Tissue distribution and activity testing suggest a similar but not identical function of fetuin-B and fetuin-A

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Fetuins are serum proteins with diverse functions including the regulation of osteogenesis and inhibition of unwanted mineralization. Besides the α_2-Heremans and Schmid glycoprotein/fetuin-A, the recently identified fetuin-B is a second member of the fetuin family [Olivier, Soury, Risler, Smih, Schneider, Lochner, Jouzeau, Fey and Salier (1999) Genomics **57**, 352–364; Olivier, Soury, Ruminy, Husson, Parmentier, Daveau and Salier (2000) Biochem. J. **350**, 589–597], which belongs to the cystatin superfamily. We compared the expressions of fetuin-B and fetuin-A at the RNA level and established that both genes are most highly expressed in liver tissue. Like fetuin-A, fetuin-B mRNA is also highly expressed in tongue and placenta tissues. We demonstrated for the first time that fetuin-B is also expressed at the protein level in sera and several organs of mouse, rat and human. We isolated contiguous genomic clones containing both fetuin-B and fetuin-A genes, indicating that these genes are closely linked at the genome level. The close proximity of both these genes may explain our observation that fetuin-B expression was decreased in fetuin-A-deficient mice. Unlike fetuin-A, the amount of fetuin-B protein in human serum varied with gender and was higher in females than in males. Functional analysis revealed that fetuin-B, similarly to fetuin-A, is an inhibitor of basic calcium phosphate precipitation, albeit less active when compared with fetuin-A. Therefore fetuin-B may have a function that is partly overlapping, if not identical, with the function of fetuin-A.

Key words: α-fetoprotein, blood protein, cystatin, fetuin-A, fetuin-B.

INTRODUCTION

In an effort to identify hepatic genes differentially expressed during acute inflammation in rats, Olivier et al. [1] identified a partial cDNA encoding a fetuin family protein, which they termed fetuin-B. Northern-blot analysis detected liver-specific expression of rat fetuin-B. By searching DNA sequence databases, Olivier et al. [2] identified ESTs (expressed sequence tags) encoding human and mouse fetuin-B. The 382-amino acid human fetuin-B protein shares 22 % sequence similarity with fetuin-A, the prototypic member of the fetuin protein family. The human, mouse and rat fetuin-B proteins share 61 % amino acid identity. Northern-blot analysis detected a developmentally regulated expression pattern for fetuin-B in mouse and rat liver that differed between species. In response to inflammatory stimuli, hepatic levels of rat fetuin-A and fetuin-B were down-regulated.

Bovine fetuin-A was described in 1944 by Pedersen as fetuin (derived from the latin word *fetus*), the most abundant globular plasma protein in foetal calf serum [3]. After the discovery of a second fetuin, called fetuin-B and analysed in the present study, the protein originally named fetuin was renamed as fetuin-A. Fetuin-A and the human species homologue α_2-Heremans and Schmid glycoprotein (α_2-HS glycoprotein/Ahsg) are generic binding proteins in mammals. Fetuin-A homologues were subsequently shown to be major serum proteins in foetal cattle, sheep, pig and goat, as well as in humans and rodents [4].

Fetuin-A is part of the cystatin superfamily of cysteine protease inhibitors, which encompasses a series of closely related proteins that are synthesized mostly in the liver. Other members of this superfamily sharing cystatin-like domains are kininogens and HRGs (histidine-rich glycoproteins) [5–8]. Fetuin-A is an acute-phase protein. It was reported that, in humans and rats, fetuin-A was down-regulated during the acute-phase response, whereas in mice and cattle it was up-regulated [4,9,10]. However, detection methodology and sampling times grossly varied in these studies, and therefore the regulation of fetuin-A genes in these species may be almost equal, in contrast with what these results suggest. Fetuin-A has been implicated in several diverse functions, including osteogenesis and bone resorption [11], regulation of insulin activity [12] and hepatocyte-growth-factor activity [13], response to systemic inflammation [14] and inhibition of unwanted mineralization [15–17].

Owing to structural similarities, we developed a working hypothesis that fetuin-A and fetuin-B may have similar biological activities and are both (secreted) liver-derived serum proteins. However, the protein expression of fetuin-B could not be studied, since no tool was available to detect fetuin-B at the protein level. To overcome this limitation, we produced antisera against mouse, rat and human fetuin-B using GST (glutathione S-transferase)–fetuin-B fusion proteins as antigens. In the present study, we show that the fetuin-B protein is indeed a serum glycoprotein and shares overlapping, but not identical, tissue distribution and functions with fetuin-A. Furthermore, we show that fetuin-B and fetuin-A genes are located as immediate neighbours within a limited region on chromosome 16 in mice.

