Molecular Basis for Defective Secretion of the Z Variant of Human Alpha-1-Proteinase Inhibitor: Secretion of Variants Having Altered Potential for Salt Bridge Formation between Amino Acids 290 and 342

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Human alpha-1-proteinase inhibitor (A1PI) deficiency, associated with the Z-variant A1PI (A1PI/Z) gene, results from defective secretion of the inhibitor from the liver. The A1PI/Z gene exhibits two point mutations which specify amino acid substitutions, Val-213 to Ala and Glu-342 to Lys. The functional importance of these substitutions in A1PI deficiency was investigated by studying the secretion of A1PI synthesized in COS cells transfected with A1PI genes altered by site-directed mutagenesis. This model system correctly duplicates the secretion defect seen in individuals homozygous for the A1PI/Z allele and shows that the substitution of Lys for Glu-342 alone causes defective secretion of A1PI. The substitution of Lys for Glu-342 eliminates the possibility for a salt bridge between residues 342 and 290, which may decrease the conformational stability of the molecule and thus account for the secretion defect. However, when we removed the potential to form a salt bridge from the wild-type inhibitor by changing Lys-290 to Glu (A1PI/SB-290Glu), secretion was not reduced to the 19% of normal level seen for A1PI/Z-342Lys; in fact, 75% of normal secretion was observed. When the potential for salt bridge formation was returned to A1PI/Z-342Lys by changing Lys-290 to Glu, only 46% of normal secretion was seen. These data indicate that the amino acid substitution at position 342, rather than the potential to form the 290-342 salt bridge, is the critical alteration leading to the defect in A1PI secretion.

Alpha-1-proteinase inhibitor (A1PI), an inhibitor of serine proteases, is one of the major serum glycoproteins synthesized in and secreted by the liver. Individuals homozygous for the Z-variant A1PI (A1PI/Z) gene have about 15% of the normal circulating level of this protease inhibitor and as a result are predisposed to pulmonary emphysema and hepatic cirrhosis (6). The decreased serum levels of A1PI in affected individuals are caused by impaired secretion of A1PI from the liver rather than by defective synthesis of the protein (5). This conclusion is supported by the observations that the levels of A1PI mRNA in affected and normal livers are essentially the same (30), the efficiencies of translation of these mRNAs are not significantly different (1, 25, 30), and A1PI/Z accumulates in the endoplasmic reticulum (ER) of the liver (4). The only demonstrated differences between the normal (A1PI/M) and variant (A1PI/Z) forms of this protein are two changes in the primary sequence. In A1PI/Z, alanine is substituted for valine at position 213 (23, 27) and lysine is substituted for glutamic acid at position 342 (13). These substitutions do not appear to cause major changes in the conformation of A1PI/Z, since the specific trypsin-inhibitory capacities, circulating half-lives, sedimentation coefficients (1, 21), immunological activities (34), and oligosaccharide structures (1, 35) of the serum forms of A1PI/Z and A1PI/M appear to be identical. Furthermore, after solubilization, the A1PI/Z that accumulates in inclusion bodies in the ER of the liver of individuals homozygous for the Z variant is fully active (1).

The observations outlined above argue that one or both of the amino acid replacements that occur in A1PI/Z directly affect secretion of the protein and that they do so by some

means other than causing gross misfolding or dramatic changes in solubility properties. The substitution of Ala for Val at position 213 seems to be relatively innocuous, a supposition supported by the data presented here. However, the substitution of Lys for Glu at position 342 is a replacement of a negative charge by a positive charge, resulting in a potentially significant change in primary structure. Several studies demonstrating characteristic differences in ER-to-Golgi transport rates for secretory proteins (7, 8, 16, 22, 26) suggest that the differences may result from the interaction of the protein with a receptor that mediates subsequent transport of the protein to the Golgi. Given such a mechanism, a change in charge could affect ligand-receptor interaction and eliminate or greatly reduce binding.

The substitution of Lys for Glu-342 destroys the potential for a salt bridge between Glu-342 and Lys-290. This salt bridge is known to exist in the form of A1PI/M that is produced by interaction with serine proteases (17); it may also be present in the native protein, but data supporting this idea are not available. The proposition that loss of this salt bridge in A1PI/Z leads to inefficient secretion, due to an altered folding pattern resulting in aggregation of the protein in the ER, has become an accepted hypothesis despite the lack of direct supporting evidence (23). This study was designed to evaluate the relative importance of the putative salt bridge and the amino acid occupying position 342 for the efficient secretion of A1PI. Our results show that the potential for salt bridge formation has some effect on the secretion of A1PI, suggesting that this ionic interaction is present in the active A1PI molecule. Furthermore, our data show that the substitution of a positively charged Lys for a negatively charged Glu at position 342 dramatically affects the secretion

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of this protein in the presence or absence of the potential to form the 342-290 salt bridge.

MATERIALS AND METHODS

Mutant construction. Oligonucleotide-directed mutagenesis of the human A1PI/M cDNA gene followed standard procedures, with modifications for use with double-stranded plasmid DNA (20). The oligonucleotides used for mutagenesis were synthesized by Paul Hagermann (University of Colorado Health Sciences Center, Denver). The human A1PI cDNA gene, subcloned into a mammalian expression vector containing the simian virus 40 origin of replication and early promoter, pSV₇ (29), was provided by Ian Bathurst (Chiron Corp., San Francisco, Calif.). DNA sequencing was done according to the dideoxy-chain termination technique for double-stranded DNA, using the Sequenase kit from United States Biochemical Corp., (Cleveland, Ohio) and synthetic oligonucleotides as primers. Restriction endonucleases, purchased from Promega Biotech (Madison, Wis.) and New England BioLabs, Inc. (Beverly, Mass.), were used according to the recommendations of the manufacturers.

