Volume 267, number 2, 277–280 FEBS 08617 July 1990

BiP expression is not increased by the accumulation of PiZ α_1-antitrypsin in the endoplasmic reticulum

Danièle Cresteil[1], Ernesto Ciccarelli[2], Théophile Soni[1], Maria A. Alonso[1], Paul Jacobs[2], Alex Bollen[2] and Fernando Alvarez[1]

[1]*INSERM U56, Hopital de Bicêtre, 94275 Le Kremlin-Bicêtre Cedex, France* and [2]*Service de Génétique Appliquée, Université Libre de Bruxelles, 24 rue de l'Industrie, 1400 Nivelles, Belgium*

Received 9 April 1990

PiZ, a mutant human α_1-antitrypsin, is associated with liver and pulmonary disease and is characterized by defective secretion and accumulation of the protein in the endoplasmic reticulum. We tested the hypothesis that BiP (a protein that binds newly synthesized protein in the endoplasmic reticulum, prevents secretion of incorrectly folded protein, and solubilizes protein aggregates), could play a part in the retention of PiZ α_1-antitrypsin in the endoplasmic reticulum. Subcellular fractions from PiM (normal) and PiZ livers were prepared and analyzed by immunoblotting. No increase of BiP was detected in the PiZ liver. In addition, when total RNA from the same livers were analyzed by slot and Northern blot hybridization, no difference was found in the level of BiP mRNA between PiM and PiZ livers. Similar results were found in clones of CHO and MDCK cells transfected with PiM of PiZ α_1-antitrypsin cDNAs. These results indicate that BiP does not play a part in the retention of PiZ α_1-antitrypsin and suggest that PiZ protein is not misfolded.

BiP; α_1-Antitrypsin; PiM; PiZ

1. INTRODUCTION

α_1-Antitrypsin (α1AT) deficienty is an autosomal recessive disorder for which many genetic variants have been reported; a point mutation in the α1AT gene resulting in the replacement of Glu by Lys in the 342 position of the polypeptide chain characterizes the PiZ α1AT variant which is associated with the lowest α1AT serum level and accumulates in the endoplasmic reticulum (ER), where it forms aggregates [1]. The mutation does not alter in vitro translation of RNA or co-translational processing of the PiZ α1AT protein by dog pancreas microsomes [2]. The presence of Lys in position 342 disrupts a salt bridge between amino acids 290 and 342, but this change is apparently not implicated in the retention of PiZ α1AT in the ER [3–5].

A protein of the ER, called BiP (immunoglobulin heavy chain binding protein) or GRP-78 has been shown to be tightly associated with newly synthesized proteins when they are incorrectly folded or polymerised [6–8]. In all those situations the level of BiP is increased. It could be hypothesized that a strong binding of PiZ α1AT to BiP would be responsible for the accumulation of PiZ α1AT in the ER. If this were the case, an increased amount of BiP would be present in livers expressing PiZ α1AT as compared with livers expressing PiM α1AT. This hypothesis was tested in the present work.

Correspondence address: F. Alvarez, INSERM U56, 78 rue du Général-Leclerc, 94275 Le Kremlin-Bicêtre Cedex, France

2. MATERIALS AND METHODS

2.1. *Human liver fractionation*

Normal human liver (PiM α1AT) was obtained from a liver transplantation (liver sample from the right lobe of a brain dead donor when only the left lobe was transplanted). Liver from a child with α1AT deficiency (PiZ phenotype) who required liver transplantation for cirrhosis was also obtained during a liver transplantation. The human livers were homogenized and subcellular fractions were prepared by differential centrifugation, according to Beaufay and Amar-Costesec [9]. Liver homogenates (E) were separated into four fractions: nuclei and large cell debris (N), mitochondria and other large granules (M), small granules or microsomes (P) and soluble constituents (S).

2.2. *Immunoblot analysis of subcellular fractions*

Forty μg of total protein from each subcellular fraction were separated by electrophoresis in a 10% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose filter and processed for immunoblot as described [10]. Antibodies used were: (i) rabbit serum anti-human-α_1-antitrypsin (Dakopatts, Sebia, France), at a dilution of 1 : 500 and (ii) rabbit serum anti-BiP kindly provided by Dr I. Haas and Dr T. Meo [11], at a dilution of 1 : 10.

