The molecular species responsible for α1-antitrypsin deficiency are suppressed by a small molecule chaperone

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Keywords
folding intermediate; polymer inhibitor; polymerisation; secretion; α1-antitrypsin

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[Correction added on 29 November 2020, after first online publication: Peer review history is not available for this article, so the peer review history statement has been removed.]

(Received 22 June 2020, revised 28 August 2020, accepted 12 October 2020)

doi:10.1111/febs.15597

Introduction

α1-Antitrypsin is the archetypal member of the serpin superfamily of proteins and a major inhibitor of neutrophil elastase in humans [1]. Plasma deficiency of this protease inhibitor predisposes to early-onset panlobular basal emphysema due to deregulated elastase activity within the lung [2]. The common severe Z deficiency variant of α1-antitrypsin (Glu342Lys) results in the formation of long chains of ordered aggregates termed polymers. These polymers accumulate within the endoplasmic reticulum (ER) of hepatocytes.

The formation of ordered Z (Glu342Lys) α1-antitrypsin polymers in hepatocytes is central to liver disease in α1-antitrypsin deficiency. In vitro experiments have identified an intermediate conformational state (M*) that precedes polymer formation, but this has yet to be identified in vivo. Moreover, the mechanism of polymer formation and their fate in cells have been incompletely characterised. We have used cell models of disease in conjunction with conformation-selective monoclonal antibodies and a small molecule inhibitor of polymerisation to define the dynamics of polymer formation, accumulation and secretion. Pulse-chase experiments demonstrate that Z α1-antitrypsin accumulates as short-chain polymers that partition with soluble cellular components and are partially secreted by cells. These precede the formation of larger, insoluble polymers with a longer half-life (10.9 ± 1.7 h and 20.9 ± 7.4 h for soluble and insoluble polymers, respectively). The M* intermediate (or a by-product thereof) was identified in the cells by a conformation-specific monoclonal antibody. This was completely abrogated by treatment with the small molecule, which also blocked the formation of intracellular polymers. These data allow us to conclude that the M* conformation is central to polymerisation of Z α1-antitrypsin in vivo; preventing its accumulation represents a tractable approach for pharmacological treatment of this condition; polymers are partially secreted; and polymers exist as two distinct populations in cells whose different dynamics have likely consequences for the aetiology of the disease.

Abbreviations
BafA1, bafilomycin A1; ConA, concanavalin A; Endo H, endoglycosidase H; ER, endoplasmic reticulum; IP, immunoprecipitation; iPSCs, induced pluripotent stem cells; mAb, monoclonal antibodies; NP-40, Nonidet P40; PAS, periodic acid–Schiff; PNGase F, peptide-N-glycosidase F; Tet-On, tetracycline-controlled transcriptional activation.
condensing as periodic acid–Schiff (PAS)-positive inclusions [1–5] that are associated with neonatal hepatitis, cirrhosis and hepatocellular carcinoma [6].

In vitro studies demonstrated that the Glu342Lys substitution perturbs folding of Z α1-antitrypsin allowing the formation of a monomeric unstable intermediate, denoted M* [7]. This state is associated with changes in β-sheet A and helix F [3,7–10] and is a precursor to oligomerisation involving an intermolecular domain swap [3,11,12]. Serpin polymerisation is a form of nonamyloid aggregation. The process involves β-sheet interactions but is distinguished by an intermediate that remains largely well-folded [13] with the sub-units of the resulting polymers exhibiting only minimal structural perturbation with respect to the native conformation [11,12,14–16]. These ordered structures most likely explain the failure of polymers to activate the unfolded protein response in cellular and animal models of the disease [17,18]. However, the accumulation of α1-antitrypsin polymers within hepatocytes results in an increase in ER volume, increased intraluminal viscosity [19] and the formation of ER-derived membrane-bound inclusions [20]. Moreover, the extent of accumulation of Z α1-antitrypsin and the associated hepatotoxicity depends on the efficiency of both ER-associated degradation (ERAD) [21–24] and lysosomal-associated degradative pathways [25–27].

The structural variability of α1-antitrypsin has resulted in the development of a toolkit of conformation-specific monoclonal antibodies (mAb) that are able to recognise different conformations of this protein. The polymer-specific 2C1 mAb [28,29] that recognises intrahepatic α1-antitrypsin polymers revealed that polymers of α1-antitrypsin are present in the circulation of all individuals with Z α1-antitrypsin deficiency [30]. The origin of these polymers is unclear, but our recent data from cell models of disease demonstrated indirectly that polymers can be secreted by cells [31].

The 5E3 mAb was used to characterise the polymerisation-prone intermediate M* [32] in vitro, but has not been evaluated in a cellular model of α1-antitrypsin deficiency.

We have used metabolic labelling and antibodies with different conformational preference (Table 1) to characterise the kinetics of polymer formation and resolution and the intracellular distribution and secretion of Z α1-antitrypsin polymers in cellular models of disease.

