The effect of amino acid substitutions at position 342 on the secretion of human α_1-antitrypsin from *Xenopus* oocytes

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A glutamic acid to lysine change in the Z variant of human α_1-antitrypsin is associated with a failure to secrete the protein from synthesising cells. The block in export of the protein may be caused either by the loss of an acidic residue or the introduction of a basic one at this point in the polypeptide chain. Site-directed mutagenesis has been used to construct novel α_1-antitrypsin mutants which show that the side chain interactions from Glu-342 are not obligatory for protein export and it is rather the introduction of a basic residue at this point which produces the intracellular accumulation of the protein.

α_1-Antitrypsin secretion; Z mutation; *Xenopus laevis*; Oocyte

1. INTRODUCTION

A variety of plasma proteins contribute towards the protease inhibitory capacity of the blood, foremost among them is the glycoprotein α_1-antitrypsin [1–3]. The protein is more systematically known as α_1-antiprotease since its normal physiological target is not trypsin but neutrophil elastase, thus the protein's main function is to protect elastic fibres in the alveoli from hydrolysis by this protease. The protein is encoded by a gene 12.2 kb in length found on human chromosome 14 [4] at a genetic locus designated Pi. The highly polymorphic nature of this locus gives rise to a large number of α_1-antitrypsin variants in the human population. The most common allele is referred to as PiM and this variant type can be further divided into six distinct sub-classes. Individuals homozygous for PiM alleles have plasma α_1-antitrypsin levels in the range 150–350 mg/dl and exhibit no apparent clinical symptoms [5]. Homozygosity for the abnormal Z variant, however, is associated with serum levels of the inhibitor <50 mg/dl and the development of emphysema due to the loss of lung elasticity and, in a proportion of cases, infantile liver disease [6]. The Z protein is synthesised in normal amounts but poorly secreted from the hepatocytes so the deficit of inhibitor in the circulation is allied to an accumulation in the endoplasmic reticulum of synthesising cells [6,7].

Two changes in primary sequence distinguish the M and Z proteins. A T-to-C transition in exon III codes for Val-213 to Ala-213 while a G-to-A change in exon V produces Glu-342 to Lys-342 [8,9]. The first of these changes is conservative and also appears in 23% of M haplotypes so it is thought to have no effect on the secretion of the protein. The second mutation has a radical effect on the charge of the molecule and profoundly influences its secretion from liver cells [10], and *Xenopus* oocytes injected with encoding mRNA [11,12]. Crystallographic measurements on a cleaved form of the normal inhibitor have suggested that Glu-342 forms a salt bridge with Lys-290 [13]. The loss of this structural feature in the PiZ variant, caused by the Glu-342 to Lys-342 substitution, has been put forward as the reason for defective secretion of the mutant protein and its intracellular accumulation [13,14]. Oligonucleotide mutagenesis has been used to demonstrate that disruption of this salt bridge by substitution of the residue at 290 has little effect on secretion of the mutant proteins [15–17]. Here we demonstrate that it is the nature of residue 342 itself, rather than its participation in a salt bridge per se which profoundly effects the secretion of α_1-antitrypsin.

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2. MATERIALS AND METHODS

All DNA- and RNA-modifying enzymes were from BRL (Bethesda Research Laboratories) or New England Biolabs. ^{35}S-labelled dATP and methionine were supplied by Amersham International plc. Sequenase reagents were from Cambridge Bioscience. Oligonucleotides were synthesised by Dr M.A. Pickett (Dept of Microbiology, Southampton General Hospital). Site-directed mutagenesis was performed using the method described by Kunkel et al. [18] using M13 vectors and bacterial strains supplied by BioRad. Other reagents were Analar grade or better and were supplied by Sigma. General methods and the use of restriction enzymes have been described elsewhere [19].