DNA transfection and enzyme-linked immunosorbent assay (ELISA). Cell culture and DNA transfections were carried out as previously described (19) except that COS cells were grown to confluence, serum starved for 24 h, and then replated at a 1:2 dilution in medium containing 10% fetal calf serum 17 h before transfection.

ELISA analyses were carried out by the sandwich technique as previously described (19), using antibody to human A1PI (no. A012-075; DAKO, Santa Barbara, Calif.) at 5 µg/ml to coat the plate and peroxidase-conjugated anti-human A1PI (no. 3201-0431; Organon Teknika, Malvern, Pa.) diluted 1:1,000 as an indicator. Standard curves for quantification were established by using purified human A1PI (no. A-6150; Sigma Chemical Co., St. Louis, Mo.).

Transfection efficiency was monitored 72 h after transfection. The cells, grown on cover slips, were fixed and treated with antibody as described below for double immunofluorescence, using tetramethylrhodamine isothiocyanate (TRITC)-WGA to identify the Golgi of all cells and anti-A1PI plus fluorescein isothiocyanate (FITC)-conjugated anti-immunoglobulin to identify transfected cells. A total of 400 to 600 cells were analyzed in each sample population for the presence of A1PI, and the ratio of the number of A1PI-positive cells to total cells counted was determined to be the transfection frequency. The sample size for counted cells was chosen to provide a 95% confidence limit for the transfection frequency.

Immunofluorescence. Immunofluorescence analyses were performed on cells 72 h after transfection. Cells growing on no. 1 glass cover slips were fixed in 3.7% Formalin for 20 min at room temperature, permeabilized with 1% Nonidet P-40, incubated with primary antibody (DAKO rabbit anti-human A1PI or rat monoclonal anti-BiP supplied by David Bole, Yale University, New Haven, Conn.) for 30 min at 37°C, washed three times with phosphate-buffered saline plus 0.1% Tween 20, and incubated with detecting antibody (FITC- or TRITC-conjugated goat anti-rabbit or anti-rat immunoglobulin G (IgG) [Organon Teknika]) for 30 min at 37°C. Cover slips were mounted with aquamount (0.1% glycerol, 0.1 M NaHCO₃, 5.5 mM phenylaminediamine), cell side down, on glass microscope slides and observed with epifluorescence on a Zeiss photomicroscope II, using a 63× oil objective. To identify the Golgi apparatus, unpermeabilized cells were

treated with wheat germ agglutinin (Sigma L-9640, 0.2 mg/ml) diluted in phosphate-buffered saline containing 1 mM CaCl₂ and 1 mM MgCl₂ to block surface binding sites, followed by permeabilization and incubation, with TRITC-conjugated wheat germ agglutinin (Sigma L-5266, 10 µg/ml) added with the primary antibody.

Immunoabsorption and endoglucosidase H digestion. Immunoabsorption and polyacrylamide gel electrophoresis were carried out by standard techniques. To radiolabel proteins, cells were incubated 62 to 74 h posttransfection in methionine-free medium for 45 min and then placed in medium containing 40 or 80 µCi of [³⁵S]methionine per ml (ICN Pharmaceuticals Inc., Irvine, Calif.), as indicated for each experiment. Cells were scraped from the dish and lysed in 2× RIPA buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS]). Immunoabsorption of medium samples (typically 3 ml) and cell lysates (from a 100-mm dish culture) was performed by using antibody bound to either CNBr-activated Sepharose 4B (Pharmacia Diagnostics, Piscataway, N.J.) according to the instructions of the manufacturer or by using Formalin-fixed *Staphylococcus aureus* (14). Before immunoabsorption with specific antibody, media and lysate were precleared overnight by rotation-incubation at 4°C with bacteria or Sepharose which carried bound immunoglobulin from nonimmune sera. The nonspecific adsorbed material was removed by centrifugation. The appropriate antibody-bound material (usually 25 µl) was added to the cleared samples and rotated for 4 to 6 h at 4°C; the immunoabsorbed material was removed from the sample by centrifugation. Stop-loading buffer (final concentration of 10 mM NaPO₄ [pH 6.5], 1% SDS, 10% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol) was added to the samples, which were then boiled for 3 min and centrifuged to pellet the bacteria or Sepharose; the immunoreactive proteins in the supernatant were resolved on polyacrylamide in a 25 mM Tris buffer containing 0.1% SDS and 186 mM glycine. Gels were fixed, stained, treated with 0.5 M salicylic acid plus 5% glycerol, dried, and exposed to Cronex film (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

For endoglucosidase H treatment, before addition of stop-loading buffer, the immunoabsorbed pellet was suspended in 25 µl of 30 mM sodium citrate (pH 5.0) containing 0.5 mM phenylmethylsulfonyl fluoride, 0.5 µg of bovine serum albumin per ml, and 0.02% SDS plus 1 mUnit of endoglucosidase H (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and incubated at 37°C for 14 to 16 h. Densitometric analyses of autoradiograms were carried out with a Hoefer GS300 scanning densitometer under conditions in which the response of the film and the instrument was in the linear range. Computer-assisted integration of the area under the peaks was used to derive a relative area per density unit for each band.

