Mol Biol Rep (2014) 41:6133–6141
DOI 10.1007/s11033-014-3492-z

Functional analysis of novel alpha-1 antitrypsin variants G320R and V321F

Mila Ljujic · Aleksandra Divac Rankov · Snezana Kojic · Elena Miranda · Dragica Radojkovic

Received: 15 July 2013 / Accepted: 17 June 2014 / Published online: 27 June 2014

Abstract Alpha-1 antitrypsin (AAT) gene is highly polymorphic, with a large number of rare variants whose phenotypic consequences often remain inconclusive. Studies addressing functional characteristics of AAT variants are of significant biomedical importance since deficiency and dysfunctionality of AAT are associated with liver and lung diseases. We report the results of the functional analysis of two naturally occurring AAT variants, G320R and V321F, previously identified in patients with lung disease. Neither of variants has been fully functionally characterized. In order to perform their functional analysis both variants were expressed in prokaryotic and eukaryotic systems and their intracellular localization, activity, stability, and polymerization were determined. The results of this study demonstrated that variants G320R and V321F have neither impaired activity against porcine pancreatic elastase nor propensity to form polymers. However, both variants had altered electrophoretic mobility and reduced thermostability when compared to M variant of the protein, indicating a slightly impaired secondary or tertiary structure.

Keywords Alpha-1 antitrypsin · Rare variants · Functional analysis · Thermostability

M. Ljujic (✉) · A. Divac Rankov · S. Kojic · D. Radojkovic
Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia
e-mail: milaljujic@imgge.bg.ac.rs

E. Miranda
Department of Biology and Biotechnologies Charles Darwin and Pasteur Institute – Cenci Bolognetti Foundation, Sapienza University of Rome, Rome, Italy

Introduction

Alpha-1 antitrypsin (AAT), the archetype member of the serine proteinase inhibitor (SERPIN) super family, is a major circulating and tissue inhibitor of serine proteases [1]. AAT is predominantly synthesized by the liver and released into the circulation where it inhibits a variety of serine proteases. The main target of AAT is neutrophil elastase, and control of its proteolytic activity in the lower respiratory tract is considered to be the main function of AAT [2].

The gene coding for AAT, *SERPINA1*, is mapped on the long arm of chromosome 14 (14q32.1) and contains seven exons and six introns [3]. Human AAT is a 52 kDa, 394 amino acids-long glycoprotein composed of nine α helices (A-I), three β sheets and an exposed mobile reactive center loop (RCL), which is a strand of the main β sheet located at the top of the molecule [4]. One of the striking features of AAT, as well as other inhibitory serpins, is that the protein's native state is metastable, which provides the basis for a dramatic conformational change upon reaction with its target protease. In the native conformation the RCL of the protein is exposed enabling binding of and proteolytic cleavage by substrate proteases [5]. Proteolysis of the RCL is followed by an irreversible transition to a very stable form where cleaved RCL is completely inserted into β sheet A, and the protease is translocated to the opposite end of the inhibitor—a mechanism known as 'loop sheet insertion' [6]. While crucial for the inhibitory function, this conformational instability at the same time entails a vulnerability to mutations, with many leading to the accumulation of misfolded protein in the endoplasmic reticulum (ER) of secretory cells and subsequent pathological consequences [7].

To date, about a hundred different alleles of AAT gene have been identified. According to their plasma concentration

and activity, the AAT variants are described as normal, deficient, dysfunctional or null [8]. The AAT variants are, also, categorized according to their prevalence as common and rare. The most common deficient variants are Z (K342E) and S (E264 V), with 95 % of individuals with severe AAT deficiency carrying the ZZ genotype [9]. Variants other than these are classified as rare due to their low allelic frequencies and are estimated to account for 2–4 % of all AAT-deficient variants [10].

The phenotypic outcome of the mutation depends on the precise site and the nature of the mutation. The AAT variants prone to polymerization and retention in the liver are associated with liver disease. Lung disease is attributed to the deficiency of circulating AAT, resulting in uncontrolled proteolytic activity. Deficiency of AAT renders individuals susceptible to early-onset emphysema, whereas the inclusions of polymerized protein in the liver lead to an increased risk of developing transient juvenile hepatitis, cirrhosis and hepatocellular carcinoma [2, 11]. Although the typical pulmonary manifestation of AAT deficiency is emphysema, lung cancer has also been linked with AAT deficiency [12, 13]. The underlying defect in Z related AAT deficiency is protein misfolding, polymerization and retention within the ER [14, 15]. Such retention results in decreased circulating levels of the protein, with homozygous carriers of Z AAT variant having only 15 % of normal protein levels. Two rare deficient variants, Siiyama (S52F) and Mmalton (F52del), are also characterized by pathological polymerization and are associated with hepatic inclusions and severe plasma deficiency. Variant S of AAT forms polymers at lower rate than the Z variant and thus can be cleared by the normal disposal pathways within the cell [16]. Homozygous carriers of S variant are estimated to have approximately 60 % of normal AAT levels. Reduced AAT levels associated with S variant result from increased intracellular degradation of S variant [17].

