Secretion of α-1-Proteinase Inhibitor Requires an Almost Full Length Molecule*

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In the human disease α-1-proteinase inhibitor deficiency, some variants of human α-1-proteinase inhibitor are not secreted. These secretory variants contain frameshift mutations leading to products with normal amino acid sequences to the points of the mutations followed by short, aberrant C-terminal sequences and then premature termination (Nukiwa, T., Takahashi, H., Brantly, M., Courtney, M., and Crystal, R. (1987) J. Biol. Chem. 262, 11999–12004; Sifers, R. N., Brashears-Macatee, S., Kidd, V. J., Muench, H., and Woo, S. L. C. (1988) J. Biol. Chem. 263, 7330–7335; Curiel, D., Brantly, M., Curiel, E., Stier, L., and Crystal, R. G. (1989) J. Clin. Invest. 83, 1144–1152). To examine possible causes for lack of secretion of these null variants, we have altered the α-1-proteinase inhibitor cDNA to encode a series of abbreviated forms of this protein that retain authentic sequences to the points of truncation. Examination of the fates of these shortened proteins in transiently transfected Cos 1 cells indicates that the aberrant C-terminal sequences in the naturally occurring variants are not responsible for their lack of secretion and show that truncation prior to Pro381 prevents movement from the endoplasmic reticulum to the Golgi apparatus and therefore secretion. These truncated forms of α-1-proteinase inhibitor do not form inclusion bodies in the endoplasmic reticulum, rather they are degraded, probably by the pre-Golgi pathway. Our results support the idea that a sequence of at least 391 of the normal 394 residues is essential for the secretion of α-1-proteinase inhibitor and suggest that residue 391 plays an especially important role, perhaps in allowing or directing proper folding or as part of a transport signal, in the secretion of this protein.

Human α-1-proteinase inhibitor (A1Pi), 1 a single chain, 52-kDa glycoprotein of 394 amino acids is the archetype of a superfamily of proteins designated serpins (4). Most serpins, including A1Pi whose major physiological function is to inhibit neutrophil elastase (5), are serine protease inhibitors, but a few have other more specialized or unknown functions (4). This family exhibits many common structural features including highly conserved regions of primary structure and, at least for A1Pi and ovalbumin, the higher orders of structure determined from x-ray crystallographic data are very similar (6, 7). A number of human genetic variants of A1Pi appear to be synthesized at normal rates, but are poorly secreted from the liver (8), their major site of synthesis. As a result, the circulating levels of A1Pi in the affected individuals are too low to adequately inhibit elastase released from activated or disintegrating neutrophils, resulting in pulmonary damage frequently culminating in emphysema (9). The sequence changes in these variants have been identified (10), but the means by which these changes affect secretion of A1Pi are unknown. A number of variants of A1Pi, designated as null due to their complete absence from the serum, are truncated forms of this inhibitor resulting from mutations that generate premature termination codons (1–3). The experiments described in this communication were designed to examine possible causes for the failure to secrete these null variants of A1Pi.

EXPERIMENTAL PROCEDURES

Cell Growth—Cos 1 cells (American Type Culture Collection, Rockville, MD) were grown to confluency and then serum-starved for 24 h in serum-free F-12 Eagle’s medium. After starvation, the growth-synchronized cells were collected, diluted, and replated at a density of 2 × 10^5 cells per 908-mm^2 well and incubated for 16 h in F-12 medium containing 10% fetal calf serum. At this point, each well of cells was ready for transient transfection as described below.

Mutagenesis and Expression—A1PiM cDNA ligated into the polylinking site of the replicative form of bacteriophage M13mp19 DNA was mutated by oligonucleotide-directed mutagenesis essentially as described by Kunkel et al. (11) using the reagents and protocols supplied in the Mutagen kit purchased from Bio-Rad. The mutagenic deoxyoligonucleotides were synthesized by Dr. Paul Hagerman (University of Colorado Health Sciences Center, Denver, CO). To confirm that the desired mutations had been produced, each variant was sequenced by the dideoxy chain termination technique using the Sequenase kit from United States Biochemical Corp. The mutated cDNAs were excised from the replicative form of the corresponding M13mp19 genomes and cloned into a pSV, expression vector using restriction enzymes and T4 DNA ligase obtained from Promega Biotec (Madison, WI). Cos 1 cells were transiently transfected with these constructs using DEAE-dextran as described previously (12, 13).

