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Human Zn-α_2-glycoprotein cDNA cloning and expression analysis in benign and malignant breast tissues

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Two cDNA clones coding for Zn-α_2-glycoprotein (Zn-α_2-gp) have been isolated from a human breast library and their nucleotide sequences determined. The deduced amino acid sequence contains the coding information for a hydrophobic signal peptide and the 278 residues of the mature protein. Comparison of this sequence with that from the protein purified from plasma reveals four differences: two amino acid changes (Gln-67 and Glu-222) and insertion of two residues (Ile-75 and Phe-76). Northern-blot analysis showed that the Zn-α_2-gp gene is expressed in liver and normal breast, but not in placenta, ovary and thyroid. A comparative analysis in mammary tissues from women with different diseases revealed enhanced expression of Zn-α_2-gp gene in benign breast lesions and a variable expression level in breast cancers.

Zn-α_2-glycoprotein; Breast cystic disease; Breast cancer

1. INTRODUCTION

Zn-α_2-glycoprotein (Zn-α_2-gp) is a human protein originally isolated from plasma and whose name derives from its electrophoretical mobility in the α_2-region and from its ability to bind zinc ions [1]. The protein shows a molecular mass of about 40 kDa, contains 12% carbohydrates and displays a considerable charge heterogeneity on agarose gel electrophoresis and on isoelectrofocusing [2,3]. The complete amino acid sequence of human plasma Zn-α_2-gp has been recently elucidated [4]. It consists of a single polypeptide chain of 276 amino acid residues, with a high degree of similarity to antigens of the major histocompatibility complex.

Although the biological function of Zn-α_2-gp is unknown, the finding of high concentrations of this protein in cyst fluid from women with gross cystic breast disease has suggested that Zn-α_2-gp may play a role in the induction of the pathological process characteristic of the disease [5]. In addition, immunohistochemistry analysis performed on tissue specimens obtained from patients with breast carcinoma has led to identifying a subtype of tumors which synthesize and secrete this protein [6,7]. A better understanding of these possible implications of Zn-α_2-gp in the development of benign and malignant breast diseases could be facilitated by the availability of the gene encoding this protein. In this work, we report the isolation and sequencing of cDNA clones for human Zn-α_2-gp. In addition, we have used these cDNAs as a probe in order to study Zn-α_2-gp expression in different normal and pathological human tissues.

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2 MATERIALS AND METHODS

2.1. *Materials*

Restriction endonucleases and other reagents used for molecular cloning were obtained from Boehringer-Mannheim. [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]ATP (3000 Ci/mmol) and [α-^{35}S]dATP (600 Ci/mmol) were purchased from Amersham International. A human breast cDNA library in λgt11 was obtained from Clontech Laboratories Inc. Oligonucleotides were synthesized by the phophoramidite method in an Applied Biosystems DNA synthesizer, model 381A and purified by polyacrylamide gel electrophoresis according to standard procedures [8]. Double-stranded DNA probes were radiolabeled to high specific activity with [α-^{32}P]dCTP by using a commercial random-priming kit from Boehringer-Mannheim.

2.2. *Isolation and analysis of cDNA clones*

Two different oligonucleotides (20-mer) with sequences AA(AG)CA(TC)GTNGT(AG)GA(TC)GTNCC and TC(TC)TC(TC)TTCCA(AG)TC(TC)TCCAT based on the amino acid sequence of Zn-α_2-gp (residues 19–25 and 61–66, respectively) were synthesized and used to screen a cDNA library made from human breast tissue. A total of 5×10^5 λ plaques were analyzed according to the method of Benton and Davis [9]. Recombinant phages from isolated positive plaques were grown and DNA was prepared from them according to Maniatis et al. [8]. The restriction map of inserts was obtained following standard procedures [8].

2.3. *Sequence analysis*

Restriction fragments were inserted in the polylinker region of phage vectors M13mp18 or M13mp19 [10]. Single-stranded templates were isolated and sequenced using either the universal M13 primer or synthetic oligonucleotide primers and the Sequenase kit (United States Biochemical Corp.). The nucleotide sequence was determinded at least twice in both strands. The DNA sequences were analysed by computer programs DM5 [11] and ALIGN [12] on an IBM PC-AT computer.