RESULTS

Construction of mutants of the human A1PI gene. Using oligonucleotide-directed mutagenesis, we constructed mutants of a normal human A1PI cDNA gene cloned into a pSV7 vector. Mutagenic oligonucleotides were designed to alter the codon of interest as well as to insert or remove unique restriction sites without altering the encoded amino acid sequence (20). To produce the amino acid change at residue 342 that is observed in the naturally occurring Z mutation of the A1PI gene, a GAG codon was changed to AAG, which resulted in the insertion of Lys instead of Glu

TABLE 1. A1PI salt bridge mutants

Gene/protein	Residue 290	Residue 342	% M secreted ^a	Salt bridge ^b
A1PI/M	CCC AAA CTC	GAT GAG AAA	100	Yes
	Pro Lys Leu	Asp Glu Lys		
A1PI/Z	CCC AAA CTC	GAT AAG AAA	19	No
	Pro Lys Leu	Asp Lys Lys		
A1PI/SB	CCC GAA CTC	GAT GAG AAA	75	No
	Pro Glu Leu	Asp Glu Lys		
A1PI/SBZ	CCC GAA CTC	GAT AAG AAA	46	Yes
	Pro Glu Leu	Asp Lys Lys		

^a Percentage of A1PI secreted by transfected COS cells, determined by ELISA on culture medium 72 h posttransfection; values are normalized for transfection frequency.

^b Potential for salt bridge formation between residues 290 and 342.

(Table 1) and thus the production of an altered form of the inhibitor (A1PI/Z-342Lys). This substitution results in a possible significant change in primary structure and destroys the potential to form a salt bridge between residues 342 and 290. A second mutant, A1PI/SB-290Glu, was constructed by altering the AAA codon specifying Lys-290 to GAA, specifying Glu. Such a substitution disrupts the potential for formation of a salt bridge between residues 290 and 342 but does not alter the Glu₃₄₂ found in the wild-type protein. The proteins encoded by the two mutants just described are similar in that they have lost the potential to form the 290-342 salt bridge, but they differ in the amino acid residues at these positions; i.e., the A1PI/Z-342Lys mutant protein has the normal Lys residue at position 290, with a Lys substitution at 342, whereas the A1PI/SB-290Glu mutant protein has the normal Glu residue at 342, with a Glu substitution at position 290 (Table 1).

A third mutant of the A1PI gene was constructed by replacing a restriction fragment of the A1PI/Z-342Lys gene which encodes the 290 residue with the same fragment from A1PI/SB-290Glu. The resulting gene (A1PI/SBZ-290Glu-342Lys) specifies a protein which is potentially capable of forming the 290-342 salt bridge but has the Z-type altered residue (Lys) at position 342. All constructs were restriction mapped and sequenced to confirm the induced mutations.

Transient transfection of COS cells with mutant A1PI genes. Introduction of A1PI genes into COS cells via the pSV7 vector provided an opportunity to study transient gene expression and secretion of A1PI. COS cells constitutively express simian virus 40 large T antigen, allowing autonomous replication of vectors containing the simian virus 40 origin of replication, and thus measurable quantities of gene product can be obtained in 48 to 72 h. To assess the secretion of A1PI by transfected COS cells, the culture medium was collected 72 h posttransfection and immediately assayed by ELISA. To control for variation in transfection efficiency between and within experiments, secretion of A1PI was normalized to transfection frequency. The percentage of positively transfected cells in a 400- to 600-cell sample of each population was determined by double-immunofluorescence analysis. Transfection frequencies ranged from 10 to 18%. Results corrected for transfection frequency, i.e., nanograms of A1PI secreted per milliliter divided by percent transfection frequency, did not significantly differ from the uncorrected data (see Table 2).

COS cells transfected with A1PI genes synthesized and secreted a protein that was immunologically reactive with antisera to human A1PI. Cells transfected with A1PI/Z-342Lys secreted only 19% of the level of A1PI secreted by

TABLE 2. A1PI secretion: ELISA analysis of media from transfected COS cells

Transfecting DNA	Uncorrected			Normalized	
	OD ₄₁₆ ± SD ^a	Avg A1PI concn (ng/ml) ^b	% of A1PI/M	Secretion/transfection frequency ^c ± SD	% of A1PI/M
Mock	0.07 ± 0.03	0	0	0	0
A1PI/M	2.25 ± 0.23	95.8	100	888 ± 0.03	100
A1PI/Z	0.39 ± 0.07	15.2	16	168 ± 0.10	19
A1PI/SB	1.66 ± 0.13	70.2	73	671 ± 0.04	75
A1PI/SBZ	1.07 ± 0.20	44.7	47	408 ± 0.03	46

^a Average from three separate experiments.

^b In medium 72 h posttransfection, calculated from sample OD₄₁₆ - mock OD₄₁₆, using straight line derived from A1PI standards ($y = 43.3x + 1.37$).

^c Average of determinations from three separate experiments, based on concentration of A1PI in medium 72 h posttransfection; measured by ELISA and calculated as described below. Transfection frequency was determined by double-immunofluorescence analysis. Transfection frequencies ranged from 0.10 ± 0.026 to 0.18 ± 0.034 . Error in the frequency determination was calculated at the 95% confidence limit, using $p \pm Z_{\alpha/2} \sqrt{pq/n}$, in which $n = 500$.

cells transfected with A1PI/M, a level similar to that observed in individuals suffering from A1PI deficiency (i.e., 15%). The same results were observed by using a similar A1PI mutant gene, A1PI/ZZ, provided by Ian Bathurst (data not shown). Cells transfected with another mutant unable to form a salt bridge between residues 290 and 342, A1PI/SB-290Glu, secreted 75% of the normal level, whereas cells transfected with the salt bridge-competent Z-type mutant, A1PI/SBZ-290Glu-342Lys, secreted 46% of the normal level (Table 2).