### Results

**Intracellular Z α1-antitrypsin polymers partition in both the NP-40-soluble and NP-40-insoluble fractions**

A previously established and validated inducible tetracycline-controlled transcriptional activation (Tet-On) cellular system [19] was used to investigate the partitioning of intracellular polymers of Z α1-antitrypsin. Chinese hamster ovary (CHO) K1 cells expressing Z or wild-type M α1-antitrypsin were induced with 0.5 μg·mL⁻¹ doxycycline for 48 h and lysed in a buffer containing 1% v/v Nonidet P40 (NP-40). Centrifugation at 12 000 g was used to separate the soluble from the insoluble fraction, and the latter was then mechanically resuspended in lysis buffer and sonicated. Biochemical analysis of these fractions and the culture medium supernatants by denaturing SDS/PAGE and immunoblot confirmed that M α1-antitrypsin was only present in the cellular soluble and secreted fractions (Fig. 1A, upper panel and graph); the wild-type variant is known to efficiently transit through the secretory pathway to the extracellular medium [19,33,34]. In contrast, Z α1-antitrypsin accumulated in both the NP-40-soluble and NP-40-insoluble fractions and was present at lower level in the cell medium compared with M α1-antitrypsin (Fig. 1A, lower panel and graph).

The NP-40-soluble and NP-40-insoluble fractions were then analysed by non-denaturing PAGE and immunoblot for total α1-antitrypsin (Fig. 1B). The soluble fraction obtained from cells expressing M α1-antitrypsin migrated as a single monomeric band, while the soluble fraction from cells expressing Z α1-antitrypsin showed a ladder of higher molecular weight forms consistent with a population of α1-antitrypsin polymers of variable size [4]. Of note, the broad profile of the soluble fraction exhibited an overall faster migration (Fig. 1B, lower) and contained distinct bands that matched, allowing for differences in N-glycosylation maturation states, with corresponding bands in the supernatant. The insoluble fraction, instead, was characterised by a high molecular weight-shifted smear and the absence of low molecular weight bands. A fraction of this sample was trapped in the stacking gel suggesting the additional presence of larger, higher molecular weight species.

The presence of α1-antitrypsin monomers and polymers in the soluble and insoluble intracellular fractions

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**Table 1. mAbs specificity.**

| mAb | mAb specificity | References |
|-----|----------------|------------|
| 2C1 | α1-antitrypsin polymers | Miranda et al. [28] |
| 3C11 | Total α1-antitrypsin | Tan et al. [30] |
| 5E3 | α1-antitrypsin folding intermediate (M*) | Irving et al. [32] |
was analysed by sequential immunoprecipitation (IP) using antibodies with differential conformational selectivity (Table 1 and Fig. 1C). First, polymers were immunoprecipitated three times with the antipolymer 2C1 mAb [28] and then residual monomer captured in one round with the non-conformation-selective 3C11 mAb [30], using the supernatant of each IP as the input for the next one (Fig. 1C, SET 1). Cells expressing M α1-antitrypsin showed a single band positive for 3C11 in the soluble fraction and no 2C1 recognition in either cellular fraction, confirming its monomeric state under the expression conditions used. The soluble
fraction from cells expressing Z α1-antitrypsin was positive for 2C1, and Z α1-antitrypsin could still be immunoprecipitated with 3C11 after three rounds of polymer depletion. In contrast, three rounds of IP of the insoluble fraction with 2C1 depleted virtually all α1-antitrypsin, with no residual nonpolymeric material recognised by the 3C11 mAb. In a control experiment, the order of the mAbs was reversed (three rounds with 3C11 and a final one with 2C1). As expected, all α1-antitrypsin was immunoprecipitated by the non-conformation-selective 3C11 mAb (Fig. 1C, SET 2).

**Z α1-antitrypsin polymers are secreted through the canonical secretory pathway**

Radiolabeling-based approaches provide a means to follow the molecular fate of a protein in a dynamic system without the perturbations that may be introduced by fluorescent tags. We investigated the kinetics of formation and accumulation of α1-antitrypsin polymers by pulse-chase experiments. CHO K1 cells expressing M or Z α1-antitrypsin were pulse-labelled for 10 min with 35S-methionine and cysteine, and at the chase times indicated in Fig. 2A, the culture medium was collected and the cells lysed in NP-40 buffer. M α1-antitrypsin samples were immunoprecipitated with 3C11 mAb to recover all α1-antitrypsin (monomeric, as demonstrated in Figs 1 and 2A, top panels, and Fig. 2B, left panel), while the Z α1-antitrypsin samples were sequentially immunoprecipitated with the 2C1 antipolymer mAb (Fig. 2A, middle panels and Fig. 2B, Z-polymers) and then with the 3C11 mAb (Fig. 2A, lower panels and Fig. 2B, Z-monomers).

As shown in Fig. 2A,B, in cells expressing the Z variant, radiolabelled monomeric α1-antitrypsin was detectable at the initial 0 h time point, but intracellular soluble polymers only became visible after a delay of 2 h and gradually increased to plateau at 8 h. Soluble extracellular polymers became detectable after 4 h of chase. In the insoluble fraction, polymers were apparent from the 8-h time point and also increased with time, without exhibiting a plateau over the duration of the experiment.

