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Fetuin: the bovine homologue of human α_2HS glycoprotein

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The fetal protein fetuin has previously been considered to be confined to species of the order Artiodactyla (cattle, sheep, etc.) in spite of demonstrable biological in vitro effects in tissues of other species [(1983) Comp. Biochem. Physiol. 76A, 241–245]. We have determined the partial amino acid sequence of bovine fetuin and compared it with the published sequence of human α_2HS glycoprotein. The N-terminal 105 residues and a segment aligned with residues 170–225 of α_2HS glycoprotein revealed 109 of 161 residues to be identical between the two proteins (68% homology). Mouse polyclonal antibodies to fetuin, and trypsin digest fragments of this protein have been prepared and used for a comparison of native and digested proteins. Polyclonal antibodies to native protein showed little if any cross reactivity. However, antibodies to trypsin digest fragments of fetuin showed obvious cross reactivity with α_2HS.

Fetuin; α_2HS glycoprotein; Development; (Fetus)

1. INTRODUCTION

The presence of high concentrations of some plasma proteins in developing tissues has suggested possible functions for these proteins [1]. In our immunocytochemical studies of developing cortex in sheep and cattle we have noted a high concentration of one particular glycoprotein, fetuin, in the immature cortical plate [2]. Recently we have obtained similar information on human developing brain; but in addition to rather weak staining for fetuin we obtained strong staining for another glycoprotein, α_2-HS glycoprotein [3]. The published first 44 amino acids of the N-terminal of fetuin [4] and the A chain of α_2HS glycoprotein [5] show about 60% homology. This prompted us to undertake a study to decide whether the two proteins (or part of them) may in fact be the same peptide. We investigated their relationship using immunological methods as well as extending the partial amino acid sequence.

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2. MATERIALS AND METHODS

2.1. *Preparation of fragments of calf fetuin*

Calf fetuin was obtained from Calbiochem (no.341506). The protein was subjected to digestion with TPCK-treated trypsin (Sigma) and V8 proteinase (Boehringer) using conditions essentially the same as those in [6]. Prior to enzyme digestion calf fetuin was reduced and *S*-carboxymethylated and in one instance lysine groups were modified by succinylation prior to trypsin digestion using procedures described [6]. Dilute acid cleavage was carried out by incubating reduced and *S*-carboxymethylated fetuin (2 mg) in 0.5 ml of 10% acetic acid/6 M guanidine HCl (pH adjusted to 2.5 with pyridine) at 40°C for 96 h.

All peptide fragments were isolated by reverse-phase HPLC, using an HPLC system similar to that in [6]. Trypsin and V8 proteinase derived peptides were subjected to initial separation on a

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Radial-Pak μBondapak C18 (0.8 × 10 cm), a Bakerbond C8 (0.46 × 25 cm) or a Vydac C18 (0.46 × 25 cm) column using a solvent system generated from 0.1% TFA and 80% CH_3CN/0.1% TFA. In some cases additional purification of peptides was achieved using a Radial-Pak μBondapak C18 column and a solvent system derived from 0.1% NH_4HCO_3 and 80% CH_3CN.

Purification of acid-cleavage peptides was achieved by injecting an aliquot (0.4 ml) of the digest onto a Bakerbond C8 column (0.46 × 25 cm) equilibrated with 20% propan-2-ol/0.1% TFA (solvent A) at a flow rate of 0.6 ml/min. A linear gradient of 140 min was applied to the column to give a final concentration of 40% solvent A, and 60% solvent B (propan-2-ol/0.1% TFA). Further purification was carried out using a Vydac-Phenyl column (0.46 × 25 cm) using a similar solvent system.

All peptides were detected by absorbance at 214 nm.

2.2. *Amino acid analysis and automated N-terminal sequence analysis*

Amino acid analysis was carried out using the procedure of Bidlingmeyer et al. [7]. Automated sequence determination was carried out using an Applied Biosystems sequencer (model 470 A) essentially as described by Hewick et al. [8]. Phenylthiohydantoin amino acid derivatives were identified by HPLC using a Nova-Pak C18 column (0.46 × 15 cm) following the procedure recommended by the manufacturers (Waters Associates, MA).