Experiments were carried out to determine whether differential turnover or uptake of secreted A1PI was affecting the value established for percent secretion. Culture medium from transfected cells was collected at 72 h posttransfection, a 700-μl portion was reserved for ELISA to quantify A1PI concentration, and the remaining conditioned medium was placed on nontransfected control COS cells. The A1PI concentration in the transferred conditioned medium after 3 days was determined in six separate experiments. The averaged results (presented as optical density at 416 nm [OD₄₁₆] at day 3 per OD₄₁₆ at day 0) were 1.04 ± 0.08 for A1PI/M, 0.95 ± 0.08 for A1PI/SB, 0.92 ± 0.08 for A1PI/SBZ, and 0.98 ± 0.12 for A1PI/Z. These results show that decreased concentrations of the variant forms of A1PI found in the growth media of the transfected COS cells were not the result of differential uptake or turnover.

Kinetics of A1PI secretion from COS cells. To study the effect of the mutations on intracellular transport of A1PI, transfected cells were pulse-labeled in the presence of 80 μCi of [³⁵S]methionine per ml for 15 min and then chased in the presence of 2.5 mM excess nonradioactive methionine for various time periods. The time course of secretion was determined by SDS-polyacrylamide gel electrophoresis analysis of immunoabsorbed A1PI from chase cell lysate and culture medium samples. The autoradiograms were scanned with a densitometer, and the area under the peaks was determined as a measure of the amount of radioactive A1PI in the sample. The results are presented in Fig. 1 and summarized in Table 3. Since half (51%) of the total A1PI secreted by cells transfected with A1PI/M was detected in the medium at 60 min, the 60-min time point was used to determine the secretion rate (percent A1PI secreted per minute) for each mutant. The secretion rate for A1PI/M was

FIG. 1. Secretion time courses for A1PI/M, A1PI/SB, A1PI/SBZ, and A1PI/Z. Transfected COS cells were pulse-labeled with radioactive methionine and chased in nonradioactive methionine for 30, 60, or 90 min. Cell lysates and medium samples from 100-mm culture dishes of cells were immunoadsorbed with rabbit anti-human A1PI. The immunoreactive proteins were separated by gel electrophoresis on 7% SDS-polyacrylamide, and the [35 S]methionine-labeled proteins visualized by radioautography. M, SB, SBZ, and Z indicate samples from COS cells transfected with pSV₇A1PI/M, pSV₇A1PI/SB-290Glu, pSV₇A1PI/SBZ-290Glu-342Lys, and pSV₇A1PI/Z-342Lys, respectively. Open arrows indicate the high-mannose form of A1PI; closed arrows indicate the complex-carbohydrate form of A1PI. Molecular size standards (m) are 95.5, 67, 55, and 43 kilodaltons.

0.85, and, as expected, the mutant proteins were secreted at very different rates. The secretion rate for A1PI/SB at 60 min was 0.60, or 71% of the rate for A1PI/M; the secretion rate for A1PI/SBZ was 0.35, or 41% of the rate for A1PI/M; and the rate of secretion for A1PI/Z was 0.17, or 20% of the rate for A1PI/M. These results are similar to and confirm the steady-state secretion data presented above.

Absence of valine 213 substitution mutation. Examination of A1PI genes for the presence of the Val-213-to-Ala mutation was accomplished by *Bst*EII restriction endonuclease digestion. The Val-to-Ala mutation alters the restriction site for the endonuclease *Bst*EII (G'GTGACC) by a point mutation to G'GCGACC (23). Vector DNA containing either the A1PI/M gene or the A1PI mutant genes was digested with *Bst*EII and resolved on 1% agarose (Fig. 2). A second restriction digest (*Eco*RI) was included to yield a diagnostic size DNA fragment. All A1PI genes in this study had the nucleotide sequence recognized by the *Bst*EII enzyme; i.e., the sequence that specified valine at position 213.

Intracellular location of A1PI in transfected COS cells. To determine the intracellular location of A1PI in transfected cells, double-immunofluorescence analyses were performed. A1PI was detected by either FITC- or TRITC-conjugated anti-rabbit IgG after treatment of the cells with rabbit anti-human A1PI. Golgi complex was identified by TRITC-conjugated wheat germ agglutinin (31), and ER was indicated by rat monoclonal antibody to BiP (3) plus FITC-conjugated anti-rat IgG. BiP is an ER-resident protein that has been exclusively localized to this organelle by immunohistochemical methods (2).

Intracellular A1PI was detected in the Golgi of cells transfected with the wild-type gene A1PI/M and in the ER of cells transfected with Z-variant gene A1PI/Z-342Lys (Fig. 3). Double-immunofluorescence analyses of COS cells transfected with A1PI/M (Fig. 3a through d) showed colocalization of A1PI/M with WGA (Fig. 3a and b, respectively) but not with BiP (Fig. 3c and d). COS cells transfected with A1PI/Z-342Lys (Fig. 3e through h) did not show colocalization of A1PI with WGA (Fig. 3e and f); however, A1PI precisely localized with the ER-resident protein BiP (Fig. 3g and h). Thus, in this system, A1PI/Z accumulates in the ER, correctly duplicating the site of accumulation of A1PI/Z in the livers of individuals homozygous for this allele.