Due to the low occurrence of rare AAT variants very little is known about their clinical phenotype and epidemiological studies are difficult to undertake. Here we report on functional analysis of two naturally occurring AAT variants denoted as G320R (Gly-320[GGG] - Arg-320[AGG]), also known as P salt lake, and V321F (Val-321[GTC] - Phe-321[TTC]), which were previously identified [18–20]. Both variants were identified in subjects with emphysema [18, 19] and variant G320R was subsequently also identified in a patient with lung cancer [20]. According to the previously published results, variant G320R does not seem to be deficient [18, 20]. However, those data are unavailable for variant V321F. Based on the data available, it is rather inconclusive whether these variants could be associated with AAT deficiency. Variant G320R has been previously shown to have reduced inhibitory activity against trypsin when compared to normal M variant of the protein [20]. However, neither of these two variants has been previously studied in more detail and their consequences on the properties of AAT are unknown.

Materials and methods

Cell culture and DNA transfections

COS-7 cells (CRL-1651) were maintained in Dulbecco's minimum essential medium (DMEM) (Gibco) containing 10 % (v/v) fetal calf serum (FCS) (Gibco) and 50 μg/mL gentamycin (PAA Laboratories GmbH) [21], whereas HepG2 cells (HB-8065) were cultured in DMEM containing 10 % (v/v) FCS, gentamycin (50 μg/mL) and non-essential amino acids (PAA Laboratories GmbH) [22]. All transfections were performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol.

Construction of alpha-1 antitrypsin expression plasmids

Total RNA was isolated from HepG2 cells using RNeasy Plus Kit (Qiagen). cDNA, synthesized by High Capacity cDNA Reverse Transcription (Applied Biosystems), was used to amplify coding sequence for wild type AAT variant M1 (M AAT in further text). AAT open reading frame was cloned into pGEM-T Easy vector using pGEM-T Easy Vector System (Promega). Variants G320R, V321F and Z were generated with the QuickChangeTM Multi-Site-Directed Mutagenesis kit (Agilent Technologies) using M AAT as the template. Primers used for amplification and mutagenesis are given in Table 1.

AAT cDNA cloned into pGEM-T Easy vector was subsequently cloned into pEGFP-N1 and pcDNA3 (Life Technologies) vectors for expression in COS-7 cells and into pQE31 (Qiagen) vector for expression in *E. coli* BL21-CodonPlus-RIL (Agilent Technologies). AAT cDNA for cloning into pcDNA3 was digested with XhoI and HindIII enzymes (New England Biolabs), and for cloning into pEGFP-N1 with KpnI and HindIII enzymes (New England Biolabs). For cloning into pQE-31, AAT cDNA was digested with BamHI and KpnI (New England Biolabs) [23, 24]. All constructs were verified by sequencing prior to experiments.

Purification of AAT expressed in *E. coli*

Purification of recombinant AAT protein was performed under native conditions, using TALON metal affinity resin according to the protocol provided by the manufacturer (Clontech Laboratories Inc.) with modified buffer compositions. Recombinant AAT was isolated from 600 mL of

Table 1 Primers used for amplification and mutagenesis

Primer sequence	Application
5′-CCAAGCTTATGCCGTCTTCTGTCTCGTGGGGCA-3′	cloning into pEGFP-N1vector
5′- CAGGTACCGCTTTTTGGGTGGG-3′	
5′-CCAAGCTTATGCCGTCTTCTGTCTCGTGGGGCA-3′	cloning into pcDNA3 vector
5′-GCCTCGAGTTATTTTTGGGTGGGATTCACCACT-3′	
5′-CCAAGCTTATGCCGTCTTCTGTCTCGTGGGGCA-3′	cloning into pQE31 vector
5′-GCGGTACCTTATTTTTGGGTGGGATTCACCACT-3′	
5′-GTCTTCTTAATGATTGACCAAAATACCAAGTCTCCCC-3′	Z mutagenesis
5′-GCTGACCTCTCCGGGTTCACAGAGGAGG-3′	V321F mutagenesis
5′-GCTGACCTCTCCAGGGTCACAGAGGAGG-3′	G320R mutagenesis

bacterial culture induced for 5 h with 1 mM IPTG at 30 °C. Cells were harvested by centrifugation and re-suspended in 50 mL lysis buffer (50 mM NaH_2PO_4 pH 7.0; 300 mM NaCl; 10 mM imidazole; 1 × protease inhibitors (Complete, EDTA free, Roche)). The cells were lysed using sonication in the presence of 0.75 mg/ml lysozyme (Sigma). The cell debris was removed by centrifugation and supernatant was then incubated with a 1 mL TALON Metal Affinity Resin (Clontech Laboratories Inc.) equilibrated with lysis buffer. After incubation, resins were washed with buffer containing 50 mM NaH_2PO_4 pH 7.0; 300 mM NaCl; 10 mM imidazol. Subsequently, the protein was eluted with 50 mM NaH_2PO_4 pH 7.0; 300 mM NaCl; 150 mM imidazole. Using Amicon Ultra 30 K centrifugal filter units (Millipore) elution buffer was replaced with buffer containing 30 mM Na-phosphate; 160 mM NaCl; pH 7.4. Protein concentration was determined using Bradford reagent (BioRad) and efficacy of purification was analyzed by denaturing polyacrylamide gel electrophoresis (PAGE).

Denaturing PAGE

Proteins were separated in 12 % (w/v) denaturing PAGE in Tris–Glycine SDS running buffer at 100 V in the stacking and 180 V in the running gel. Analysis of difference in protein mobility was performed in the presence of 5 M urea as an additional denaturing agent, in both gel and running buffer. Sample volume was 30 µL. The protein bands were visualized by Coomassie Brilliant Blue staining.