MATERIALS AND METHODS

DNA cloning and protein expression

Fetuin-A-deficient C57BL/6-129/Sv hybrid mice were backcrossed for at least ten successive generations to pure C57BL/6 and DBA/2 genetic background mice as described previously [17]. Control mice were littermate fetuin-A wild-type mice obtained from heterozygous fetuin-A ($\pm$) matings.

Abbreviations used: α_2-HS glycoprotein, α_2-Heremans and Schmid glycoprotein; BCP, basic calcium phosphate; DTT, dithiothreitol; EST, expressed sequence tag; GST, glutathione S-transferase; HRG, histidine-rich glycoprotein; MBP, maltose-binding protein; PAC, P1-derived artificial chromosome; PNGase F, peptide N-glycosidase F.

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Total RNAs from the livers of mouse and rat were extracted by using PeqGold RNApure (Peqlab Biotechnology, Erlangen, Germany). Total RNA from human liver was purchased from Ambion (Huntingdon, Cambs., U.K.). cDNA was synthesized from 2 μg of total RNA by reverse transcription under standard conditions [5 mM $MgCl_2$/50 mM KCl/10 mM Tris/HCl (pH 8.3)/1 mM each of dGTP, dATP, dTTP, dCTP/1 unit/μl RNase inhibitor/2.5 μM random hexamer or 2.5 μM oligo(dT) primer as indicated/2.5 units/μl MuLV reverse transcriptase (PerkinElmer LifeSciences, Boston, MA, U.S.A.)/2 μg each of total RNA isolated from the liver of mouse, rat and human] for 15 min at 42 °C in a total volume of 25 μl. PCR was performed with 2 μl of the reverse transcription product as template under the following conditions: 1 × PCR buffer, 20 μM dGTP, 20 μM dATP, 20 μM dTTP, 20 μM dCTP, 0.6 pmol/μl forward primer and 0.6 pmol/μl reverse primer, 25 m-units/μl Taq (Amersham Biosciences, Freiburg, Germany) and 1 M betaine. The program for the PCR was 1 min at 71 °C to add the template to the PCR mixture (10 s, 96 °C; 2 min, 62 °C; 2 min, 71 °C) for 35 cycles and 6 min at 71 °C to complete the reactions. Primers were synthesized for the following fetuin-B genes (accession numbers of the template and primer sequences are given in parentheses in the following order: forward primer, reverse primer, calculated length of the amplified fragment and position; in the primer sequences, boldface letters identify nucleotides added to the primers to allow in-frame cloning into the vectors pGEX-2T-1 and pMal C using *Bam*HI and *Eco*RI restriction endonucleases): mouse fetuin-B (accession no.: NM_021564, 5′-**CGGGAT**CCCTCTCACCTCTGCATC-3′, 5′-**GGAAT**TCTCAGGGTGGGACCAG-3′, 1092 nt, positions 285–1365), rat fetuin-B (accession no.: NM_053348, 5′-**CGGGAT**CCTTCGCACCTCTGCGTCC-3′, 5′-**GGAAT**TCAGGGGGTTCTTTGCTTTTC-3′, 1060 nt, positions 198–1246) and human fetuin-B (accession no.: NM_014375, 5′-**CGGGAT**CCCTCAACCCCTCGGCTC-3′, 5′-**GGA**ATTCTCATGGCGGAAGGACAAG-3′, 1080 nt, positions 175–1254). The PCR products were cloned into the pGEM T vector (Promega, Mannheim, Germany), digested with *Bam*HI and *Eco*RI, and ligated in-frame into the *Bam*HI–*Eco*RI sites of pGex-2T-1 (Amersham Biosciences), resulting in plasmids pGEX2T1mFetB, pGEX2T1rFetB and pGEX2T1hFetB respectively. To confirm their proper insertion into the vector, DNA sequences were determined by automated DNA sequencing (Applied Biosystems, Lincoln, CA, U.S.A. and PerkinElmer Life Sciences). The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) and induced at an absorbance of 0.5 at 600 nm by the addition of isopropyl 1-thio-β-D-galactopyranoside (final concentration of 0.3 mM) to express the GST–mouse fetuin-B, GST–rat fetuin-B and GST–human fetuin-B fusion proteins. After induction for 2 h at 37 °C, cells were harvested, and GST–mouse fetuin-B, GST–rat fetuin-B and GST–human fetuin-B fusion proteins were prepared and purified by an FPLC procedure using glutathione–Sepharose 4B columns (Amersham Biosciences) according to the manufacturer's instructions. The same *Bam*HI–*Eco*RI fetuin-B fragments were subcloned into the pMal C vector (New England Biolabs, Frankfurt, Germany) to express MBP (maltose-binding protein)–mouse fetuin-B, MBP–rat fetuin-B and MBP–human fetuin-B fusion proteins according to the manufacturer's instructions.