Endoglucosidase H digestion analysis was used to confirm the intracellular location of the accumulated A1PI/Z. This enzyme cleaves the high-mannose form of the three N-linked carbohydrates added to A1PI in the ER (28). The core carbohydrates are modified in the Golgi to a complex form that is insensitive to endoglucosidase H activity. For these studies, A1PI was biosynthetically labeled by exposure of transfected cells to 40 μ Ci of [35 S]methionine per ml during the last 6 h of transient transfection. Culture medium was collected, and cells were harvested by scraping and then lysed in RIPA buffer. All samples were immunoadsorbed by using antibody bound to *S. aureus*; the immunoreactive proteins were treated with endoglucosidase H and analyzed by autoradiography of SDS-polyacrylamide gels (Fig. 4) as described in Materials and Methods. Intracellular A1PI/Z was sensitive to endoglucosidase H digestion, which confirmed the immunofluorescence data indicating that intracellular A1PI/Z accumulates in the ER.

Association of the A1PI/Z protein with the ER-resident protein BiP. Further evidence of deficient secretion and accumulation of A1PI/Z in the ER comes from studies to assess the association of A1PI/Z with BiP. The glucose-regulated heavy-chain-binding protein (BiP) appears to be

TABLE 3. Secretion time courses of A1PI/M, A1PI/SB, A1PI/SBZ, and A1PI/Z

Transfecting DNA	Band area/density unit ^a at chase time (min) of:							% A1PI secreted ^b at chase time (min) of:				
	0 (lysate)	30		60		90		30	60	90	Secretion rate ^c	% of A1PI/M secretion rate
		Medium	Lysate	Medium	Lysate	Medium	Lysate					
A1PI/M	13,576	3,192	10,110	7,281	6,832	9,231	2,845	24	51	76	0.85	100
A1PI/SB	21,634	2,463	17,689	6,898	12,014	9,450	4,151	12	36	69	0.60	71
A1PI/SBZ	16,818	1,145	20,832	3,100	11,920	6,292	9,442	5	21	40	0.35	41
A1PI/Z	13,306	589	18,347	1,426	12,738	2,779	12,907	3	10	18	0.17	20

^a Amount of A1PI in the sample based on scanning densitometry and computer-facilitated calculations of area under the curves. Data are from two experiments, corrected for transfection frequency.

^b Determined as units of A1PI in medium per total medium-plus-lysate units.

^c Percent of A1PI secreted per minute at the 60-min time point.

involved in the ER retention of unassembled immunoglobulin molecules and some improperly folded proteins (3, 10, 11). To study the possibility that BiP has a role in preventing the transport of A1PI/Z from the ER, we examined the ability of A1PI/Z to associate with BiP in transfected COS cells. Proteins were biosynthetically labeled by exposure of transfected cells to 40 μ Ci of [³⁵S]methionine per ml during the last 6 h of transient transfection. Culture medium was collected, cells were harvested by scraping and lysed in RIPA buffer, and the samples were immunoadsorbed by using antibody bound to Sepharose. Lysate samples were first immunoadsorbed with Sepharose bound to anti-BiP, followed by immunoadsorption with Sepharose bound to anti-A1PI. The immunoreactive proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography (Fig. 5). A1PI was not present in samples immunoadsorbed with antibody against BiP, and neither BiP nor any other labeled protein was present when the samples were immunoadsorbed with antibody against A1PI. Thus, under the conditions of these experiments, association of A1PI/Z with BiP could not be demonstrated. Furthermore, we did not detect an increase in the quantity of BiP in cells showing ER accumulation of A1PI/Z (compare lanes with anti-BiP-adsorbed lysates in Fig. 5). In addition, these studies showed deficient secretion of A1PI/Z (compare culture

medium lanes for A1PI/Z and A1PI/M in Fig. 5) and intracellular accumulation of A1PI/Z (compare anti-A1PI-adsorbed lysate lanes in Fig. 5). Also, the secreted form had a higher molecular weight, which indicated normal carbohydrate processing before secretion.

DISCUSSION

One objective of this study was to determine whether both the Lys-for-Glu-342 and the Ala-for-Val-213 amino acid replacements must occur to produce an A1PI that is inefficiently secreted and, if both replacements are not required, to establish which one has the dominant effect on the secretion of this protein. Our results show that the Lys-for-Glu-342 substitution alone is sufficient for the retention of A1PI in the ER, which indicates that the Ala-for-Val-213 substitution is silent with respect to effects on the secretion of A1PI. These data also show that A1PI deficiency is not the result of a mutation in a cosegregating allele which affects some other component required for the efficient secretion of this protein; i.e., the single amino acid replacement at position 342 is sufficient to cause the defective secretion of A1PI.

The major objective of this study was to test the idea that disruption of a salt bridge between residues 290 and 342 in A1PI/Z is responsible for the defective secretion of this protein. A salt bridge between residues 290 and 342 has been identified by X-ray crystallographic analysis of the cleaved form of A1PI/M (17). Whether this salt bridge exists in the native form of the protein is unknown. A popular belief is that the Lys-for-Glu-342 substitution in A1PI/Z disrupts the interaction between residues 290 and 342, resulting in a less stable molecule, possibly with very different folding properties, which causes retention of A1PI/Z in the ER (17). Our studies clearly show this is not the case. The genetically engineered A1PI designated A1PI/SB contains a Glu-for-Lys-290 substitution and lacks the potential to form the 290-342 salt bridge. This altered protein is secreted at 75% of the level of the normal protein. Furthermore, when we returned the potential to form the 290-342 salt bridge to A1PI/Z by constructing another mutant, A1PI/SBZ-290Glu-342Lys, the mutant protein was secreted poorly (46% of normal) and accumulated in the ER. Together, these data show that the major cause for the defective secretion of A1PI/Z is not the result of a loss of the potential to form the 290-342 salt bridge.

