding and TAT complex formation. Bock *et al.* (*14*) reported that the Utah-mutant in the patient's plasma showed the same affinity for heparin as normal antithrombin, but did not form a TAT complex. This might be due to the presence of normal antithrombin as well as the Utah-mutant in the patient's plasma, and normal antithrombin may interact with thrombin more strongly than the Utah-mutant. Pro407 (P14′ position) of antithrombin is conserved among serpins, and the mutation of Pro407 to Leu is thought to disturb the flexibility of the distal hinge region (Fig. 7) (*30-32*). The proband of an analogous abnormality, α_1-antitrypsin M Heerlen (Pro369 →Leu), is also known to have a decreased plasma level (*39*). Interestingly, however, two other families with a mutation of Pro407 to Thr have 86 and 100% antigen levels (*6*). Collectively, the hydrophobic property of Leu may cause impaired secretion and partial intracellular degradation of serpins. To support this possibility, we found that among various mutants of Arg15 in the γ-carboxyglutamic acid domain of protein C, a vitamin K-dependent plasma anticoagulant protein, the secretion efficiency depended on the hydrophobicity of the substituted amino acid residues (*40*).

In this study, we showed that each of three antithrombin mutants, Oslo, Kyoto, and Utah, having a single amino acid replacement in the region between strands 1C and 4B of the polypeptide chain and grouped as the pleiotropic effect-type, exhibits different properties as to secretion, intracellular degradation and functional activities. Verpy *et al.* (*41*) reported that mutants of C1 inhibitor, having an amino acid replacement in COOH-terminal strand 4B or 5B, such as Leu459→Pro or Arg, or Pro467→Arg, resulted in complete intracellular retention or degradation in COS-7 cells. A Val451→Met mutant in strand 1C, corresponding to the mutation site (P11′ position) of the antithrombin Oslo-mutant, was partially secreted, but the secreted C1 inhibitor similar to antithrombin Oslo was dysfunctional, and only a trace amount of the Phe455→Ser mutant of C1 inhibitor, corresponding to the site (P15′ position) next to that of the Utah-mutant, was secreted. They concluded that certain residues in the conserved COOH-terminal region of serpins play important roles in protein folding, transport and inhibitory activity. Our results agree with their conclusion.

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