2.3. *Transfection of CHO and MDCK cells with cDNA for PiM and PiZ α 1AT*

To obtain the PiZ α1AT cDNA the cDNA, for PiM α1AT was mutated in vitro (In vitro Mutagenesis Kit, Amersham, Les Ulis, France), replacing G by A at the 1096 nucleotide position [12], to obtain the cDNA α1AT PiZ. These two cDNAs were inserted separately into the Tig Hbb expression vector. Plasmids Tig Hbb containing PiM α1AT cDNA and Tig Hbb containing PiZ α1AT cDNA were transfected into CHO and MDCK cells by electroporation. Clones expressing human α1AT were selected with the antibiotic G-418 (Sigma, La Verpilliere, France) by expression of the 'neo' gene in vector Tig Hbb. The presence of α1AT in the cells and in the supernatants was assayed by ELISA and immunoprecipitation with specific anti-human α1AT antibodies, after labelling of cells with [^{35}S]methionine.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2.4. *Total RNA extraction and analysis*

RNA extraction from human liver was made with guanidiniun. isothiocyanate followed by centrifugation in cesium chloride [13]. RNA extraction from transfected CHO and MDCK cells was made as described by White and Bancroft [14]. Analysis of 1–5 μg of total RNA from each liver sample was carried out by Northern and slot blotting [11], using labelled antisense RNAs for BiP and α1AT (Riboprobe, Promega Biotec, Coger, France) as probes. In addition, 4 μg of total RNA from CHO and MDCK cells, expressing α1AT PiM or PiZ, were tested by slot blotting, using the same probes. After autoradiography, the Northern blot film was scanned with an autoscanning apparatus (Cottel, Osi, France) and results expressed in arbitrary units (integrated optical density/μg of RNA). Spots from slot blots were cut and radioactivity measured in scintillation mixture (Picofluo, Packard, Rungis, France) in a Packard Tri-Carb 460 C counter. In the case of normal and pathological livers results were expressed in cpm/μg of RNA. In the case of transfected cells results were expressed for 4 μg of total RNA. The ratio hybridized RNA probe for PiM α1AT/hybridized RNA probe for BiP in clones of a given cell type and the ratio of hybridized RNA probe for PiZ α1AT/hybridized RNA for BiP in clones of the same cell type, were compared using the Mann-Whitney test.

3. RESULTS AND DISCUSSION

In normal human liver α1AT was detected, as expected, in the microsomal fraction (Fig. 1). Two bands of 50 kDa and 56 kDa, respectively, were visible. It could be suggested that these bands correspond to the partially glycosylated form (in the endoplasmic reticulum) and to the terminally glycosylated form (in Golgi or post-Golgi subcellular compartments), respectively. In the PiZ α1AT liver α1AT was also detected in the microsomal fraction but only one band of 50 kDa was visible and the reactivity was more intense than in the PiM liver (Fig. 1); in addition, in the PiZ liver, α1AT was also present in the mitochondrial fraction. It is known that most of the newly synthesized PiZ α1AT forms aggregates in the lumen of the endoplasmic reticulum (ER). The aggregates change the density of ER vesicles [15] and this very likely explains the large amounts of PiZ α1AT found in the mitochondrial fraction after differential centrifugation.

When anti-BiP antibodies were used on immunoblots of the microsomal fractions from PiZ and PiM livers, a faint band of 78 kDa was detectable in microsomes from PiM α1AT liver only (Fig. 2). Forty μg of total microsomal proteins from PiM and PiZ livers were used in each experiment; but in the case of microsomes from the PiZ liver, α1AT accounted for a larger fraction of the total proteins. Therefore, to detect protein BiP in PiZ liver, it might have been necessary to load the gel with more of the total microsomal proteins, because there was not a parallel increase in the amounts of BiP and α1AT in these livers.

Total RNA from PiM and PiZ livers was analyzed by slot and Northern blotting using radiolabelled antisense RNA probes. Autoradiography of Northern blots (Fig. 3) or evaluation by scanning (Table I) showed no differences between PiM and PiZ livers for α1AT mRNA or BiP mRNA when evaluated by scanning

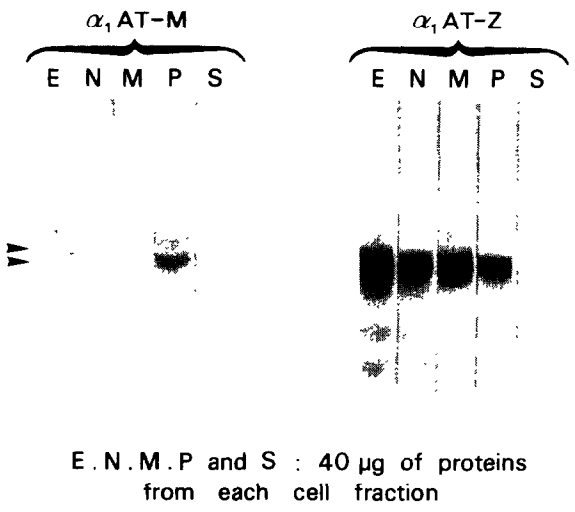

Fig. 1. Immunoblot of cell fractions from normal (α1AT M) and pathological (α1AT Z) livers. E, homogenate; N, nuclei; M, mitochondria and large granules; P, microsomes and small granules; and S, soluble constituents. Nitrocellulose filters were incubated with anti-α1AT antibodies. Two bands of 50 kDa and 55 kDa were seen in normal (PiM) liver (arrows) and only a band of 50 kDa in the pathological (PiZ) liver.

