Intracellular Accumulation of Antithrombin 
Morioka (C95R), a Novel Mutation Causing Type I Antithrombin Deficiency*

Yuki Tanaka‡, Kazue Ueda‡, Tetsuo Ozawa‡, Nobuo Sakuragawa§, Sadaki Yokota¶, Ryuichiro Sato†, Shoji Okamura‡, Masashi Morita‡, and Tsuneo Imanaka‡**

From the ‡Department of Biological Chemistry, Laboratory Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, and §Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan

Antithrombin (AT) is a major plasma protease inhibitor with three intramolecular disulfide bonds, and its deficiency is associated with increased venous thrombosis. Recently, we found a novel missense mutation named AT Morioka (C95R), which causes the loss of one of the three disulfide bonds. In this study, we prepared Chinese hamster ovary cells stably overexpressing wild type or mutant AT and examined the intracellular fate of the ATs. In pulse-chase experiments, newly synthesized wild type AT was secreted into the medium with a half-life of ~1.5 h. In contrast, most of the mutant type AT was not secreted during the chase period of 9 h and, surprisingly, was not degraded in the cells. The kinetics of the secretion suggests that the mutant was secreted about 50 times more slowly into the medium. Most of the mutant AT in the cells had high mannose type oligosaccharides, suggesting that it was retained in the endoplasmic reticulum (ER). In addition, half of the mutant AT existed in a dimeric form with an intramolecular disulfide bond. On immunoelectron microscopy, the mutant AT was found to have accumulated in variously sized structures surrounded by a single membrane in the cytoplasm. Immunogold particles exhibiting calnexin immunoreactivity were detected on the membranes. Ribosomes were attached to some of the small structures that had accumulated the mutant AT. Further, we prepared Chinese hamster ovary cells stably overexpressing another mutant AT in which two cysteine residues at 21 and 95, responsible for disulfide bond formation, were substituted for arginines. In pulse-chase experiments, the mutant AT (C21C,C95R) was secreted faster than that of AT Morioka (C95R) into the medium. These results suggest that AT Morioka remained for a long time in ER without being degraded and accumulated in newly formed membrane structures derived from the ER. The dimerization of AT Morioka (C95R) through Cys-21 seems to be critical for its intracellular accumulation.

Received for publication, October 7, 2002
Published, JBC Papers in Press, October 23, 2002, DOI 10.1074/jbc.M210231200

Antithrombin (AT) is the major plasma inhibitor of thrombin and other coagulation proteases and is important for the maintenance of normal hemostasis in that it prevents activated coagulation reactions (1, 2). Human AT is a glycoprotein of 58 kDa existing in plasma. It is synthesized by hepatocytes as a 464-amino acid propeptide from which the N-terminal 32 amino acids constituting the signal peptide are cleaved to give the mature protein of 432 residues. AT contains four potential glycosylation sites at asparagine residues 96, 135, 155, and 192 and three intramolecular disulfide bonds between cysteine residues 8–128, 21–95, and 247–430 (3–6). AT has two functional domains; the N-terminal heparin-binding domain and the C-terminal protease-binding domain including the reactive site (7, 8). Intramolecular disulfide bonds are thought to be important for the correct folding of nascent AT polypeptide.

An inherited deficiency in AT is associated with a predisposition to familial venous thromboembolic diseases (9, 10). Two major forms of AT deficiency have been identified from the results of functional and immunological assays (11, 12). Type I deficiency, which is found only in heterozygous patients, is characterized by a reduction in immunological and functional AT levels to ~50% of normal. In contrast, type II deficiency is characterized by the presence of a dysfunctional protein in the plasma of affected individuals, and this AT may be present in either normal or reduced amounts. In the case of type I deficiency, intracellular degradation of mutant ATs is thought to be the reason for the deficiency of plasma AT (11, 12).

The major site for quality control within the secretory pathway is the endoplasmic reticulum (ER). Within the ER, newly synthesized secretory polypeptides are associated with resident chaperone proteins until they are fully folded and covalently modified with oligosaccharides and assembled into appropriate oligomers, at which point they are packed into ER-to-Golgi complex transport vesicles (13, 14). Most proteins that fail to retain the correct conformation in the ER are degraded. In many cases, the ER-associated degradation is carried out by cytoplasmic proteasomes (15–17). On the other hand, misfolded proteins with hydrophobic structures form aggresomes (18, 19). However, the intracellular fate is not well characterized in the case of misfolded proteins, which escape degradation in the ER.

