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ORIGINAL ARTICLE

Aberrant disulphide bonding contributes to the ER retention of alpha1-antitrypsin deficiency variants

Riccardo Ronzoni[1], Romina Berardelli[1], Daniela Medicina[2], Roberto Sitia[3], Bibek Gooptu[4,5] and Anna Maria Fra[1,*]

[1]Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy, [2]Department of Pathology, Spedali Civili, Brescia, Italy, [3]Università Vita-Salute San Raffaele, Milan, Italy, [4]Institute of Structural and Molecular Biology/Crystallography, Birkbeck College, University of London, London, UK and [5]Division of Asthma, Allergy and Lung Biology, King's College, London, UK

*To whom correspondence should be addressed at: Department of Molecular and Translational Medicine, University of Brescia, Viale Europa, 11, 25123 Brescia, Italy. Tel: +39 0303717468; Fax: +39 0303701157; Email: annamaria.fra@unibs.it

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Abstract

Mutations in alpha1-antitrypsin (AAT) can cause the protein to polymerise and be retained in the endoplasmic reticulum (ER) of hepatocytes. The ensuing systemic AAT deficiency leads to pulmonary emphysema, while intracellular polymers are toxic and cause chronic liver disease. The severity of this process varies considerably between individuals, suggesting the involvement of mechanistic co-factors and potential for therapeutically beneficial interventions. We show in Hepa1.6 cells that the mildly polymerogenic I (Arg39Cys) AAT mutant forms aberrant inter- and intra-molecular disulphide bonds involving the acquired Cys39 and the only cysteine residue in the wild-type (M) sequence (Cys232). Substitution of Cys39 to serine partially restores secretion, showing that disulphide bonding contributes to the intracellular retention of I AAT. Covalent homodimers mediated by inter-Cys232 bonding alone are also observed in cells expressing the common Z and other polymerising AAT variants where conformational behaviour is abnormal, but not in those expressing M AAT. Prevention of such disulphide linkage through the introduction of the Cys232Ser mutation or by treatment of cells with reducing agents increases Z AAT secretion. Our results reveal that disulphide interactions enhance intracellular accumulation of AAT mutants and implicate the oxidative ER state as a pathogenic co-factor. Redox modulation, e.g. by anti-oxidant strategies, may therefore be beneficial in AAT deficiency-associated liver disease.

Introduction

Alpha1-antitrypsin (AAT) is an acute phase glycoprotein produced and secreted by liver cells. It inhibits neutrophil elastase, protecting lung tissue from excessive damage during inflammatory responses. Mutations in the SERPINA1 gene (OMIM 107400) encoding AAT cause the hereditary autosomal disorder alpha-1 antitrypsin deficiency (AATD, OMIM 613490) (1). Pathological AAT variants are either 'null' (no detectable levels of AAT in the plasma, e.g. due to premature stop codons) or 'deficient', where missense mutations or small deletions result in synthesis of conformationally altered AAT and retention within the endoplasmic reticulum (ER). These mutants engage with a number of ER quality-control mechanisms.

The most frequent genotype associated with severe AATD is homozygosity for the Z (Glu342Lys) allele. This is associated with plasma AAT levels that are 10–15% of normal and early-onset emphysema in adults due to protease:antiprotease imbalance in the lung. A subset of ZZ patients also develop liver diseases, e.g. neonatal hepatitis, liver cirrhosis and hepatocellular carcinoma. Liver disease is associated histopathologically with accumulation of Z AAT protein inclusions in the ER of hepatocytes (2). The majority of synthesized Z AAT is degraded

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by ER-associated degradation (ERAD) (3,4). However, a proportion escapes degradation and self-associates to form ordered polymeric structures that accumulate in dilated cisternae of the ER as inclusion bodies. Liver manifestations vary significantly among ZZ patients, implicating additional genetic and environmental factors in the onset of liver pathologies associated with AATD. Hypomorphic variants of ERAD or autophagy pathway components have been proposed as co-factors predisposing to accumulation of Z AAT polymers to cause hepatotoxicity (5).

Besides Z, many other AAT mutants have been identified in AATD patients. The S variant (Glu264Val) is relatively frequent in South-West European countries and is associated with a milder secretory deficiency. Rarer variants are increasingly identified in AATD patients, often compound heterozygotes who also carry the Z allele (1,6). A subset of rare variants form polymeric structures and intracellular inclusions similar to Z AAT and so contribute to both liver disease risk and severe secretory defect. Examples include Mmalton (ΔPhe52), Siiyama (Ser53Pro), King's (His334Pro) and Pbrescia (Gly225Arg) (1).

A further variant in this class is the I variant (Arg39Cys), that is now well-recognized in Europe (7). It was first reported in a IZ heterozygous patient with emphysema (8). A child with IZ genotype was then reported with evidence of liver disease. *In vitro* cell-free assays indicated that purified I AAT was as polymerogenic as the S variant. This similarity may be rationalized by reference to the X-ray crystallographic structure of AAT; Arg39, the site of the I mutation, lies within part of the A helix of AAT that interacts directly with Glu264, the site of the S mutation (9).

The amino acid substitution of the I variant causes AAT to acquire a second cysteine residue in addition to Cys232 that is present in the wild-type (M) AAT. Cysteines normally form disulphide bonds in the ER environment (10). This process is catalyzed by members of the protein disulphide isomerase (PDI) family in appropriate redox conditions (11,12). Non-native intra- or intermolecular disulphide bonds may form transiently during folding but can be isomerized by PDI family members to more stable native disulphides. Mutations affecting cysteine residues (e.g. causing their gain or loss) may result in abnormal intra- or interchain disulphides with ER-resident or cargo proteins that prevent export of the unfolded/misfolded proteins. We investigated the role of such events in the transport and aggregation of the I variant and other deficiency mutants in Hepa 1.6 cells. Our results show that aberrant intra- and inter-molecular disulphide bonding between disease-variant AAT molecules contributes to ER retention and impedes transport to distal compartments of the secretory pathway. The effects are greatest for the I variant, but this process also affects the more common Z variant.

