Intracellular Events Determine the Fate of Antithrombin Utah

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We sought to determine whether intracellular or extracellular events contribute to the decrease in circulating antithrombin (AT) levels that is seen in subjects with the Utah mutation (Pro 407 to Leu). Site-directed mutagenesis was used to recreate this mutation within a previously characterized rabbit AT cDNA. Cell-free expression of the mutated cDNA yielded an AT protein that failed to react with thrombin. Expression of the rabbit AT-Utah protein in transiently transfected Cos cells resulted in a 10-fold decrease in the amount of AT antigen detected in the conditioned media, as compared with that seen with the wild-type recombinant AT. This effect was not caused by variations in transfection efficiency, because AT levels were normalized to the product of a cotransfected plasmid, chloramphenicol acetyl transferase. Moreover, on Northern blot analysis, AT mRNA levels were comparable in cells expressing either the rabbit AT-Utah or wild-type recombinant rabbit AT. Immunoblots of conditioned media from the two populations of transfected cells showed that the recombinant AT-Utah protein was intact. The results obtained with Cos cells were reproduced using permanently transfected Chinese hamster ovary (CHO) cells. Pulse-chase experiments with the CHO lines showed that both initial levels of rabbit AT-Utah after the pulse labeling and the rate of subsequent secretion during the chase period were reduced compared with that seen with cells expressing the wild-type AT. The observed reduction in AT secretion was also observed for the AT-Oslo mutation (Ala 404 to Thr) when recreated in the rabbit AT background, and expressed in Cos cells. In these experiments, the media levels of mutant AT were reduced by 50%, compared with wild-type. These results show that intracellular events, as opposed to accelerated clearance or other extracellular causes, contribute to the paucity of AT secretion seen in these strand 1C AT mutants.

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ANTITHROMBIN (AT) is a 432 amino acid glycoprotein found in plasma, previously known as antithrombin III (AT3). Its primary function is the regulation of thrombin and other procoagulant serine proteases activated in the coagulation cascade. AT achieves this control by acting as a suicide substrate inhibitor; AT forms stable complexes highly resistant to denaturation, after recognition by one of its cognate proteases of its reactive site loop, which includes the reactive center, Arg393-Ser394. The interaction of AT with thrombin or factor Xa is greatly accelerated in the presence of heparin or related glycosaminoglycan molecules. At least two models have been proposed to explain the mechanism of action of AT and related serine protease inhibitor (serpin) molecules with target proteases: the acyl intermediate model, in which thrombin is proposed to be covalently linked to AT cleaved at the reactive center, and the tetrahedral intermediate model, in which a kinetically stable, covalently linked complex is proposed to form in which both inhibitor and enzyme are intact.

Inherited deficiencies of AT predispose affected individuals to thrombotic disease, particularly in combination with additional triggering factors. In many instances, the molecular genetic basis of such deficiencies has now been determined. Deletions or short frameshifts in the AT gene are responsible for type I (quantitative) deficiency, in which the gene product of the affected allele is absent. Qualitative, or type II deficiencies, on the other hand, result from point mutations that alter a single amino acid residue and abolish or decrease either AT-thrombin or AT-heparin interactions. Mutations affecting AT-thrombin interactions are found predominantly toward the C-terminus of the molecule, near the reactive center, whereas mutations affecting heparin-binding cluster in the N-terminal third of AT.

Recently, a group of mutations was described that confer multiple, or pleiotropic, effects upon the resulting mutant AT polypeptides. These mutations cluster between residues 402 and 407 of AT. They are associated with alterations to thrombin (AT) levels that is seen in subjects with the Utah mutation (Pro 407 to Leu) is one of the most extreme examples of these mutations, in that it was originally thought to represent a Type I deficiency; however, on subsequent analysis, the presence of a small amount of nonfunctional, aberrantly migrating mutant gene product was detected immunologically. Two explanations have been proposed to explain the phenotype of these pleotropic mutants, with respect to the circulating levels of AT. The first proposes that extracellular events are responsible for the paucity of circulating AT mutant molecules, in that the mutation leads to accelerated clearance of the mutant AT via hepatic receptors ordinarily specific for inhibitor-protease complexes. The second proposes that intracellular events, such as a block to secretion in the endoplasmic reticulum, account for the decreased circulating levels. This question has been addressed using recombinant rabbit AT molecules in which the Utah mutation has been recreated. The results show that these mutant AT proteins fail to be secreted normally in cell culture systems.

