# A multifunctional protein: involvement of the alpha-1 serum protease inhibitor in SDS and high salt-stable DNA-protein complexes

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**Abstract Occasionally new and intriguing roles arise for proteins with well established functions. The alpha-1 serum protease inhibitor (α-1 PI) represents another example. Sequence identities exist in the α-1 PI and in a nuclear 52-kDa glycoprotein which is involved in resistant DNA-polypeptide complexes. The results of Western blots support the identity of the two proteins and immunocytochemical studies indicate the nuclear location of the α-1 PI. Consistently, e.g. Ehrlich ascites tumor cells express the α-1 PI, and the fusion protein between the α-1 PI and the green fluorescent protein from *Aequorea victoria* shows intracellular accumulation and partly nuclear location.**



*Key words:* Multifunctional proteins; Alpha-1 antitrypsin; Alpha-1 serum protease inhibitor; Nuclear protein; Green fluorescent protein

## 1. Introduction

Human alpha-1 antitrypsin (α-1 AT, swiss: a1at_human, 46.7-kDa polypeptide, glycosylated) is known to be a serum protease inhibitor which is secreted by liver cells and which is primarily involved in the control of elastolytic activity. Lowering of the blood concentration of this protein induces pulmonary emphysema [1]. The homologous murine protein (82% similarities, 65% identities) is expectedly involved in the same function and has been termed alpha-1 protease inhibitor-1 (α-1 PI, swiss: a1a1_mouse, 46-kDa polypeptide, glycosylated). However, these protease inhibitors may have additional, e.g. not yet characterized and even more important intracellular functions. Previous immunocytochemical studies pointed to a potential intracellular accumulation of α-1 AT in normal and in tumor cells [2–5]. However, these results were not verified by other methods and the significance of this potential intracellular location of α-1 protease inhibitors and their subcellular distributions have not yet been investigated. Studying tight DNA-protein complexes we characterized a supramolecular structure consisting of three major glycosylated polypeptides with apparent molecular masses of 62 kDa, 52 kDa and 40 kDa [6,7]. Complexes of this type were found to be attached to DNA isolated from human placenta and from murine tumor cells [6]. The DNA-protein complexes survive treatments designed to dissociate DNA-protein complexes, including cell lysis in the presence of SDS and proteinase K, salting out steps, prolonged incubation in 1% SDS or 4 M urea at 56°C and ethanol precipitations [6,7]. Further characterization of the 52-kDa nuclear glycoprotein by partial sequencing and by application of immunochemical and recombinant DNA techniques indicates that it is identical with the α-1 protease inhibitor which points to a nuclear function of this protein.

## 2. Materials and methods

### 2.1. Preparation of the 52-kDa nuclear protein for microsequencing

DNA isolation by the 'salting out' procedure and optional modifications have been described previously in detail [6–8]. Briefly, cell pellets of the Ehrlich ascites tumor were suspended in buffer (400 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl, pH 8.2) containing 50 μg/ml proteinase K. Cell lysis was induced by SDS to obtain a final concentration of 0.63%. The lysates were incubated at 37°C for 18–25 h. Following dilution, the undigested material was 'salted out' by addition of 6 M NaCl. After centrifugation the DNA contained in the supernatant was precipitated with ethanol and resuspended in 1 vol of Tris/EDTA 0.1 (0.1 mM EDTA, 1 mM Tris-HCl, pH 8.0) containing 5 μg per ml of DNase I-free pancreatic ribonuclease (ribonuclease I A, Pharmacia). Pellets of DNA (1 mg) prepared by ethanol precipitation were digested in 50 μl benzonase buffer (1 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.0) with Benzonase (100 units), mixed with 50 μl of 2× sample buffer [9] and submitted to 10% (w/v) SDS-polyacrylamide gel electrophoresis [9].

### 2.2. Microsequencing

The proteins were blotted from gels to PVDF membranes (ProBlot, Applied Biosystems) [10] by means of a SemiDry blotting chamber (BIO-RAD). Proteins were stained with Ponceau S (Sigma) and regions of interest were cut out and submitted to the microsequencing procedure [11]. Sequence analysis was performed on an ABI 477 A gas-phase sequencer equipped with on-line 120 PTH analyzer and 610 A data-acquisition system using standard programs. The FastN data base searching program of Lipman and Pearson [12] was used for sequence identification in data bases.

