Human Z α_1-antitrypsin accumulates intracellularly and stimulates lysosomal activity when synthesised in the *Xenopus* oocyte

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Microinjection of human liver mRNA from a patient homozygous for α_1-antitrypsin deficiency (PiZZ) into *Xenopus* oocytes led to a 2–10-fold increase in lysosomal activity. Stimulation of lysosomal activity was not observed when mRNA from a normal human liver (α_1-antitrypsin PiMM), or water was injected into the oocyte. This lysosomal activity was oocyte derived and was not due to translation products of the human liver mRNA. Thus a protein that accumulates intracellularly in the secretory pathway is capable of stimulating lysosomal activity.

Lysosomal protein *Oocyte injection* *Protein synthesis* *Protein secretion* *Z human α_1-antitrypsin*

1. INTRODUCTION

The human liver synthesizes and secretes most of the proteins found in plasma. One of these proteins, α_1-antitrypsin, has a genetic variant (the Z variant) that results in a plasma level 10–15% of normal [1] and in accumulation of the protein in the endoplasmic reticulum in a high mannose form [2].

The mechanism underlying the intracellular accumulation is a matter of debate. One view [3] is that there is normal production of the mRNA and polypeptide, the defect occurring at the final stage of processing just prior to entry into the Golgi vesicles. A small proportion (15%) is fully processed and secreted but the bulk of the material accumulates in the endoplasmic reticulum. An essential feature of this proposal is that there is an accompanying proteolysis of the accumulated material which accounts for the finite and variable size of the intracellular inclusions. The alternative view [4] assumes a markedly decreased production of Z variant mRNA; the hepatocyte inclusions representing the aggregated total of a much reduced production of Z α_1-antitrypsin. The translation and secretion of human α_1-antitrypsin have been studied in the oocyte system, and the synthesis of Z α_1-antitrypsin results in the intracellular accumulation [5] of a high mannose form of the protein which is compatible with a blockage at the final processing stage in the endoplasmic reticulum (D.M.E. and I.C.B., unpublished). During this work, it was noted that a small, but consistent, rise in intracellular protein synthesis occurred in the oocytes injected with mRNA from Z human liver. This rise could not be accounted for by α_1-antitrypsin accumulation, so an investigation was undertaken in order to determine whether the accumulation of a foreign protein in the endoplasmic reticulum could result in increased lysosomal protein activity.

2. MATERIALS AND METHODS

Liver samples obtained at autopsy within 1 h of death, or as a result of a liver transplant, were

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stored and the mRNA isolated as described [5,6]. Oocytes of *Xenopus laevis* were maintained in Barths' medium and microinjected as described by Colman [7]. Injected oocytes were cultured in unlabelled Barths' medium overnight at 20°C and the unhealthy oocytes discarded. The remaining oocytes were either cultured in Barths' medium containing 2 mCi/ml [^{35}S]methionine for a further 16 h, or used directly for lysosomal assays. Preparation and use of the wheat germ cell-free translation system was as described in [18].

Purified α_1-antitrypsin mRNA was prepared from total M and Z liver mRNA by hybridization to cloned human α_1-antitrypsin cDNA (obtained from J. Riley and R.K. Craig, Courtauld Institute of Biochemistry, London) linked to DBM paper, according to the methods of Alwine et al. [8] and Wahl et al. [9].

Oocytes were homogenised according to [7]. Immunoprecipitation of oocyte extracts and incubation media, polyacrylamide gel electrophoresis, and subsequent fluorography were as in [5].

After homogenising the oocytes (5 oocytes/100 μl) in a 20 mM Tris-HCl (pH 7.4) buffer containing 100 mM NaCl and 0.1% Triton-X100, lysosomal activity was measured by assaying the following enzymes.

β-Glucurondiase: 10 μl oocyte extract was added to a reaction mixture containing 0.4 ml of 0.1 M sodium acetate buffer, pH 4.5, and 0.03 mM phenolphthalein glucuronidate. This mixture was incubated overnight at 25°C and the reaction stopped and colour developed by the addition of 2.5 ml of 0.2 M glycine-NaOH, pH 10.45, containing 0.2 M NaCl. The colour absorbance was read at 540 nm [10].