In the autoradiographs (Fig. 2A), the secreted material (both monomeric and polymer Z α1-antitrypsin, white arrowheads) had a higher molecular mass compared with the intracellular species (black arrowheads), as is characteristic of mature glycoproteins secreted through the canonical secretory pathway [28,31]. Analysis of the N-linked glycosylation state was performed to assess the type of glycans present in the extracellular polymers. Polymers immunoprecipitated by 2C1 mAb from culture media of Z α1-antitrypsin cells pulsed as in Fig. 2A were subjected to digestion with peptide-N-glycosidase F (PNGase F), which removes all types of N-linked glycans, or endoglycosidase H (endo H), which cleaves pre-Golgi glycans only. All secreted α1-antitrypsin was sensitive to digestion by PNGase F but resistant to treatment with endo H (Fig. 2C). This demonstrated that at all time points only mature glycans were present in extracellular polymers and therefore that their presence in the media was the result of passage through the secretory pathway and not a cell lysis-related artefact.

**Insoluble Z α1-antitrypsin polymers have a longer intracellular half-life than soluble polymers**

Soluble Z α1-antitrypsin polymers reached a peak at approximately 4 h and remained stable until 12 h, while insoluble polymers were still increasing after 12 h of chase (Fig. 2A). The kinetics of clearance of these polymers was assessed with a long metabolic labelling experiment. CHO K1 cells expressing Z α1-antitrypsin were metabolically labelled with 35S methionine and cysteine for 24 h. At the end of the labelling time, the radioactive culture medium was replaced with normal medium and the cells were grown for a further 12 h. This prolonged 'conversion time' was designed to promote the incorporation of all the radioactive monomeric Z α1-antitrypsin molecules into polymers. Following the conversion period, cells were chased for up to 72 h and polymeric α1-antitrypsin was immunoprecipitated with the 2C1 mAb (Fig. 2D). This analysis demonstrated that soluble polymers were cleared more rapidly from cells than the insoluble counterpart. The half-time for the clearance of intracellular soluble polymers was 10.9 ± 1.7 h, while the half-time for the insoluble polymers was 20.9 ± 7.4 h (Table 2). It is notable that while soluble polymers were almost entirely cleared following the 72-h chase, a residual insoluble component remained.

In order to verify the data obtained in the inducible CHO K1 cell lines and to expand our analysis to a more physiologically relevant context, the same approach was applied to induced pluripotent stem cell (iPSC)-derived hepatocytes. These had been generated previously using fibroblasts from an individual homozygous for the Z variant of α1-antitrypsin [35,36]. iPSC-derived hepatocytes predifferentiated for 23 days were revived from frozen stocks and kept in specific medium and under hypoxic conditions in order to promote further hepatic differentiation [37]. At day 35 postdifferentiation, cells were starved, radioactively labelled with 35S methionine and cysteine for 24 h and...
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incubated for 12 h in nonradioactive medium to allow the conversion of radioactive $\alpha_1$-antitrypsin monomers into polymers. Culture media were collected and cells processed every 12 h up to 108 h postpulse. Intracellular soluble and insoluble fractions of $\alpha_1$-antitrypsin polymers were immunoprecipitated with the 2C1 mAb and analysed as above. In this cell system, soluble polymers also exhibited a shorter half-time $14.6 \pm 6.2$ h compared with $19.1 \pm 4.9$ h for insoluble polymers (Table 2).

**Circulating polymers of $\alpha_1$-antitrypsin have a predominantly intracellular origin**

Our data provide direct evidence that polymers are actively secreted by cells. This has potential implications for the interpretation of polymers identified in patient plasma [30]. However, while plasma contains a complex mixture of molecules, including sugars and proteins such as serum albumin which are known to act as stabilisers against denaturation [38], it is unknown whether polymers can form spontaneously in the circulation. A previous experiment in which plasma samples from $\alpha_1$-antitrypsin homozygotes were incubated *in vitro* at 37 °C for 3 days showed no increase in polymer content [30]. To extend this study, plasma samples from three different $\alpha_1$-antitrypsin homozygote individuals were incubated at 20, 37, 41, 45 and 50 °C for 24 h and polymer formation was assessed by ELISA with the 2C1 mAb relative to recognition of the total $\alpha_1$-antitrypsin population by the 3C11 mAb (Fig. 3A, first bar). There was no significant increase in the $\alpha_1$-antitrypsin polymer signal following 24-h incubation over this range of temperatures (Fig. 3A, bars 3 to 7). Plasma samples from the same patients were also incubated at 37 °C for 1, 3 and 10 days, representing 0.2-, 0.6- and twofold the half-life of $\alpha_1$-antitrypsin in the circulation [39], and analysed again by 2C1 mAb ELISA. Again, no significant difference in polymer signal was observed after 3 and 10 days of incubation (Fig. 3B).

The plasma samples were also concentrated by concanavalin A (ConA) pull-down and analysed on a nondenaturing gel followed by western blot with the 2C1 mAb (Fig. S1). This analysis showed a small but nonsignificant increase in polymer content after 10 days. Together, these data support the conclusion that $\alpha_1$-antitrypsin has marked stability in plasma and accordingly the primary source of circulating polymer is most likely secretion from cells.