Formation of SDS-stable complexes between AAT and elastase

Complex formation of AAT with elastase from porcine pancreas (SERVA) was examined by monitoring the appearance of SDS resistant inhibitor–protease complex in SDS-PAGE [25]. AAT activity was measured as residual porcine pancreatic elastase activity employing 1 mM N-succinyl-(Ala)3-p-nitroanilide as a chromogenic substrate. The concentration of active porcine pancreatic elastase was determined by measuring the initial rates of hydrolysis of 1 mM N-succinyl-(Ala)3-p-nitroanilide with increasing concentrations of elastase. Reactions were performed in a volume of 50 µL, in assay buffer containing 30 mM NaH_2PO_4; 160 mM NaCl; 0.1 % PEG600; 0.1 % TritonX100; pH 7.4. Dilutions of elastase solution were prepared in assay buffer. Samples were incubated at 37 °C for 10 min and reactions were stopped by tenfold dilution with the assay buffer. All readings were performed at 410 nm using Multiskan RC (Labsystems). Determined concentration of active porcine pancreatic elastase was afterwards used to ascertain concentration of active AAT.

For SDS analysis of AAT-elastase complexes, 3 µg of purified AAT was incubated with the elastase in 30 µL reactions at the following molar ratios of inhibitor to protease—1:0; 1:0.3; 1:0.6 and 1:1. The reaction products were analyzed by 10 % SDS-PAGE and protein bands were visualized by Coomassie Brilliant Blue staining.

Heat stability assays

The effect of the tested mutations on stability of the protein was assessed by heat stability assays [26]. These assays were carried out by incubation of 3 µg of *E. coli* expressed protein in 20 µL of phosphate-buffered saline (PBS) containing 13.7 mM NaCl; 0.34 mM KCl; 0.18 mM H_2PO_4; 1 mM Na_2HPO_4; pH 7.2. Incubations were performed for 1 h at following temperatures: 4, 45, 50, 55 and 60 °C. Heat treated samples were analyzed by non-denaturing PAGE and samples were visualized by Coomassie Brilliant Blue staining. Simultaneously, 1 µL from each sample was taken for assessment of remaining AAT activity against porcine pancreatic elastase. Reactions were performed as described above. Values were normalized to the inhibitory activity of the corresponding protein variant incubated for 1 h at 4 °C.

Statistical analysis

Data regarding relative inhibitory activity of AAT were analysed by paired *t* tests using the GraphPad Prism 6.0

software (GraphPad Software, San Diego, CA, USA). For each protein variant four paired t-tests were performed (one for each temperature). Results are expressed as mean ± SE and differences were considered significant when the p value was < 0.05.

Non-denaturing PAGE

Non-denaturing PAGE was performed in a 7.5 % (w/v) gel with a discontinuous buffer system with cathodic buffer containing 53 mM Tris; 68 mM glycin; pH 8.9; and anodic buffer containing 0.1 M Tris; pH 7.8 [27]. Sample buffer was 1 M Tris–HCl pH 6.8; 20 % glycerol; 0.02 % (w/v) bromophenol blue. Electrophoretic conditions were as previously described [28].

Immunoblot analysis

COS-7 cells were transfected with AAT/pcDNA3 constructs, culture media were collected 24 h after transfection and subjected to non-denaturing PAGE, followed by transfer onto PVDF membrane (Millipore) and western blotting as described [28]. Membranes were probed with anti-AAT Mab 2G7 and HRP-conjugated anti-mouse antibody (Sigma-Aldrich). Antibody complexes were detected by enhanced chemiluminescence, using ECL SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology).

Immunofluorescence and confocal microscopy

Given that intracellular retention is one of the pathological features of mutated AAT, we tested this property for variants G320R and V321F. Intracellular localization of tested AAT variants, as well as well-characterized M and Z variants was determined by immunofluorescence of transiently transfected COS-7 cells. Their localization was monitored with respect to that of the Golgi apparatus and the ER. COS-7 cells were grown on coverslips pretreated with rat tail collagen type I (BD Biosciences) and transiently transfected with AAT/pEGFP-N1 constructs. 24 h after transfection, cells were washed with PBS, fixed with 3 % (w/v) paraformaldehyde and permeabilised with 1 % (v/v) TritonX100. After blocking with PBS containing 2 % (w/v) bovine serum albumin, cells were labeled with either mouse anti-golgin-97 antibody (Molecular Probes) or mouse anti-PDI (Stressgen) followed by anti-mouse biotinylated secondary antibody (Vector Laboratories) and Cy3-streptavidin tertiary antibody (Jackson ImmunoResearch). Cells were imaged by Leica TSC SP8 confocal microscope (Leica Microsystems) using 63/1.4 NA oil immersion lens.

Results

Electrophoretic mobility of AAT variants

Denaturing PAGE revealed that all variants, expressed and purified from bacterial cells, were present in satisfactory amounts and purity for further experiments. This analysis also revealed that both G320R and V321F variants migrated faster when compared to the M AAT. Altered electrophoretic mobility was observed both for proteins expressed in *E. coli* (Fig. 1a, left panel) as well as for proteins expressed in COS-7 cells (Fig. 1c, left panel).

To elucidate if changes in electrophoretic mobility were due to alterations in charge and conformation, electrophoresis was performed in the presence of 5 M urea. The differences in electrophoretic migration were abolished, indicating that these amino acid substitutions change the electrophoretic mobility of AAT (Fig. 1a, right panel).