Results

AAT variants form intra-cellular disulphide-linked complexes

We set out to investigate whether the additional cysteine found in I AAT besides Cys232 in the wild-type (M) sequence could mediate aberrant disulphide bonds and contribute to secretion defects. We used a liver-derived cellular model previously used to characterize other rare deficiency AAT variants (13,14). We compared the I mutant with M AAT, the common Z (Glu342Lys) mutant and two further mild deficiency variants containing an extra cysteine residue: the previously reported F variant (Arg223Cys) (15) and a novel, naturally occurring mutant we have named Brixia (Phe35Cys). The Brixia allele was identified in a 40-year-old male heterozygote referred at Spedali Civili (Brescia, Italy) because of mildly reduced plasma AAT levels (89 mg/dl; 90–220 mg/dl normal range). The new allele shows a T>G transversion in exon2 (c.176T>G) resulting in Phe35Cys substitution.

We first analysed the five AAT variants by metabolic labelling of Hepa1.6 transient transfectants. Importantly, post-lysis, artefactual disulphide bonding interactions were prevented by N-ethylmaleimide (NEM) treatment. All fractions were immunoprecipitated by a polyclonal anti-AAT antibody and analysed by sodium dodecyl sulphate-polyAcrilamyde gel electrophoresis (SDS-PAGE) under non-reducing conditions in order to detect the presence of disulphide-linked complexes (Fig. 1A, upper panels).

Wild-type M AAT showed the expected pattern: detected mainly as an immature high-mannose monomer in the NP40-soluble cellular fraction and efficiently secreted in the cell media as a mature glycosylated form. All mutants were secreted as fully glycosylated monomers but at lower levels compared with M, with I being the most deficient among the rare variants (by densitometric analysis Z, F, Brixia and I were, respectively, 8, 49, 55 and 31% of the M levels). The I mutant forms multiple high molecular weight complexes both in soluble and insoluble cellular fractions. These dissociate upon reduction (Fig. 1A, lower panels), confirming that they are stabilized by disulphide bonds. The faster migrating complex has an apparent molecular weight of 100 kDa, followed by higher molecular weight complexes. The Brixia mutation occurs close to that of the I variant on the A helix and shows similar disulphide-linked complexes with the 100 kDa form prevalent. A 140 kDa covalent complex was also observed in F, Z and other disease associated AAT mutants (Supplementary Material, Fig. S1), but not in M AAT, suggesting formation of dimers by Cys232 in variants with abnormal conformation and ER retention. Similar results were obtained by immunoblot analysis with anti-AAT antibodies (not shown).

To further investigate the nature of the disulphide-linked complexes formed by AAT mutants, we performed diagonal gels, consisting of non-reducing SDS-PAGE in the first dimension followed by a reducing SDS-PAGE in the second dimension (Fig. 1B). As expected, the wild-type AAT M (top left panel) shows the monomeric form and runs in the second dimension as a train of spots along the diagonal, indicating that neither intra- nor inter-chain disulphide bonds are formed as the protein undergoes glycan processing. The disulphide complex of the Z mutant with an apparent molecular weight of 140 kDa migrates in the reducing dimension as a high-mannose immature monomer, suggesting that the complex is a covalently linked Z AAT homodimer. I and Brixia mutants show similar behaviour, with all major disulphide complexes dissociating into monomeric AAT. No other proteins were found associated with AAT in this analysis, under the experimental conditions adopted, even at longer exposure times. This is consistent with the retained higher mass species being composed entirely of AAT subunits with a range of disulphide linkage patterns. We cannot formally exclude interactions of AAT with additional long-lived proteins or methionine/cysteine-poor proteins, due to the nature of the pulse-chase radiolabelling approach. However, our findings suggest that if they occur, heterotypic interactions are a minor contributor to the observed profile of disulphide-adducted AAT species. Importantly, a proportion of I and Brixia AAT runs faster than fully extended monomers in the first dimension (Fig. 1A and B, asterisks) and then migrates as a full-length monomer in the reducing second dimension (arrows), consistent with the presence of an intra-chain disulphide bond. An intra-chain form was not evident for the F mutant. In this case, the acquired Cys223 lies within the same β-strand as Cys232 in the native structure. Formation of an intra-chain bond would likely necessitate major disruption of the protein backbone and stabilising interactions relative to the native structure with kinetically and

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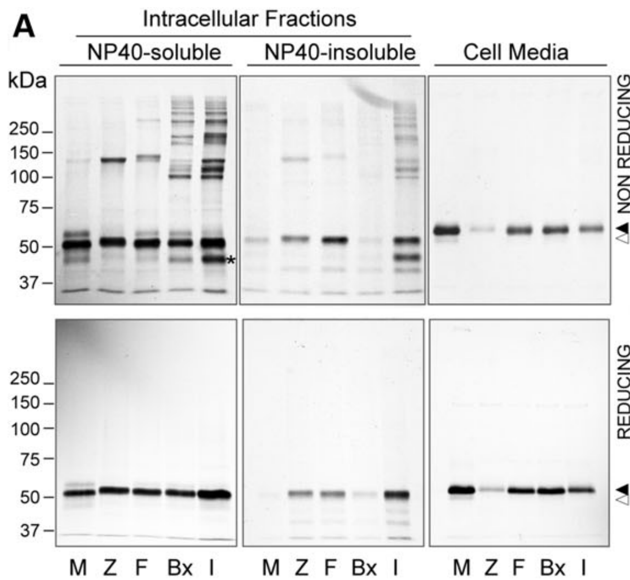