Recently, we found a novel missense mutation, which we named AT Morioka (20). A single base mutation leads to the replacement of cysteine (Cys; TGT) 95 with arginine (Arg;
Intracellular Accumulation of Mutant Antithrombin

CGT). To examine the molecular and cellular mechanisms of AT deficiency, we transfected CHO cells with the cDNA of AT Morioke and compared its intracellular fate to that of wild type AT. We found that the mutant AT is not transported to the Golgi apparatus and accumulates without degradation in novel structures surrounded by a single membrane derived from the ER.

EXPERIMENTAL PROCEDURES

Materials—PRO-MIXTM, t-[35S]-in vitro cell labeling mix (70% l-[35S]methionine and 30% l-[35S]cysteine, >37 TBq/nmol), concana- 
vlin A (ConA)-Sepharose, and ECL-Plus, a Western blotting detection system were purchased from AmerHAM Biosciences. Rabbit anti- 
man AT, sheep anti-human AT, and rabbit anti-rat GRP78 (Bip) were obtained from Gelco Diagnostics, Inc. (Shreveport, LA), Cedarlane Lab- 
oratories Ltd., (Victoria, Canada), and Affinity Bioreagents, Inc. (Gold- 
en, CO), respectively. Rabbit anti-canine calnexin, rabbit anti-human calreticulin, mouse anti-protein disulfide isomerase, rabbit anti-
mouse ERp72, rabbit anti-human Erp57, and rat anti-chicken GRP94 were from StressGen Biotechnologies Corp. (Victoria, Canada). Protein A- 
and Protein G-Sepharose CL-4B, brefeldin A (BFA), phenylmethyl- 
sulfonyl fluoride, and human AT were from Sigma. Endoglycosidase H (Endo H) was purchased from Seikagaku Kogyo Co., LTD (Tokyo, 
Japan). Antipain, chymostatin, leupeptin, and pepstatin A were from Peptide Institute Inc. (Osaka, Japan).

Construction of an AT Expression Vector—The plasmid pBluescript KS(-)/AT in which the human cDNA sequence encoding AT was cloned into the EcoRI site of pBluescript was described in Ref. 21. From this, the full-length AT was excised with BamHI and XhoI and ligated into pCDNA 3.1(+)(Invitrogen) at the corresponding sites, to obtain 
pCDNA3.1(+)/AT.

Construction of Mutant cDNA—A mutant version of AT containing the mutation C(TGT/95R/CTG) (the underlined letters indicate the single base mutation leading to an amino acid replacement of cysteine to arginine) was constructed with a QuiK ChangTM site-directed mutagenesis kit (Stratagene) using pcDNA 3.1(+)/AT(C5R). Two BsgI fragments (the substitution site is underlined), 5′-TATGACCAACTGGTGGCCCGGAGATG- 
ACACC-3′ and 5′-GGGTCTATTACGGGCACCCAGCTTGGTCATA-3′, were used as forward and reverse primers, respectively. Another mut- 
ant version of AT containing Cys(TGC/21Arg/GCG) and Cys(TGT/95A- 
r-g/CCTG) was also constructed using pcDNA 3.1(+)/AT(C5R). Two BsgI fragments (the forward primer is underlined), 5′-TATGACCAACTGGTGGCCCGGAGATGACACC-3′ and 5′- 
GGGTCTATTACGGGCACCCAGCTTGGTCATA-3′, were used as forward and reverse primers, respectively. The mutation in the construct was confirmed by DNA sequencing.

Transfection of AT cDNAs and Selection of Cells Overexpressing AT—CHO cells were transfected using lipofectamine. CHO cells were cultured with Ham's F-12 medium (100 units/ml of penicillin and 100 μg/ml of streptomycin) and transfected with 5.0 μg of 
pCDNA3.1(+)/AT, which had been mixed with Trans FastTM (Promega). The procedure was essentially the same as described in Ref. 22. Su-
viving isolated colonies were removed by the cylinder technique and subjected to analysis for immunodetection and immunoprecipitation of AT. The same procedure was carried out to obtain cells overexpressing 
the mutant ATs.

Pulse-Chase Experiments—CHO cells were plated at a concentration of 2 × 10^5 in 6-well plates and cultured at 37 °C for 18 h with Ham's F-12 medium containing 10% (v/v) feral calf serum. The culture medium was then exchanged for 10 mM dithiothreitol or non-reducing conditions without dithiothreitol. When the molecular size of AT was compared under reducing conditions (S) and non-reducing conditions (N), the size difference was due to the carbohydrate moieties, which were smaller in the non-reducing (N) than in the reducing (S) condition.