Northern-blot analysis

Total RNA was extracted from liver, heart and tongue tissues as indicated, using PeqGOLD RNAPure (Peqlab Biotechnology) according to the manufacturer's instructions. The yield of the total RNA was calculated by measuring the absorbance at 260 nm. The quality of RNA was routinely checked by electrophoretic analysis of an RNA sample (1 μg) using denaturing agarose–formaldehyde gels and ethidium bromide staining. In Northern-blot experiments, 20 μg of total RNA/lane was separated by electrophoresis on denaturing 1 % agarose–formaldehyde gels and transferred by capillary blotting with 20 × SSC (3 M NaCl/0.3 M sodium citrate, pH 7.0) on to nylon membranes (BrightStar-Plus Nylon Membrane; Ambion) according to the manufacturer's instructions. The RNA was UV-cross-linked to the membrane (120 mJ burst over a 30 s time period). Alternatively, FirstChoice Northern Blot, mouse Blot I, or FirstChoice Northern Blot, human Blot I (both from Ambion) were used for tissue-specific mRNA-expression analysis. Filters were hybridized with random prime-labelled cDNA probes using [α-^{32}P]dCTP (DecaPrime labelling kit; Ambion). As indicated in the Figures, cDNA fragments used for hybridization were mouse fetuin-B cDNA *Bam*HI fragment (828 bp), human fetuin-B cDNA *Eco*52I fragment of clone pGEM T hFetB (1122 bp), mouse fetuin-A cDNA *Pvu*II fragment (508 bp) and human fetuin-A cDNA *Bst*XI fragment (525 bp). At least 2×10^6 c.p.m./ml of labelled probe was used for the hybridization. The blots were prehybridized for 30 min at 42 °C in 5 ml of ULTRAhyb (Ambion). The denatured probe (for 5 min at 95 °C) was added to the prehybridization mix and incubated for 16 h at 42 °C. The hybridized filters were washed four times for 30 min each in 2 × SSC/0.1 % SDS at 42 °C and twice in 0.1 × SSC/0.1 % SDS at 42 °C. Filters were exposed to an X-ray film at −70 °C using intensifying screens and exposure times varied between 2 and 16 h.

Preparation of antisera/tissue protein extract

Polyclonal antisera against mouse fetuin-B, rat fetuin-B and human fetuin-B were raised in rabbits by standard immunization methods using the mouse, rat and human GST–fetuin-B fusion proteins respectively.

Tissue protein extracts from mice were prepared according to published methods [18]. Briefly, mice were anaesthetized using 0.5 mg avertin/g body weight and the vena cava inferior was ligated. Then 5 units of heparin/g body weight were injected into the portal vein, followed by perfusion (flow rate 10 ml/min) starting with 25 ml of isolation buffer (150 mM NaCl/5.6 mM KCl/5.5 mM glucose/20 mM Hepes/25 mM $NaHCO_3$, pH 7.4) containing 1 mM EDTA, and then with 50 ml of isolation buffer containing 2 mM $CaCl_2$. Subsequently, the tissues were excised from the animal, trimmed into small pieces, and placed in cold buffer A [50 mM Tris/HCl (pH 7.5)/2 mM EDTA/150 mM NaCl/0.5 mM DTT (dithiothreitol)]. The tissues were washed three times in buffer A, the excess buffer was drained, and the tissues were weighed. They were homogenized in 2.5 vol. (by wt.) of buffer B [50 mM Tris/HCl (pH 7.5)/10 % (v/v) glycerol/5 mM magnesium acetate/0.2 mM EDTA/0.5 mM DTT/1.0 mM PMSF]. Samples were cleared by centrifugation at 15 000 ***g*** for 30 min. The supernatant (protein extract) was carefully removed, snap-frozen in aliquots, and stored at −70 °C. For immunoblot analysis, 8 μl of protein extract was used.