Lysozyme: To 1 ml of a cell suspension containing *Micrococcus lysodeikticus* (9 μg/30 ml in 0.1 M potassium phosphate, pH 7.0), 20 μl of the oocyte extract was added and the change in absorbance at 450 nm recorded [11].

Cathepsins B and H were measured by the change in absorbance at 400 nm of the substrate benzoylarginine-*p*-nitroanilide HCl at 0.4 mg/ml in 0.1 M potassium phosphate, pH 5.0, containing 2 mM cysteine and 0.5 mM EDTA [12]. β-Hexosaminidase was assayed by adding 25 μl oocyte extract to 100 μl of 1 mM 4-methylumbelliferol dissolved in 0.1 M sodium citrate, pH 4.5, and incubation at 25°C overnight. The reaction was stopped by the addition of 1 ml 0.5 M glycine-NaOH, pH 10.4, and read on a fluorometer at 450 nm with the excitation at 300 nm [13].

Acid phosphatase was estimated by a modification of the method of Ewen and Spitzer [14]. 20 μl oocyte extract was added to 0.2 ml of 0.1 M sodium acetate, pH 5.4, containing 1% Brij 35 and 1.5 mM disodium thymolphthalein monophosphate. After overnight incubation at 25°C, the reaction was stopped and the colour developed by the addition of 2 ml of 1 M Na_2CO_3/NaOH.

3. RESULTS AND DISCUSSION

It has already been shown that the Z variant of human α_1-antitrypsin accumulates in oocytes injected with mRNA from affected livers, whilst the normal protein is readily secreted [5].

In this work we show that injection of the Z mRNA but not of the M variant causes an increase in the activity of the oocyte lysosomal enzymes.

A human α_1-antitrypsin cDNA clone was used to isolate pure α_1-antitrypsin mRNA from M and Z liver total poly(A^+) RNA. These purified mRNAs were translated in a wheat germ cell-free system. The 47-kDa bands produced comigrated with α_1-antitrypsin which was immunoprecipated after translation of total liver polyA^+ RNA (fig.1). In extracts from oocytes injected with Z mRNA (total or purified) the activities of 4 different lysosomal enzymes rose from 2- to 10-fold. Fig.2 also shows that both preparations of M mRNA failed to increase lysosomal enzyme activity above that measured in control oocytes.

Lysosomal enzyme activity was the same in oocytes injected with total Z liver mRNA or purified Z α_1-antitrypsin mRNA, implying that the increased enzyme activity was oocyte derived and not due to coinjection of message for liver lysosomal proteins. This fact was confirmed by measuring the heat stability of β-glucuronidase activity. It is known [16] that amphibian β-glucuronidase is more temperature sensitive than the mammalian enzyme. Fig.3A shows that the oocyte enzyme was destroyed at 45°C while the mammalian form was stable at 55°C and was only inactivated by heating to 70°C. A preincubation of 15 min at 55°C had no effect on the mammalian enzyme but destroyed greater than 90% of the oocyte enzyme activity (fig.3B). All the oocyte ex-