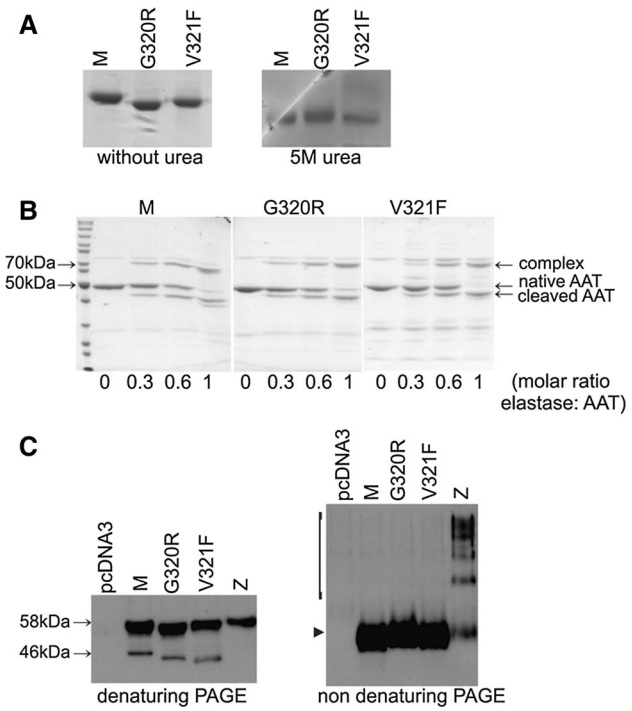

Fig. 1 Analysis of migration, inhibitory properties and polymerization of AAT variants G320R and V321F. **a** SDS–PAGE of M, G320R and V321F AAT expressed in *E. coli* without urea (*left panel*) and with 5 M urea (*right panel*). **b** SDS-PAGE analysis of SDS-stable complexes formed between M, G320R and V321F AAT expressed in *E. coli* and porcine pancreatic elastase at indicated molar ratios of elastase over AAT. **c** SDS–PAGE (*left panel*) and non-denaturing PAGE (*right panel*) followed by immunoblot analysis of culture media from COS-7 cells transiently transfected with M, G320R, V321F, Z and pcDNA3; *square bracket:* AAT polymers; *arrowhead*: AAT monomers. All experiments were performed at least three times and representative images were presented

Inhibitory activity of AAT variants

In order to test if the activity of AAT was impaired due to the presence of the tested mutations, inhibitory activities of AAT variants G320R and V321F were assessed by observing the formation of a covalently bound inhibitory complex with a target protease—porcine pancreatic elastase. Increasing amounts of elastase were added to a constant, empirically determined amount of AAT and formation of stable complexes with elastase was monitored. In both tested variants the proportions of cleaved AAT and AAT in complex with elastase were comparable to that of the M variant (Fig. 1b). In reactions where the molar ratio of AAT to elastase was 1:1 there was no native AAT detected, indicating that all protein reacted with protease. These results show that both tested variants form SDS-stable complexes with elastase as well as M AAT, and imply unimpaired inhibitory activity.

Thermal stability and polymerization of AAT variants

The ability of G320R and V321F variants to form polymers was tested with both *E. coli* and COS-7 expressed proteins. Polymerization of AAT variants expressed in *E. coli* was induced by thermal denaturation for 1 h and their thermal stability was assessed in two ways—by non-denaturing PAGE and monitoring the change in inhibitory activity. Same as M AAT, variants G320R and V321F did not show a change in thermal stability until heated to non-physiological temperatures. However, these variants were slightly more unstable under thermal denaturation. While M AAT was stable until 55 °C, loss of stability for variants G320R and V321F was observed at 50 °C (Fig. 2a). Relative inhibitory activity of heat treated protein against porcine pancreatic elastase compared to the protein incubated at 4 °C followed the same pattern—significant decrease in inhibitory activity for variants G320R and V321F started at 50 °C and for M variant at 55 °C (Fig. 2b). Although polymers could not be detected using this approach, our results show that variants G320R and V321F are present in monomeric and functionally active form at physiological temperatures.

In order to confirm that these variants are not prone to polymerization, culture media from transiently transfected COS-7 cells expressing variants G320R and V321F were tested by non-denaturing PAGE and immunoblot analysis. AAT variants M and Z were analyzed as controls. At 24 h after transfection the Z variant formed detectable polymers. On the other hand, variants G320R and V321F, as well as M AAT, were detected as monomers, clearly confirming that these variants are secreted in the monomeric form (Fig. 1c, right panel).