Results

Preparation of the Soluble and Insoluble Fractions from Cell Lysate—CHO cells were washed twice with PBS containing 20 mM N-ethylmale- 

lamine and incubated with 10 mM Tris/HCl, pH 7.4, containing 0.2% SDS, and 200 mM NaCl, 200 μg/ml of bovine serum albumin, 10 μg/ml of antipain, chymostatin, leupeptin, and pepstatin A. A-Methyl-β-mannose was added to the samples, and the cell lysates were centrifuged at 13,000 × g for 15 min and separated into soluble and insoluble fractions.

Electron Microscopy—For transmission electron microscopy, CHO cells were fixed with 4% (w/v) paraformaldehyde, 2% (w/v) glutaralde- 
hyde in 0.1 M cacodylate hydrochloride, pH 7.4, for 1 h at room temper- 
ature and washed with PBS. Cells were then detached from culture 
plates. The cell monolayers were washed two times in PBS containing 
0.2% (w/v) glutaraldehyde and 0.2% (v/v) glycine, and then treated with 
20 mM N-ethylmaleimide/PBS for 30 min at 4 °C to block free sulfhydryl groups in the proteins. The gels were dried, and the band of AT was quantified with a Fuji BAS 2000 imaging analyzer (Fuji Film).

Co-immunoprecipitation—CHO cells were transfected with 3 μg of 
[35S]methionine and cysteine and followed for 3 h. The [35S]AT in the 
culture medium was removed, and the cells were labeled with 925 kBq of [35S]methionine and cysteine, and the cells were lysed with 
Triton buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 10% gly- 
cerol, 10 mM sodium pyrophosphate, and 17.5 mM β-glycerophosphate) 
(25). The protease inhibitors described above were added to avoid pro- 
teolysis. The cellular lysates were centrifuged for 10 min in a microcen- 
trifuge. The supernatants were incubated at 4 °C for 2 h with the antibodies, and the immunoprecipitates were separated by SDS-
PAGE. The supernatants were then put on 10% slab gels and subjected to autoradiography.
tions, and calnexin was detected by rabbit anti-calnexin antibody and 15-nm gold coupled to goat anti-rabbit IgG applied to the other side of the same sections.

Other Methods—Lactate dehydrogenase was assayed using a lactate dehydrogenase-UV test kit (Wako, Osaka, Japan). Immunoblotting was done by the method of Small et al. (28) using ECL/H11001 Plus, a Western blotting detection system.

RESULTS
Preparation of CHO Cells Expressing Wild and AT Morioka (C95R)—The expression plasmid pcDNA3.1(+)/AT and pcDNA3.1(+)/AT(C95R) were transfected into CHO cells, and stable transformants were selected based on Geneticin resistance. The clones were first examined for the expression of wild and mutant ATs by immunofluorescence microscopy. Several cell lines transfected with pcDNA3.1(+)/AT and pcDNA3.1(+)/AT(C95R) showed a punctuated staining pattern, but control cells and cells transfected with pcDNA3.1(+) did not exhibit any immunofluorescence (data not shown). The cells expressing wild and mutant ATs were then labeled with [35S]methionine and cysteine overnight, and the medium and cell fraction were immunoprecipitated with anti-human AT antibody. As shown in Fig. 1, a band of 52 kDa corresponding to premature AT was detected in the cell fraction and a band of 58 kDa corresponding to mature AT was detected in the medium of the cells expressing wild type AT. In contrast, mutant AT with a molecular mass of 52 kDa was only detected in the cell fraction, and neither the 52- nor the 58-kDa bands were detected in the medium. These results suggest that newly synthesized wild type AT was processed in CHO cells and secreted into the medium, but the mutant type AT was not. For subsequent experiments, we chose #23 (w-AT) and #13 (AT/C95R) cells, because these cells synthesized relatively large amounts of the wild type and mutant ATs compared with the other cell lines.

Intracellular Transport of Wild and Mutant AT—To examine the intracellular fate of the wild type and mutant ATs, #23 (w-AT) and #13 (AT/C95R) cells were labeled for 1 h with [35S]methionine and cysteine, and the radioactive activity was followed for up to 3 h in #23 (w-AT) and up to 9 h in #13 (AT/C95R) cells. As shown in Fig. 2A, after the labeling of the #23 (w-AT) cells, the [35S]-mature wild type AT was detected in the medium and increased up to 3 h. The amount of [35S]-premature AT detected in the cell fraction decreased during chase periods of 1 to 3 h. This secretion pattern suggests that newly synthesized wild type AT was folded properly with oligosaccharides and secreted into the medium in the CHO cells expressing wild type AT.
AT. On the other hand, mutant AT was not secreted into the medium in the period up to 3 h, and only a very small amount of AT of mature size was secreted into the medium in the chase period of 9 h (Fig. 2B). The amount of $^{35}$S-premature AT detected in the cells was almost constant over a chase period of up to 9 h. Quantification of the radioactivity was carried out with a BAS 2000 imaging analyzer as shown in Fig. 2C. The newly synthesized wild type AT was secreted into the medium with a half-life of $1.5/h$, whereas mutant AT was secreted only very slowly and was retained in the cells rather than undergoing rapid degradation.