MATERIALS AND METHODS

Materials

Human α-thrombin (>3,300 NIH U/mg; >93% active) was kindly provided by Dr J. Fenton (New York State Division of Biologicals, Albany, NY). Plasmid vector pCMV5 was generously provided by Dr J. Fenton (New York State Division of Biologicals, Albany, NY). Plasmid vector pCMV5 was generously provided by

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Submitted February 16, 1995; accepted June 29, 1995.

Supported in part by Canadian Red Cross Society Grant No. HA 02 92. W.P.S. is a Miled Canadian Red Cross Society/Medical Research Council of Canada Scholar.

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Dr Mark Stinski (University of Iowa, Iowa City, IA) All enzymes for the modification of DNA or RNA, as well as ribonucleotides, deoxyribonucleotides, and mRNA CAP analog methylguanosylguanosine triphosphate [m7G(5')ppp(5')G] were purchased from Promega Biotec (Madison, WI), as was rabbit reticulocyte lysate. The thrombin inhibitor D-phenylalanyl-L-propyl-arginine chloromethyl ketone (PPACK) (>99% active) was purchased from Calbiochem (La Jolla, CA). T7 sequencing kits were from Pharmacia LKB Biotechnology (Baie d’Urfé, Quebec, Canada). Bethesda Research Laboratories (Burlington, Ontario, Canada) was the supplier of competitor Escherichia coli DH5α cells, Lipofectin reagent, and penicillin/streptomycin for cell culture. COS-1 and Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Rockville, MD). Translation-grade (28S) methionine (>1,000 Ci/mmol, 10 μCi/μL) was from New England Nuclear (NEN) Dupont (Mississauga, Ontario, Canada), while mixture of (28S) methionine and (35S) cysteine (TranSlabel) suitable for metabolic labeling of cultured cells was bought from ICN (Mississauga, Ontario, Canada), as were methionine and cysteine-deficient Dulbecco’s media. Protein molecular-weight standards were from BioRad Laboratories (Oakville, Ontario, Canada). Recombinant endoglycosidase F (PNGase F) was from New England Biolabs (Beverly, MA). A kit for site-directed mutagenesis was purchased from Amersham (Oakville, Ontario, Canada). Oligonucleotides were synthesized at the Institute for Molecular Biology and Biotechnology, McMaster University (Hamilton, Ontario, Canada). All other chemicals and reagents were of the highest quality available.

Methods

Site-directed mutagenesis and plasmid construction. A plasmid similar to the previously described pGEM-7Zf(+)-rAT-III[32,43,5] and containing the identical DNA insert, was used to provide single-stranded starting material for mutagenesis, which was performed using the gapped-duplex method in accordance with the kit manufacturer’s instructions (Amersham). The mutagenic oligonucleotide 2369 (5′-GCGAANCGCTTCCTCGTT-3′) was used to alter codon 408 from Pro to Leu. The alteration was confirmed by sequencing of the resulting plasmid, designated pSDM14. The altered AT cDNA was excised from this plasmid with EcoRI and inserted into the EcoRI site of pCMV5, using standard procedures of ligation and transformation of E. coli DH10B. A clone, containing the insert in the appropriate orientation, was isolated after diagnostic restriction digestions of candidate miniprep DNA and designated pCMV5rab (Utah). The same procedure, using nonmutagenized AT cDNA, was used to produce plasmid pCMV5rab(WT).

Construction of pSG5rab(Oslo). To produce an expression vector capable of transient expression of the rabbit AT equivalent of the Oslo mutation (Ala 405 to Thr), a polymerase chain reaction (PCR)-based approach was used. Oligonucleotides 3715 (5′-AGGTTGACCTTCAGGACCAACAGG-3′) and the M13 universal primer were used in a PCR reaction, using pMAT2 plasmid DNA as a template. The 247-bp PCR product was restricted with BseEI and BamHI and combined with a 1,342-bp EcoRI-BseHI restriction fragment of pCMV(rab)WT, and vector pSG5 was cut with EcoRI and BamHI in a three-part ligation, as described. The resulting plasmid was designated pSG5rab(Oslo). The portion of pSG5rab(Oslo) generated by PCR was sequenced in its entirety, and found to contain no changes other than those that were designed. An expression plasmid encoding the wild-type rabbit AT cDNA was constructed by inserting the entire cDNA into the EcoRI site of pSG5. A clone containing the insert in the appropriate orientation was isolated after diagnostic restriction analysis, and designated pSG5rab(WT).