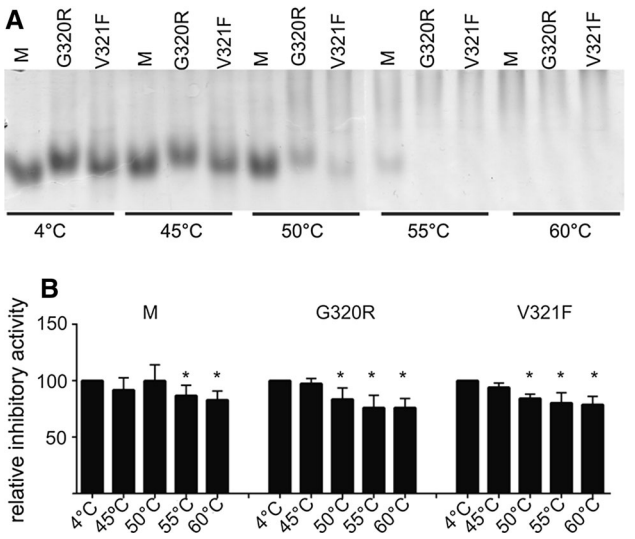

Fig. 2 Thermal stability of AAT variants G320R and V321F. **a** Non-denaturing PAGE analysis of M, G320R and V321F AAT expressed in *E. coli* and incubated for 1 h at indicated temperatures. **b** Relative inhibitory activity of heat treated AAT variants M, G320R and V321F against porcine pancreatic elastase. Proteins were pretreated at indicated temperatures for 1 h, their inhibitory activities were measured and values were represented as percentage of the activity of the protein incubated at 4 °C. Data are the mean ± SD of results from at least three experiments (*$P < 0.05$)

Intracellular localization of AAT variants

At 24 h after transfection variants G320R and V321F were, same as M AAT, predominantly localized in the Golgi apparatus and also present in a region that corresponds to the ER (Fig. 3). At the same time, poorly secreted Z variant was predominantly found in a region that corresponds to the ER and was located in the Golgi apparatus to a lesser extent (Fig. 3). High concentration of AAT variants G320R and V321F in the Golgi apparatus indicates their unimpaired secretion and shows that these variants behave in a similar manner to the M variant.

Discussion

Human AAT is a very polymorphic gene with a large number of rare variants whose effects on protein function and clinical phenotype are poorly understood. Elucidating the mechanism by which mutation affects the protein is of importance in providing information on its structural and functional consequences, as well as in estimating the risk for disease development. Naturally occurring AAT variants G320R and V321F were previously identified and in order to determine whether they could be classified as 'at risk' for disease development, we analyzed their intracellular localization, activity, stability and polymerization propensity.

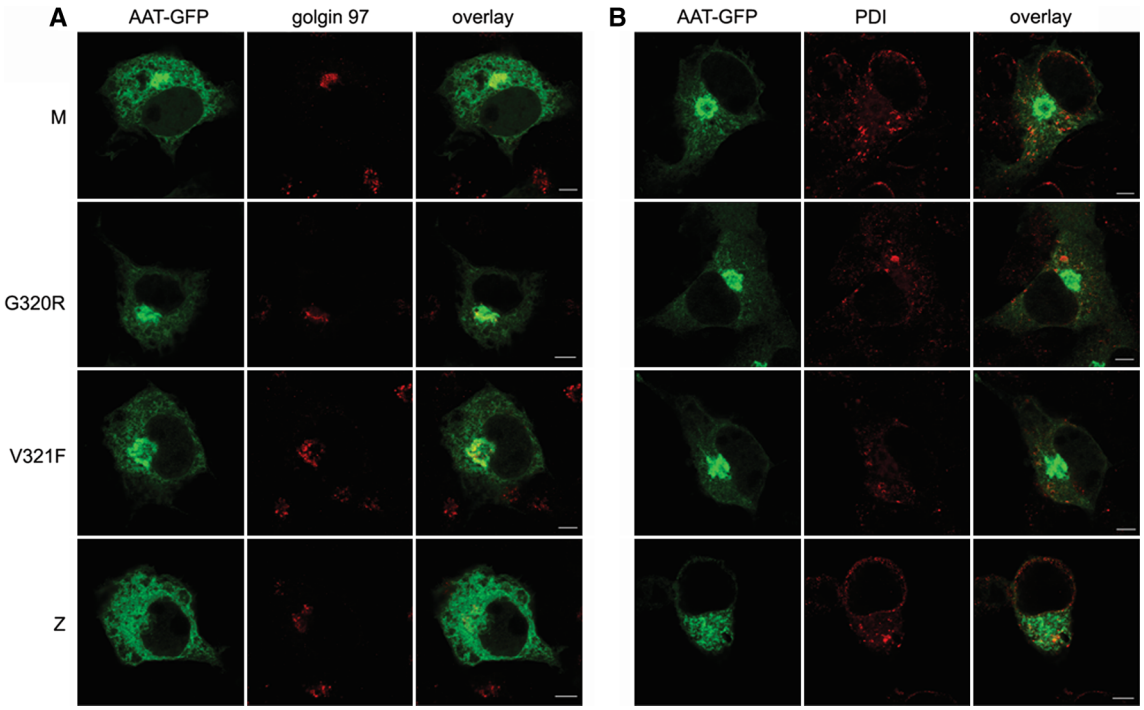

Fig. 3 Intracellular localization of AAT variants G320R and V321F. Confocal microscopy images of intracellular localization of AAT in COS-7 cells transiently transfected with AAT/pEGFP-N1 constructs—M, G320R, V321F and Z. **a**, **b** Cells were fixed 24 h after transfection and stained against either Golgi apparatus resident protein golgin 97 or endoplasmic reticulum marker protein disulfide isomerase (PDI) respectively. Three independent experiments were performed and representative images were presented. Scale bar 5 μm

AAT amino acids G320 and V321 are located in a loop between the α helix I (αI) and strand 5 of β sheet A (s5A) [29]. According to the secondary structure available from Uniprot (uniprot.org/uniprot/P01009) they contribute to the small turn region composed of three amino acids—S319, G320 and V321. In the case of variant G320R, small, flexible and neutral glycine is replaced with bulkier and positively charged arginine, which could affect the region's flexibility. In the case of variant V321F both amino acids are hydrophobic but differ in size, with the mutant residue being larger than the wild type residue. Both mutations are predicted to be damaging by SIFT algorithm [30], and G320R substitution is predicted to be probably damaging and V321F possibly damaging by PolyPhen 2 [31]. The serpin sequence alignment available from SMART (smart.embl.de) [32, 33] shows that over 60 % of compared sequences contain a small amino acid (G, S, A) conserved at position G320 and aliphatic amino acid (I, L, V) conserved at position V321.