**Soluble and insoluble polymers are cleared by different mechanisms of degradation**

Endoplasmic reticulum-associated degradation, mediated by the proteasome, has previously been identified as the major contributor to turnover of the $\alpha_1$-
antitrypsin variant [9,22,40–43]. CHO K1 cells expressing Z α1-antitrypsin were subjected to pulse chase in the presence of the reversible inhibitor MG132 in order to investigate the effect of proteasomal degradation on the kinetics of accumulation and secretion of the polymer populations. Treatment with the inhibitor, compared with the kinetics of untreated cells shown in Fig. 2 (and reported in Fig. 4, lower panel, NT), resulted in increased accumulation of polymers in the soluble but not in the insoluble intracellular fractions, and increased polymeric and total Z α1-antitrypsin in the culture medium (Fig. 4A, upper panel and Fig. 4B, upper panels). MG132 did not appreciably alter the accumulation of monomer in the supernatant over time, but instead led to the intracellular retention of a monomeric component (Fig. 4B, lower panels).

Recent work has shown that in addition to an autophagy-mediated mechanism [43], Z α1-antitrypsin polymers are degraded via a distinct lysosome-associated system [27]. We thus evaluated the clearance of soluble and insoluble intracellular polymers by lysosomes in our cell model. CHO K1 cells expressing Z α1-antitrypsin were induced with doxycycline for 48 h, labelled for 15 min with 35S methionine and cysteine and then treated for 16 h with either 50 nm bafilomycin A1 (BafA1), a drug capable of inhibiting the lytic activity of lysosomes, or with a DMSO control. Cells were processed at different time points as shown in Fig. 4C, and intracellular fractions were immunoprecipitated first with the 2C1 mAb for polymers and then with the 3C11 mAb for residual nonpolymeric α1-antitrypsin. Treatment with BafA1 caused a small but nonsignificant increase in the quantity of insoluble polymers but had no effect on soluble polymers or monomeric Z α1-antitrypsin.

An experimental compound, c716, prevents intracellular polymer formation and increases secretion

We have developed a small molecule inhibitor of polymerisation, c716, that is active in vivo and that prevents the formation of M* in vitro [44]. To investigate its behaviour under the experimental conditions considered here, CHO K1 cells were seeded in the presence of DMSO or c716 and subsequently induced to produce Z α1-antitrypsin for 48 h before being starved, pulsed and processed as described in Fig 1. Intra-Z α1-antitrypsin and extracellular Z α1-antitrypsin were sequentially immunoprecipitated with 2C1 and 3C11 mAbs. The experimental compound abolished the formation of both soluble and insoluble polymers (Fig. 5A, upper panels and graphs) and increased the secretion of Z α1-antitrypsin (Fig. 5A, lower right panel and graphs). Indeed, treatment with the compound resulted in rates of secretion similar to those of the wild-type M α1-antitrypsin (compare Fig. 5A, right graph with Fig. 2B, left graph), increasing the amount secreted after 1 h by ~5-fold from 5.2 ± 0.2% (± SD, n = 2) to 28.0 ± 5.3% (± SD, n = 2).
The monomeric intermediate M* is the key species for Z α1-antitrypsin intracellular polymer formation

In vitro experiments have shown that α1-antitrypsin folds via an intermediate ensemble [43] before reaching its native monomeric state [13,45,46]. Folding is rapid for wild-type M α1-antitrypsin but is delayed for the Z mutant, increasing the population of these intermediate conformations [47]. One of these conformations, that we have termed M*, is a precursor to polymer formation and elongation [3,7,48,49]. This molecular species, or a monomeric by-product thereof, is sufficiently persistent to be detectable in vitro [11,46,49] and is recognised by the 5E3 mAb [32] (Table 1). The presence of M* in a cellular environment has not been established; we therefore made use of the 5E3 mAb and c716 to determine its role in intracellular polymer formation.

CHO K1 cells expressing Z α1-antitrypsin were induced with doxycycline and treated for 8 h with DMSO, c716 or MG132. Intra- and extracellular fractions were collected and subjected to sequential IPs, first with the 2C1 antipolymer mAb (four rounds to guarantee complete depletion, Fig. S2), once with the anti-M* 5E3 mAb and finally with the 3C11 antitotal α1-antitrypsin mAb. 2C1-positive polymers were efficiently immunoprecipitated from the NP-40-soluble intracellular fraction (Fig. 5B, left panels). However, a fraction remained that was immunoprecipitated by the 5E3 mAb and a residual amount of α1-antitrypsin that was immunoprecipitated by the 3C11 mAb, corresponding to the native monomer. Inhibition of ERAD with the proteasomal inhibitor MG132 slightly increased the quantity of polymers recognised by 2C1 mAb with a corresponding decrease in monomers detected by the 3C11 mAb.