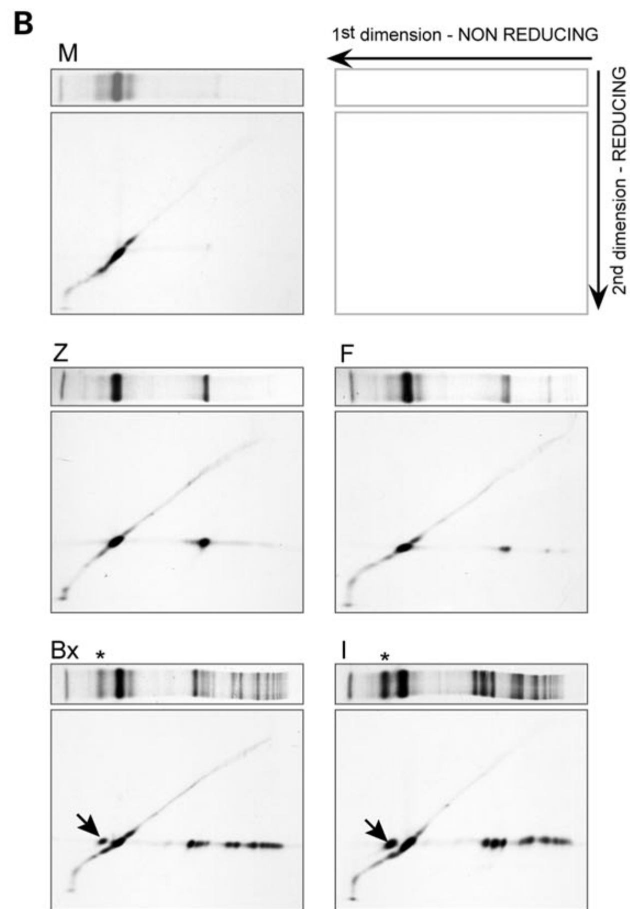

Figure 1. AAT mutants form intracellular disulphide-linked complexes. Hepa1.6 cells were transfected with vectors encoding the AAT variants M, Z, F, Brixia (Bx) or I. Twenty-four hours after transfection the cells were metabolically labelled for 4 h with ^{35}S Met/Cys. Cell media were collected, the cells lysed in 1% NP40 buffer and the NP40-insoluble fractions solubilized in 1% SDS buffer. (**A**) AAT was immunoprecipitated from the culture media and the intracellular fractions and analysed by 8% SDS-PAGE under non-reducing conditions (upper panels) or reducing conditions (lower panels), followed by autoradiography. White arrowhead: immature AAT. Black arrowhead: mature AAT. (**B**) NP-40 soluble cell lysates obtained as in (A) were analysed by diagonal gels. The samples were first resolved by non-reducing 8% SDS-PAGE; for the second dimension, individual lanes were cut, reduced with Laemmli buffer containing 100 mM DTT and further subjected to 8% SDS-PAGE analysis and autoradiography. First dimension lanes are shown at the top of each panel. Asterisks indicate the I and Brixia AAT conformers with an intrachain disulphide bond, which run in the second dimension as reduced monomeric AAT (arrows). The results shown are representative of two independent experiments.

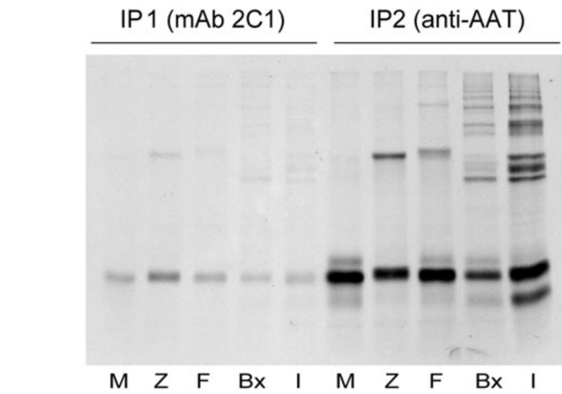

Figure 2. 2C1 fails to immunoprecipitate disulphide-linked complexes. NP-40 soluble cell lysates of Hepa1.6 cells expressing M, Z, F, Brixia (Bx) or I, metabolically labelled for 4 h with ^{35}S Met/Cys as in Figure 1A, were immunoprecipitated with the 2C1 mAb (IP1, left side), and the leftover further immunoprecipitated by an anti-total AAT polyclonal antibody (IP2, right side). Immunoprecipitated samples were analysed by non-reducing 8% SDS-PAGE and autoradiography. The results shown are representative of two independent experiments.

energetically unfavourable consequences. We therefore favour this over the alternative interpretation that a disulphide bridge can form but does not result in a significant change in migration on SDS-PAGE. The presence of a second cysteine in the F variant is also associated with less extensive formation of higher molecular weight disulphide species than the other AAT mutants containing two cysteines.

Disulphide-linked complexes are distinct from classical polymers

Inclusions formed in the hepatocytes of Z homozygotes are composed of polymers that are well-recognized by the conformation-specific monoclonal antibody (mAb) 2C1 (16). To date all AAT polymers that have been assessed in tissue or cell models have also been recognized by the 2C1 mAb. To investigate whether disulphide-linked complexes are associated with 2C1-positive polymeric structures, we performed immunoprecipitation of NP40-soluble fractions obtained as in Figure 1 with the 2C1 mAb (Fig. 2, left). The residual solution was then subjected to immunoprecipitation for total AAT using a polyclonal antibody (Fig. 2, right). About 25% of total Z variant migrating in SDS-PAGE as monomer is immunoprecipitated by 2C1 mAb reflecting the fraction of intracellular Z in polymeric forms. Instead the disulphide-linked dimers and the other disulphide-linked species were less efficiently immunoprecipitated by 2C1 (<10%). These observations suggest that the disulphide-linked species are not incorporated into classical polymers recognized by 2C1 mAb, but may be incorporated in distinct aggregation states.