When analyzed in denaturing PAGE both G320R and V321F showed altered electrophoretic mobility compared to M AAT. SDS-PAGE is a standard in determining protein's molecular weight since the SDS coating equalizes the charge to mass ratio for all proteins and their relative mobilities are proportional to the molecular weight. However, in some cases membrane proteins load twofold greater amounts of SDS than globular polypeptides [34, 35] and protein tertiary structure may affect both detergent-loading levels and SDS-PAGE migration rate [36]. Electrophoretic analysis of different CFTR fragments showed that hydrophobic mutants bind more SDS molecules and have reduced electrophoretic mobility [36]. Electrophoresis of G320R and V321F variants in the presence of 5 M urea abolished the differences in migration between wild type and mutant proteins. This result could indicate that these mutants are less hydrophobic than the M AAT and that they bind less SDS molecules, causing increased electrophoretic mobility. Further studies are needed to test this hypothesis.

Plasma AAT is normally fully glycosylated at three asparagine residues (46, 83 and 247) [37]. Alterations of the normal glycosylation pattern impair protein secretion and increase its degradation [38]. In case of variants G320R and V321F, a change in electrophoretic mobility was observed in variants expressed in both prokaryotic and eukaryotic systems, clearly suggesting that this alteration was rather due to the properties of these single amino acid substitutions than to the post-translational modifications such as glycosylation.

As for other inhibitory serpins, the inhibition pathway of AAT is a branched one and can be described as a 'suicide'

substrate mechanism [39]. Upon antiprotease binding to the protease, reaction partitions between formation of stable protease-antiprotease complex and cleavage of antiprotease [40, 41]. The outcome of the reaction is dependent on the affinity of the antiprotease for the protease and can be affected by mutations. Most of the mutations affecting inhibitory activity reside either near the RCL or at the loop insertion site on beta sheet A [41]. Mutations in the hinge region of the molecule, which provides mobility essential for the conformational transition, often convert inhibitory serpins into substrates [42]. The inhibitory activity of variants G320R and V321F was monitored according to their ability to form SDS-stable complexes with pancreatic elastase and showed no difference when compared to M AAT. Increase in the amount of elastase added to the constant amount of AAT led to the decrease in the detection of native protein in both variants implying that they readily react with elastase. In all reactions a certain amount of cleaved AAT was present, which can be explained by lower reactivity of AAT with pancreatic elastase than with its primary target, neutrophil elastase [43]. This result is also consistent with the previous studies which have demonstrated that complex formation was favored in reactions with human neutrophil elastase and AAT cleavage is more apparent in reaction with pancreatic elastase [25, 44, 45]. However, the inhibitory activity of the variant G320R differs from the previously published result which revealed that this variant was associated with reduced inhibitory activity against trypsin when compared to M variant [20]. This discrepancy could be explained by the difference in initial material that was used for analysis—while there were no differences in inhibitory activity when recombinant protein was analyzed, G320R AAT from serum showed impaired activity. This could imply that presence of other factors present in serum could affect and interfere with the activity of AAT against trypsin and use of recombinant protein excluded presence and influence of other risk factors. On the other hand, this also emphasizes the importance of other inherited or acquired factors that could affect protein's activity in vivo.

Native conformation of AAT and other inhibitory serpins is not thermodynamically the most stable conformation, which is essential for their inhibitory function. Stabilization of the protein is achieved upon inter- or intra-molecular insertion of RCL into β sheet A and can be induced under denaturing conditions, i.e. temperatures above the physiological one [14, 46, 47]. The loop-sheet polymerization hypothesis provides a structural basis of in vivo aggregation and accounts for retention of AAT in the ER and subsequent deficiency of the protein. Incubation of recombinant AAT at physiological temperatures has been shown to lead to spontaneous polymerization of Z variant but not of M variant [14, 48]. In vitro and in vivo analysis of polymerization of G320R and V321F variants showed that they do not form polymers under physiological conditions. Unlike the Z variant, G320R and V321F variants formed no detectable polymers when expressed in COS-7 cells. Although thermostability of G320R and V321F variants expressed in *E. coli* was mildly reduced when compared to M AAT, both variants were stable at physiological temperatures. Relative inhibitory activity of heat treated protein compared to protein incubated at 4 °C was consistent with the observed decrease in thermostability, reflecting the amount of native and functional protein. Intracellular localization of examined variants was also comparable to that of the M variant with relatively high concentrations of the protein in the Golgi apparatus. This result shows that under normal physiological conditions the tested AAT variants remain in their monomeric form and are steadily secreted.