To analyze the intracellular fate of mutant AT in detail, #13 (AT/C95R) cells were labeled with $[35S]$methionine and cysteine for 3 h, and the radioactivity was followed for up to 72 h. As shown in Fig. 3B, a small amount of $^{35}$S-mature AT was detected in the medium after a chase period of 9 h, and the amount increased up to 72 h. In addition, $^{35}$S-premature AT was also detected in the medium after a chase period of 48 h and increased up to 72 h. After a chase period of 72 h, $30\%$ and $20\%$ of the newly synthesized AT had been secreted into the medium as mature and premature forms, respectively (Fig. 3C). Most intracellular AT existed in a premature form, and the amount decreased with a half-life of 75 h (Fig. 3C). However, the total AT radioactivity remained virtually constant throughout the chase period. These results suggest that the secretion of

---

Fig. 3. Pulse-chase analysis of newly synthesized wild and mutant type ATs in cells and medium followed for an extended period. A, #23 (w-AT) cells. B, #13 (AT/C95R) cells. Cells were pulse-labeled with $[35S]$methionine and cysteine for 3 h and followed for the times indicated. AT was examined following immunoprecipitation and SDS-PAGE. C, kinetics of intracellular transport and secretion of AT after pulse-chase labeling. The open circles depict the amount of radio-labeled AT in the cells, and the closed circles and squares depict the amount of mature and premature AT in the medium, respectively.

---

Fig. 4. Endo H digestion of AT and binding of AT to ConA-Sepharose. #23 (w-AT) and #13 (AT/C95R) cells were labeled for 3 h with $[35S]$methionine and cysteine and followed for 3 h. A, the $^{35}$S-mutant AT (C95R) in the cells or $^{35}$S-wild type AT (wild) in the medium fractions was immunoprecipitated, and the isolated ATs were incubated with Endo H and then subjected to SDS-PAGE. Purified human AT (2 $\mu$g) was also incubated with Endo H and subjected to SDS-PAGE followed by immunoblot analysis by anti AT antibody. The arrows indicate the positions of the mature AT and premature AT. The arrowhead indicates the position of AT reduced in molecular size by Endo H treatment. B, the cell lysates were diluted in the binding buffer, and the final concentration of $\alpha$-methyl-$\beta$-mannose was adjusted to 0, 20, and 200 mM. After incubation with ConA-Sepharose, the samples were then spun for 10 min by microcentrifuge. The resulting supernatants were immunoprecipitated with rabbit anti-human AT antibodies. As a control experiment, the binding of purified human AT to ConA-Sepharose was examined under the same conditions, and the AT remaining in the supernatant fraction was analyzed by immunoblotting.
mutant AT is ~50 times slower, and as a result, the mutant AT accumulates in the cells as mostly the premature form.

It is possible that $^{35}$S-premature AT in the medium is released from cells damaged during the longer chase periods. To exclude this possibility, lactate dehydrogenase activity was measured in the medium and cell fractions under the same pulse-chase conditions. As shown in Table I, lactate dehydrogenase activity was detected in the medium after any chase periods in #13 (AT/C95R) cells, suggesting that in fact the mutant AT was not transported into the Golgi apparatus and did not bear complex oligosaccharide. To test this hypothesis, #13 (AT/C95R) and #23 (w-AT) cell cultures were pulse-labeled with $^{35}$S-methionine and cysteine for 3 h and followed for 3 h. The cell lysates were subjected to non-denaturing immunoprecipitation with the several antibodies indicated in the figure or preimmune IgG. The immunoprecipitates were diluted 3 h and then processed with a second immunoprecipitation with anti AT antibody. PDI, protein disulfide isomerase.