A study by Forman (9), published during the preparation of this paper, supports this conclusion. His results demonstrated that normal and salt bridge mutant (analogous to our A1PI/SB) proteins were efficiently secreted by *Xenopus*

FIG. 2. Agarose gel analysis of the Val-213-to-Ala mutation by *Bst*EII digestion. Lanes: 1, uncut pSV₇A1PI/M; 2, *Eco*RI linearization of pSV₇A1PI/M; 3a, 4a, 5a, 6a, and 7a, DNA restricted with *Bst*EII; 3b, 4b, 5b, 6b, and 7b, DNA restricted with both *Bst*EII and *Eco*RI; 3, pSV₇A1PI/M; 4, pSV₇A1PI/SB-290Glu; 5, pSV₇A1PI/SBZ-290Glu-342Lys; 6, pSV₇A1PI/Z-342Lys; 7, pSV₇A1PI/ZZ; 8, *Hind*III-plus-*Eco*RI-restricted lambda DNA marker.

FIG. 3. Intracellular location of A1PI in transfected COS cells by double immunofluorescence. A1PI is identified in panels a and e by FITC-conjugated goat anti-rabbit IgG and in panels c and g by TRITC-conjugated goat anti-rabbit IgG after treatment of fixed cells with rabbit anti-human A1PI. Golgi complex is identified in panels b and f by TRITC-conjugated WGA. ER is identified in panels d and h by rat anti-BiP plus FITC-conjugated goat anti-rat IgG. (a, b, c, and d) Cells transfected with pSV₇A1PI/M; (e, f, g, and h) cells transfected with pSV₇A1PI/Z-342Lys. Each photomicrograph set (i.e., left and right panels) contains the same cells visualized at different wavelengths. Bar, 10 μ m.

FIG. 4. Effect of endoglucosidase H treatment of intracellular and secreted A1PI. Autoradiography of immunoadsorbed [35 S] methionine-labeled proteins after separation by electrophoresis on 12% SDS-polyacrylamide. Cell lysates (L) and medium samples (M) from COS cells transfected with pSV $_7$ A1PI/Z-342Lys were treated with endoglucosidase H (LE and ME) for 14 h at 37°C. The four bands in lane LE represent removal of each of the three carbohydrate chains from A1PI/Z. The same results were seen when intracellular A1PI/M was treated with endoglucosidase H (data not shown).

oocytes injected with mRNA synthesized in vitro from cloned genes. There are at least two potentially important differences in the results of Forman and the data presented here. First, the oocytes failed to secrete detectable levels of A1PI/Z. In contrast, the transiently transfected COS cells secreted about the same proportion of A1PI/Z, relative to A1PI/M, as did individuals suffering from A1PI deficiency. These observations suggest that the COS cell system may be better suited for studies on the secretion of A1PI/Z than are *Xenopus* oocytes. Second, we consistently observed a moderate (25%) inhibition of secretion of A1PI/SB by COS cells.

Because this COS cell model system secretes the same relative amounts of A1PI/M and A1PI/Z as are observed in vivo, these data suggest that a salt bridge between residues 342 and 290 does occur in the native inhibitor and that its disruption in mutant A1PI/SB results in a conformational change which minimally affects secretion. In addition, return of the potential to form a salt bridge in A1PI/Z by changing Lys-290 to Glu did result in an increase in secretion from 19% of the wild-type level for A1PI/Z to 46% for A1PI/SBZ. The idea that disruption of a salt bridge between residues 290 and 342 causes a conformational change in A1PI is consistent with recent data suggesting that A1PI/Z has a reduced affinity for elastase, which has been attributed to slight conformational changes caused by disruption of the Lys-290-Glu-342 salt bridge (24). Our observations suggest that the Lys for Glu-342 substitution that occurs in A1PI/Z does disrupt a salt bridge in the native molecule and that the resulting conformational change does affect secretion but not enough to account for the drastically reduced rates of secretion observed in individuals with A1PI deficiency.

FIG. 5. Analysis of association of BiP with A1PI by immunoadsorption. Autoradiography of [35 S]methionine-labeled proteins after separation by electrophoresis on 7% SDS-polyacrylamide. Total cell lysates (L) or culture medium (M) from COS cells transfected with pSV $_7$ A1PI/M or pSV $_7$ A1PI/Z-342Lys were immunoadsorbed with anti-BiP (B) or anti-A1PI (A). A1PI-i, High-mannose, major intracellular form of A1PI; A1PI-s, complex-carbohydrate, secreted form of A1PI. Molecular weight standards on the right are in kilodaltons.

In view of the results of these independent studies showing that a mutant form of A1PI which lacks the potential to form the salt bridge between residues 290 and 342 is efficiently secreted, it seems unlikely that this structural feature of A1PI is a major factor in determining the rate of secretion of this protein. On the other hand, in both cases where Lys was substituted for Glu-342 (A1PI/Z-342Lys and A1PI/SBZ-290Glu-342Lys), we found that A1PI secretion was significantly decreased and that the altered proteins accumulated in the ER. On the basis of these observations, we conclude that the major cause for the defective secretion of A1PI/Z is the replacement of Glu-342 with Lys.

The accumulation of A1PI/Z in the ER of individuals suffering from A1PI deficiency and, as shown in our studies, in COS cells transiently transfected with appropriately modified genes raises the possibility that this altered protein may interact with resident ER proteins and thereby be retained. The ER-resident protein BiP appears to be responsible for preventing transport of unassembled immunoglobulin molecules from the ER (3), associates with improperly folded proteins (10), is induced by the presence of malformed proteins (15), and therefore may be involved in the retention of these proteins in the ER. In our experiments, BiP was not induced by the presence of accumulated A1PI/Z. Furthermore, A1PI/Z was not precipitated by anti-BiP, and neither BiP nor any other protein coprecipitated with A1PI/Z when cellular extracts were treated with anti-A1PI. It seems unlikely, therefore, that A1PI/Z is retained in the ER because of an association with resident proteins. The recent results of Kozutsumi et al. (15) indicate that BiP specifically interacts with improperly folded proteins and that these malformed proteins induce the synthesis of BiP. These

results and our observations argue that A1PI/Z is essentially correctly folded and that the defective secretion of this protein does not result from gross structural changes.