Enzyme-linked Immunosorbent Assay—After incubation for 48 h post-transfection, the growth media on the Cos 1 cells were collected. The amounts of the truncated forms of A1Pi secreted in the preceding 48 h period were then measured by a quantitative enzyme-linked immunosorbent assay of aliquots of growth media using peroxidase-conjugated anti-A1Pi as described previously (12, 13). The optical densities were read on a Cambridge Technologies Inc. model 700 plate reader (Watertown, MA).

Pulse Chase—Following transfection and incubation for 48 h as described above, the Cos 1 cells were incubated for 45 min in methionine-free medium and then for 30 min in medium containing 50 μCi of [35S]methionine per ml (Du Pont-New England Nuclear). At this point, the cells were transferred to standard growth medium, and, at
appropriate times, growth media were collected, and cells were harvested and lysed in buffer containing detergent and protease inhibitors (25 mM Tris-HCl (pH 7.5), 20 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.2% aprotinin). Subsequent analyses by immunoprecipitation, electrophoresis, autoradiography, and scanning densitometry were carried out as described previously (12, 13).

Endogluccosidase H Digestion (Endo-H)—The washed and dried immunoprecipitates were suspended in 45 μL of 50 mM sodium citrate buffer (pH 5.5). 2.2 μL of 2.2% sodium dodecyl sulfate (SDS) was then added to the immunoprecipitates followed by a 5-min incubation at 100°C. Upon cooling, 0.45 μL of 100 mM phenylmethylsulfonyl fluoride (in ethanol) was added to each reaction. Prior to enzymatic digestion, the contents of each tube were divided into two 21-μL aliquots (± Endo-H), one of which received 0.9 μL of 0.005 unit/μL Endo-H stock solution (Sigma) and was incubated for 20 h at 37°C. After this incubation, sample buffer for SDS-PAGE on 10% gels was added, and the samples were separated using SDS-PAGE on 10% gels. The low molecular weight gel standards utilized were from Pharmacia LKB Biotechnology Inc. which we labeled with 14C essentially as described by Sheehan and Yang (31) with the only notable exception being that [14C]Citic acid anhydride was utilized as opposed to [14C]formic acid.

Immunofluorescence—Cos 1 cells were transfected and grown as described above, but on coverslips. After the 48-h incubation, the cells were washed with PBS and fixed by successive 10-min treatments at -20°C first with methanol followed by acetone. The fixed cells were washed with PBS and incubated with affinity-purified rabbit anti-human AlPi for 1 h in the dark at 37°C in a humid chamber. After thorough washing with PBS, the samples were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit-IgG (United States Biochemical Corp.) as before, washed with PBS, mounted with antifade (5 μM p-phenylendiamine dihydrochloride, 100 mM NaHCO3, 70% glycerol), and examined using an Olympus BH-2 microscope. Photography was with Tmax P3200 at 3200 ASA.

RESULTS

To determine whether the aberrant C-terminal sequences of amino acids identified in the null AlPi variants prevent secretion and to establish the minimum length required for secretion of this protein, we constructed and expressed the series of termination mutants, Term 1-9 (Fig. 1). AlPiM and AlPiT9, but not the other truncated variants, were efficiently secreted. These results are compatible with the conclusions that: the minimum length required for secretion of AlPi is 391 amino acids, lack of secretion of the truncated forms of AlPi produced in the naturally occurring null variants is not a consequence of their aberrant C-terminal sequence of amino acids, and a contiguous sequence of amino acids between the N terminus and residue 390 is not sufficient to direct the secretion of AlPi.