**Mutant AT (C95R) Possesses High Mannose Type Oligosaccharides in the Cells**—The finding that the molecular mass of the mutant AT in the cells was ~5 kDa smaller than that of wild type AT secreted in the medium suggested that the mutant was not transported into the Golgi apparatus and did not bear complex oligosaccharide. To do this, we examined the binding of wild type and mutant ATs to ConA-Sepharose. Human AT contains four complex oligosaccharides with two branched chains in one molecule (29). It is known that this type of oligosaccharide with a free hydroxy residue in the C-2 position of the two mannoses is bound to ConA-Sepharose and that high mannos type oligosaccharides are released by more than 100 mM $\alpha$-methyl-D-mannose and that high mannos type oligosaccharides are released by more than 100 mM $\alpha$-methyl-D-mannose (30). Using this selectivity of ConA-Sepharose to the complex or high mannose oligosaccharides, the binding of wild type and mutant ATs to the resin was examined. As shown in Fig. 4B, the mutant AT (C95R) bound to ConA-Sepharose was released by 200 mM but not 20 mM $\alpha$-methyl-D-mannose. In comparison, wild type and purified
human AT bound to the resin were released by as low as 20 mM α-methyl-β-mannos. These results suggest that mutant AT (C95R) bears the high mannose type oligosaccharides.

Next, we examined the inhibitory effect of BFA on the processing of oligosaccharide chains of AT in #13 (AT/C95R) and #23 (w-AT) cells. BFA is well known to block transport between the ER and Golgi apparatus by inhibiting the exchange of GDP to GTP on ADP-ribosylation factor 1 (ARF1) (31, 32). In #23 (w-AT) cells, mature AT was observed in the medium in the absence of BFA, whereas premature AT, whose oligosaccharide chain has not yet been processed in the Golgi apparatus, was detected in the cell fraction in the presence of BFA (Fig. 5). The size of the premature AT in the #13 (AT/C95R) cells was not altered by the presence or absence of BFA. Furthermore, the size of the premature AT in #13 (AT/C95R) cells was the same as that of the wild type AT in the #23 (w-AT) cells incubated with BFA. These results suggest that mutant AT was not transported to the Golgi apparatus but rather remained in the pre-Golgi compartments.

Properties of Mutant AT (C95R) in the Cells—A cysteine at residue 21 in wild type AT forms a disulfide bond with a cysteine at residue 95. If AT lacks a cysteine at residue 95, a dimer might be able to form with another cysteine at residue 21. To examine this possibility, #13 (AT/C95R) and #23 (w-AT) cells were labeled with [35S]methionine and cysteine for 3 h followed for 3 h. The cell fractions were immunoprecipitated with anti-AT antibody, and the resulting immunocomplexes were subjected to SDS-PAGE under non-reducing conditions. The wild type molecular mass did not change under non-reducing conditions (compare Fig. 6A with Fig. 2A), but mutant AT (C95R) exhibited dimeric and oligomeric structures. Both cell fractions were also subjected to immunoblot analysis to determine steady state forms of the mutant AT (C95R). As shown in Fig. 6B, the dimeric form of AT was detected in the mutant cell fraction, and the amount was almost equal to that of monomeric form. Next we examined whether mutant AT (C95R) formed insoluble aggregates. The cell lysates from #23 (w-AT) and #13 (AT/C95R) cells were separated into soluble and insoluble fractions, and immunoblot analysis was carried out. As shown in Fig. 7, there is no detectable AT in the pellet from CHO cells expressing wild type or mutant AT (C95R). The lack of mutant AT (C95R) in the insoluble fraction suggests that the formation of insoluble aggregates is not essential for the accumulation of the mutant AT in these cells.

The prolonged retention of mutant AT (C95R) in the absence of aggregation suggests that it associates with ER chaperones. To investigate this possibility, #13 (AT/C95R) cells were labeled with [35S]methionine and cysteine and then lysed in non-denaturing lysis buffer. The lysates were first immunoprecipitated with several anti-chaperone antibodies, and the resulting immunoprecipitates were diluted, and a second immunoprecipitation was then performed with anti-AT antibody. As shown in Fig. 8, mutant AT (C95R) was detected in the immunoprecipitate with GRP78 but not with calnexin, calreticulin, GRP94, Erp72, protein disulfide isomerase, or Erp57. These results suggest that mutant AT (C95R) associated with at least the chaperone GRP78 in the ER.