The extracellular fraction was characterised by the presence of polymers and native folded monomers (Fig. 5B, right panel, NT lanes); treatment with MG132 increased the secretion of α1-antitrypsin polymers and decreased the accumulation of monomer (Fig. 5B, right panel, MG132 lanes). It is interesting to note that little extracellular material could be immunoprecipitated by 5E3 mAb, demonstrating the intracellular nature of this folding intermediate and the specificity of the mAb. Stabilisation of a native-like state using c716 reduced the formation of M* and polymers, favouring the secretion of the monomeric form of Z α1-antitrypsin (Fig. 5B, right panel). The level of insoluble polymers remained unchanged upon treatment with MG132, while treatment with c716 completely abolished the presence of polymers and M* in this fraction (Fig. 5A).

Discussion

The Z (Glu342Lys) mutant of α1-antitrypsin misfolds and forms linear unbranched polymer chains in the ER. It has been shown in cell models of disease that proteostatic degradative mechanisms – ERAD, autophagy and lysosomal-associated degradation – compensate to some degree for this intracellular burden of misfolded and aggregated protein [18,21,22,27,42,43]. However, a hallmark of α1-antitrypsin deficiency is the deposition of polymers of mutant α1-antitrypsin as PAS-positive inclusions within hepatocytes [3]. The processes that result in this retention of α1-antitrypsin are incompletely characterised.

Polymers of α1-antitrypsin partition into components of cellular extracts that can be defined as ‘NP-40-soluble’ and ‘NP-40-insoluble’ [42,50], but it has not been determined whether these reflect a single population of molecules or two populations with distinct characteristics. Here, we investigated in detail the kinetics of formation, deposition and secretion of Z α1-antitrypsin polymers. Our data show that the two populations of polymers with differential solubility do indeed exhibit different characteristics: the NP-40-soluble fraction contained a mixture of monomers and lower molecular weight polymers, with a shorter cellular half-life and whose levels increased in the presence of the proteasomal inhibitor MG132. In contrast, the NP-40-insoluble fraction lacked monomers, had a generally larger polymer size distribution, exhibited a longer half-life and was unaffected by MG132. Notably, the latter population appeared to include a component that was not degraded by the cellular proteostatic mechanisms over the course of a 72 h of experiment. We speculate that this may represent the species that form intractable inclusions within the liver of Z α1-antitrypsin individuals. A limited subset of polymers appeared in the culture medium at a similar rate to their appearance in the intracellular soluble fraction and with a similar size profile, suggesting progression from formation to secretion. These extracellular polymers were sensitive to PNGase F but resistant to endo H digestion, in keeping with the maturation of N-glycans during transit through the Golgi complex along the canonical secretory pathway [51]. Additionally, the faster migration of the bands under electrophoresis of the soluble fraction from 4 h onwards is consistent with trimming of N-glycans that occurs in the ER or in the cis-Golgi [52].
A

B

C

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Inhibition of the proteasome increases soluble intracellular Z α1-antitrypsin polymers. (A) CHO-K1 Tet-On cells expressing Z α1-antitrypsin were induced with doxycycline for 48 h, treated with 80 µM MG132, labelled for 10 min with [35S] Met/Cys and chased at the reported times. Culture media were collected and cells lysed in 1% v/v NP-40 buffer. Intracellular fractions and culture media were immunoprecipitated twice with the polymer-specific mAb 2C1 and then with the 3C11 mAb against total α1-antitrypsin. Samples were resolved by 4–12% w/v acrylamide SDS/PAGE and detected by autoradiography. White and black arrowheads indicate the mature and immature glycosylated forms of α1-antitrypsin, respectively. Representative of three independent experiments. (B) Graphs show densitometric analysis of MG132 pulse-chase experiments in (A) (mean ± SEM, n = 3). Densitometry of the bands was performed by IMAGE STUDIO software. Statistical analysis (two-way ANOVA) was calculated using GRAPHPAD PRISM. Dotted lines are intended to indicate the trend of the data only. Protein precipitated in the first round of IP with the 2C1 mAb is denoted ‘polymer’. ‘Monomer’ refers to residual α1-antitrypsin immunoprecipitated by the 3C11 mAb after two rounds of IP with the 2C1 mAb. (C) CHO cells expressing Z α1-antitrypsin were induced with 0.5 µg/mL doxycycline and treated with either 50 nM BafA1 or 0.1% v/v DMSO (NT) for 16 h. After lysis at the indicated times, 1% v/v NP-40-soluble and NP-40-insoluble fractions were separated and sequentially immunoprecipitated with the 2C1 antipolymer mAb and once with the 3C11 mAb that detects all conformers of α1-antitrypsin. The eluted samples were resolved on 4–12% w/v acrylamide SDS/PAGE followed by immunoblotting for total α1-antitrypsin. The top panel is representative of three independent experiments, and the graph shows mean ± standard error of the mean (± SEM, n = 3). Densitometry of the bands was performed by IMAGE STUDIO software. ‘Polymer’ denotes the protein precipitated in the first round of IP with the 2C1 mAb. ‘Monomer’ refers to residual α1-antitrypsin immunoprecipitated by the 3C11 mAb after two rounds of IP with the 2C1 mAb.

These modifications were not seen in insoluble polymers, suggesting that they are inaccessible to the enzymes that process N-glycans. Treatment with the proteasome inhibitor MG132 increased the formation of intracellular soluble and secreted polymers but had no effect on insoluble intracellular polymers. This supports the hypothesis that while soluble polymers are trafficked through the secretory pathway and into the extracellular space, insoluble polymers represent a molecular endpoint that cannot be resolved by proteasomal degradation but that must be resolved by alternative cellular homeostatic processes such as autophagy.