Cysteine-mediated interactions contribute to ER retention of AAT mutants

To dissect the individual roles of the two cysteines in determining the intracellular fate of I AAT, we generated two I AAT

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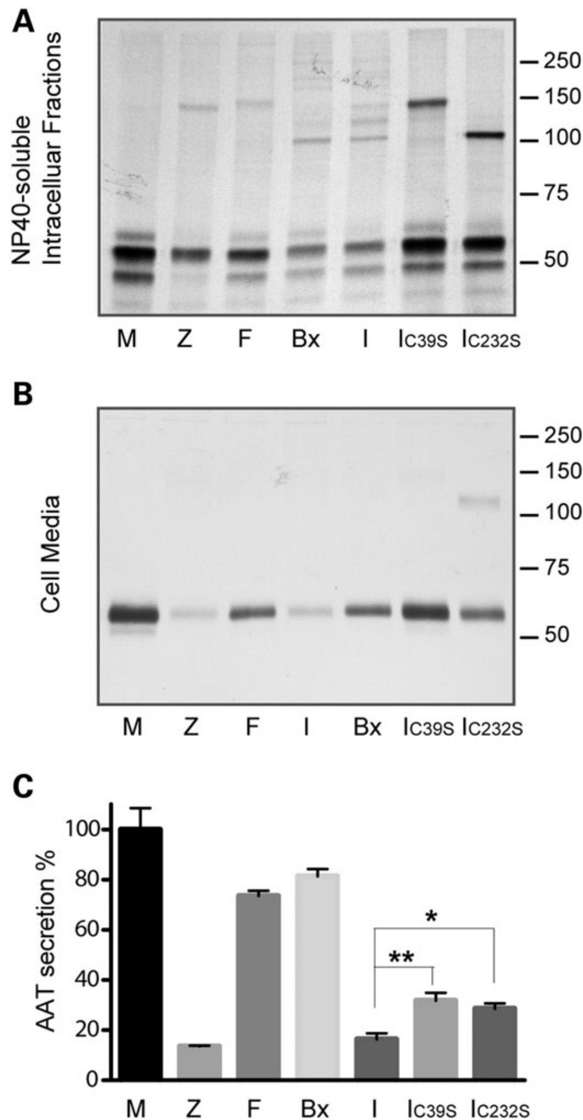

Figure 3. Effects of Cys39 and Cys232 substitution. Hepa1.6 cells were transfected with vectors encoding the AAT variants M, Z, F, Brixia (Bx) or I, and the artificial mutants IC39S and IC232S. (**A** and **B**) Transfected cells were metabolically labelled for 4 h and processed as in Figure 1. Immunoprecipitated AAT from the NP-40-soluble cellular fractions (panel A) and cell media (panel B) were analysed by non-reducing 8% SDS-PAGE and autoradiography. (**C**) Transfected Hepa1.6 cells were incubated for 6 h in serum-free culture medium. Secreted AAT levels in the media were quantified by sandwich ELISA using a standard curve of purified AAT and expressed as percentages of the wild-type M levels. Results are presented as means and SD of three independent transfection experiments. Statistical significance was determined by one-way ANOVA followed by the Bonferroni's post-hoc test using GraphPad Prism version 5 (Graphpad software Inc., San Diego, CA, USA): *$P < 0.05$; **$P < 0.01$.

variants (I_{C39S} and I_{C232S}) with Cys39 or Cys232, respectively, substituted with serines. Serine residues are chemically similar to cysteines but cannot form disulphide bonds. We expressed these artificial mutants in Hepa1.6 cells and compared their behaviour with that of M, Z and I AAT. Cells were metabolically labelled and analysed by non-reducing SDS-PAGE (Fig. 3). I_{C39S} AAT formed a Cys232-Cys232 disulphide-bonded dimer that migrated close to the molecular weight marker of 140 kDa. A dimer with identical behaviour was observed in cells expressing Z AAT. On the other hand, I_{C232S} expression formed a Cys39-Cys39 disulphide-linked dimer that migrated more rapidly (apparent mass 100 kDa) likely due to a compact structure and was also found in the cell media (Fig. 3B). A further dimer species was apparent in cells expressing the I variant that migrated intermediate between these points and likely involves both cysteine residues. Formation of higher order complexes was indicated by slower migrating species.

The disulphide-linked pattern observed for Brixia was more consistent with that seen in I_{C232S} than in I variant containing Cys232. Most likely, therefore, Brixia dimer formation was mediated by Cys35 rather than Cys232. The variation in disulphide bonding between the I and Brixia variants, which both have two cysteines in near-identical positions, indicates differences in dynamic folding and/or conformational behaviour.

Levels of AAT secreted by cells expressing different AAT variants were then analysed by quantitative enzyme-linked immunosorbent assay (ELISA) (Fig. 3C). F and Brixia mutant secretion was mildly deficient relative to the M AAT control (75 and 80%, respectively) while Z and I showed severe deficiency (respectively, 13 and 20%). Notably, secretion of the I_{C39S} and I_{C232S} mutants was significantly higher than that of I, indicating that the formation of heterogeneous disulphide complexes in the ER contributes to the secretory defect of the I variant. Interestingly, intracellular co-aggregation and disulphide bonding of I and Z AAT were demonstrated when they were co-expressed to model hepatocyte behaviour in IZ compound heterozygotes (Supplementary Material, Fig. S2). Such individuals are more likely to be encountered in clinical practice than I homozygotes. The observation that co-expression of a second, milder deficiency variant can result in complex formation with the Z variant in the ER environment validates a previous proposal based upon biophysical studies in cell-free conditions (9).