How then is A1PI/Z retained in the ER? The results of our studies with mutants of A1PI indicate that defective transport of A1PI/Z is largely due to the substitution of a positively charged Lys residue for the negatively charged Glu-342. Although there are other plausible mechanisms, we favor the idea that this substitution alters the affinity of A1PI/Z for a receptor that is involved in the movement of this protein from ER to Golgi. There are no confirmed examples of receptors that channel proteins from ER to Golgi, but on the basis of the kinetics of movement of secretory and membrane proteins (7, 8, 16, 22, 26, 33) and the increased rate of secretion of C-reactive protein during the acute-phase response (18), other investigators suggest that carrier-mediated transport between these organelles may exist. We also suspect that the 19% of normal secretion of A1PI/Z from transfected COS cells reported here and the 15% of normal secretion of A1PI/Z in individuals homozygous for the A1PI/Z allele reflect the amount which exits the ER by nonselective bulk flow (32) and that this process is not efficient enough to prevent the accumulation of A1PI/Z in the ER. Analysis of the A1PI amino acid sequence by hydrophobicity profiles (12) shows that Glu-342, which occurs in the sequence Asp Glu Lys, is located in a hydrophilic region that is likely to be available for interaction with a receptor. Further experiments using oligonucleotide-directed mutagenesis to alter the other residues in this hydrophilic sequence and to test the effects of these changes on the secretion of A1PI are in progress.

Taken together, our results indicate that the previously well-accepted hypothesis, that loss of a salt bridge in A1PI/Z leads to the observed secretory defect, is invalid. Conversely, the data presented here are consistent with the signal-receptor concept of selective export from the ER and suggest that strategically placed charged amino acids may serve as signals from such interactions. Further experimentation, however, will be necessary before firm conclusions regarding these ideas can be reached.

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ADDENDUM IN PROOF

After this article was accepted for publication, Brantly et al. (Science 242:1700-1702, 1988) reported that the secretion defect seen for A1PI/Z could be repaired by a second mutation that changes the residue Lys-290 to Glu (the A1PI/SBZ gene construct reported here). Perhaps the basis for the differences between our data and those reported by Brantly et al. is that our studies (this paper and a preliminary report [J. Cell Biochem. 2B(Suppl.):286, 1988]) include both steady-state and kinetic analyses of secretion, quantified by two different methods and normalized for both sample size and transfection frequency. Our view, that the inefficient secretion of A1PI/Z is a direct result of the replacement of Glu-342 with Lys rather than the result of secondary effects due to the presence or absence of a salt bridge, is unchanged.

LITERATURE CITED

1. Bathurst, I. C., J. Travis, P. M. George, and R. Carrell. 1984. Structural and functional characterization of the abnormal Z alpha-1-antitrypsin isolated from human liver. *FEBS Lett.* 177: 179-183.
2. Bole, D. G., M. Doriaux, and J. D. Jamieson. 1987. Immunocytochemical localization of the immunoglobulin heavy chain binding protein, BiP. *J. Cell. Biol.* 105:77a.
3. Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Post-translational association of immunoglobulin heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102: 1558-1566.
4. Callea, F., J. Fevery, G. Massi, C. Lievens, J. de Groote, and V. J. Desmet. 1984. Alpha-1-antitrypsin (AAT) and its stimulation in the liver of PiMZ phenotype individuals. A "recruitment-secretory block" ("R-SB") phenomenon. *Liver* 4:325-337.
5. Carrell, R. W., J. O. Jeppsson, C. B. Laurell, S. O. Brennan, M. C. Owen, I. Vaughan, and D. R. Boswell. 1982. Structure and variation of human alpha-1-antitrypsin. *Nature (London)* 298: 329-334.
6. Fagerhol, M. K., and D. W. Cox. 1981. The Pi polymorphism genetic, biochemical, and clinical aspects of human alpha-antitrypsin. *Adv. Hum. Genet.* 11:1-62.
7. Fitting, T., and D. Kabat. 1982. Evidence for a glycoprotein "signal" involved in transport between subcellular organelles. Two membrane glycoproteins encoded by murine leukemia virus reach the cell surface at different rates. *J. Biol. Chem.* 257:14011-14017.
8. Fries, E., L. Gustafsson, and P. Peterson. 1984. Four proteins synthesized by hepatocytes are transported from endoplasmic reticulum to Golgi complex at different rates. *EMBO J.* 3: 147-252.
9. Forman, R. C. 1987. Disruption of the Lys-290-Glu-342 salt bridge in human alpha-1-antitrypsin does not prevent its synthesis and secretion. *FEBS Lett.* 216:79-82.
10. Gething, M.-J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell* 46:939-950.
11. Hendershot, L., D. G. Bole, G. Kohler, and J. F. Kearney. 1987. Assembly and secretion of heavy chains that do not associate post-translationally with immunoglobulin heavy chain-binding protein. *J. Cell Biol.* 104:761-767.
12. Hopp, T. P., and K. R. Woods. 1981. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* 78:3824-3828.
13. Jeppsson, J. O. 1976. Amino acid substitution Glu-Lys in alpha-1-antitrypsin P-1-Z. *FEBS Lett.* 65:195-197.
14. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* 115:1617-1624.
15. Kozutsumi, Y., M. Segal, K. Normington, M.-J. Gething, and J. Sambrook. 1988. The presence of misfolded proteins in endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature (London)* 332:462-464.
16. Lodish, H. F., N. Kong, M. Snider, and G. J. A. M. Strous. 1983. Hepatoma secretory proteins migrate from rough endoplasmic reticulum to Golgi at characteristic rates. *Nature (London)* 304:80-83.
17. Loebermann, H., R. Tokuoka, J. Deisenhofer, and R. Huber. 1984. Human alpha-1-proteinase inhibitor crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. *Mol. Biol.* 177:531-556.
18. Macintyre, S. S., I. Kushner, and D. Samols. 1985. Secretion of C-reactive protein becomes more efficient during the course of the acute phase response. *J. Biol. Chem.* 260:4169-4173.
19. McCracken, A. A., and N. F. Fishman. 1986. Secretion of rat serum albumin by COS cells transfected with a spliced cDNA gene. *J. Biol. Chem.* 261:508-511.
20. McCracken, A. A., K. B. Kruse, and J. L. Brown. 1988. A rapid enrichment selection for mutants resulting from oligonucleotide