Construction of pMAT-cfu. To express the Utah mutation in a rabbit AT background in a cell-free system, the 450-bp Nsi I-EcoRI fragment of pCMV5(Utah) was used to replace that of pMAT2, and the resulting plasmid was designated pMAT-cfu.

In vitro transcription of plasmid templates and cell-free translation. In vitro transcription reactions using SP6 polymerase were performed as described, after linearization with EcoRI. Resulting transcripts were translated in an mRNA-dependent rabbit reticulocyte lysate as described. After overnight dialysis against TRIS-buffered saline, the translation products were reacted with excess thrombin (0.85 μmol/L) as previously described, and as shown in Fig 1.

Expression in COS-1 cells. COS-1 cells were grown in Dulbecco’s minimum essential medium supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin. Cells grown in 100-mm dishes were transfected at 60% to 80% confluency with 10 μg of AT expression plasmid, either wild-type or mutant, 1 μg of...
radiolabel into protein by trichloroacetic acid (TCA) precipitation, and immunoprecipitated using the sheep-antirabbit AT antibody described below, using a commercially available preparation of heat-inactivated protein G-bearing Streptococcus cells (Omnisorb; Calbiochem, La Jolla, CA).

Isolation of RNA and Northern blot analysis. RNA was isolated essentially by the method of Chomczynski and Sacchi, using the TriZOL reagent as directed by the manufacturer (Canadian Life Technologies, Burlington, Ontario, Canada). Northern blots of formaldehyde agarose gels were made using capillary transfer onto Zetaprobe nylon membranes, and RNA cross-linked onto the membrane by ultraviolet irradiation. A random primer-based probe was prepared, using either the gel-purified entire EcoRI flanked rabbit AT cDNA, or a HindIII-EcoRI fragment of a human glyceraldehyde-3-phosphate dehydrogenase cDNA (Ambion, Austin, TX). Prehybrid-

pSV,CAT and 50 µg of Lipofectin reagent, using a procedure we have previously described. In the experiments whose results are depicted in Figs 2 and 3, the same plates were used for both RNA extraction and enzyme-linked immunosorbent assay (ELISA)-based determination of AT and chloramphenicol acetyl transferase (CAT) levels.

Establishment of permanently transfected CHO cell lines. CHO cell lines were maintained in a-modified essential medium supplemented with 10% FCS and penicillin/streptomycin. Transfections were performed as described above, except that 1 µg of pSV,neo was used as a cotransfecting plasmid. Cells were allowed to recover for 24 hours after transfection, then trypsinized and split 1:3 into 150-mm plates and selected in the presence of 1.0 mg/mL G418, for 10 to 12 days. Colonies were subcultured and expanded, with those expressing both neo resistance and AT selected for future study.

Pulse-chase experiments. CHO cell lines permanently transfected with pCMV5rab(Utah) or pCMVVrab(WT) were grown to 75% to 90% confluence, in 60-mm dishes. Cells were washed twice in phosphate-buffered saline and once with media lacking methionine and cysteine and then starved for these amino acids for 45 minutes. At this point, 35S-methionine and 35S-cysteine (Translabel; ICN, Lachine, Quebec, Canada) was added to a final concentration of 0.1 mCi/mL. The cells were pulse-labeled for 30 minutes. The medium was then removed and replaced with complete media including serum. At various time points, aliquots of the supernatant were removed, and/or the cells were procured from duplicate plates. Cells were lysed in situ with radioimmunoprecipitation assay (RIPA) buffer, supplemented with Triton X-100 to a final concentration of 2% (vol/vol). Extracts and supernatants were normalized to reflect similar levels of incorporation of

Fig 2. Quantification of secreted AT relative to intracellular CAT produced by transfected Cos cells. The amount of AT antigen detected by quantitative ELISA of conditioned media from Cos cells transfected with pCMV5rab(Utah) or pCMVrab(WT) (WT) is shown graphically. Each determination was normalized to the amount of AT antigen detected by quantitative ELISA of cellular protein-containing extracts, expressed as pg CAT per microgram total cellular protein. Error bars, SE of the mean; n, number of transfection experiments performed.