This study combined analysis of AAT expressed in prokaryotic and eukaryotic cells in order to assess its structural and biochemical properties. The approach used here could be also applied for the analysis of other uncharacterized AAT variants. We have demonstrated that G320R and V321F variants have altered electrophoretic mobility and slightly reduced thermostability in comparison to M AAT. Their reduced thermostability could be associated with changes known to account for altered electrophoretic mobility and detailed structural studies are needed to investigate this hypothesis. In conclusion, the results of this study indicate that inhibitory activity, polymerization and intracellular distribution were unaltered in naturally occurring AAT variants G320R and V321F. Considering that mainly changes in these properties are relevant for pathological effect of AAT mutations it is most likely that presence of variants G320R and V321F does not increase the risk for the disease. Although these two variants show no clear disease association it should be taken into account that the clinical phenotype in AAT deficiency could be affected by modifier effects of other genetic or environmental factors, and therefore their deleterious effects cannot be completely excluded. It would also be useful to determine their frequency in general population as well as in larger patient population.

Acknowledgments This work was supported by the grant 173008 from the Ministry of Education and Science, Republic of Serbia. EM is supported by Pasteur Institute – Cenci Bolognetti Foundation and by Telethon Foundation (GGP11057). We gratefully acknowledge the generous gifts of the anti-AAT Mab 2G7 antibody from Professor David A. Lomas from Faculty of Medical Sciences, University College London, UK, and the pEGFP-N1 vector from both dr Wei-shan Chang from College of Animal Science and Technology, Shandong Agricultural University, Tai'an, China and dr Tatjana Arsenijevic, Laboratory of Pathophysiological and Nutritional Biochemistry, Université Libre de Bruxelles, Brussels, Belgium.