The CHO cell model faithfully recapitulates the handling of mutant variants of α1-antitrypsin seen in other cellular models [23,53], with the benefits of inducible and titratable expression and robust characteristics in cell culture. We confirmed our findings using an iPSC model of Z α1-antitrypsin deficiency [35,36], which showed similar half-times for the clearance of soluble and insoluble polymers. In both cellular models, a longer turnover time for insoluble polymers, together with the differing effect of proteasomal inhibition on polymer pools, suggests that soluble and insoluble polymers are handled in different ways; smaller and more soluble polymers can be secreted, whereas larger ones, which we hypothesise represent the precursors of the inclusion bodies seen in pathological specimens, are more persistent and only partially degraded by an alternative pathway related to lysosomal degradation [43,54].

We have shown previously that polymers of α1-antitrypsin can be detected in the circulation of all subjects who carry a Z allele [55]. Our observation that polymers of Z α1-antitrypsin can be secreted by cellsin vitro, coupled with the inability to induce polymer formation in plasma samples incubated at either high temperature or for prolonged times, allows us to conclude that the polymers found in circulation are most likely the result of secretion of soluble polymers from hepatocytes. Circulating polymer levels therefore represent a potential ‘window’ into the efficacy of the proteostatic processes that regulate the formation and accumulation of α1-antitrypsin polymers in the liver of individuals with α1-antitrypsin deficiency.

Many in vitro studies have established that α1-antitrypsin folds via an intermediate ensemble [13,27,32,56] that samples a polymerisation-prone intermediate state that we have termed M* [3,7,11,13,33,47,49]. However, the presence of M* within the ER as an intermediate on the Z α1-antitrypsin secretion pathway has not been established. Using the 5E3 mAb that detects M* [43] and a small molecule that stabilises α1-antitrypsin against M* formation, we identified three different conformational species within the NP-40-soluble fraction in cells expressing Z α1-antitrypsin: α1-antitrypsin polymers, the M* intermediate and a residual conformer that is most likely native monomer. Using MG132, the M* population was found to be unaffected by the ERAD pathway, indicating that this molecular state is either not recognised as aberrant or assimilated sufficiently rapidly into polymers to evade degradation. In contrast, the formation of M* was suppressed almost completely by c716, resulting in an increase in native monomer and inhibition of both soluble and insoluble polymer formation. Thus, both soluble and insoluble species have a common origin and are not formed by alternative pathways. The compound also restored the secretion profile of Z α1-antitrypsin to that of the wild-type protein [19,34].
demonstrating that M* is a key point in the process of Z α1-antitrypsin folding, degradation and polymerisation within the ER of cells.

In summary, our data allow us to propose a model for the handling of Z α1-antitrypsin within the ER. The nascent α1-antitrypsin polypeptide folds via M* to native monomer to become incorporated into a polymer in the soluble fraction of the cell. This polymer can in turn become insoluble through mechanisms that have yet to be elucidated, can be secreted or be degraded. As these secreted polymers are a product of the intracellular processes of expression, M* formation, oligomerisation, soluble–insoluble partition and secretion, they potentially represent a useful reporter of intrahepatic polymerisation for polymer-blocking therapies in individuals with α1-antitrypsin deficiency.
Materials and methods

Inducible cell lines and iPSC-derived hepatocytes

Chinese hamster ovary (CHO K1) cell lines expressing M or Z α1-antitrypsin under the tetracycline-inducible promoter [19] were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% v/v tetracycline-free FBS (Takara Bio, Saint-Germain-en-Laye, France), 1% w/v penicillin/streptomycin, 200 μg·mL⁻¹ gentamicin and 500 μg·mL⁻¹ hygromycin B (both selective antibiotics from Invitrogen, Carlsbad, CA, USA) at 37 °C and 5% v/v CO₂. Before each experiment, cells were seeded at a density of 16.3 × 10⁴ cells·cm⁻² and induced to express α1-antitrypsin with 0.5 μg·mL⁻¹ doxycycline for 48 h.

iPSC-derived hepatocytes and supplements for cell media were supplied by DefiniGEN (DefiniGEN Ltd, Cambridge, UK). Cells were revived and maintained as advised by the supplier. Cells were differentiated in mature hepatocytes after 10 days of culture in hypoxic conditions (5% v/v CO₂, 5% v/v O₂, 90% v/v N₂) in their recommended supplemented medium.