Fig 3. Northern blots of RNA from transfected Cos cells. Northern blotting of a 1% formaldehyde-agarose gel was performed, initially using AT probes (AT) and subsequently, after stripping of the blot, using glyceraldehyde-3-phosphate dehydrogenase (GAP) probes. A shows autoradiograms of blots in which total RNA (30 µg) was electrophoresed per lane, from cells transfected with pCMV5rab(WT) (lanes 1 and 2), with pCMVrab(Utah) (lanes 4 and 5), or from cells transfected with no AT expression plasmid (lane 3). All cells were cotransfected with pSV,CAT. The positions of the 28S and 18S ribosomal RNAs are shown, at right. Quantification of radiographic signals are shown in B, expressed as the ratio of the AT signal to that of GAP.
ization, hybridization, and washing conditions were as recommended by the manufacturer of the membrane (BioRad, Mississauga, Ontario, Canada).

ELISAs. Rabbit AT was purified to apparent homogeneity by the method of Carlson et al 23 and used both to immunize a sheep and to prepare a column of the protein immobilized on CNBr-activated Sepharose. Partially purified IgG was affinity-purified using this column. Aliquots of this preparation were subsequently biotinylated. Trapping ELISAs were performed, using the affinity-purified IgG as the capture antibody, and the biotinylated material as the detecting antibody. Results were quantified using a standard curve for purified rabbit AT. For ELISA determination of CAT, soluble cell extracts were made and quantified according to the manufacturer of the CAT-ELISA assay (Boehringer Mannheim, Mississauga, ON).

Miscellaneous. Previous publications from this laboratory have described routine protocols for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), fluorography, and autoradiography, and concentration of conditioned cell media using Centricon (Amicon, Beverly, MA) units.5,17 All protein samples analyzed by SDS-PAGE in this study were reduced with 2-mercaptoethanol before electrophoresis.

RESULTS

Cell-Free Expression of Rabbit AT-Utah

Initially, we planned to investigate the clearance and hepatic uptake of mutant AT molecules in vivo. To minimize cross-species effects, we decided to produce recombinant rabbit AT molecules, with a view toward purifying these proteins, labeling them, and reintroducing them into the circulation. Although this strategy clearly necessitated the production of recombinant glycosylated forms of AT, we first needed to verify that introducing the Utah mutation into the rabbit AT molecule would indeed lead to dysfunctional AT. Accordingly, we performed site-directed mutagenesis, and manipulated the altered cDNA such that we could produce either wild-type recombinant rabbit AT, or mutant rabbit AT-Utah, in a rapid cell-free expression system. In this system, the AT produced is heterogenous, with only a subpopulation of the AT produced is heterogenous, with only a subpopulation being capable of forming complexes with thrombin. Nevertheless, expression of the AT-Denver, Utah and retained a physical property that distinguished its rabbit AT molecule would indeed lead to dysfunctional AT. According to the results of these experiments, the rabbit-Utah polypeptide were electrophoresed in a 10:1 ratio to take into account the expected difference in the amount of secreted protein between the wild-type and mutant constructs was mediated at the transcriptional or posttranscriptional level. Northern blotting was performed. As shown in Fig 3, both wild-type and AT-Utah—transfected Cos-1 cells harbored intact AT RNA transcripts of ~2.0 kb in size, consistent with that expected from a 1.5-bp cDNA introduced into this vector. In contrast, nontransfected Cos-1 cells did not produce AT-related transcripts. The blot was stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase probes, to control for possible gel-loading differences. Quantification of the AT and GAP signals (Fig 3B) showed that the ratio of AT transcripts to GAP transcripts varied by less than 50% between mutant and wild-type producing cells, with the AT-Utah cells actually producing more AT mRNA than the cells transfected with the wild-type construct (1.39 ± 0.16 v 0.93 ± 0.11). Thus, transcriptional differences were slight and do not account for the difference in amounts of secreted protein that was observed.