- directed mutagenesis of double stranded DNA. *Biotechniques* **6**:332-339.
21. Miller, R. R., M. S. Kuhlenschmidt, C. J. Coffee, I. Kuo, and R. H. Glew. 1986. Comparison of the chemical, physical and survival properties of normal and Z-variant alpha-1-antitrypsins. *J. Biol. Chem.* **251**:4751-4757.
 22. Morgan, E. H., and T. Peters. 1985. The biosynthesis of rat transferrin: evidence for rapid glycosylation, disulfide bond formation, and Z-variant alpha-1-antitrypsins. *J. Biol. Chem.* **251**:4751-4757.
 23. Nukiwa, T., K. Satoh, M. L. Brantly, F. Ogushi, G. A. Fells, M. Courtney, and R. G. Crystal. 1976. Identification of a second mutation in the protein-coding sequence of the Z type alpha-1-antitrypsin gene. *J. Biol. Chem.* **261**:15989-15994.
 24. Ogushi F., G. Fells, R. Hubbard, S. Straus, and R. Crystal. 1986. Z-type alpha-1-antitrypsin (ATT) is less competent than M1-type as an inhibitor of neutrophil elastase. *Am. Rev. Respir. Dis.* **133**(Suppl.):A218.
 25. Perlmutter, D. H., R. M. Kay, F. S. Cole, T. H. Rossing, D. Van Thiel, and H. R. Colten. 1985. The cellular defect in alpha-1-proteinase inhibitor (alpha-1-PI) deficiency is expressed in human monocytes and in *Xenopus* oocytes injected with human liver mRNA. *Proc. Natl. Acad. Sci. USA* **82**:6918-6921.
 26. Scheele, G., and A. Tartakoff. 1985. Exit of nonglycosylated secretory proteins from the RER is asynchronous in the exocrine pancreas. *J. Biol. Chem.* **260**:926-931.
 27. Sifers, R. N., J. A. Carlson, S. M. Clift, F. J. DeMayo, D. W. Bullock, and S. L. Woo. 1987. Tissue specific expression of the human alpha-1-antitrypsin gene in transgenic mice. *Nucleic Acids Res.* **15**:1459-1475.
 28. Tarentino, A. L., R. B. Trimble, and F. Maley. 1978. Endo-N-acetylglucosaminidase from *Streptomyces plicatus*. *Methods Enzymol.* **50**:574-580.
 29. Truett, M. A., R. Blacher, R. L. Burke, D. Caput, C. Chu, D. Dina, K. Hartog, C. H. Kuo, F. R. Masiarz, J. P. Merryweather, R. Najarian, C. Pachl, S. J. Potter, J. Puma, M. Quiroga, L. B. Rall, A. Randolph, M. S. Urdea, P. Valenzuela, H. H. Dahl, J. Favalaro, J. Hansen, O. Nordfang, and M. Ezban. 1985. Characterization of the polypeptide composition of human factor VIII:C and the nucleotide sequence and expression of the human kidney cDNA. *DNA* **4**:333-349.
 30. Verbanac, K. M., and E. C. Heath. 1986. Biosynthesis, processing and secretion of M and Z variant human alpha-1-antitrypsin. *J. Biol. Chem.* **261**:9979-9989.
 31. Vertanen, I., P. Ekblom, and P. Laurila. 1978. Subcellular compartmentalization of saccharide moieties in cultured normal and malignant cells. *J. Cell Biol.* **85**:429-434.
 32. Wieland, F. T., M. L. Gleason, T. A. Serafini, and J. E. Rothman. 1987. The rate of bulk flow from the endoplasmic reticulum to the cell surface. *Cell* **50**:289-300.
 33. Williams, D. B., S. J. Swiedler, and G. W. Hart. 1985. Intracellular transport of membrane glycoproteins: two closely related histocompatibility antigens differ in their rates of transit to the cell surface. *J. Cell Biol.* **101**:725-734.
 34. Yosheda, A., J. Liegerman, L. Gaidulis, and C. Ewing. 1976. Molecular abnormality of human alpha-1-antitrypsin variant (PiZZ) associated with plasma activity deficiency. *Proc. Natl. Acad. Sci. USA* **73**:1324-1328.
 35. Yosheda, A., and T. Mega. 1979. Carbohydrate composition of normal and variant human alpha-1-protease inhibitor. *Arch. Biochem. Biophys.* **195**:591-595.