Comparisons of the rates of secretion and the disappearance of the intracellular forms of AlPiT8, AlPiT9, AlPiP, and AlPiM are shown in Fig. 2. Similar amounts of AlPiM and AlPiT9 were secreted after 2 h and secretion of both occurred with nearly identical kinetics. While neither AlPiT8 nor AlPiP is efficiently secreted, only about 60% of the initial amounts of these variants could be accounted for after 120 min of chase. In other experiments in which the chase period was extended to 6 h, none of the AlPiT8 was secreted, but a continued slow secretion of AlPiT9, up to about 30% of the initial level, was observed. Significant loss of the intracellular forms of these variants began about 45 min after the start of the chase; after 3.5 h, approximately 50% of both had disappeared; and, after 6 h, less than 20% of their initial levels remained in the cells. We assume that the loss of AlPiT8 and of the AlPiP that was not secreted was due to degradation. On the other hand, essentially all of the AlPiM and AlPiT9 present at the start of the chase was present in the growth medium after 6 h of incubation confirming that these forms are efficiently secreted and indicating that little or no degradation of these variants occurs.

The intracellular locations of AlPiM, AlPiT9, AlPiP, and AlPiP were examined by indirect immunofluorescence

FIG. 1. Construction and expression of a series of termination mutants of AlPi. The nanograms secreted/24 h represent data from two experiments in which AlPi secreted from 5 × 10^6 cells was analyzed in duplicate. These values are corrected for transfection efficiency (12). The open bars indicate authentic AlPi amino acid sequences, and the shaded areas represent the aberrant sequences reported for the null variants. 3' TC deletion from the CTC codon specifying Leu388 initiating a frameshift and generating a nucleotide sequence specifying a sequence of 15 aberrant amino acids followed by a termination signal (TAG) as codon 334 (2). T insertion in the TTA codon specifying Leu392 initiating a frameshift and generating a nucleotide sequence specifying 22 aberrant amino acids followed by a termination signal (TAG) as codon 375 (3). The lack of secretion of AlPi(Hong Kong) and AlPi(Mattawa) has been documented by other investigators (2, 3).

FIG. 2. Rates of secretion of AlPiT9, AlPiM, AlPiP, and AlPiT8 from transfected Cos 1 cells. AlPiM is the most common human form of this inhibitor and is associated with normal serum levels of 20 to 53 pM (8). AlPiP is a relatively common (allelic frequency of about 0.01 to 0.02 (14)), poorly secreted variant in which a single amino acid, Glu52, is replaced by Lys (29). This amino acid replacement appears to limit the transport of AlPi from endoplasmic reticulum and to be directly involved in the decreased secretion of the Z variant (12, 30). AlPiT8 and AlPiT9 are described in Fig. 1. The data are from a typical experiment and are expressed as percent of the intracellular 35S-labeled AlPi present at the zero time point. (O, secreted AlPi; ☆, intracellular AlPi; △, total AlPi, i.e. the summation of secreted and intracellular material.) These measurements were performed at least 3 times on each of the variants shown with essentially the same results. Results identical with those shown for Term 8 were obtained when these procedures were repeated using the plasmids containing the cDNAs specifying Term 1 through Term 7 (data not shown).
(Fig. 3). Based on our earlier observations, the patterns of fluorescence observed for AlPiM and AlPiT9 and for AlPiZ and AlPiT8 correspond to localization in the Golgi apparatus and the endoplasmic reticulum (ER), respectively (12). To confirm these conclusions, the sensitivities of the secreted and intracellular forms of these four variants to digestion by endoglucoosidase H (Endo-H) were examined (Fig. 4). Aliquots of $^{35}$S-labeled material precipitated by anti-AlPi at various times after initiation of the chase (see Fig. 2) were digested with Endo-H prior to analysis. The results show that all of the secreted AlPiM, AlPiT9, and AlPiZ were resistant to digestion by this enzyme. Of the material that remained in the cells after 2 h of chase, $>90\%$ of both AlPiM and AlPiT9, about 20\% of the AlPiZ, and none of the AlPiT8 had developed resistance to Endo-H. These results show that AlPiM and AlPiT9 are efficiently transported from ER to the Golgi apparatus, that at least a small fraction of AlPiZ reaches this organelle, and that AlPiT8 is not transported to the Golgi apparatus.