Effects of disulphide bonding on maturation and secretion of I AAT

To further investigate the kinetics of formation of the disulphide-linked complexes and their effects on secretion, we performed pulse-chase experiments (Fig. 4). The electrophoretic patterns of the I mutant revealed that intra-chain disulphides and Cys39-mediated homodimers form very soon after synthesis. Inter-chain Cys232-Cys232 bonds appear to form more slowly, and the three dimer isoforms are similarly represented from the 30 min time point. Intra-chain bonding is associated with reduced persistence of monomeric AAT since the population of this species attenuates steadily. This likely reports abnormal conformational behaviour, favouring degradation and/or polymerization. Higher order forms become prevalent at later time points. As expected, I_{C39S} and I_{C232S} AAT form only homodimers and show increased secretion with faster intracellular maturation to the 56 kDa glycosylated form that acquires Golgi processing. Some I_{C232S} homodimers were secreted, whilst only I_{C39S} monomers accumulated extracellularly. The distribution of AAT in the intracellular fractions and in the cell media at different time points was quantified by densitometry on reducing SDS-PAGE gels and are shown in Supplementary Material, Figure S3. The effects of aberrant disulphide bonding upon I AAT secretion were determined by densitometric analysis of data from two independent pulse-chase experiments (Fig. 4A, lower panel). At 240 min of chase, when about 60% of the wild-type M was found in the culture medium, 10, 26 and 13%, respectively, of I, I_{C39S} and I_{C232S} AAT were secreted. The significant increase in secretion associated with Cys39Ser substitution further confirms that Cys39-mediated disulphide bonding promotes I AAT retention.

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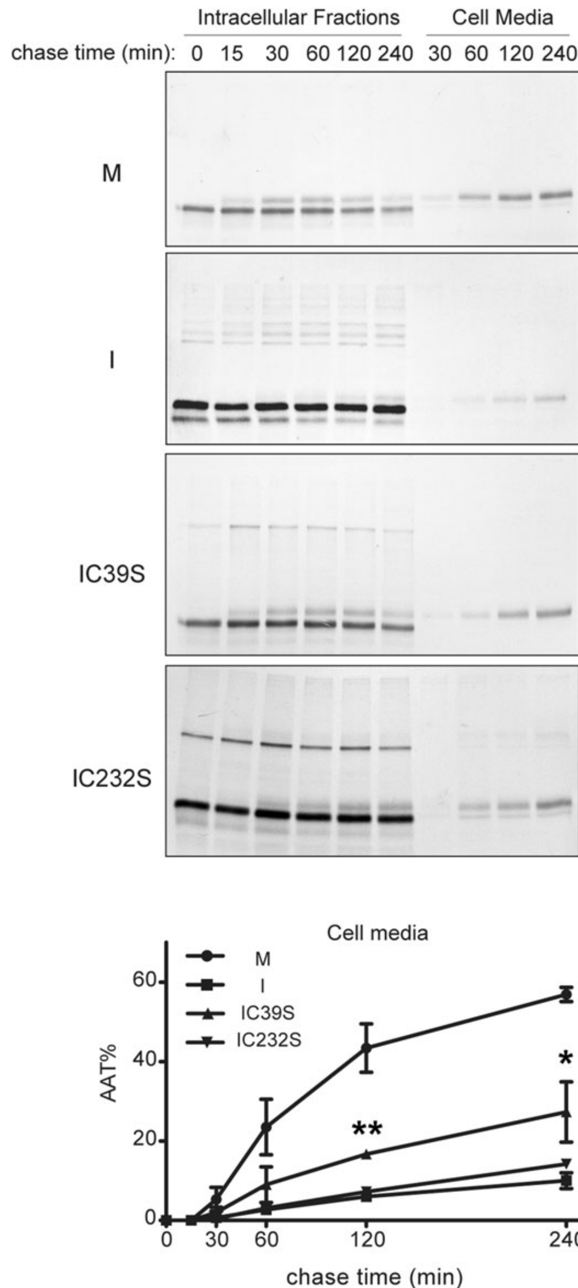

Figure 4. Effects of disulphide bonding on maturation and secretion of I AAT. Hepa1.6 cells expressing M, I, IC39S or IC232S were pulsed with ^{35}S Met/Cys for 10 min and chased for the indicated times. AAT was immunoprecipitated from the culture media and the intracellular fractions and analysed by non-reducing 8% SDS-PAGE and autoradiography. The kinetics of secretion of the mutants, determined by densitometric analysis as in Supplementary Material, Figure S3, is represented in the graphs as means and SD of two independent experiments. Statistical significance of IC39S versus I secretion was determined at each chase time point by *t*-test analysis: $^{*}P < 0.05$; $^{**}P < 0.01$.

Preventing aberrant disulphide bond formation increases secretion of Z AAT

To specifically address the role of aberrant Cys232 thiol interactions in the retention of Z AAT, we performed four independent pulse-chase experiments, comparing Z with a Z mutant in which Cys232 was substituted for a serine (Z_{C232S}). Cys232Ser substitution significantly increased Golgi maturation and secretion of Z AAT (Fig. 5A), indicating that Cys232-mediated disulphide bonding plays a role in the intracellular retention of Z AAT.