References

1. Karnaukhova, E (2012) Recent advances in the research and development of alpha-1 proteinase inhibitor for therapeutic use. In: Dr. Elvisegran Malcolm Irusen (Ed) Lung Diseases -Selected State of the Art Reviews. In Tech, Winchester
2. Crystal RG (1990) Alpha 1-antitrypsin deficiency, emphysema, and liver disease. Genetic basis and strategies for therapy. J Clin Investig 85:1343–1352
3. Long GL, Chandra T, Woo SL, Davie EW, Kurachi K (1984) Complete sequence of the cDNA for human alpha 1-antitrypsin and the gene for the S variant. Biochemistry 23:4828–4837
4. Whisstock J, Skinner R, Lesk AM (1998) An atlas of serpin conformations. Trends Biochem Sci 23:63–67
5. Im H, Woo MS, Hwang KY, Yu MH (2002) Interactions causing the kinetic trap in serpin protein folding. J Biol Chem 277:46347–46354
6. Yamasaki M, Sendall TJ, Harris LE, Lewis GM, Huntington JA (2010) Loop-sheet mechanism of serpin polymerization tested by reactive center loop mutations. J Biol Chem 285:30752–30758
7. Lomas DA (2005) Molecular mousetraps, alpha1-antitrypsin deficiency and the serpinopathies. Clin Med 5:249–257
8. Society AT, Society ER (2003) American Thoracic Society/European Respiratory Society statement: standards for the diagnosis and management of individuals with alpha-1 antitrypsin deficiency. Am J Respir Crit Care Med 168:818–900
9. Blanco I, de Serres FJ, Fernandez-Bustillo E, Lara B, Miravitlles M (2006) Estimated numbers and prevalence of PI*S and PI*Z alleles of alpha1-antitrypsin deficiency in European countries. Eur Respir J 27:77–84
10. Zorzetto M, Ferrarotti I, Campo I, Balestrino A, Nava S et al (2005) Identification of a novel alpha1-antitrypsin null variant (Q0Cairo). Diagn Mol Pathol 14:121–124
11. Eriksson S, Carlson J, Velez R (1986) Risk of cirrhosis and primary liver cancer in alpha 1-antitrypsin deficiency. N Engl J Med 314:736–739
12. Yang P, Wentzlaff KA, Katzmann JA, Marks RS, Allen MS et al (1999) Alpha1-antitrypsin deficiency allele carriers among lung cancer patients. Cancer Epidemiol Biomarkers Prev 8:461–465
13. Yang P, Bamlet WR, Sun Z, Ebbert JO, Aubry MC et al (2005) Alpha1-antitrypsin and neutrophil elastase imbalance and lung cancer risk. Chest J 128:445–452
14. Lomas DA, Evans DL, Finch JT, Carrell RW (1992) The mechanism of Z alpha 1-antitrypsin accumulation in the liver. Nature 357:605–607
15. Lomas DA (2006) The selective advantage of alpha1-antitrypsin deficiency. Am J Respir Crit Care Med 173:1072–1077
16. Elliott PR, Stein PE, Bilton D, Carrell RW, Lomas DA (1996) Structural explanation for the deficiency of S alpha 1-antitrypsin. Nat Struct Mol Biol 3:910–911
17. Curiel DT, Chytil A, Courtney M, Crystal RG (1989) Serum alpha 1-antitrypsin deficiency associated with the common S-type (Glu264—Val) mutation results from intracellular degradation of alpha 1-antitrypsin prior to secretion. J Biol Chem 264:10477–10486
18. Bornhorst JA, Calderon FR, Procter M, Tang W, Ashwood ER et al (2007) Genotypes and serum concentrations of human alpha-1-antitrypsin "P" protein variants in a clinical population. J Clin Pathol 60:1124–1128
19. Ljujic M, Topic A, Divac A, Nikolic A, Petrovic-Stanojevic N et al (2008) Isoelectric focusing phenotyping and denaturing gradient gel electrophoresis genotyping: a comparison of two methods in detection of alpha-1-antitrypsin variants. Transl Res 151:255–259
20. Ljujic M, Topic A, Nikolic A, Divac A, Grujic M et al (2010) Identification of a rare p. G320R alpha-1-antitrypsin variant in emphysema and lung cancer patients. Genet Mol Biol 33:5–8
21. Gluzman Y (1981) SV40-transformed simian cells support the replication of early SV40 mutants. Cell 23:175–182
22. Djurovic J, Stevanovic M (2004) Structural and functional characterization of the human SOX14 promoter. Biochim Biophys Acta 1680:53–59
23. Dufour EK, Denault JB, Bissonnette L, Hopkins PC, Lavigne P et al (2001) The contribution of arginine residues within the P6-P1 region of alpha 1-antitrypsin to its reaction with furin. J Biol Chem 276:38971–38979
24. Courtney M, Buchwalder A, Tessier LH, Jaye M, Benavente A et al (1984) High-level production of biologically active human alpha 1-antitrypsin in Escherichia coli. Proc Natl Acad Sci USA 81:669–673
25. Lee KN, Im H, Kang SW, Yu MH (1998) Characterization of a human alpha1-antitrypsin variant that is as stable as ovalbumin. J Biol Chem 273:2509–2516
26. Jung CH, Na YR, Im H (2004) Retarded protein folding of deficient human alpha 1-antitrypsin D256 V and L41P variants. Protein Sci 13:694–702
27. Lomas DA, Elliott PR, Chang WS, Wardell MR, Carrell RW (1995) Preparation and characterization of latent alpha 1-antitrypsin. J Biol Chem 270:5282–5288
28. Miranda E, Römisch K, Lomas DA (2004) Mutants of neuroserpin that cause dementia accumulate as polymers within the endoplasmic reticulum. J Biol Chem 279:28283–28291
29. Whisstock JC, Skinner R, Carrell RW, Lesk AM (2000) Conformational changes in serpins: I. the native and cleaved conformations of alpha(1)-antitrypsin. J Mol Biol 296:685–699
30. Kumar P, Henikoff S, Ng PC (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 4:1073–1081
31. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A et al (2010) A method and server for predicting damaging missense mutations. Nat Methods 7:248–249
32. Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci USA 95:5857–5864
33. Letunic I, Doerks T, Bork P (2012) SMART 7: recent updates to the protein domain annotation resource. Nucleic Acids Res 40:D302–D305
34. Grefrath SP, Reynolds JA (1974) The molecular weight of the major glycoprotein from the human erythrocyte membrane. Proc Natl Acad Sci USA 71:3913–3916
35. Miyake J, Ochiai-Yanagi S, Kasumi T, Takagi T (1978) Isolation of a membrane protein from R rubrum chromatophores and its abnormal behavior in SDS-polyacrylamide gel electrophoresis due to a high binding capacity for SDS. J Biochem 83:1679–1686
36. Rath A, Glibowicka M, Nadeau VG, Chen G, Deber CM (2009) Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. Proc Natl Acad Sci USA 106:1760–1765
37. Carrell RW, Jeppsson JO, Vaughan L, Brennan SO, Owen MC et al (1981) Human alpha 1-antitrypsin: carbohydrate attachment and sequence homology. FEBS Lett 135:301–303
38. Samandari T, Brown JL (1993) A study of the effects of altering the sites for N-glycosylation in alpha-1-proteinase inhibitor variants M and S. Protein Sci 2:1400–1410
39. Patston PA, Gettins P, Beechem J, Schapira M (1991) Mechanism of serpin action: evidence that C1 inhibitor functions as a suicide substrate. Biochemistry 30:8876–8882
40. Gettins PG (2000) Keeping the serpin machine running smoothly. Genome Res 10:1833–1835
41. Seo EJ, Im H, Maeng JS, Kim KE, Yu MH (2000) Distribution of the native strain in human alpha 1-antitrypsin and its association with protease inhibitor function. J Biol Chem 275:16904–16909
42. Irving JA, Pike RN, Lesk AM, Whisstock JC (2000) Phylogeny of the serpin superfamily: implications of patterns of amino acid

conservation for structure and function. Genome Res 10:1845–1864
43. Beatty K, Bieth J, Travis J (1980) Kinetics of association of serine proteinases with native and oxidized alpha-1-proteinase inhibitor and alpha-1-antichymotrypsin. J Biol Chem 255:3931–3934
44. Jung CH, Chae YK, Im H (2004) Suppression of the facile latency transition of alpha(1)-antitrypsin variant M(malton) by stabilizing mutations. Biochem Biophys Res Commun 325:744–750
45. Im H, Yu MH (2000) Role of Lys335 in the metastability and function of inhibitory serpins. Protein Sci 9:934–941
46. Lomas DA, Evans DL, Stone SR, Chang WS, Carrell RW (1993) Effect of the Z mutation on the physical and inhibitory properties of alpha 1-antitrypsin. Biochemistry 32:500–508
47. Dafforn TR, Mahadeva R, Elliott PR, Sivasothy P, Lomas DA (1999) A kinetic mechanism for the polymerization of alpha1-antitrypsin. J Biol Chem 274:9548–9555
48. Levina V, Dai W, Knaupp AS, Kaiserman D, Pearce MC et al (2009) Expression, purification and characterization of recombinant Z alpha(1)-antitrypsin–the most common cause of alpha(1)-antitrypsin deficiency. Protein Expr Purif 68:226–232