Expression of Rabbit-AT Utah in Cos-1 Cells

The pCMV5 expression vector was selected for use in this study because its CMV promoter is active both in Cos-1 and CHO cells. Although CHO cell production was necessary to produce the 100-μg quantities necessary for in vivo analysis, transient expression experiments in Cos-1 cells were performed to assess the efficacy of expression. Initial experiments, in which no cotransfecting plasmid was used, produced unexpectedly low levels of AT antigen in conditioned media from cells transfected with pCMV5rAB(Utah), as compared with those transfected with the wild-type plasmid (data not shown). To investigate this paucity of secreted protein, these experiments were repeated, using cotransfection with CAT plasmids as a means of controlling for transfection efficiency between replicate plates. As shown in Fig 2, 10-fold less AT-Utah than wild-type AT was detected in conditioned media from Cos-1 cells, after normalization to intracellular levels of CAT antigen, to adjust for variations in transfection efficiency (1.0 ± 0.1 v 11.9 ± 2.3 μg AT/pg CAT/μg total protein). The effect was reproducible in five transfection experiments per construct, yielding a highly significant difference (P < .01, using a two-tailed Student’s t-test).

Analysis of AT RNA Expression

To determine whether the difference in AT expression between the wild-type and mutant constructs was mediated at the transcriptional or posttranscriptional level, Northern blotting was performed. As shown in Fig 3, both wild-type and AT-Utah—transfected Cos-1 cells harbored intact AT RNA transcripts of ~2.0 kb in size, consistent with that expected from a 1.5-bp cDNA introduced into this vector. In contrast, nontransfected Cos-1 cells did not produce AT-related transcripts. The blot was stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase probes, to control for possible gel-loading differences. Quantification of the AT and GAP signals (Fig 3B) showed that the ratio of AT transcripts to GAP transcripts varied by less than 50% between mutant and wild-type producing cells, with the AT-Utah cells actually producing more AT mRNA than the cells transfected with the wild-type construct (1.39 ± 0.16 v 0.93 ± 0.11). Thus, transcriptional differences were slight and do not account for the difference in amounts of secreted protein that was observed.

Immunoblot Analysis of Recombinant AT Proteins

Concentrated conditioned media samples containing either the recombinant AT-Utah or the recombinant wild-type protein were electrophoresed in a 10:1 ratio to take into account the concentration difference, and immunoblot analysis was performed. As shown in Fig 4, no gross differences in band pattern were apparent between the two AT preparations. A slight increase in mobility of both intact and enzymatically deglycosylated recombinant AT-Utah, as compared with equivalently treated wild-type protein, was apparent; this is seen most clearly for the coelectrophoresed intact AT-Utah and wild-type recombinant AT (Fig 4, compare lanes 7 and 8). However, no proteolytic fragments of AT-Utah were detected, and prolonged incubation of conditioned media at
Fig 4. Immunoblot analysis of recombinant rabbit AT species secreted from transfected Cos cells. Conditioned media from Cos cells transfected with pCMVrab(WT) (lanes 1, 4, and 5) were analyzed directly, using an affinity-purified sheep-antirabbit AT IgG. Samples from cells transfected with pCMVrab(Utah) (lanes 2, 3, and 6) were concentrated 10-fold before immunoblot analysis, whereas those from cells transfected with pCMVrab(WT) were not. The samples shown in lanes 3 and 5 were deglycosylated with endoglycosidase F before electrophoresis. Lanes 7 and 8 are replicates of lanes 1 and 2 electrophoresed in a central portion of a second gel to minimize edge effects and highlight the difference in mobility. The arrows to the left of the figure show the position of prestained low molecular-weight markers (BioRad Laboratories, Oakville, ON). All samples were reduced before electrophoresis.

37°C before immunoblotting did not significantly change the banding pattern obtained (not shown).

Pulse-Chase Experiments

Permanently transfected CHO cell lines expressing either the recombinant wild-type rabbit AT or the recombinant rabbit AT-Utah were established to obtain a cell line in which AT-Utah production was elevated, as might be expected if the construct was integrated in a particularly favorable genomic location. However, all 20 lines generated expressed AT-Utah at roughly 10% the wild-type AT levels, with a single exception; the U8 line expressed AT-Utah at roughly 25% of wild-type (not shown). Efforts to purify AT-Utah from conditioned media of this cell line were unsuccessful.