Glycosylation and immunoblot analysis

Tissue protein extracts from mice were deglycosylated using the PNGase F (peptide N-glycosidase F) deglycosylation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Two reactions were performed for each sample both with and without PNGase F. Samples were analysed by

immunoblotting. To this end, sera or protein extracts were diluted in reducing SDS/PAGE sample buffer containing 5 % (v/v) 2-mercaptoethanol, separated by SDS/PAGE and transferred on to nitrocellulose membrane (Optitran BA-S83; Schleicher and Schuell, Dassel, Germany) by semi-dry electroblotting (1 h, 1.5 mA/cm^2). After blocking the membranes with blocking buffer [PBS containing 1.5 % (w/v) non-fat milk] for 30 min at 37 °C, membranes were washed once with washing buffer (PBS containing 0.05 % Nonidet P40), followed by incubation for 45 min at 37 °C with the primary antiserum dissolved in blocking buffer. Membranes were washed four times with washing buffer and incubated for 45 min at 37 °C with peroxidase-conjugated goat anti-rabbit IgG dissolved in blocking buffer (Jackson ImmunoResearch, Westgrove, PA, U.S.A.). After washing four times with washing buffer, proteins were detected by enhanced chemiluminescence (ECL® reagent; Amersham Biosciences).

Two-dimensional PAGE

Two-dimensional PAGE was performed essentially as described in [19] and detailed in the Swiss two-dimensional PAGE web pages (http://expasy.ch/ch2d).

Serum samples (6.25 μl) were incubated at 95 °C for 5 min with 10 μl of 10 % (w/v) SDS/2.3 % (w/v) DTT. These sample mixtures (2.9 μl) were diluted to 350 μl with rehydration solution containing 8 M urea, 4 % CHAPS, 0.5 % immobilized pH gradient buffer (IPG buffer, pH 3–10 NL; Amersham Biosciences), 65 mM DTT, also containing a trace of Bromophenol Blue dye. For isoelectric focusing, non-linear gradient strips (Immobiline DryStrips pH 3–10, 18 cm; Amersham Biosciences) were rehydrated with sample volumes of 350 μl (equivalent to 1.1 of μl serum and containing approx. 40 μg of total protein) in focusing trays for 12 h. Samples were electrofocused for 30 min at 500 V, 30 min at 1000 V, 60 min at 10 000 V and finally at 10 000 V for a total of 40 000 V · h (PROTEAN IEF Cell; Bio-Rad, München, Germany) at 20 °C. After the isoelectric focusing, sample strips were equilibrated for 15 min at 20 °C in equilibration buffer [50 mM Tris/HCl (pH 8.8)/6 M urea/2 % SDS/30 % (w/v) glycerol/1 % DTT], followed by a 15 min incubation in the same buffer containing 4 % (w/v) iodoacetamide instead of DTT. The two-dimensional electrophoresis was done using SDS/PAGE [20]. Strips were transferred on to the top of a slab gel [18.5 cm × 20 cm, 1.5 mm thick, 10 % (w/v) polyacrylamide gel] and fixed with 0.5 % agarose dissolved in running buffer (25 mM Tris/192 mM glycine/0.1 % SDS). Gels were run at 20 mA/gel for the first 20 min followed by 32 mA/gel in a Bio-Rad Protean IIxi apparatus until the Bromophenol Blue marker reached the bottom of the gel (approx. 6 h).

The separated proteins were detected by silver staining according to the published methods [21]. For immunoblotting, proteins were transferred on to nitrocellulose membranes by semi-dry blotting [22] and identified by enhanced chemiluminescence as described previously [23].

Genomic analysis and DNA cloning by screening cosmid/PAC (P1-derived artificial chromosome) library

Mouse genomic cosmid and PAC libraries were screened with cDNA probes for fetuin-B and fetuin-A respectively by the RZPD (Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin, Germany, http://www.rzpd.de/). Mouse genomic DNA was prepared from mouse tails according to standard procedures and was digested with the indicated restriction endonucleases. Conditions for Southern blotting were as described in [24].