### 2.3. Antibodies and Western blots

An antiserum to the 52-kDa nuclear protein has been described previously [6]. The antiserum to α-1 AT was a commercial product (Paesel). Following SDS-polyacrylamide gel electrophoresis (10%, w/v) the polypeptides released from DNA, α-1 AT (Serva), and molecular weight markers were electro-blotted to nitrocellulose membranes (Schleicher and Schüll, BA85). Parallel gel sections were stained with Coomassie brilliant blue. After blocking the membranes with TTBS (Tris-buffered saline, 0.5% Tween 20) and 5% dry milk they were incubated with the diluted primary antibodies (1:500 v/v, TTBS/dry milk) at 4°C, minimum 1 h. After several washes with TTBS the immunocomplexes were detected by means of a peroxidase-conjugated secondary antibody and the ECL-detection kit (Amersham) which was performed according to the instructions of the producer.

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*2.4. Preparation of specimen for immunofluorescence microscopy*

Ehrlich ascites cells settled on poly-prep slides (Sigma) were fixed in ethanol (4°C), washed with PBS and incubated with the antiserum to human α-1 AT (Paesel, dilution 1: 500, PBS/1% BSA, 60 min at room temperature). After several washes with PBS the cells were incubated under the same conditions with FITC affinity-pure anti rabbit IgG (Jackson, dilution 1: 200, PBS/1% BSA, 60 min at room temperature). After washes with PBS, the specimen were incubated with H 33342 (0.1 μg/ ml, Sigma). After a final wash in PBS the wet specimen were covered with cover slips and directly inspected in the fluorescence microscope, and photographed (Kodak Elite, 400).

*2.5. Expression of the alpha-1 protease inhibitor in Ehrlich ascites cells*

Plasmid DNA from an Ehrlich ascites cell RNA-based cDNA library prepared in the pBluescript vector (Stratagene) was linearized by *Sca*I and used as template for PCR amplification of the α-1 PI-encoding cDNA (Clontech, advantage PCR kit, #k1906-1). Primer pairs were used fitting to the coding sequence, e.g. to the first 30 nucleotides at the 5′ end and to 30 bases preceding the stop codon. The amplification product was submitted to agarose gel electrophoresis.

*2.6. Expression constructs and transfection*

The cDNA sequence encoding the enhanced green fluorescent protein (EGFP, Clontech) was PCR amplified (Clontech, advantage PCR kit, #k1906-1), and the ends were modified during PCR by addition of restriction sites: *Sal*I (5′ end) and *Hin*dIII (3′ end). The product was cloned into the *Sal*I/*Hin*dIII sites of the pBluescript KS+ vector (Stratagene) to obtain the plasmid pBlue-EGFP. The authentic coding sequence of the murine α-1 PI is comprised in an EST clone (IMAGE clone ID 332638) which was kindly supplied to us by the 'Ressourcenzentrum im Deutschen Humangenomprojekt', Berlin. Using this template the complete coding region for the α-1 PI was PCR amplified with cDNA-fitting primer pairs and restriction sites were added: *Kpn*I (5′ end) and *Sal*I (3′ end). Cloning into the pBlue-EGFP (*Kpn*I/*Sal*I) resulted in inframe fusion (pBlue-PI-EGFP). The fused sequence encoding α-1 PI-EGFP was released (*Kpn*I/*Not*I) and recloned in the *Kpn*I/*Not*I sites of the eukaryotic expression vector pcDNA3 (Invitrogen). For controls the EGFP sequence was recloned in the pcDNA3 vector using the same restriction sites. Purified plasmids (Qiagen) were used to transfect cultured Ehrlich ascites cells (electroporation, Gene Pulser II, Biorad, 366 V, 950 μF, 0.8 ml PBS, d = 4 mm). Expression was documented by fluorescence microscopy using an FITC filter system.