Pulse-chase experiments were performed to compare the relative rates of synthesis and secretion of the wild-type AT and AT-Utah polypeptides in two CHO cell lines. The two cell lines selected for analysis maintained the 1:10 (Utah:wild-type) ratio of AT secretion observed in our transient experiments with Cos cells. Immunoprecipitates (shown in Fig 5) of intracellular AT species show a reduction over time in the wild-type recombinant AT band, as expected for a secreted protein. Despite the fact that equal amounts of TCA-precipitable counts were immunoprecipitated, the AT-Utah band was barely detectable and varied little with time. As illustrated in Fig 5B, 5 hours after the pulse, levels of intracellular AT had dropped by 70%. In contrast, no decrease in intracellular AT-Utah was seen; initial levels of intracellular AT-Utah were ~20% that of the wild-type AT.

Levels of Secreted Rabbit AT-Oslo

The same type of experiments were performed with another of the pleiotropic AT mutants, AT-Oslo (Ala 404 to Thr). In this instance, PCR was used to alter the relevant codon and the pSG5 vector used. Cotransfection with pSV2CAT was again performed, and the results obtained with the AT ELISAs from the conditioned media were normalized to intracellular CAT levels and total internal protein, using four transfection experiments per construct. A significant reduction (P < .01) in recombinant rabbit AT-Oslo secretion was observed, in that AT secretion and/or synthesis was reduced by approximately twofold, from 10.3 ± 0.4 to 5.2 ± 0.3 μg AT/pg CAT/μg total cellular protein (reported as the mean ± SE of the mean).

Fig 5. Pulse-chase analysis of CHO cell lines. CHO cell lines stably transfected with pCMVrab(Utah) (lanes 1 through 4) or pCMVrab(WT) (lanes 5 through 8) were pulse-labeled for 30 minutes as described in Materials and Methods. The radioactivity was then chased in the presence of complete media for 0 to 5 hours. The immunoprecipitates from cell lysate (intracellular) or cell supernatant (extracellular) samples were electrophoresed on 10% SDS gels as shown in A. B shows a quantification of the amount of intracellular AT, expressed as a percentage of the initial level of intracellular wild-type AT. Samples were adjusted to contain identical amounts of TCA-precipitable radioactivity before immunoprecipitation and reduced before electrophoresis on 10% SDS gels.
AT is a member of the serpin superfamily of proteins, and AT-thrombin complexes have been shown to interact with the putative serpin-enzyme complex receptor.25-28 The minimum sequence necessary to interact with this binding site is a pentapeptide FVFLM, a sequence similar to that found in AT between residues 408 and 412.28 The close proximity of this site to the sites of pleiotropic mutants like Utah (Pro 407 to Leu) suggested that the low circulating levels of AT-Utah and other pleiotropic-type mutants could reflect an accelerated interaction with the receptor. Because of the low levels of these mutants in the plasmas of the members of kindreds with such mutations, studies supporting such a hypothesis have not been done.

Our intent when we began this study was to study the in vivo interaction of mutant forms of AT with the putative hepatic receptor. However, it quickly became apparent that intracellular, rather than extracellular events, were most relevant in determining the level of AT-Utah in the conditioned media in our experimental system. Having shown that the positioning of the Utah mutation within the rabbit AT molecule, rather than its human counterpart, still produced a dysfunctional AT with aberrant electrophoretic migration suggestive of misfolding (Fig 1), we examined the production of this molecule in Cos-1 cells. As shown in Fig 2, there was a 10-fold difference in the amount of AT antigen detected in the conditioned media of cells transfected with the AT-Utah expression plasmid as compared with that of those obtained in cells transfected with the wild-type plasmid. This 10-fold reduction was not caused by differences in transfection efficiency because intracellular CAT levels were used to control for this parameter. Differences in RNA levels, caused by either a transcriptional or RNA stability effect, were discounted by Northern blot analysis (Fig 3), which established that RNA levels differed by less than 50% and that there was actually more mutant RNA produced than wild type. The possibility that recombinant AT-Utah was less stable outside of the cell was rendered unlikely by immunoblot analysis, showing no detectable degradation products, and a semiquantitative concordance between the amounts of antigen detected by ELISA with the intact AT protein levels observed (Fig 4).