Web-based sequence analysis included: 'Basic Local Alignment Search Tool' (BLAST) [25] (http://www.ncbi.nlm.nih.gov/blast/), 'PROSITE' [26] (http://www.expasy.org/prosite/) and 'MatInspector V 2.2' [27] (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl).

Precipitation inhibition assay

The precipitation inhibition assay was performed as described in [15,16]. Briefly, a buffered salt solution [50 mM Tris/HCl (pH 7.4)/4.8 mM $CaCl_2$/2 × 10^6 c.p.m. of [^{45}Ca]Cl_2/1.6 mM Na_2HPO_4], containing test proteins as indicated was incubated at 37 °C for 90 min. Precipitates were collected by centrifugation at 15 000 ***g*** for 5 min at room temperature (22 °C), dissolved in 1 % acetic acid and quantified by liquid-scintillation counting. All incubations were performed in triplicate. BSA (Roth, Karlsruhe, Germany) and bovine fetuin-A (Sigma, Steinheim, Germany) were used as negative and positive control proteins respectively.

RESULTS

Fetuin-B cDNA can be generated from mRNA of mouse, rat and human liver

Computer analysis of gene libraries resulted in the discovery of EST sequences coding for a second member of the fetuin family, called fetuin-B [2]. To isolate the corresponding fetuin-B cDNA from mouse, rat and human liver, we used published EST sequences to design oligonucleotide primers. The primers were used for PCR amplification of reverse-transcribed liver mRNA primed with oligo(dT) or random hexamer oligonucleotides (results not shown). PCR amplification resulted in cDNA fragments of 1092 bp length for mouse fetuin-B, 1060 bp for rat fetuin-B and 1080 bp for human fetuin-B. In each case, the amplicon size matched exactly with the predicted size for PCR products originating from the contiguous published cDNA sequence flanked by the fetuin-B-specific primers as described in the Materials and methods section. Identical products were obtained, irrespective of whether oligo(dT) or random hexamer oligonucleotide was employed during reverse transcription.

Fetuin-B mRNA is expressed predominantly, but not exclusively in the liver

Figure 1 illustrates the tissue-specific expression of fetuin-B in human and mouse, studied by Northern-blot analysis of commercial RNA blots. This analysis of mRNA revealed a single transcript of approx. 1.6 kb for human fetuin-B (Figure 1A). This fetuin-B transcript could be detected in the liver (Figure 1A, lane 7) and also at a lower level in the placenta (Figure 1A, lane 2). No human fetuin-B-specific transcript was detectable in brain, skeletal muscle, heart, kidney, pancreas, lung, spleen and thymus mRNA. A similar expression pattern was obtained for human fetuin-A mRNA, which revealed a single transcript of approx. 1.5 kb (Figure 1D), confirming the published expression pattern and extending it to placenta tissue.

In mice, a single fetuin-B transcript of approx. 1.5 kb was detected in liver samples (Figure 1B, lane 3; 1C, lane 1) and lower amounts also in kidney, embryo, lung, ovary and tongue (Figure 1B, lanes 5–7, 10; 1C, lane 3). No mouse fetuin-B mRNA was detected in heart, brain, spleen, thymus and testes. Mouse fetuin-A, similar to mouse fetuin-B, was expressed in liver, embryo, kidney and tongue, but unlike mouse fetuin-B, mouse fetuin-A could not be detected in lung and ovary (Figures 1E and 1F).