(Table I). Similar results were found using slot blotting and radioactivity measurement of hybridized probe (Table I, Fig. 4). The relative abundance of mRNA coding for α1AT in PiM and PiZ livers has already been shown not to differ significantly [16]. Comparable results were obtained when 4 μg of total RNAs from transfected cells were tested using the same methods (Fig. 5). These experiments indicate that retention of

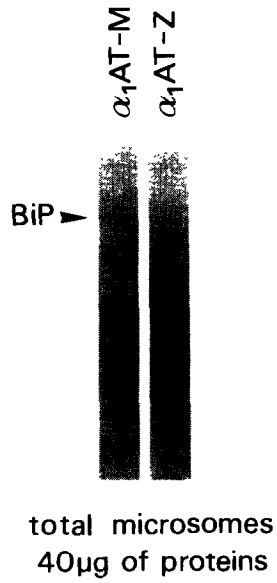

Fig. 2. Immunoblot of microsomal fraction from livers expressing PiM α1AT and PiZ α1AT. Filters were incubated with anti-BiP. One band of 78 kDa was shown in microsomes from PiM α1AT.

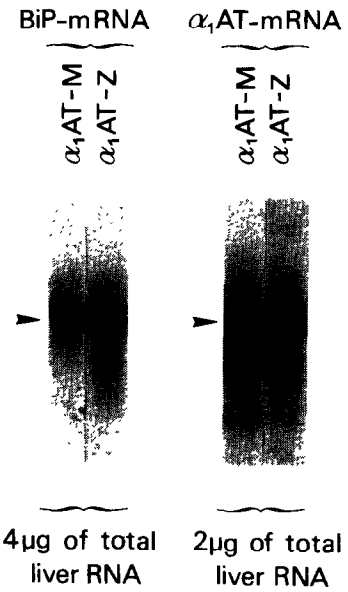

Fig. 3. Northern blots of total RNA from PiM and PiZ α1AT livers. Specific mRNAs (arrows) were hybridized with antisense RNA probes.

PiZ α1AT in the ER is not associated with a detectable increase of BiP mRNA. Minor differences in the level of mRNA for PiM and PiZ α1AT in each type of transfected cells were probably due to differences in the efficacy of transcription of α1AT cDNA between clones. However, no significant differences were seen in the ratios of hybridized RNA probes for PiM α1AT/hybridized RNA probe for BiP and the ratios of hybridized RNA for PiZ α1AT/hybridized RNA probe for BiP between clones of a given cell type.

Induction of transcription and translation of the BiP gene occurs when an improperly folded mutant of the influenza virus hemagglutinin is expressed in CV-1 cells [17] and accumulates in the ER [8]. Although PiZ α1AT accumulates in large amounts in the ER of hepatocytes, a similar increase in the expression of the BiP gene could not be demonstrated here.

Experiments with the hemagglutinin mutant and other experiments led us to suggest that BiP could prevent normal sorting of newly synthesized proteins when

Table I

Radioactivity and scanning measurements of slot and Northern blots

Total RNA from liver (probe)	Slot blot (cpm/μg RNA)	Northern blot[a]
α1AT PiM (α1AT)	1812 ± 601	1174
α1AT PiZ (α1AT)	1732 ± 773	1163
α1AT PiM (BiP)	1332 ± 699	837
α1AT PiZ (BiP)	1251 ± 395	819

[a] Northern blot autoradiography films were scanned and results expressed in arbitrary units

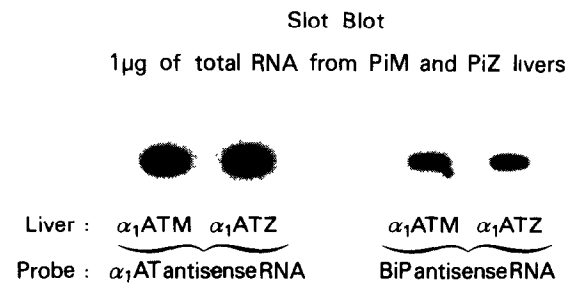

Fig. 4. Slot blots of total RNA from PiM and PiZ α1AT livers. Antisense RNA of BiP and α1AT were used as probes.

they are incorrectly folded or incompletely assembled [6–8]. It was further suggested that BiP could promote folding or assembly of proteins and could solubilize protein aggregates in the lumen of the ER [6–8,17,18]. Because no increase of BiP could be detected in PiZ α1AT livers, we might speculate that accumulated α1AT is free from BiP and correctly folded. Therefore, the BiP-α1AT binding would not be the reason why α1AT accumulates in the ER. Similarly, BiP was not found to be associated with PiZ α1AT in homogenates from COS cells transfected with PiZ α1AT cDNA [5].