*2.7. Electron microscopy*

DNA associated with the residual complexes was spread by direct adsorption to mica (10 mM Tris pH 7.3, 1 mM EDTA, 50 mM $MgCl_2$) and stained with 1% $UO_2$-acetate. Shadowcasting was performed under 5°C by Pt/C = 95/5). Supramolecular structures released from DNA by nuclease digestion were visualized by negative staining with $UO_2$-acetate.

## 3. Results

Cell lysis in presence of SDS and proteinase K followed by 'salting out' of residual peptides with saturated sodium chloride solution resolves efficiently deproteinized DNA [8]. However, this DNA is still associated with a characteristic set of polypeptides (Fig. 1C). The residual DNA-polypeptide complexes can be visualized on spread DNA by electron microscopy (Fig. 1A). The protein complexes are released during digestion of DNA as globular 12 nm particles (Fig. 1B) which become gradually disassembled resulting in smaller but still supramolecular structures [7]. The latter process is accompanied by the activation of an encrypted ATPase/phosphatase [7]. Fig. 1C shows the characteristic electrophoretic pattern of the major polypeptides (62 kDa, 52 kDa and 40 kDa) involved in the SDS and salt-stable DNA-polypeptide complexes.

Preliminary N-terminal sequence analysis of the 52-kDa protein blotted to PVDF membranes showed identities with protease inhibitors [6]. By improved methods and by repeated assays we now identified reliably 12 amino acid residues which are 100% identical with an N-terminal section of the murine α-1 PI (Fig. 2). The α-1 PI precursor protein has an N-terminal signal peptide. Consistently, the mature serum protein α-1 PI starts downstream of this signal peptide with a glutamate residue at aa position 25 of the precursor protein (swiss: a1a1_mouse). In contrast, the first residue of the nuclear 52-kDa protein comprised in the resistant complexes is an aspartate residue at aa position 31 of the precursor protein. Accordingly, the N-terminal peptide EDVQET of the mature serum protein is missing in the 52-kDa nuclear protein. At present it is unknown whether this difference reflects an alternative post-translational processing of the precursor protein or a site-specific cleavage of the nuclear protein during the cell lysis in presence of proteinase K.

The identity between the 52-kDa nuclear protein and the α-1 protease inhibitor is further supported by immunochemical cross-reactions. The antibody to the 52-kDa nuclear protein detects specifically the 52-kDa component among the polypeptides released by digestion of DNA isolated by the 'salting out' procedure ([6] and Fig. 3B). Moreover, it forms an immuno complex with human α-1 AT (Fig. 3B). Vice versa, the commercially available α-1 AT antiserum shows a significant and specific immuno reaction with the 52-kDa component of the polypeptides released from DNA (Fig. 3A). This interspecies cross-reactivity of the two antigens revealed by the two sera further supports the relation of the 52-kDa nuclear protein and the α-1 PI and the relative intensities of the Western blot signals reflect well the 82% similarity between the human and the murine protease inhibitors.

The DNA-polypeptide complexes shown in Fig. 1 were isolated from Ehrlich ascites cells grown in the peritoneal cavity

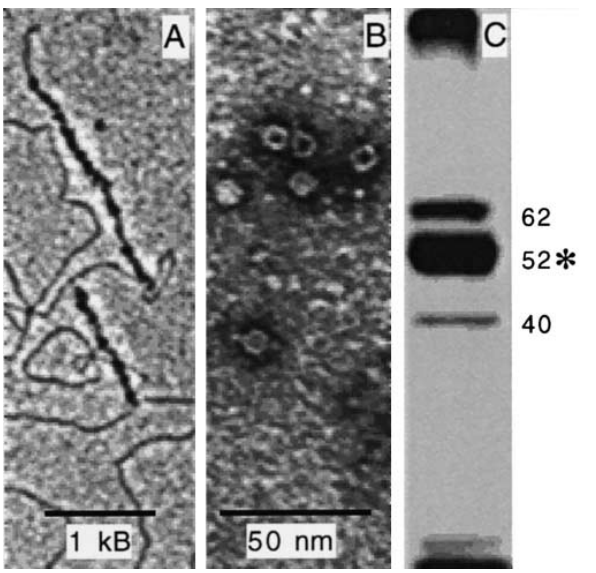


Fig. 1. Release of the nuclear 52-kDa protein from resistant DNA-associated protein complexes. (A) Electron microscopy of DNA isolated by the 'salting out' procedure [6–8]. Discontinuities are seen which reflect non-DNA material. (B) Supramolecular structures released from DNA after nuclease digestion. (C) SDS polyacrylamide gel electrophoresis of polypeptides released from DNA during nuclease digestion. The 52-kDa band marked with an asterisk was submitted to the microsequencing procedure (see Fig. 2).