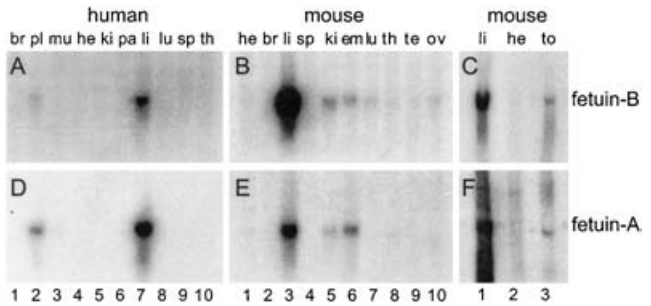

Figure 1 Northern-blot analysis using tissue RNA blots

Commercial multiple human (**A**, **D**) and mouse (**B**, **E**) tissue RNA blots (Ambion) as well as blots with 20 μg of total RNA isolated from mouse liver (positive control – **B** and **E**, lane 3), heart (negative control – **B** and **E**, lane 1) and tongue of fetuin-A-deficient mice (**C**) or wild-type mice (**F**) were hybridized with random-primed ^{32}P-labelled cDNA probes against human (**A**) and mouse (**B**, **C**) fetuin-B, and random-primed ^{32}P-labelled cDNA probes against human (**D**) and mouse (**E**, **F**) fetuin-A. Tissues analysed include brain (br), placenta (pl), skeletal muscle (mu), heart (he), kidney (ki), pancreas (pa), liver (li), lung (lu), spleen (sp), thymus (th), embryo (em), testes (te), ovary (ov) and tongue (to).

Fetuin-B is secreted into the serum

Until now, for lack of tools of detection, fetuin-B could not be studied at the protein level. Therefore we generated rabbit antisera against the fetuin-B protein from human, rat and mouse, using recombinant GST–fetuin-B fusion proteins as antigens. First, the antisera were tested against recombinant GST–fetuin-B fusion proteins to show their cross-reactivity against fetuin-B from different species (results not shown). Antiserum against human fetuin-B reacted with human and mouse GST–fetuin-B, but not with rat GST–fetuin-B fusion protein. Antiserum against rat fetuin-B reacted with rat GST–fetuin-B and less strongly with mouse GST–fetuin-B fusion protein. Antiserum against mouse fetuin-B reacted with mouse and rat GST–fetuin-B fusion proteins and weakly with human GST–fetuin-B (results not shown). To determine whether fetuin-B is secreted into the serum like fetuin-A, human, rat and mouse sera were analysed by immunoblotting. Figure 2(A) illustrates that fetuin-B-immunoreactive bands migrating at approx. 60 kDa were present in human, rat and mouse sera. Thus fetuin-B was secreted into the sera of all the three species. The fetuin-B antisera and the corresponding antisera against fetuin-A were tested for cross-reactivity in immunoblot analysis using sera from human, rat and mouse (Figure 2A). The rabbit antiserum raised against human fetuin-B reacted with human and mouse fetuin-B, confirming the cross-reactivity pattern observed with GST–fetuin-B fusion proteins (Figure 2A, top panel, lanes 1, 3 and 4). However, the antiserum was highly specific for fetuin-B in that it did not react with other serum proteins or with purified human fetuin-A protein (Figure 2A, top panel, lane 5). Antisera raised against rat and mouse fetuin-B detected fetuin-B in sera of both rodent species (Figure 2A, middle and bottom panels, lanes 2–4). Additionally, the antiserum raised against mouse fetuin-B weakly cross-reacted with the human fetuin-B (Figure 2A, bottom panel, lane 1). No further cross-reaction with serum proteins was detected. All three sera were specific for fetuin-B in that they failed to cross-react with recombinant fetuin-A from each species (results not shown). The fetuin-B specificity of the antisera is further illustrated by the fact that a fetuin-B band is still detected in serum of null mutant mice that no longer produce fetuin-A (labelled −/− in Figure 2A, lane 4). Antiserum against human fetuin-A proved highly specific for human fetuin-A in serum and in a purified human fetuin-A sample (Figure 2A, top panel). The two human fetuin-A bands in