Subcellular Localization of Mutant AT—The above evidence indicates that the intracellular transport of mutant AT is blocked before the Golgi apparatus. The localization of mutant AT in #13 (AT/C95R) cells was examined by both immunoelectron and transmission electron microscopy. As shown in Fig. 9B, a large number of gold particles corresponding to mutant AT was observed in structures of various size surrounded by a single membrane, different from any subcellular organelles. A large number of gold particles recognizing AT were also de-
Fig. 10. **Immunoelectron micrographs of #23 (w-AT) and #13 (AT/C95R) cells.** Immunostaining with anti-AT (A and B). A, gold particles are present in endoplasmic membrane structures and the intermembrane space of the nucleus (N) (long arrows). B, ribosomes associate with some membrane structures that accumulate mutant AT (small arrows). The small arrows in A also indicate ribosomes. C, double immunostaining with anti-AT (small gold particles; thin arrows) and anti-calnexin (large gold particles; bold arrows). Calnexin co-localized with AT in the same membrane structures. The bars are 0.5 μm.
Intracellular Accumulation of Mutant Antithrombin

Lactate dehydrogenase activity in the cells and the medium fraction in long chase experiments

Control (Cont), #23 (w-AT), and #13 (AT/C95R) cells (5 × 10^6) were seeded on 6-cm^2 dishes and cultured under the same conditions as in the pulse-chase experiments. After 24, 48, and 72 h of culture, the cell and medium fractions were separated by centrifugation. Lactate dehydrogenase activity was measured in both fractions, as a marker enzyme of cytosol and measure of cell damage. Activity in the medium was calculated by subtracting the activity in the cell-free culture medium at each time point.

| Cells, mU | 24 | 48 | 72 |
|-----------|----|----|----|
| Cont      | 59 | 117| 248| 4.0| 0 | 0 |
| #23 (w-AT)| 45 | 98 | 220| 3.0| 0 | 0 |
| #13 (AT/C95R) | 45 | 95 | 210| 5.0| 0 | 0 |

Intracellular Transport of Another Mutant AT (C21R,C95R)—The dimerization of mutant AT (C95R) with cysteine at residue 21 appears crucial for the retention of the AT in the cells. To test this, CHO cells overexpressing another mutant AT (C21R,C95R) with double mutations of cysteine residues at 21 and 95 were prepared. #8 (AT/C21R,C95R) cells were labeled for 1 h with [35S]methionine and cysteine, and the radioactivity was followed for up to 6 h. As shown in Fig. 11, after the labeling of #8 (AT/C21R,C95R) cells, the 35S-mature mutant AT was detected in the medium after a chase period of 0.5 h, and the amount increased up to 6 h. After a chase period of 6 h, ~25% of the newly synthesized AT had been secreted into the medium. The result suggests that the mutant AT (C21R,C95R) is secreted faster than that of AT Morioka (C95R) (compare Fig. 11B to Fig. 3C).

**DISCUSSION**

In this study, we prepared CHO cells expressing AT Morioka and characterized the molecular mechanism of AT deficiency by pulse-chase experiments and morphological observation. AT Morioka has a single base mutation leading to the amino acid replacement of cysteine 95 with arginine. As the cysteine is responsible for the forming an intramolecular disulfide bond, the mutation ostensibly affects the folding of AT molecules.

Prior to this study, we had compared the secretion of AT in #23 (w-AT) cells to that in human hepatoma HuH7 cells, which are commonly used as a model of hepatocytes. In a pulse-chase study, the newly synthesized AT in HuH7 cells was secreted with a half-life of ~1.5 h (data not shown). The rate of secretion is essentially the same as that in #23 (w-AT) cells (Fig. 2C), suggesting that the protein secretion can be studied using these CHO cells.

The present study shows the following unique features of intracellular fate of mutant type (C95R) (1). The mutation caused accumulation of AT in the cells without degradation (2). The sites where mutant AT accumulated were novel cell compartments.

**The Intracellular Fate of Mutant AT**—Nascent proteins that fail to fold correctly are usually removed rapidly from the ER. Misfolded proteins in the lumen of the ER are thought to be sent back to the cytoplasm through a translocon composed of Sec 61, where they are ubiquitinated and degraded by proteasomes (16, 33, 34). We had expected AT Morioka to be degraded rapidly because of abnormal folding in the lumen of the ER, because the molecule lacks the cysteine residue required for the correct disulfide bond formation. However, AT Morioka accumulated in the cells. Most of the newly synthesized mutant AT (C95R) remained in the cells over a chase period of 9 h and was not degraded in the pre-Golgi compartments (Fig. 2). The presence of the AT in the pre-Golgi compartments was supported by several lines of evidence, namely that the mutant AT was sensitive to Endo H treatment (Fig. 4A) and exhibited selective association with ConA-Sepharose (Fig. 4B), and the molecular mass of the mutant was the same as wild type AT prepared from #23 (w-AT) cells incubated with BFA (Fig. 5). The mutant AT evidently escapes proteolytic degradation by proteasomes.
in the compartments. Furthermore, prolonged pulse-chase experiment revealed that the accumulation of mutant AT correlated with a decline in the rate of secretion of AT. The rate decreased to about $\frac{1}{3}$ that of the wild type AT (Fig. 3). It is quite unlikely that the accumulation of mutant AT in the cells is because of an overexpression of the protein. Expression levels of the mutant and wild type ATs were comparable (Fig. 1).