To examine the possibility that the intracellular forms of AlPiZ and AlPiT8 accumulate due to aggregation and subseuent precipitation (14, 15), cells transfected with the appropriate vectors were analyzed for insoluble forms of AlPi essentially as described by Graham et al. (16). After incubation with $[^{35}]$S-methionine for 48 h, the cells were lysed, and non-aggregated proteins were extracted with buffer containing 1% Nonidet P-40. Proteins insoluble in this buffer were isolated by centrifugation, and the pellets were examined for AlPi by immunoprecipitation and subsequent analysis (see Fig. 2) of material solubilized in 1% (final concentration) sodium deoxycholate. By this criterion, no aggregates of AlPiT8 were detected, but a small fraction of the intracellular AlPiZ ($<0.3\%$ of the total) was insoluble. These observations indicate that retention of AlPiZ and AlPiT8 in the ER is not due to the formation of inclusion bodies.

**DISCUSSION**

Our results show that forms of AlPi differing in length by only one amino acid (390 versus 391 residues) have very different fates. The shorter molecule is not transported from ER to the Golgi apparatus, instead it is degraded, probably by the pre-Golgi pathway reported for other AlPi variants (17) and different proteins that accumulate in the ER (18–20). On the other hand, the longer molecule, extended by 1 residue to include Pro$^391$, is secreted to the same extent and at the same rate as AlPiM and does not undergo significant degradation. Comparison of the sequences of 20 serpins reveals a highly conserved, 9 amino acid sequence at or near their C termini corresponding to residues 385 through 391 in AlPi (4). We$^1$ observed a 50\% decrease in the secretion of AlPi when Lys$^{390}$, present in AlPiM, was replaced by Leu suggesting that changes in this conserved region may have significant effects on the secretion of this protein. Examination of the structure of AlPi predicted from x-ray crystallographic data shows that the first 8 residues of this conserved sequence, found in sheet 5B, are largely internal and that Pro$^{391}$ emerges from the surface to initiate a one-turn C-terminal helix (4). The crystallographic structure does not indicate that Pro$^{391}$ participates in the stabilization of AlPi by interaction with other residues, and it is not apparent that removal of this residue should result in structural changes which may lead to improper folding and consequently defective secretion (21, 22). Truncation of AlPi prior to residue 391 may cause subtle structural changes which lead to a structure that is not competent to exit the ER. We (12) and others (17, 23) have not detected interaction of the poorly secreted AlPiZ, which is inefficiently transported to the Golgi apparatus, with heavy chain binding protein or any other resident ER protein, nor have we observed co-immunoprecipitation of any other protein along with AlPiT8 or the other truncated variants that are not secreted. These observations suggest that failure of the forms of AlPi truncated prior to residue 391 to move to the Golgi apparatus is not linked to interaction with resident proteins which bind and retain these variants in the ER. The current popular notion that proteins exit the ER via bulk flow suggests that aberrant proteins may not be transported due to interaction with resident ER proteins which prevent their movement (24). Our results are not compatible with this idea nor are they compatible with the suggestion that precipitation or inclusion body formation (14, 15) is responsible for the failure of the truncated AlPi variants to exit the ER. We (12, 13) and a number of other investigators (25–28) have proposed that movement from the ER to the Golgi apparatus may be receptor-mediated and that mutations.

\[^2\] In AlPi, this conserved sequence is: Leu-Phs-Met-Gly-Lys-Val-Val-Asn-Pro.

\[^3\] T. Samandari and J. L. Brown, manuscript in preparation.
which alter the interaction of a protein with the receptor result in insufficient or no movement from the ER to the Golgi apparatus. The results presented in this communication suggest that Pro$^{391}$ and perhaps the entire conserved sequence from residue 383 through 391 plays an important role in the secretion of A1Pi and possibly the other serpins. Since the crystallographic structure for A1Pi does not suggest obvious, significant differences in the structure of molecules of 390 and 391 residues, it seems possible that Pro$^{391}$ serves as at least part of a ligand recognized for signal-mediated transport of A1Pi from ER to the Golgi apparatus. Alternatively, truncation prior to residue 391 may cause the A1Pi variants to fold into structures that are not recognized by the putative receptor. Experiments to test the idea that truncation of A1Pi prior to residue 391 prevents interaction with a receptor and thereby prevents secretion are in progress.

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