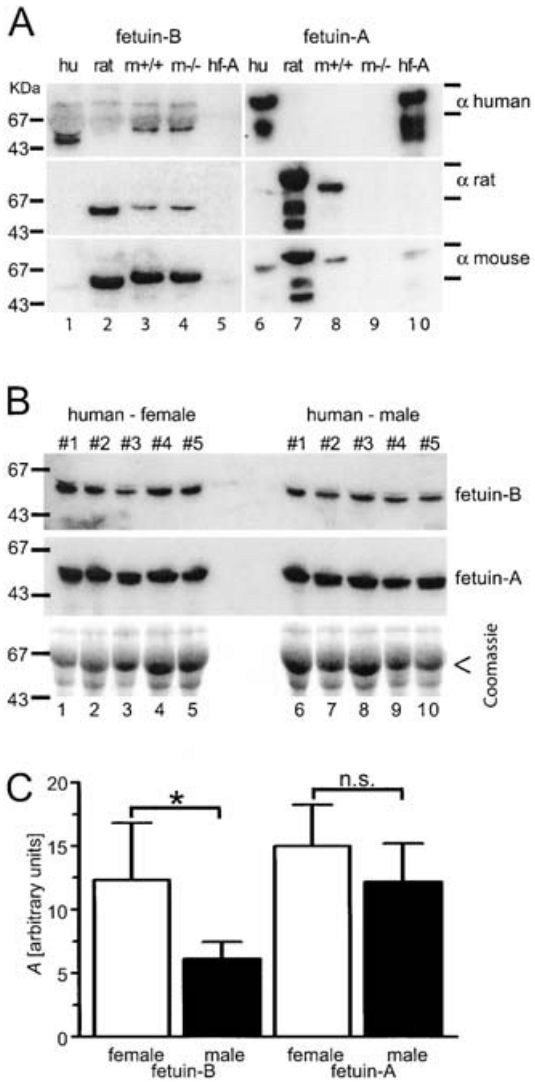

Figure 2 Immunoblot analysis of antisera directed against fetuin-B and fetuin-A

(**A**) Specificity of antisera prepared against fetuin-B and fetuin-A. Sera (0.1 μl) from human (hu), rat (rat), wild-type mouse (m +/+), fetuin-A-deficient mouse (m −/−) as well as 500 ng of purified human fetuin-A (hf-A) were separated by SDS/PAGE, transferred on to nitrocellulose membrane and probed with antisera against human (α human), rat (α rat) or mouse (α mouse)-specific antisera to fetuin-B and fetuin-A as indicated. Bound antibody was visualized by chemiluminescence. The mass of marker proteins is indicated in kDa. (**B**) Sera from five human females (lanes 1–5) and five human males (lanes 6–10) were analysed by immunoblotting using antisera against human fetuin-B (top panel) and human fetuin-A (middle panel). In the bottom panel, the corresponding Coomassie-stained gel with the albumin band (<) is shown. (**C**) Quantification of the amount of fetuin-B and fetuin-A proteins. Immunoreactive bands of fetuin-B (**B**, top panel), and fetuin-A (**B**, middle panel) were analysed densitometrically, and normalized to the Coomassie-stained albumin band (**B**, <, bottom panel). Data were analysed using the unpaired Student's t test. The bars represent means ± S.D. ($n = 5$, $^*P = 0.0187$); n.s. = not statistically significant.

serum are the heavy chain and the A-chain [23,28]. Antibodies raised against recombinant rat fetuin-A also cross-reacted with mouse fetuin-A in sera of wild-type mice, but not with any proteins contained in serum of fetuin-A-deficient mice or in human serum (Figure 2A, middle panel). Antiserum against mouse fetuin-A cross-reacted with rat and weakly with human fetuin-A (Figure 2A, bottom panel, lanes 6 and 10). The antisera against rat fetuin-A and mouse fetuin-A neither cross-reacted with fetuin-B [no band in serum of fetuin-A-deficient mice (−/−) detectable;

Figure 2A, middle and bottom panels, lane 9] nor with any other serum proteins in any serum tested. Multiple bands observed in the rat serum might represent degradation products of rat fetuin-A (Figure 2A, middle and bottom panels, lane 7). Thus our antisera were highly specific for each species analysed.

For lack of purified fetuin-B, we used recombinant MBP–fetuin-B fusion protein as a standard to quantify the fetuin-B concentration in sera of male mice, rats and humans. We compared the signal strength of a dilution series of serum with known amounts of the corresponding MBP–fetuin-B fusion protein and determined that mouse serum contains 156 ± 3 μg/ml ($n = 3$), rat serum 262 ± 51 μg/ml ($n = 3$) and human serum 5 ± 1 μg/ml ($n = 3$).

Fetuin-B protein is more abundant in the human serum of females than males

In a screen for fetuin-B protein content of archived human serum samples, we noticed significant variation between individual donors. Analysing age and sex-matched donor groups, we determined that this difference was due to differential fetuin-B expression in males and females. Figure 2(B) illustrates a representative immunoblot showing that fetuin-B is more abundant in female serum than in male serum. Following normalization to the respective Coomassie-stained albumin band of each serum sample, the mean fetuin-B content in the sera of females was twice as high as in sera of males (Figure 2C, $n = 5$, $P = 0.0187$). In contrast with fetuin-B, the amount of fetuin-A protein was not significantly different (Figure 2C, $n = 5$, not statistically significant) in both genders, confirming published results [29].