In addition, secretion of newly synthesized proteins except for the mutant AT was normal in #13 (AT/C95R) cells and in control CHO cells (data not shown).

Why did the mutant AT (C95R) accumulate in the pre-Golgi compartments? One explanation is that it forms structures such as aggresomes. It has been reported recently (18, 19) that certain misfolded proteins aggregated in pericentriolar structures, termed aggresomes. When the mutant cystic fibrosis transmembrane conductance regulator (CFTR) was overexpressed in human embryonic kidney 293 cells, it accumulated in cytoplasm as aggregations in which the protein molecules were mult ubiquitinated (18, 35). A cytosolic protein chimera (GFP-250) composed of green fluorescent protein fused at the C terminus to a 250-amino acid fragment of the cytosolic protein, p115, has also been shown to form aggresomes on overexpression in COS 7 cells (36). However, the mode of AT accumulation does not appear to be because of aggregation for the following reasons. When #13 (AT/C95R) cells were treated with 2% Triton X-100 (Fig. 7) or 1% Nonidet P-40 and 0.5% deoxycholate (date not shown) and then separated into detergent-soluble and -insoluble fractions, the mutant AT was recovered completely in the detergent-soluble fraction under conditions where CPTRA508 and GFP-250 were recoverable in the detergent-insoluble fraction (18, 36).

Another explanation other than the polymerization mechanism is that there are mechanisms by which mutant AT (C95R) is retained in the ER. Although we cannot be certain of the exact mechanism at present time, dimerization of the mutant AT (C95R) and/or association with GRP78 likely might be involved in the prolonged retention of the mutant AT (C95R) in the ER for the following reasons (1). A portion of the mutant AT (C95R) existed in a dimeric form and was not degraded in the cells (see Figs. 2B and 6). Furthermore, another mutant AT (C21R,C95R) with a double mutation of the cysteines at residues 21 and 95 was secreted faster than that of mutant AT (C95R) (Fig. 11). These results demonstrate that the cysteine residue at 21 plays a critical role in the retention of mutant AT in the pre-Golgi compartment (2). Association of mutant AT with resident protein(s) of the ER would contribute to the retention of mutant AT (C95R), and we have shown that the mutant AT (C95R) was bound to GRP78 (Fig. 8). In contrast, wild type AT did not (date not shown). It is known that GRP78 binds to unfolded or unassembled proteins, and a role for GRP78 in preventing the secretion of a number of misfolded proteins has been proposed (37–39). Interestingly, the amount of GRP78 in #13 (AT/C95R) cells increased about 4-fold compared with those in either #23 (w-AT) or control CHO cells (date not shown). Taken together, AT Morioka (C95R) misfolds in the ER because of an incorrect disulfide bond and forms dimer. The monomeric or dimeric form of misfolded AT is recognized and bound by GRP78. Such modified structure(s), perhaps along with the resident chaperone proteins that can recognize them, are capable of preventing the AT from being sent back to the translocon composed of Sec 61 for degradation by proteasomes. As a result, the mutant AT remains and is accumulated in the ER.

With regard to inherited AT deficiency, many mutations of the AT gene have been identified (12), but only a relatively small number of studies have examined the cellular basis of the pathology. In the case of type I deficiency, AT (E313) and AT (P429Stop) are degraded rapidly by proteasomes when these cDNAs are expressed in baby hamster kidney cells (40). The mutations AT (C128Y) and AT (C430F) were identified recently (41, 42), but the intracellular fate of these ATs has not yet been elucidated. From our investigation, these ATs are likely to have a similar intracellular fate as AT (C8SR). In the case of type II deficiency, AT Oslo (A404T) and AT Kyoto (R406M) have been shown to be secreted at a similar rate to wild type AT. On the other hand, AT Utah (P407L) was degraded rapidly in the cell by proteasomes, and its secretion was reduced (43, 44). Therefore, AT Morioka (C95R) has been shown to have a novel and unique intracellular fate among the mutant ATs yet known. Morphological Observation Sites of Accumulation Sites of the Mutant AT in the Cells—The preponderance of mutant AT did not undergo carbohydrate modifications associated with the Golgi apparatus, such as conversion to the Endo H-resistant form, suggesting that the AT might accumulate in ER. However, the mutant AT showed a quite different localization. The majority of the mutant AT was found in variously sized structures surrounded by a single membrane with a rather electron-dense morphology (see Figs. 9 and 10). The structures at times occupied more than 50% of the cytoplasm and were different from any other organelles present such as mitochondria, peroxisomes, and lysosomes. In addition, immunogold particles against proteasomes were not detectable in these structures (data not shown). Careful examination of the morphology and immunogold localization of the AT immunoreactive sites revealed that the mutant AT located in the intermembranous spaces of the nuclear envelope and ribosomes were associated with the membrane structures that accumulated the mutant AT (Fig. 10, A and B). In addition, immunogold particles against calnexin were shown to co-localize with those against AT (Fig. 10C). These observations suggest that the structures were derived from the ER.