Cell treatment with reducing agents has been used previously to shift the ER to a more reducing state and impair the formation of disulphides on cargo proteins (17,18). Therefore, we next assessed whether such treatment could thereby increase the secretion of Z AAT from Hepa1.6 cells similarly to the effect of the Cys232Ser mutation. A significant increase in secretion of Z AAT was observed upon treatment with 0.5 mM dithiothreitol (DTT) (Fig. 5B). This was of similar magnitude to the increase in secretion resulting from the introduction of the Cys232Ser mutation. To assess how far this increase could be attributed to the rescue of Cys232-mediated aggregation, as opposed to the known pleiotropic effects of DTT upon cells, we similarly treated cells expressing Z_{C232S} AAT. Treatment of these control cells did not increase AAT secretion whatsoever, indicating that this effect of DTT was indeed due to inhibition of Cys232-mediated Z AAT aggregation within cells. Only fully glycosylated AAT was detected in the cell media upon treatment with DTT and we did not observe major effects on the intracellular degradation of the proteins. Consistent results were obtained by measuring secreted AAT by ELISA in the culture media of Hepa1.6 cells expressing Z, treated with either 2ME or DTT. Notably, the change in I AAT secretion was smaller and did not achieve significance, consistent with the more extensive disulphide bonding patterns observed in this mutant.

Discussion

The oxidizing environment of the ER lumen favours disulphide bonding during the biosynthesis and maturation of secreted proteins. The process is assisted by numerous disulphide isomerases and oxidases, working in a tightly controlled redox environment (11). The interaction between free exposed cysteines and disulphide isomerases also serve to prevent unfolded and unassembled proteins from exiting the ER, and so are fundamental in protein quality-control within the early secretory compartment (10). Such thiol-dependent ER retention was originally described for unassembled IgM subunits (19,20) and was then shown for other substrates (21,22). Moreover proteins exposing free cysteine residues may be retrieved from ER-Golgi intermediate compartment to the ER by pH-dependent interactions with ERp44 (23,24). The stringency of thiol-mediated retention can be modulated by the local pK_a of the thiol group, as demonstrated by the lower reactivity of the IgA C-terminal cysteine due to the presence of an adjacent negatively charged amino acid (25).

Human AAT appears to represent an exception to this general model of thiol-dependent quality-control: it contains a single cysteine residue (Cys232) at a solvent-exposed site yet is an efficiently secreted protein. The reason for this discrepancy is unclear but does not obviously relate to masking of Cys232 that might render it poorly reactive and/or not accessible in the native structure. Biochemical studies have demonstrated that Cys232 of recombinant AAT has a pK_a of 6.86 and can be efficiently oxidized *in vitro* (26). Moreover, high resolution crystal structures of AAT indicate that the thiol group of Cys232 is highly exposed to the solvent and electron density maps are consistent with an oxidized state (PDB IDs: 2QUG and 3NE4 (27,28). Whether Cys232 interacts with protein isomerases in the secretory pathway, however, remains to be elucidated.

Here we report that the Z mutant as well as other transport-incompetent AAT variants form disulphide-linked homodimers in the ER. This suggests that Cys232 participates in disulphide bonding events that report abnormal conformational behaviour of AAT and stabilize aberrant conformations in pathogenic AAT

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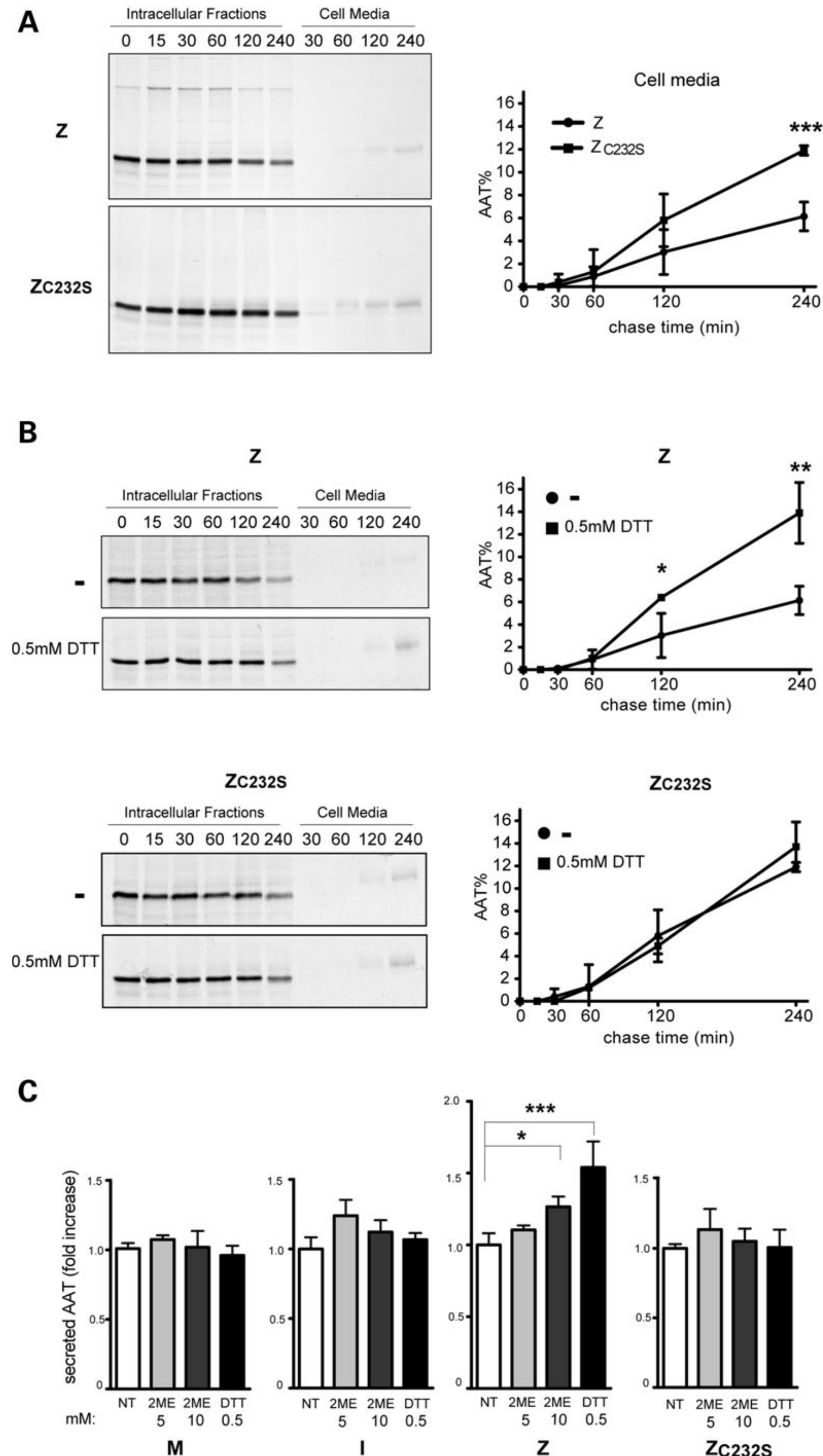