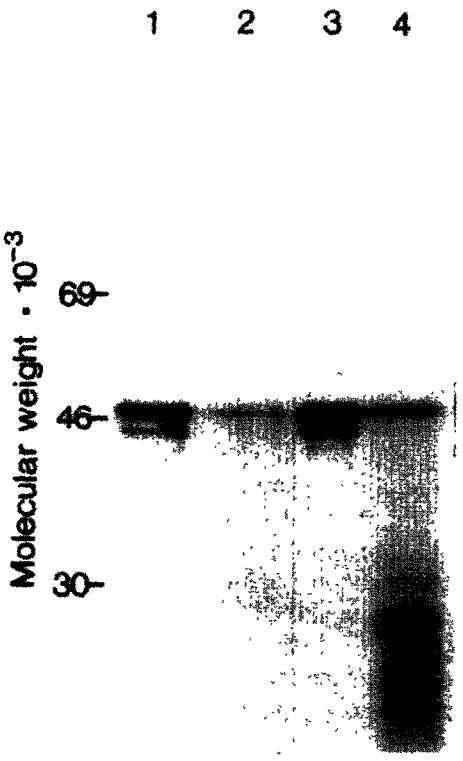


Fig.1. SDS-polyacrylamide gel analysis of α_1-antitrypsin synthesised in the wheat germ system. Lanes 1 and 3, ^{35}S-labelled proteins from translation of total M and Z liver mRNA followed by immunoprecipitation with antiserum specific for human α_1-antitrypsin. Lane 2, translation product of clone purified M liver mRNA. Lane 4, translation product of clone purified Z liver mRNA. M_r values on the left of the figure were derived from the comigration of ^{14}C-labelled markers, carbonic anhydrase (M_r 30000), ovalbumin (M_r 46000) and bovine serum albumin (M_r 69000). (Limited proteolysis of the translation product gives rise to a minor band visible in some tracks.)

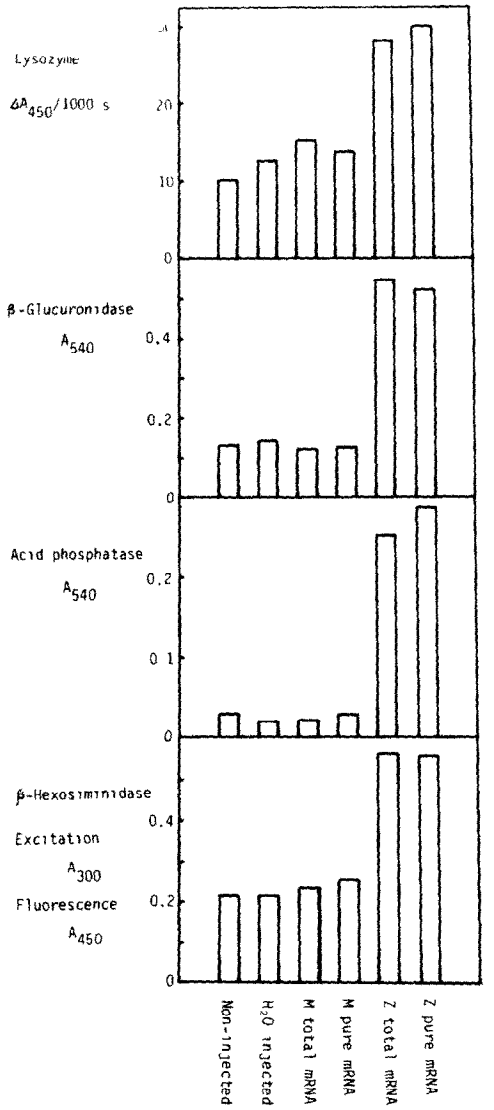


Fig.2. Change in lysosomal activity in the oocyte after injection of human liver mRNA. The lysosomal activity in the oocyte was estimated by measuring (a) lysozyme, (b) β-glucuronidase, (c) acid phosphatase, and (d) β-hexosaminidase as described in section 2. Columns 1 and 2 represent the activity in non-injected and water-injected oocytes. Columns 3 and 4 represent the activity in oocytes injected with M human liver mRNA and M α_1-antitrypsin mRNA. Columns 5 and 6 represent the activity in oocytes injected with Z human liver mRNA and Z α_1-antitrypsin mRNA.

tracts were preincubated at 55°C as described then reassayed for β-glucuronidase activity (fig.3). In every case the activity measured in injected oocytes was reduced to that of the control by heat treatment. This confirmed that the increased enzyme activity measured in oocytes injected with Z mRNA came solely from the oocytes own enzymes.