2.3. *Limited proteolysis of calf fetuin and human α_2HS glycoprotein with trypsin*

Limited proteolysis was carried out by dissolving fetuin and α_2HS glycoprotein at a concentration of 5 mg/ml in 0.1 M NH_4HCO_3 and incubating with TPCK-treated trypsin (Sigma, enzyme to substrate ratio of 1:100, v/v) for 2 h at 37°C. Digests were either stored frozen or aliquots were freeze-dried.

2.4. *Immunological comparison of bovine fetuin and human α_2HS*

2.4.1. Antibodies against fetuin and fetuin fragments

Balb/c mice were injected intraperitoneally either with 50 μg of 2 h trypsin digested fetuin (antiserum D) or 100 μg of native protein (antiserum F) in Freund's complete adjuvant on day 0, 14 and 28. 3 days after the last injection the mice were bled and the specificity of the antiserum checked in crossed electrophoretic plates as described [9]. Rabbit anti-calf fetuin was obtained from Dakopatts (Denmark) and rabbit anti-human α_2HS from Hoechst (FRG).

2.4.2. SDS-polyacrylamide gel electrophoresis and immunoblotting

Samples of fetuin (5 μg), α_2HS glycoprotein (5 μg) and limited tryptic digests of these proteins (20 μg) were each subjected to electrophoresis on 15% acrylamide gels under reducing conditions in the presence of SDS [10]. Following electrophoretic transfer of protein bands to nitrocellulose [11] additional protein binding sites were blocked by incubation with 3% gelatin in TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.4) for 1 h. The paper was then incubated with a 1:100 dilution of mouse D or F antiserum for 1 h and finally with anti-mouse IgG antiserum conjugated with horseradish peroxidase (Dakopatts, Denmark). Antibody dilutions were in TBS containing 1% gelatin and 0.05% Tween 20. In between antibody incubations the nitrocellulose was washed, briefly, with distilled water and two 10 min washes with TBS/0.05% Tween 20. After the final antibody incubation the nitrocellulose was washed with TBS and peroxidase activity visualized using 0.2 mg/ml diaminobenzidine/0.005% H_2O_2 in TBS.

3. RESULTS

3.1. *Amino acid sequences of fetuin*

The partial sequence of fetuin was obtained by automated amino acid sequence of intact fetuin and fragments generated by trypsin, V8 proteinase and dilute acid cleavage and this is compared with that of human α_2HS glycoprotein in fig.1.

The previously published N-terminal sequence of calf fetuin [4] has been extended to 105 residues and of these, 75 residues were also found in the α_2HS glycoprotein sequence (71% homology). Peptide T26 A3 was positioned by homology with α_2HS glycoprotein; this is supported by the amino acid composition of peptide V8 40, which corresponds to residues 21–58 of fetuin (not shown).