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Mutant cDNAs were cloned into the modified vector SP64T for in vitro transcription using SP6 polymerase [15]. The preparation and micro-injection of *Xenopus* oocytes was as described by Colman [20]. Oocyte extracts and incubation media were immunoprecipitated using anti-human α_1-antitrypsin (DAKO Immunoglobulins a/s) and the products analysed on 12.5% SDS-polyacrylamide gels followed by fluorography using 'Amplify' (Amersham International plc) [15].

Constructs of the mutagenised cDNAs ligated into the eukaryotic expression vector pSV2 were transfected into NIH 3T3 cells by calcium phosphate co-precipitation [21]. Transfection efficiency was determined by counting the number of G418-resistant colonies 10 days after G418 selection compared to no selected cells. Stable transfected cells and an equivalent aliquot of culture medium were analysed by gel electrophoresis and Western blotting [19] and indirect ELISA [22].

3. RESULTS AND DISCUSSION

Oligonucleotide directed mutagenesis was used to construct a series of α_1-antitrypsin mutants that encoded single amino acid substitutions at position 342. These mutants were designed to introduce alternatively charged side chains at this position and disrupt the putative salt bridge between the glutamic acid residue found at this position in the normal, PiM inhibitor and the lysine residue at 290 (Table I). After DNA sequencing to verify the desired mutation was in place the α_1-antitrypsin cDNAs were ligated into a modified form of the vector SP64T for transcription of viable mRNA in vitro.

To compare the rates of secretion of different mutant α_1-antitrypsins equivalent amounts of translationally active mRNA were injected into *Xenopus* oocytes. Secretion of the mutant proteins was accessed by incubating injected oocytes in medium containing L-[^{35}S]methionine. Radiolabelled α_1-antitrypsin was immunoprecipitated from incubation media and oocyte extracts and analysed by SDS gel electrophoresis (Fig. 1). All of the injected transcripts directed the synthesis of a 54 kDa intracellular protein which represents partially glycosylated inhibitor. The incubation media contained a 58 kDa secreted protein formed as a result of further glycosylation of the intracellular species before export [11]. To quantitate secreted versus intracellular inhibitor radiolabelled bands were excised from the gel and incorporation measured by liquid scintillation counting.

Table I

Mutant forms of human α_1-antitrypsin and their ability to form a salt bridge with Lys-290

DNA construct	Amino acid position 342	Potential for salt bridge
Pi M	Glu	Y
Mut Z	Lys	N
Mut A	Ala	N
Mut B	Arg	N

M type α_1-antitrypsin and mutant A are equally well secreted from oocytes. The absolute level of secretion varies among batches of oocytes (e.g. see Table II); however, in all experiments the majority of M type and Mut A inhibitor synthesised by oocytes was subsequently secreted. The insertion of a basic residue at position 342 markedly reduces the level of secretion. This applies for both Arg-342 and the naturally occurring Z mutation, Lys-342, although the effect of the Glu to Arg substitution is less pronounced.

In addition to the Glu-342 to Lys-342 coding base change, a second point mutation is present in the