Figure 5. Preventing aberrant disulphide bond formation increases secretion of Z AAT. (**A**) Hepa1.6 cells expressing Z or Z_{C232S} were pulsed with ^{35}S Met/Cys for 10 min and chased for the indicated times. AAT was immunoprecipitated from the culture media and the intracellular fractions, and analysed by non-reducing 8% SDS-PAGE and autoradiography. The kinetics of secretion of the AAT mutants, determined by densitometric analysis, is represented in the graph as means and SD of four independent experiments. Statistical significance of ZC232S versus Z secretion levels was determined at each chase time point by *t*-test analysis: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (**B**) The kinetics of secretion of Z and ZC232S in three independent pulse-chase experiments performed in the absence or presence of 0.5 mM DTT are represented in the graphs as means and SD. Statistical significance of DTT effect on secretion levels was determined at each chase time point by *t*-test analysis: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Autoradiographies of one of the experiments quantified are shown in the left panels. (**C**) Hepa1.6 cells expressing M, I, Z or ZC232S were incubated for 6 h in serum-free culture medium in the absence (NT) or presence of 2-ME or DTT at the indicated mM concentrations. AAT levels in the cell media were quantified by sandwich ELISA using a standard curve of purified AAT and expressed for each variant as fold increase relative to NT sample, set as 1. Results from two independent experiments, each one in duplicates, are presented as means and SD. Statistical significance was determined by one-way ANOVA followed by Bonferroni's post-hoc test using GraphPad Prism version 5 (GraphPad software Inc, San Diego, CA, USA): *$P < 0.05$; ***$P < 0.001$.

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mutants. These interactions may be favoured by cellular oxidative conditions such as those observed in liver tissues of PiZ mice (29). Under our experimental conditions, we did not detect major disulphide interactions of AAT mutants with resident ER isomerases. However this does not preclude their existence, since such interactions are highly dynamic and may form transiently during protein folding. In our view such interactions with isomerases and other ER protein folding quality-control proteins are likely to occur. These could be further interrogated in future high sensitivity (e.g. mass spectrometry-based) interactome studies involving immunoprecipitation of intracellular aggregates of AAT variants characterized here. Proteins identified as potential interactors could then be validated by reciprocal immunoprecipitation and functional studies.

The Cys232-mediated dimers we observe for the Z and other polymerogenic variants are reminiscent of those previously reported for the truncated AAT variant NHK (334stop), whose degradation requires EDEM (ER-degradation enhancing alpha-mannosidase-like protein) and ERdJ5 (30,31). Moreover, the intracellular accumulation of such dimers as NP40-insoluble aggregates is consistent with their incorporation into high molecular weight polymers. However, disulphide-linked dimers were not immunoprecipitated effectively by the anti-polymer mAb 2C1 raising the possibility that *in vivo* these disulphide-linked states are incorporated in non-classical polymers that may co-exist alongside polymers recognized by the 2C1 mAb. It is now known that AAT and other members of the serpin protein superfamily to which it belongs can assemble into a range of distinct polymeric conformations (1,32). The different polymer architectures share many characteristics, but vary in the degree to which each protein subunit is folded relative to the native state. This could account for the differential recognition of AAT polymers by the 2C1 mAb described here, with differently linked polymers having different predisposition to abnormal disulphide bond formation. Alternatively, the polymers may all be similar in structure but the observed disulphide bonding may cause disruption of the 2C1 epitope or render it less accessible due to local effects.

Aberrant disulphide bonding by gain- or loss-of-cysteine mutants expressed in the ER has been previously reported for other genetic diseases (33–36). Cysteine-mediated retention of the I AAT mutant is a striking example of the consequences of a non-native cysteine in the protein maturation in the ER. The presence of an additional cysteine residue (Cys39) in the I mutant leads to the formation of an intra-chain and multiple inter-chain disulphide-linkages involving both Cys39 and Cys232. Gain-of-cysteine AAT mutants may therefore be regarded as a subset of AAT deficiency variants, with a distinctive molecular pathogenesis characterized by increased aberrant disulphide binding events. Notably, our studies have also provided the first proof of co-aggregation of different AAT deficiency variants in the ER. Specifically, we observed formation of mixed disulphide complexes by the co-expressed I and Z mutants. Such heteropolymerization was previously hypothesized on the basis of biophysical data in a cell-free model (9). Since polymer load correlates with disease severity, this has implications for risk stratification of compound heterozygotes such as the IZ cases reported in the literature.