Taken together, these results suggest that posttranscriptional, intracellular events are responsible for the low levels of AT-Utah secreted from the cells. Although we cannot eliminate the possibility that the Utah mutation renders its transcript less capable of being translated, this seems unlikely given that the synthetic mRNA that was produced was translated without apparent difficulty by reticulocyte lysate (Fig 1). To explore further the intracellular blockage, pulse-chase experiments were performed. Although the signal from intracellular wild-type AT was readily detectable at early time points, as the chase period progressed, it decreased to 30% of initial levels (Fig 5). In contrast, the intracellular AT-Utah signal never achieved wild-type levels; intracellular AT-Utah levels remained low (at 20% of initial intracellular wild-type AT levels), with little change throughout the chase.

The well-characterized Z mutation of α1-antitrypsin (PiZ) arises because of the replacement of Lys342 by Glu, and is associated with intracellular accumulation at the site of synthesis, giving rise to liver damage.29,30 Pulse-chase experiments with hepatoma cells in culture show normal levels of PiZ synthesis compared with the wild-type at early time points.11 However, intracellular PiZ fails to decrease with time, but instead remains at high levels indicative of a block in secretion, a blockage either caused or exacerbated by extensive aggregation.31 The situation we report in this study differs from that of PiZ in that, after the pulse but before the chase, AT-Utah levels are low. This observation could derive from a number of possible explanations: the nascent AT-Utah polypeptide may be unstable within the secretory pathway and is rapidly proteolyzed; the AT-Utah mutation could interfere with release of the nascent polypeptide from the transmembrane proteins of the protein translocation machinery of the endoplasmic reticulum; or the AT-Utah mRNA could be less efficiently translated than its wild-type counterpart. As mentioned above, the latter possibility cannot be excluded, but appears unlikely from the cell-free expression data. The rate of secretion of AT-Utah also appears to be decreased, as indicated by the fact that the intracellular AT-Utah band changes little with time, whereas the wild-type decreased at least 60% over the same time period. Therefore, our results suggest that the paucity of AT-Utah in the circulation of affected individuals derives from intracellular, rather than extracellular causes.

Similar results were also found with the recombinant form of another pleiotropic mutant, AT-Oslo,13 when it was recreated in a rabbit AT background and expressed in Cos-1 cells. In this instance, the reduction in secretion was only twofold. This finding of reduced secretion in another human mutation expressed in the rabbit background suggests that our results are general and do not derive from an artifact of using the rabbit, rather than human, AT in our experiments.

Carrell et al37 have recently described the structure of a crystallized human AT dimer. One molecule is thought to be in an active conformation, whereas the second is in an inactive conformation resembling that of latent plasminogen activator inhibitor.38 In the inactive molecule, a proximal portion of the reactive center loop (P3-P7) of the “active” molecule has displaced strand IC of the inactive molecule. Strand IC includes residues 400 through 404. The Utah mutation, P407L, lies immediately C-terminal to this strand on the turn leading to the next structural element, strand 4B. Therefore, the crystal structure suggests that strand IC is readily displaced and that this displacement is likely to perturb the nearby reactive center loop. Indeed, movement of strand IC away from sheet C has been proposed to be necessary to allow the rearrangement of the reactive center thought to be essential for complex formation.39 Both strand 1C mutations and more C-terminal alterations that perturb the Cys 247-Cys 430 disulfide bridge can result in pleiotropic effects on AT.11 Recent interpretations of crystallographic data suggest that the structural defect in both cases is the resulting unnatural mobility of strand 1C; freed from constraint, it adopts a conformation that is unable to participate in protease complexing.35 This inappropriate conformation may also be susceptible to loop-sheet polymerization, as seen in the dimeric AT crystal structure. Indeed, loop-sheet polymeriza-
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Although we have not identified the precise mechanism of intracellular reduction in AT-Utah levels, our results clearly exclude extracellular explanations of the paucity of circulating AT-Utah, and suggest that intracellular events also contribute to the reduction in circulating AT levels seen with other pleiotropic AT mutants. These results fit well with the recent observation that C-terminal substitutions in another serpin, C1 inhibitor, result in only partial secretion of the mutant, dysfunctional proteins.

REFERENCES


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