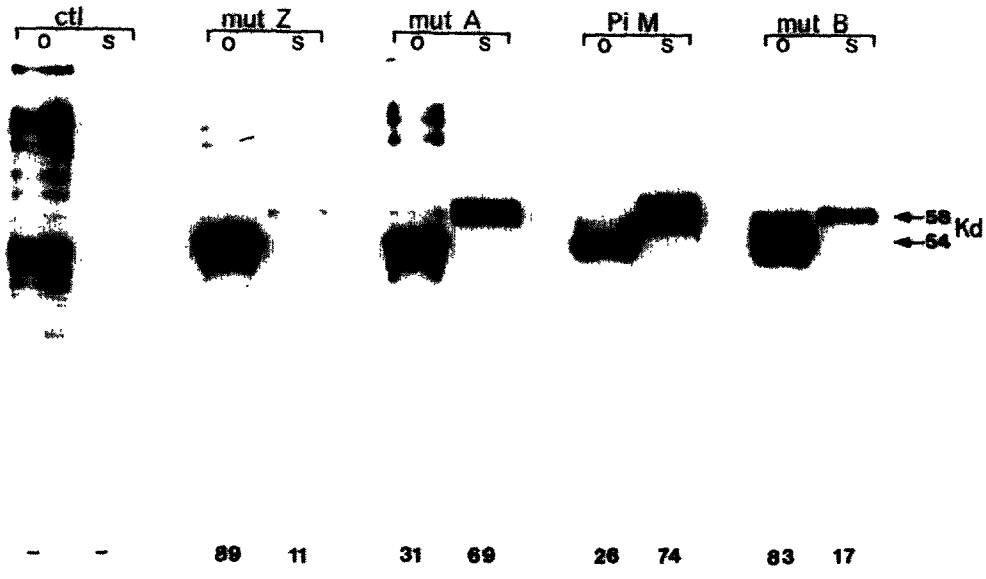

Fig. 1. Secretion of mutant α_1-antitrypsins from *Xenopus* oocytes. S, secreted protein; O, protein from oocyte extracts. Control tracks are from water-injected oocytes. Immunoprecipitation and gel electrophoresis were as described in section 2.

Table II

Secretion of mutant α_1-antitrypsins from NIH 3T3 cells and *Xenopus* oocytes

DNA construct	% α_1-AT secreted	
	Oocytes	NIH 3T3 cells
Pi M	69	100
Mut Z	19	14
Mut A	77	89
Mut B	28	n.d.

human Pi Z allele which codes for a Val to Ala substitution at residue 213 [9]. The Z mutant construct described here does not carry this second mutation yet exhibits the secretory defect found in the natural mutant. This result suggests that in oocytes, as in other cell types [16], the Glu-342 to Lys-342 change is the sole cause of the failure in Z protein export.

A comparison of the secretory properties of the various mutant α_1-antitrypsins in oocytes and the murine embryonic fibroblast cell line NIH 3T3 is shown in Table II. Cultured cells were transfected with the modified eukaryotic expression vector pSV2 containing cloned mutant α_1-antitrypsin cDNAs. Antiprotease in the medium and cells was assayed by ELISA. Pi M and Mut A proteins are secreted more efficiently from transfected mammalian cells than from amphibian oocytes. However, while absolute amounts of the various mutant antiproteases secreted from the two cell types may vary, it is apparent that the qualitative effect of the various point mutations on the secretory properties of the resultant protein products is constant regardless of the cell type used for expression.

Disruption of the Lys-290/Glu-342 salt bridge by mutagenesis of codon 290 does not radically affect the secretion of mutant α_1-antitrypsins from *Xenopus* oocytes [15] or transfected mammalian cells [16,17]. However, substitution of Glu at position 290 will partially alleviate the affect of the Z mutation and produce a level of protein secretion intermediate between Z and wild-type [16,23]. The results presented here show that it is rather the positive nature of Lys-342 in the Z mutation which causes the secretory defect. Substitution of a positively charged arginine residue at this point produces a similar, if less extensive, reduction in protein export (Fig. 1, mut B). The introduction of a neutral alanine residue at position 342 prevents salt bridge formation but has no negative effect on protein secretion (Fig. 1, mut A), the introduction of Gln at position 342 has a similar effect [16]. Successful export of the alanine mutant also suggests that an acidic residue at 342 is not obligatory for protein transport.

The proximity of residues 290 and 342 is evident from the crystallographic structure of α_1-antitrypsin [13]. The conservation of Lys-290 and Glu-342 in many other members of the serpin protein family further underscores the importance of these residues in overall protein structure [14]. One possibility is that two highly basic side chains in a sterically restricted space are incompatible with correct folding of the protein or with its correct interaction with some cellular element which mediates in secretion. However, results presented here and by other groups indicate that the interaction between these residues might be more subtle than first predicted.

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