The structures observed in this study resemble Russell bodies (RB). RB were described originally in plasma cells and are thought to be dilated ER cisternae containing condensed IgM (19). Their biogenesis has been attributed to the synthesis of a mutated Ig, which is neither secreted nor degraded, and which, by itself, is sufficient to induce RB formation in cells of different species and histotype (19). In this type RB, mutant IgM exists as insoluble aggregates (45, 46). Similar structures have been reported in the hepatocytes of an individual carrying mutated α1-antitrypsin alleles (PiZ; the glutamic acid at position 342 being substituted by lysine) and transgenic mice (47–49), although the immunogold that reacted with PiZ α1-antitrypsin localized exclusively in rough ER, and the PiZ α1-antitrypsin seemed to be highly polymerized.

In RB formation, the specific molecular structure of insoluble lattice is thought to be important. However, mutant AT (C95R) does not exist as aggregates but forms RB like structures (see Figs. 7, 9, and 10). Very recently a nonpolymerogenic mutant of α1-antitrypsin was shown to have prolonged retention in the ER (50). Therefore, protein aggregation does not appear to be necessary for the biogenesis of RB. Taking these observations into consideration, along with our studies, the prolonged accumulation of mutant AT (C95R) appears to lead to an unusual expansion and budding of the ER membrane so as to segregate misfolded mutant AT from the ER proper. This response of the ER seems to be one of the systems by which cells protect their essential functions.

Another important finding of the present study is that a portion of the premature mutant AT was secreted from #13 (AT/C95R) cells. One explanation for this is that the mutant accumulated in the RB-like structures is secreted into the
medium by exocytosis. Another possibility is that the RB-like structures are segregated from the cells when the cells divide. In fact, #13 (AT/C95R) cells grow as well as #23 (w-AT) and control cells (Table I), and no cell damage was observed even after more than 10 passages. The content of mutant AT in #13 (AT/C95R) cells was only about 5 times higher than that of wild type AT in #23 (w-AT) cells although the secretion of newly synthesized AT was decreased to ~1/50. This provides insight into the role of ER and RB in the quality control of misfolded proteins.

In this study, we report that AT Morioka is not transported to the Golgi apparatus but accumulates without degradation in newly formed, membranous structures derived from the ER. The mechanism by which mutant AT escapes degradation, as well as the biogenesis and turnover of the RB-like structures, for the time being remain subjects for future research.

REFERENCES
1. Travis, J., and Salvesen, G. S. (1983) Annu. Rev. Biochem. 52, 655–709
2. Bjork, I., and Danielsson, A. (1999) in Proteinase Inhibitors (Barrett, A. J., and Salvesen, G., eds) pp. 489–513, Elsevier Science Publishers B.V., Amsterdam
3. Sun, X. J., and Chang, J. Y. (1989) J. Biol. Chem. 264, 11288–11293
4. Egeberg, O. (1965) Thromb. Diath. Haemorrh. 15, 1563–1570
5. Ellgaard, L., Molinari, M., and Helenius, A. (1999) Nature 397, 7286–7293
6. Rapoport, T. A., and Ploegh, H. L. (1996) Nature 383, 7293–7298
7. Williams, G., and Milstein, C. (1990) Cell 61, 1239–1254
8. Schuck, P., Trapman, J., van Deurs, J., Cold, J., and van der Horst, G. (1997) FEBS Lett. 412, 15–20
Intracellular Accumulation of Antithrombin Morioka(C95R), a Novel Mutation Causing Type I Antithrombin Deficiency
Yuki Tanaka, Kazue Ueda, Tetsuo Ozawa, Nobuo Sakuragawa, Sadaki Yokota, Ryuichiro Sato, Shoji Okamura, Masashi Morita and Tsuneo Imanaka

J. Biol. Chem. 2002, 277:51058-51067.
doi: 10.1074/jbc.M210231200 originally published online October 23, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M210231200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 20 of which can be accessed free at http://www.jbc.org/content/277/52/51058.full.html#ref-list-1