We also measured the lysosomal enzymes cathepsins B and H using benzoylarginine-*p*-nitroanilide as substrate. We detected enzyme activity in control oocytes but, surprisingly, there was no measurable activity in oocytes injected with the liver mRNAs. This was attributed to the presence of intracellular α_1-antitrypsin, in the process of be-

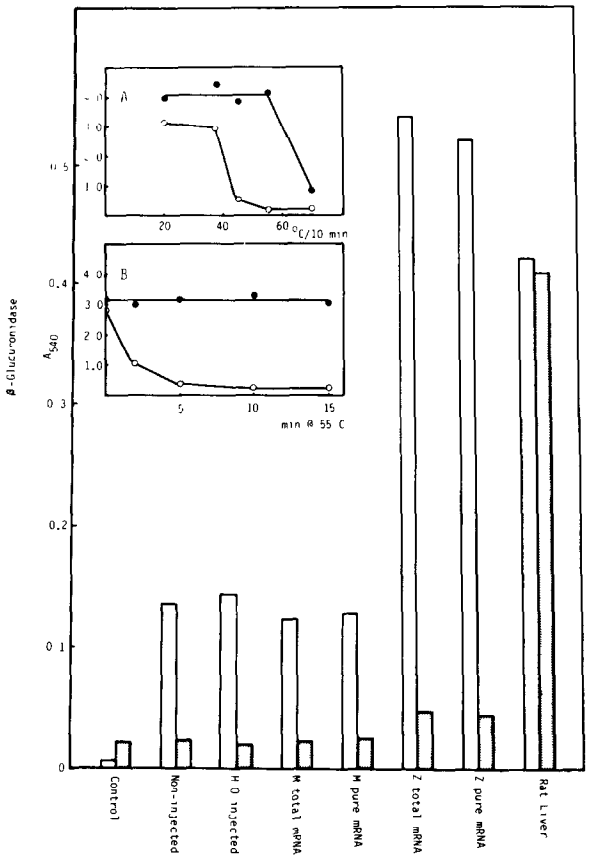


Fig. 3. Heat stability of oocyte β-glucuronidase. (A) Temperature sensitivity of oocyte vs rat liver β-glucuronidase. β-glucuronidase activity was measured as described in section 2 with pre-incubation for 10 min at the temperature described instead of 25°C overnight. (B) Temperature stability of oocyte vs rat liver β-glucuronidase. β-glucuronidase activity was measured after incubation at 55°C for an increasing period of time. The sensitivity of the β-glucuronidase to heat was measured by pre-incubating a duplicate reaction at 55°C for 15 min prior to the overnight incubation at 25°C. Column 1 represents a control reaction with the homogenate buffer. Columns 2 and 3, non-injected and water-injected oocytes. Columns 4 and 5, M human liver mRNA and clone purified M a_1-antitrypsin mRNA. Columns 6 and 7, Z human liver mRNA and clone purified Z α_1-antitrypsin. Column 8 is the β-glucuronidase activity in a rat liver preparation from 10 μl of 2.5 × 10^7 cells/ml treated in an identical way to the oocyte fraction. The open columns represent the non-heated sample while the hatched columns represent the heat-treated sample.

ing secreted, which inactivated the cathepsins. This inhibition could be overcome by the addition of trypsin to the assay mixture. Approx. 4-times the amount of trypsin was added to the Z injected oocyte extract than the M injected oocyte extract before any cathepsin activity could be detected.

Our results demonstrate that synthesis of a protein that accumulates intracellularly is capable of stimulating lysosomal enzymes. The main question is what constitutes the signal for increased lysosomal activity and what is its mode of transmission? It is interesting that many lysosomal proteins are stimulated together, suggesting a coordinated response, and implying that all of the lysosomal protein genes may be induced simultaneously. These results do not directly indicate the fate of Z α_1-antitrypsin in the human liver. However, we believe that we have demonstrated a plausible mechanism for the removal of much of the protein which could otherwise accumulate if Z mRNA is present and is translated at the same rate as the normal M mRNA [3,6].

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