2.4. *Preparation and analysis of RNA*

Human tissues were obtained at surgery or from autopsies performed within 15 h after death; human placenta was obtained immediately after delivery. Total RNA was isolated according to Chomeczynski and Sacchi [13] and samples of about 10 μg were separated by electrophoresis in 1.4% agarose denaturing gels and blotted onto Hybond N nylon filters (Amersham International). Filters were prehybridized for 3 h at 42°C with 50% formamide, 5 × SSPE (1 × =150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4) 2× Denhardt's solution (1 × =0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 0.1% SDS and 0.1 mg/ml of denatured herring sperm DNA and then hybridized for 48 h under the same conditions, using as a probe the *Eco*RI fragment 847 bp long which contains the 3′ portion of the Zn-α_2-gp cDNA. Filters were washed with 0.2 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7), 0.5% SDS at 68°C for 2 h and exposed to autoradiography.

3. RESULTS AND DISCUSSION

In order to identify Zn-α_2-gp encoding clones, a human breast cDNA library was screened with mixed oligonucleotides derived from the amino acid sequence of the protein. Two out of approximately 5×10^5 λ-phage clones were selected on the basis of their strongly positive hybridization to the probes. The nucleotide sequence of inserts corresponding to these clones was determined following the strategy outlined in Fig. 1. Both cDNA clones shared the same 3′ sequence, containing the polyadenylation sequence AATAAA and a poly (A) tail. However, they presented clear differences in the length of their 5′ regions. Clone Zn102 was an incomplete cDNA while clone Zn133 contained the complete coding information for Zn-α_2-gp. Analysis of nucleotide sequence of this latter clone revealed that it contained at its 5′-end a region with a high degree of similarity to human elongation factor-1α, which probably resulted from a cloning artifact.

The nucleotide sequence derived from analysis of the different Zn-α_2-gp cDNA clones is shown in Fig.2. The sequence includes an open reading frame encoding a hydrophobic signal peptide and the 278 residues of the mature protein. Comparison of the amino acid sequence deduced from the nucleotide sequence with that determined for the protein isolated from plasma reveals some discrepancies. There are two substitutions: Gln and Glu are present at positions 65 and 222 of the mature polypeptide chain instead of the Glu and Gln residues determined at these positions by protein sequencing. In addition, we have detected the insertion of an Ile–Phe pair between residues located at positions 75 and 76 of the previous sequence. These changes could reflect genetic polymorphisms, although the possibility of protein sequencing errors cannot be excluded.

The nucleotide sequence described herein has also provided additional information on the relationship of Zn-α_2-gp to human class I major histocompatibility antigens (HLA). Zn-α_2-gp and HLA antigens show a significant homology at the protein level (36% identities), which is also detected in this work at the nucleotide level (56% identities to HLA-B7). However, Zn-α_2-gp lacks

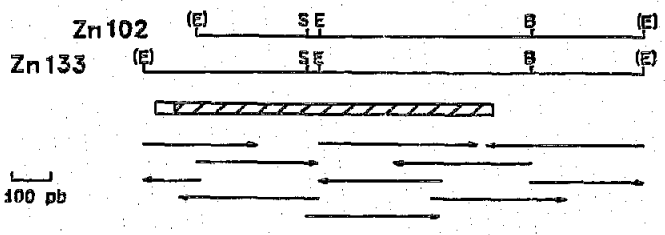

Fig. 1. Sequencing strategy for the Zn-α_2-gp cDNA clones. The restriction map is shown. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; (E), cloning *Eco*RI sites; S, *Sspl*. The boxed region represents the coding sequence and the hatched part shows the region coding for the mature protein.

the transmembrane and cytoplasmatic domains characteristic of HLA antigens [4]. These domains are removed by proteolytic enzymes to yield solubilized antigens, suggesting that Zn-α_2-gp might be derived through cleavage by proteases present in plasma. However, the finding of an in phase stop codon immediately adjacent to the Ser residue identified as C-terminal in Zn-α_2-gp, demonstrates that this protein is not generated by a protease-mediated mechanism and suggests that Zn-α_2-gp may play a role as a soluble HLA-like antigen.

The availability of the cDNA clones allowed us to study the expression of the Zn-α_2-gp gene in different human tissues. Total RNA was isolated from the collected samples and subjected to Northern-blot analysis,