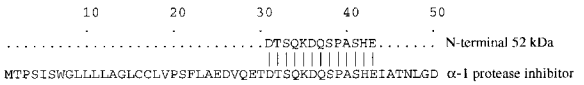


Fig. 2. Amino terminal peptide sequence of the 52-kDa nuclear polypeptide aligned with the peptide sequence of the alpha-1 protease inhibitor-1 precursor protein (α-1 PI, swiss: a1a1_mouse).

of mice. Although these cells were pelleted before cell lysis the antigens released from DNA could reflect serum proteins becoming associated with the DNA during the isolation procedure. However, such an artificial contamination of the DNA with serum α-1 PI is ruled out by the results of immunocytochemical studies. It has been shown previously that the antiserum to the 52-kDa polypeptide immuno stains mainly a nuclear antigen in whole cells [6]. Fig. 4 shows that the antibody to the α-1 AT immuno stains as well mainly the nuclei of whole cells which proves the intracellular origin of the antigen and eliminates the potential contamination of isolated DNA with the serum protein.

Closely related but non-identical proteins could comprise short sequence identities resulting in immunochemical cross-reactions. Thus, the 52-kDa nuclear protein and α-1 PI could still represent very similar but non-identical proteins. However, by PCR and recombinant DNA technology it can be shown that the authentic α-1 PI is expressed in Ehrlich ascites cells, and that significant amounts of this protein accumulate in cells. The α-1 PI is expressed in Ehrlich ascites cells because its coding sequence (1239 Bp) is present in Ehrlich ascites cell RNA-based cDNA libraries. This is shown by PCR amplification of a ≈1200 Bp DNA fragment by means of primer pairs (each 30 nucleotides) encoding the N-terminal and C-terminal sections of the α-1 PI (Fig. 5).

Moreover, the intracellular accumulation of the α-1 PI can be visualized by recombinant DNA technology. The green fluorescent protein from the jelly fish *Aequorea victoria* is a reporter molecule suitable for monitoring protein localization and transport in vivo because chimeric proteins consisting of a functional protein subunit and the EGFP-tag behave like the functional protein [13], e.g. a fusion protein between a secretory protein and the green fluorescent protein does not accumulate in the cell [14]. This system allowed to trace the fate of the α-1 PI expressed in Ehrlich ascites cells. Fig. 6 shows that Ehrlich ascites cells transfected with the reporter construct express the chimeric protein which accumulates inside the cells. In agreement with the immunocytochemical studies it appears that at least a portion of the protein is transported into nuclei where it forms a punctuate fluorescence pattern. However, 48 h post transfection a fraction of the α-1 PI still resides in the cytoplasm which may reflect either a slow transport of the (overexpressed) protein into nuclei or an additional cytoplasmic function of the protease inhibitor.

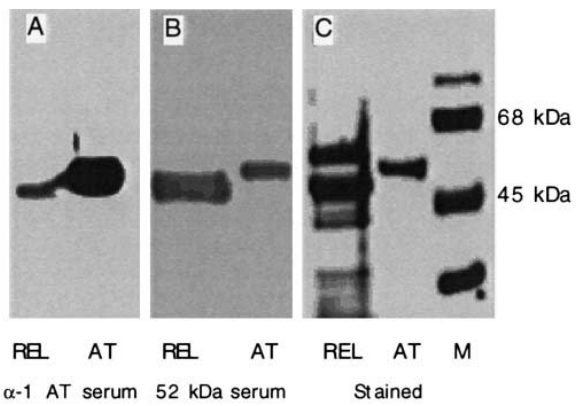


Fig. 3. Immunochemical cross-reaction of the (murine) 52-kDa nuclear protein and the (human) α-1 AT. An SDS polyacrylamide gel (10% w/v) was loaded with the polypeptides released from DNA by nuclease digestion (REL), and with commercially available α-1 AT (AT). Lane M shows marker proteins. Sections A and B were electroblotted to nitrocellulose. The blot section A was incubated with a commercially available antiserum to the human α-1 AT. The blot section B was incubated with the antiserum to the 52-kDa nuclear protein [6]. After several washes the immuno complexes were detected by the ECL system (Amersham). The gel section C was stained with Coomassie brilliant blue.