It could be further speculated that after folding PiZ, α1AT aggregates because it fails to recognize a protein of the cell translocation apparatus. If this were the case, the PiZ mutation could alter a signal necessary for the sorting out of the ER.

Although an increased synthesis of some proteins of the heat shock/stress gene family was recently shown in the liver of patients with PiZ α1AT deficiency and liver disease [19], the results reported here do not support that BiP is included in this particular feature; indeed, although BiP is considered a protein of the heat shock/stress gene family, it is already known that BiP is not stimulated by heat shock but rather by glucose starvation. The present results add another difference between stimulus provoking increased BiP synthesis and synthesis of other proteins in the same family.

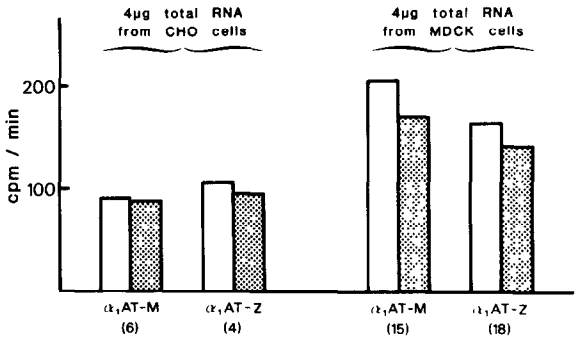

Fig. 5. Results of slot blots for CHO and MDCK cells transfected with PiZ α1AT and PiZ α1AT cDNAs. This figure represents some of the clones analyzed: CHO cell clone 6 (expressing PiM α1AT) and clone 4 (expressing PiZ α1AT). MDCK cells clone 15 (expressing PiM α1AT) and clone 18 (expressing PiZ α1AT).

Acknowledgements: This work was supported by CEE (Communauté Economique Européenne) and CNAMTS (Caisse Nationale d'Assurance Maladie). E.C. and M.A. are supported by CEE. We thank Dr O. Bernard for fruitful discussions. We thank Dr M. Raff (Smith Kline Beckman, Philadelphia, USA) for providing the Tig vector.

REFERENCES

[1] Yoshida, A., Lieberman, J., Gaidulis, L. and Erwing, C. (1976) Proc. Natl. Acad. Sci. USA 73, 1324–1328.
[2] Verbanac, K.M. and Heath, E.C. (1986) J. Biol. Chem. 261, 9979–9989.
[3] Sifers, R.N., Hardck, C.P. and Woo, S.L.C. (1989) J. Biol. Chem. 264, 2997–3001.
[4] Brontly, H., Courtney, H. and Crystal, R.G. (1988) Science 242, 1700–1702.
[5] McCraken, A.A., Kruse, K.B. and Brown, J.L. (1989) Mol. Cell Biol. 9, 1406–1414.
[6] Haas, I.G. and Wabl, M. (1983) Nature 306, 387–389.
[7] Bole, D.G., Hendershot, L.M. and Kearney, J.F. (1986) J. Cell Biol. 102, 1558–1566.
[8] Gething, M.J., McCammon, K. and Sambrook, J. (1986) Cell 46, 939–950.
[9] Beaufay, H. and Amar-Costesec, A. (1976) in: Methods in Membrane Biology, vol. 6 (Korn, E.D. ed.) pp. 1–100, Plenum, New York.
[10] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[11] Haas, I.G. and Meo, T. (1988) Proc. Natl. Acad. Sci. USA 85, 2250–2254.
[12] Bollen, A., Herzog, A., Cravador, A., Hérion, P., Chuchana, P., Vander Straten, A., Loriau, R., Jacobs, P. and Van Elsen, A. (1983) DNA 2, 255–264.
[13] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in: Molecular Cloning, p. 196, Cold Spring Harbor, New York, NY.
[14] White, B.A. and Bancroft, F.C. (1982) J. Biol. Chem. 257, 8569–8572.
[15] Eriksson, S. and Larsson, Ch. (1974) N. Engl. J. Med. 292, 176–180.
[16] Schwarzenberg, S.J., Sharp, H.L., Manthei, R.D. and Seelig, S. (1986) Hepatology 6, 1252–1258.
[17] Kozutsumi, Y., Segal, M., Normington, K., Gething, M.J. and Sambrook, J. Nature 332, 462–464.
[18] Munro, S. and Pelham, H.R.B. (1986) Cell 46, 291–300.
[19] Pelmutter, D.H., Schlesinger, M.J., Pierce, J.A., Punsal, P.I. and Schwartz, A.L. (1989) J. Clin. Invest. 84, 1555–1561.