Cell lysis, polymer extraction and immunoprecipitation

Both CHO K1 cells and iPSC-derived hepatocytes were lysed at a concentration of 2.5 × 10⁶ cells·mL⁻¹ in 1% v/v NP-40 buffer (10 mM Tris, pH 7.4, 300 mM NaCl, 1% v/v NP-40) supplemented with protease inhibitors (Roche Ltd, Hertfordshire, UK). Cell lysates were collected and mixed for 30 min at 4 °C on a rotator mixer. 1% v/v NP-40-insoluble and NP-40-soluble fractions were separated by centrifugation at 16 000 g for 15 min at 4 °C. The supernatant was collected (1% v/v NP-40-insoluble fraction), while the pellet (1% v/v NP-40-insoluble fraction) was washed twice in 1% v/v NP-40 buffer and mechanically resuspended in an equal volume of 1% v/v NP-40 buffer supplemented with protease inhibitors. The 1% v/v NP-40-insoluble fraction was finally solubilised by sonication at 1.15 KHz (5 μm amplitude) for 15 s at RT (Soniprep 150, MSE Ltd, London, UK).

For analysis in nondenaturing PAGE, total α1-antitrypsin from cell lysates and culture medium samples was concentrated using ConA-conjugated agarose beads (Sigma-Aldrich Co, Dorset, UK). Samples were diluted 1 : 2 with binding buffer (20 mM Tris, pH 7.0, 0.5 M NaCl) and incubated overnight on the rotator at 4 °C. Elution was performed by incubating the beads with 1 M methyl-α-D-mannopyranoside at 37 °C for 2 h. The eluates were then resolved on 3–8% w/v nondenaturing PAGE (Bio-Rad Laboratories Ltd, Hertfordshire, UK) and immunoblotted with a rabbit polyclonal anti-α1-antitrypsin (only the first 2C1 IP is shown). The top panels are representative of two independent experiments, and the graphs show mean ± standard deviation (± SD, n = 2). Asterisk (*) indicates signals from intracellular material released by dead cells. Dotted lines do not show the fit but indicate the trend of the data.

Immunoprecipitation

For IP, cell lysates or culture media were mixed on the rotator with 1 μg of purified antipolymer 2C1 mAb [28] for 1 h at 4 °C. Recombinant protein G agarose beads (Thermo Fisher, Loughborough, UK) were then added and the sample incubated on the rotator overnight at 4 °C. The supernatant was collected and subjected to a further two rounds of IP with the 2C1 mAb, and the residual monomeric species of α1-antitrypsin were immunoprecipitated with the antitotal α1-antitrypsin 3C11 mAb [55]. For the experiment shown in Fig. 5B, the culture media and cell lysate samples were sequentially immunoprecipitated four times with the antipolymer 2C1 mAb, to ensure that all the polymers had been captured, once with anti-M* intermediate 5E3 mAb [32] and finally with antitotal α1-antitrypsin 3C11 mAb. At the end of each IP step, beads were collected, washed three times with 1% v/v NP-40 buffer and once with 10 mM Tris, pH 7.4, and eluted in loading buffer.

Fig. 5. Detection of the folding intermediate M* in cells. (A) CHO-K1 Tet-On cells expressing Z α1-antitrypsin were induced with doxycycline and treated either with the small molecule polymerisation inhibitor (c716) or with 0.1% v/v DMSO for 48 h and labelled for 10 min with 35S Met/Cys. Culture media were collected and cells lysed in 1% v/v NP-40 buffer as described above. Intracellular fractions and cell media were immunoprecipitated with the 2C1 antipolymer mAb and then the 3C11 total α1-antitrypsin mAb. Samples were resolved by 4–12% w/v acrylamide SDS/PAGE and the proteins detected by autoradiography. Densitometry of the bands was performed using IMAGE STUDIO software. ‘Polymer’ indicates the protein precipitated in the first round of IP with the 2C1 mAb. ‘Monomer’ refers to residual α1-antitrypsin immunoprecipitated by the 3C11 mAb after two rounds of IP with the 2C1 mAb. NT represents the densitometric analysis of untreated cells, while ‘polymer inhibitor’ refers to cells treated with the experimental small molecule (c716). The top panels are representative of two independent experiments, and the graphs show mean ± standard deviation (± SD, n = 2). Dotted lines do not show the fit but indicate the trend of the data. (B) CHO cells expressing Z α1-antitrypsin were induced with 0.5 μg·mL⁻¹ doxycycline and treated with either the small molecule inhibitor of polymerisation (c716) or 0.1% v/v DMSO for 48 h. Cells treated with proteasome inhibitor were incubated with 80 μM MG132 1 h before the beginning of the secretion assay. After washing and incubation in Opti-MEM, the NP-40-soluble and extracellular fractions were separated and sequentially immunoprecipitated four times with the 2C1 antipolymer mAb, once with the 5E3 anti-M* mAb and once with the 3C11 mAb that detects all conformers of α1-antitrypsin. The eluted samples were resolved on 4–12% w/v acrylamide SDS/PAGE followed by immunoblotting with a rabbit polyclonal anti-α1-antitrypsin (only the first 2C1 IP is shown). The top panels are representative of two independent experiments, and the graphs show mean ± standard deviation (± SD, n = 2). Asterisk (*) indicates signals from intracellular material released by dead cells. Dotted lines do not show the fit but indicate the trend of the data.
performed with 35S Cys/Met for 12 h in the presence of
samples from patients were analysed by sandwich ELISA
to IP or concentration by ConA-agarose resin. The serum
5 min and 48 h. Long metabolic labelling (0.9 MBq/10⁶ cells) was
then chased in normal culture medium for 0, 0.5, 1, 2, 4, 8
and 12 h. Radiolabelled α1-antitrypsin into polymers.
Cells were pulsed (0.45 MBq/10⁶ cells) for 10 min with 35S
hepatocytes after differentiation to the hepatocyte stage.
Cells were washed twice in prewarmed PBS and then
incubated at 37 °C with Opti-MEM (Gibco, Thermo Fisher
Scientific Ltd, Loughborough, UK). After a 12-h incubation,
culture medium was collected, centrifuged at 300 g for
5 min and 4 °C, transferred into a clean tube and subjected
to IP or concentration by ConA-agarose resin. The serum
samples from patients were analysed by sandwich ELISA
as previously described [28].