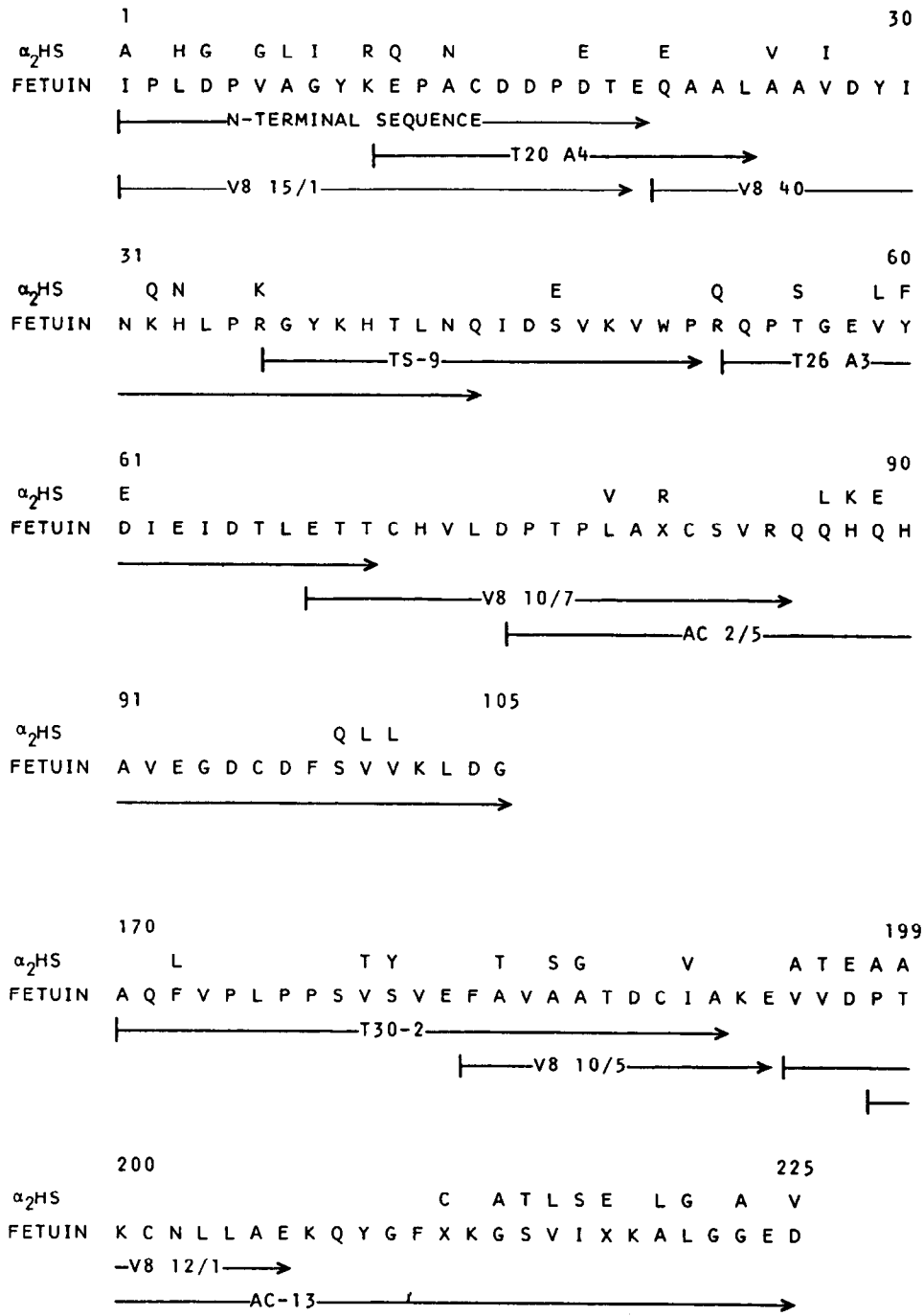

Fig.1. Comparison of partial sequence of calf fetuin with human α_2HS glycoprotein. T, tryptic peptides; TS, tryptic peptide from succinylated fetuin; V8, V8-protease peptide; AC, peptide generated by dilute acid cleavage. Residues are in the single letter code: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Glu; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, unknown; Y, Tyr. Residues in the α_2HS glycoprotein sequence [5] which differ from fetuin are noted above the fetuin sequence.

A further segment of 56 residues was homologous to residues 170–225 of the human α_2HS glycoprotein sequence with 34 out of 56 residues (61%) being identical. The positioning of peptide V8 12/1 was based on homology.