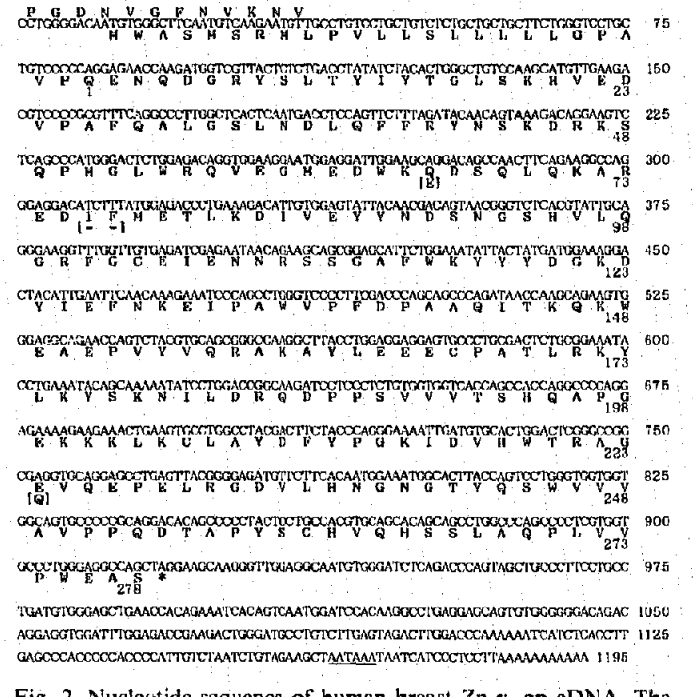

Fig. 2. Nucleotide sequence of human breast Zn-α_2-gp cDNA. The deduced amino acid sequence is shown below the mucleotide sequence with numbering starting with residue 1 of the mature protein. Residues which are different in the protein sequence of plasma Zn-α_2-gp are shown in brackets. The amino acid sequence from the 5′-flanking region with homology to elongation fator 1-α is shown above the nucleotide sequence. The polyadenylation signal is underlined.

using as a probe the *Eco*RI restriction fragment which contains the 3'-end of the cDNA. As shown in Fig. 3,a single hybridizing band corresponding to a mRNA species of about 1300 nucleotides was identified in breast, liver and in both benign and malignant breast tumors. The strongest signal was obtained with RNA from patients with fibroadenoma, being the result in Fig. 3, a representative example of 4 different specimens. According to this, enhanced expression of Zn-α_2-gp could be considered as a marker for benign mammary lesions. The variable intensity of the band detected at breast carcinoma tissues may be related to specific behavioural characteristics of the tumors and agrees with immunohistological studies indicating the existence of a subset of tumors which maintain the ability to produce this protein [6].

The molecular basis for the increased levels of Zn-α_2-gp in benign tumors or in cyst fluid from breast gross cystic disease are presently unknown, but the mechanism is probably mediated by hormonal factors, since several data suggest that these lesions result from an hormonal imbalance [5]. In relation to this, it is remarkable that Zn-α_2-gp and other major cyst fluid proteins like apolipoprotein D [14] are induced by androgens in breast cancer cells [15,16] which suggests that androgens could be direchtly involved in the enhanced expression of Zn-α_2-gp.

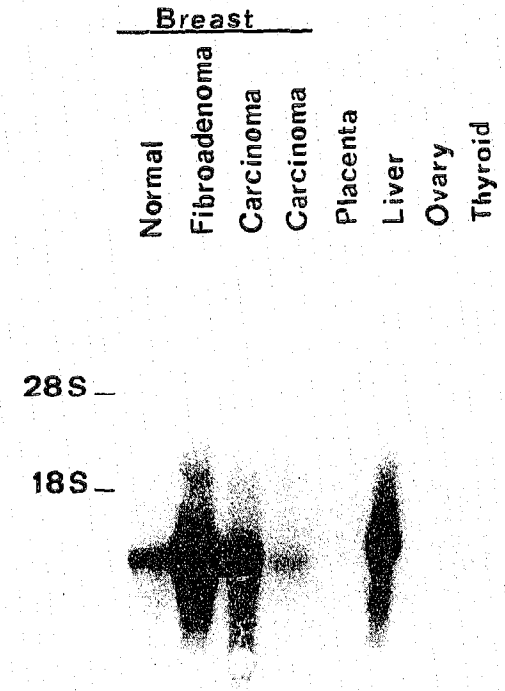

Fig. 3. Expression of Zn-α_2-gp mRNA in human tissues. About 10 μg of total RNA from the tissues indicated in the upper part of the figure were analysed by Northern-blot using a Zn-α_2-gp probe. The positions of 28 S- and 18 S-rRNA bands are indicated.

During the preparation of this manuscript, a report describing the characterization of a Zn-α_2-gp clone from a prostate cDNA library has been published [17]. Examination of this nucleotide sequence revealed some minor discrepancies with that presented in this work (changes at positions 26 and 35 and variable extensions at the 3'-flanking region). The absence of data on the 5'-untranslated sequence precludes a more detailed comparison of this region with the corresponding one to the breast cDNA clone characterized in this work. The isolation and characterization of genomic DNA clones containing this part of the gene will be useful to elucidate its structure as well as the mechanisms controlling the expression of the gene in normal and pathological breast tissues.

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