Our mutagenesis data formally demonstrate that aberrant disulphide bonding can contribute to the secretory deficiency of AAT mutants in cells. This effect is particularly evident for I AAT. Here aberrant and complex patterns of intra- and inter-molecular thiol interactions mediated by the two cysteines combine with the inherent polymerogenic tendency of cysteine or serine mutations at position 39. This combination enhances intracellular aggregation of I AAT in a way that makes in relatively refractory to treatment with reducing agents at concentrations previously used to modulate the ER folding and ER-associated degradation (30,37,38). On the other hand, a simpler pattern of aberrant disulphide bonding contributes to accumulation of the most clinically important variant, Z AAT, within cells. This is more tractable to anti-oxidant treatments to improve secretion. Our data are consistent with the previous understanding that non-covalent polymerization is the primary mechanism of intracellular AAT retention, with abnormal disulphide bond formation playing an exacerbating role. Anti-oxidant strategies would therefore be highly complementary to other approaches in development to block or reverse polymerization (39).

Concluding remarks

Previous studies have established that misfolding and aberrant conformational behaviour of AAT mutants determine their intracellular retention, degradation and tendency to form ordered polymers. Here we show that cysteine-mediated interactions within the ER contribute to the intracellular retention of the AAT deficiency mutants. The ER redox state may therefore be regarded as a modifier factor for AATD and represent a useful therapeutic target.

Materials and Methods

Reagents and antibodies

Unless stated otherwise, reagents and culture media were from Sigma-Aldrich. The rabbit polyclonal anti-AAT was from DAKO; the 2C1 mAb (16) was kindly provided by David Lomas (CIMR, University of Cambridge, UK). The rabbit polyclonal anti-HA (H6908) was from Sigma-Aldrich.

Expression vectors for the AAT variants

The Brixia allele was identified in a male heterozygous subject by sequencing all coding exons (II–V) of the SERPINA1 gene (RefSeq: NG_008290), as previously described (14). The expression vectors encoding for human M1(Val213) and Z AAT were reported previously (14). The vectors for the tagged AAT variants M-myc, M-HA and Z-HA (40) were kind gifts of Dr Giulia Baldini (University of Arkansas for Medical Sciences, Little Rock, AR, USA). The vectors encoding the Brixia, I and F variants, and the artificial mutants I_{C39S}, I_{C232S}, Z_{C232S}, I-myc and I-HA, were obtained by site-directed mutagenesis, using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) and the primers listed in Supplementary Material, Table S1.

Cell culture and transfections

Hepa1.6 is a mouse hepatoma cell line (ATCC CRL-1830) grown in dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum. Transient transfections were performed with Polyethyleneimine 'MAX' (PEI) (Polysciences). For a 10 cm^2 well, 20 µg PEI and 3 µg plasmid were incubated for 20 min in 400 µl DMEM, diluted to 1.5 ml with normal culture medium and added to the cell layer for 5 h.

Metabolic labelling experiments

Transiently transfected Hepa1.6 cells were pulsed for 10 min with ^{35}SCys/Met (EasyTag™ Express Protein Labelling, Perkin Elmer) in DMEM without Cys/Met-1% dialysed FCS, and then chased in

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normal culture medium for 0, 15, 30, 60, 120 and 240 min. Long metabolic labelling was performed with ^{35}S Cys/Met for 5 h. After labelling, the media were centrifuged at 600*g* for 5 min and supplemented with 10 mM NEM. Cells were washed with phosphate buffered saline (PBS)/10 mM NEM and lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP40, 10 mM NEM and protease inhibitors. The NP40-insoluble fractions were separated by centrifugation at 12 000 *g*, dissolved in a buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 0.2% NP40, 0.1% SDS, 1 mM $MgCl_2$ and 20 µg/ml DNAse I, and finally diluted 10-fold with 1% NP40 buffer. The radiolabelled AAT in the cell lysates and media was immunoprecipitated with an anti-AAT polyclonal antibody (DAKO) and analysed by 8% SDS-PAGE under reducing or non-reducing conditions followed by autoradiography. Densitometric analysis of AAT bands was performed by Image Quant 5.2 (Molecular Dynamics).

Diagonal gels

The NP40-soluble cell extracts from metabolically labelled Hepa1.6 cells were resolved by SDS-PAGE in non-reducing conditions. All the lanes were cut and reduced by incubation with 2× Laemmli Sample Buffer containing 100 mM DTT for 10 min. For the second dimension, lane slices were placed onto a 8% acrylamide gel and run as a conventional SDS-PAGE followed by autoradiography of the dried gels.

Western blot analysis

For immunoblot analysis, NP40-soluble cell extracts were obtained from transfected Hepa1.6 cells as described above for pulse experiments, resolved by SDS-PAGE on pre-casted 7.5% mini PROTEAN TGX gels (Biorad) and transferred to LF-PVDF membranes by the Turbo Blot Transfer System (Biorad). Membranes were saturated in low-fat milk, probed with the indicated primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare), revealed by ECL (LiteAblot® plus, Euroclone).

Secretion assays and sandwich ELISA

To quantify secreted AAT, Hepa1.6 transfected cells were washed to remove serum and incubated for 6 h in serum-free culture medium, supplemented with 2-mercaptoethanol (2-ME) or DTT when indicated. ELISA plates (half-volume, Costar) were coated overnight at 4°C with 1 µg/ml anti-AAT antibodies (DAKO), washed with PBS/0.1% Tween 20 (PBS-T) and then blocked with 0.25% BSA/PBS-T for 75 min at 37°C. Serial dilutions of cell media were added to the plate and incubated at 37°C for 1 h. For the standard curve, we used serial dilutions of purified AAT (Millipore). Bound AAT was revealed by incubating for 1 h with 1 µg/ml HRP-conjugated goat anti-hAAT (Abcam) followed by TMB-plus substrate (Thermo Scientific). After blocking the reaction with 3 M HCl, absorbance was measured at 450 nm using an ELISA plate reader.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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