3.2. *Immunological comparison of fetuin and α_2HS glycoprotein*

In order to determine immunologically how closely α_2HS and fetuin are related we used mouse polyclonal antisera to native and peptide fragments of fetuin. It has previously been shown, using crossed immunoelectrophoresis, that rabbit anti-bovine fetuin cross reacts with α_2HS but rabbit anti-α_2HS does not cross react with fetuin [3]. Intact fetuin and α_2HS glycoprotein and fragments generated from these proteins by limited proteolysis with trypsin were compared by immunoblotting using mouse polyclonal D and F antisera (fig.2). Limited proteolysis resulted in the appearance of a 30 kDa fragment of fetuin which was recognized by both antisera. Native α_2HS glycoprotein showed no cross reactivity with the F antiserum but reacted clearly with D antiserum which was raised against a sample of fetuin that had been subjected to limited proteolysis with trypsin. On the other hand, both D and F antisera recognized partially digested α_2HS.

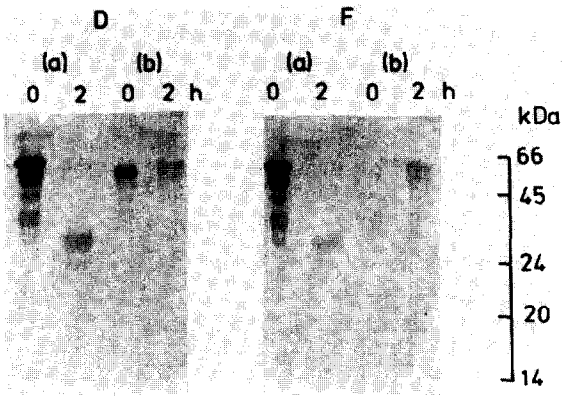

Fig.2. Comparison of calf fetuin and human α_2HS and their limited trypsin digests by immunoblotting. Samples of untreated proteins and trypsin digest were run on 15% acrylamide gels in the presence of SDS and proteins transferred to nitrocellulose. Immunological detection used mouse anti-fetuin D and F sera as the primary antisera. Molecular mass marker proteins were bovine serum albumin (66 kDa), ovalbumin (44 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and lactalbumin (14 kDa). (a) Fetuin, (b) α_2HS. 0, native protein; 2 h, 2 h trypsin digested protein; D, antiserum against trypsin digested fetuin; F, antiserum against native fetuin.

4. DISCUSSION

Sequence homologies amongst different proteins have revealed interesting relationships between structure and function [1]. In other cases a high degree of homology at the level of amino acid and DNA sequence has been used to group proteins into families although there is no obvious similarity in their known functions (e.g. albumin, α-fetoprotein and Gc-globulin [12]). In the present paper a striking similarity in amino acid sequence between a human plasma protein, α_2HS glycoprotein and a bovine fetal protein, fetuin, is demonstrated. We have previously commented on the similarity between the amino acid composition of these two proteins and their similar distributions in the developing brain [3]. The complete amino acid sequence of α_2HS has been published [5]: we have now obtained the partial sequence of fetuin including the first 105 residues and a segment which was aligned with residues 170–225 of α_2HS glycoprotein. The homology between the A chain of HS and the partial sequence of fetuin is 68%. Cysteine and tryptophan residues are conserved and there are few substitutions which introduce charge differences between the two proteins. Most of the substitutions (38 out of 49) can be explained by single base pair changes. From the degree of homology it is probable that fetuin and α_2HS have originated from a common ancestral gene [1].

The antigenic relationships between fetuin and α_2HS glycoprotein have been investigated. In order to distinguish whether the tertiary rather than primary structure of the protein is responsible for the antibody response to fetuin [3], we have obtained antibodies to trypsin digested fetuin. Using these antibodies it was possible to demonstrate clearly that fetuin and human α_2-HS are related proteins, although the antigenic determinants of both proteins seem to be different.

Comparison of the sequence of fetuin fragments and α_2HS A chain with protein sequences in a data bank demonstrated some similarity to the C-terminus of human α_2-macroglobulin [13]

although at a low level of significance (26% identity in 62 amino acids overlap). However, this may be of considerable interest, since our D antiserum (against trypsin digested fetuin) cross reacts strongly with human α_2-macroglobulin, a finding that is currently being investigated.

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