Secretion assay and sandwich ELISA
After 48-h induction with 0.5 µg·mL⁻¹ doxycycline, CHO
K1 cells were washed twice in prewarmed PBS and then
incubated at 37 °C with Opti-MEM (Gibco, Thermo Fisher
Scientific Ltd, Loughborough, UK). After a 12-h incubation,
culture medium was collected, centrifuged at 300 g for
5 min and 4 °C, transferred into a clean tube and subjected
to IP or concentration by ConA-agarose resin. The serum
samples from patients were analysed by sandwich ELISA
as previously described [28].

Metabolic labelling and pulse chase
Chinese hamster ovary K1 cells were labelled after 48-h
induction with 0.5 µg·mL⁻¹ doxycycline and iPSC-derived
hepatocytes after differentiation to the hepatocyte stage.
Cells were pulsed (0.45 MBq/10⁶ cells) for 10 min with 35S
Cys/Met (EasyTag™ Express Protein Labelling: Perkin
Elmer, Beaconsfield, UK) in DMEM without Cys/Met and
then chased in normal culture medium for 0, 0.5, 1, 2, 4, 8
and 12 h. Long metabolic labelling (0.9 MBq/10⁶ cells) was
performed with 35S Cys/Met for 12 h in the presence of
cold methionine and cysteine. Cells were then cultured for
12 h in normal medium to promote the incorporation of all
the radioactive monomeric α1-antitrypsin into polymers.
After the pulse, cells were chased at 0, 3, 12, 24, 36, 48, 60
and 72 h for CHO K1 cells and up to 108 h for iPSC-
derived hepatocytes. Radiolabelled α1-antitrypsin was iso-
lated by IP and resolved by SDS/PAGE followed by
autoradiography. Densitometric analysis of α1-antitrypsin
bands was performed with IMAGE STUDIO LITE software (LI-COR Biosciences, Cambridge, UK). Statistical analysis was performed using the GRAPHPAD PRISM program.

Human plasma samples
All human biological samples were obtained with informed
consent under an IRB/EC protocol approved by NRES
Committee London-Hampstead (REC Ref. 13/LO/1085,
IRAS Project ID 130158, Study Title: Targeting Dysfunc-
tional Mechanisms in α1-antitrypsin deficiency).

Acknowledgements
This work was supported by GlaxoSmithKline, the
Medical Research Council (UK) (grant number MR/
NO24842/1) and the NIHR UCLH Biomedical
Research Centre. This work was funded in part by a
grant from Alpha-1 Foundation (USA) (grant number
615837) to RR. E. Miranda is funded by the Alpha-1
Foundation (USA) and the Pasteur Institute-Cenci
Bolognetti Foundation (Italy). D. Lomas is an NIHR
Senior Investigator.

Conflict of interest
David Lomas is an inventor on patent PCT/GB2019/
051761 that includes the small molecule inhibitor of
polymerisation c716. The intellectual property has been transferred from GlaxoSmithKline to UCL Busi-
ness who have licensed it to a third party.

Author contributions
RR, ACP, JAI and DAL designed research; RR, NH-
C and JAI analysed data; RR, NH-C and MR per-
formed the research; and RR, AF, EM, JAI and DAL
wrote the paper.
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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Plasma samples from Fig. 3B were pulled down with concanavalinA (ConA), eluted and resolved on 4-12% w/v acrylamide PAGE. Proteins were then transferred to a PVDF membrane and detected with 2C1 mAb. Densitometric analysis of the signals was performed with ImageStudio software and analysed with Graphpad Prism software. Graph shows mean ± standard error of the mean (±SEM, n=2) (One-way ANOVA, Bonferroni multiple comparisons test, df=4).

Fig S2. CHO cells expressing Z α1-antitrypsin were induced with 0.5 µg/mL doxycycline and treated with either the polymerisation inhibitor or 0.1% v/v DMSO for 48 h. 1 h before the beginning of the secretion assay, cells were treated with 80 µM MG132, washed and incubated in OptiMEM for 4 h at 37°C. The second, third and fourth IP with 2C1 mAb for 1% v/v NP-40 soluble, insoluble and extracellular fractions were resolved after elution, on 4%-12% w/v acrylamide SDS-PAGE followed by immunoblotting for total α1-antitrypsin. No 2C1 positive signal was detectable in the intracellular fraction after the third IP. One set of IP with 2C1 mAb was sufficient to deplete all the polymeric component in the EC.