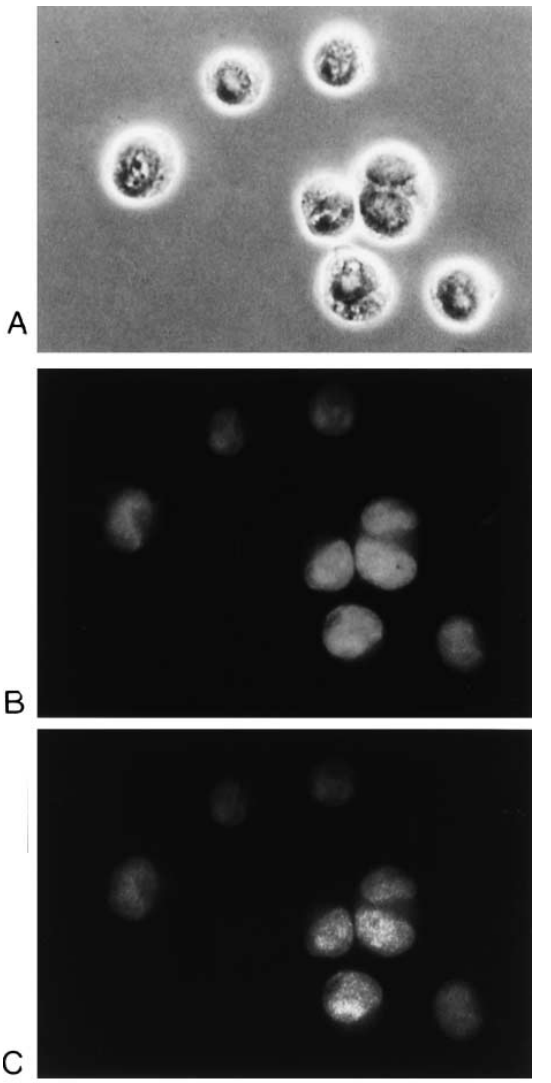


Fig. 4. Intracellular location of the antigen detected by the antiserum to α-1 AT. Ehrlich ascites cells were sequentially treated with the antiserum to human α-1 AT, FITC-anti-rabbit IgG, and with H 33342. (A) Phase contrast microscopy. (B) The same section as shown in A, H 33342 fluorescence specific for DNA. (C) The same section as shown in A, FITC-fluorescence specific for the antigen detected by the α-1 AT antiserum.

## 4. Discussion

During the last few years the serum α-1 protease inhibitors (human α-1 AT and murine α-1 PI) met minimal interest because these proteins had been well characterized long ago on the molecular and functional level [1]. However, well characterized proteins may be utilized in unrelated biological processes. A prominent example for a multifunctional protein is the glycolytic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which can serve also in other fundamental biological processes including DNA repair, translational control of gene expression, DNA replication and in endocytosis [15]. The α-1 protease inhibitor seems to represent another example for a protein involved in unrelated functions depending on its location.

The electrophoretic mobilities, the glycoprotein nature [1,7], the overlapping 12mer microsequence (Fig. 2) and the immunochemical data (Figs. 3 and 4) point to a close similarity between a nuclear 52-kDa protein and the α-1 PI. Moreover, since the authentic α-1 PI is expressed in Ehrlich ascites cells (Fig. 5), and since it accumulates in cells (Fig. 6) our results rather reflect the identity of the two proteins than their strong similarity.

Of course, the intrinsic activity of this protein is expectedly independent of its location, e.g. it should act inside the cell like in the serum as a serine protease inhibitor. This view is justified because even the non-glycosylated recombinant α-1 PI shows inhibition of serine proteases [1]. Accordingly, its intracellular fraction is suggestively involved in the regulation of pathways governed by serine proteases including serum protease-mediated apoptosis [16,17]. In such pathways it could serve as an antagonist to those serine proteases involved in apoptotic death events. Accordingly, the identification of the intracellular target protease for the α-1 protease inhibitors is of high interest. Possible candidates are those polypeptides (62 kDa, 40 kDa) which are found together with the 52-kDa nuclear protein in the globular complexes shown in Fig. 1 and which are presently characterized on the sequence level.

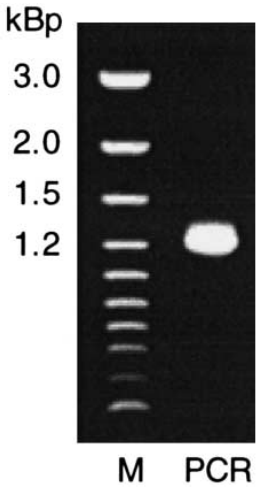


Fig. 5. Expression of the α-1 protease inhibitor in Ehrlich ascites cells. cDNAs (1239 bp) corresponding to transcripts encoding the full length α-1 PI are present in Ehrlich ascites cell RNA-based cDNA libraries. A single PCR product (PCR) of the expected size is resolved by 1% (w/v) agarose gel electrophoresis after PCR using the *Sca*I-linearized plasmid population of the cDNA library as template, and primer pairs encoding the N- and C-terminal sections of the α-1 PI. 30 cycles, annealing temperature was 60°C. Lane M shows DNA marker fragments.

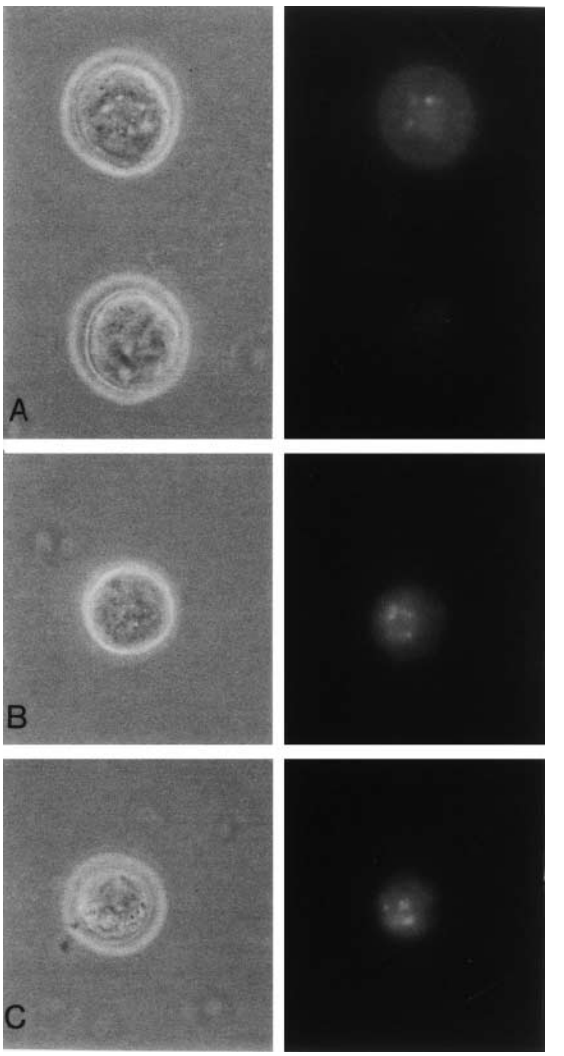


Fig. 6. Intracellular accumulation of the chimeric protein consisting of the authentic α-1 PI and the enhanced green fluorescent reporter protein (EGFP). Living Ehrlich ascites cells 48 h post transfection with the expression construct encoding a chimeric protein consisting of the α-1 PI and the EGFP-tag. The left panels show cells in phase contrast. The right panels show the same sections under UV light in combination with an FITC filter system. Panel A shows two cells, one of which was obviously not transfected with the plasmid. Such cells serve as control for negative background fluorescence. The fluorescent chimeric protein accumulates in transfected cells. The fraction deposited in nuclei shows a punctuate fluorescence pattern. Transfection with the control plasmid encoding only the EGFP results in a uniform fluorescence in all cellular compartments (not shown).

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