Tissue distribution of fetuin-B protein in mice

To determine the tissue distribution of fetuins in mice, we analysed sera and protein extracts from cecum, brain, colon, heart, kidney, liver, lung, placenta, skeletal muscle, skin, small intestine, spleen, stomach and tongue using immunoblots and the mouse-specific antisera (Figure 3A). Coomassie Blue-stained replicas of all blotted gels were prepared to estimate the amount of protein extracted and its complexity. It is noteworthy that a prominent serum albumin band was absent in all tissue extracts, demonstrating that the perfusion protocol effectively removed blood before tissue harvesting. Therefore, in Figure 3(A), the amounts of mouse fetuin-B and mouse fetuin-A proteins detected truly reflect the amount of these proteins associated with the extracellular matrix or contained within the cells of these tissues. As shown in Figure 3(A) (top panel), fetuin-B-immunoreactive bands migrating at approx. 60 kDa were detected in all tissues tested with the exception of brain. In some tissues, fetuin-B and fetuin-A migrated as clearly separated bands, probably reflecting differential secondary modifications, including limited proteolysis [28], differential glycosylation and phosphorylation [23]. In liver and tongue protein extracts, two separate fetuin-B-immunoreactive bands were always detected (Figure 3A, top panel, lanes 8 and 17). In sera and in all other organs analysed with the exception of brain, fetuin-B migrated as a single band. The positive detection of fetuin-B protein in peripheral tissues, rinsed by perfusion liquid, suggests that fetuin-B is sequestered by the peripheral tissues from the serum. Therefore no fetuin-B forms, other than the serum form, were detected in any tissues except liver and tongue even after longer exposure of the immunoblots (results not shown). Fetuin-B was detected in various amounts depending on the tissue. In cecum, kidney, placenta, skin and tongue, the amount of fetuin-B was comparatively higher than

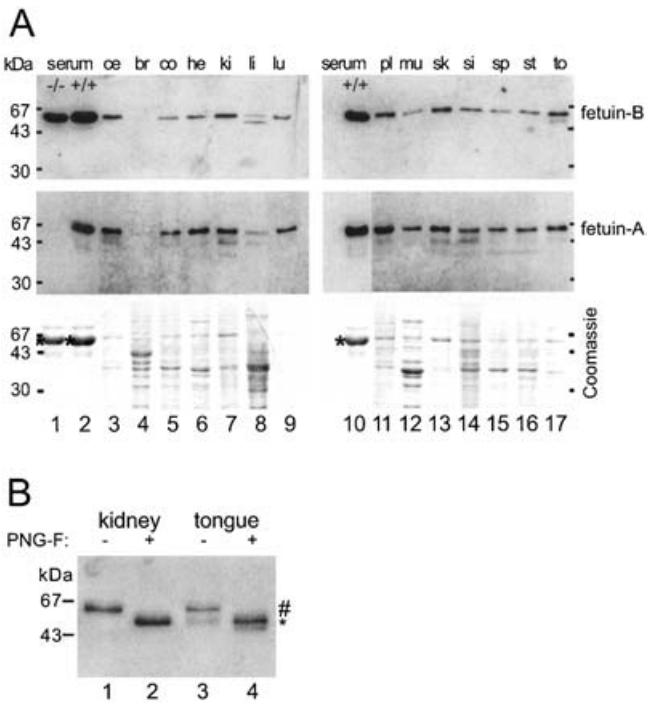

Figure 3 Tissue distribution of fetuin-B protein in mice

(**A**) Detection of fetuin-B and fetuin-A proteins in mouse tissues. Sera from fetuin-A-deficient (−/−) and from wild-type mice (+/+) as well as protein extracts from cecum (ce), brain (br), colon (co), heart (he), kidney (ki), liver (li), lung (lu), placenta (pl), skeletal muscle (mu), skin (sk), small intestine (si), spleen (sp), stomach (st) and tongue (to) were separated by SDS/PAGE, transferred on to nitrocellulose membrane, and probed with antisera against mouse fetuin-B (top panel) and mouse fetuin-A (middle panel). In the bottom panel, identical amounts of protein extract as used in the immunoblot analysis were separated by SDS/PAGE and stained with Coomassie Blue. Serum albumin is indicated by ⋆ (lanes 1, 2 and 10). (**B**) Deglycosylation of fetuin-B. Protein extracts from kidney and tongue were left untreated (−) or were treated with PNGase F (+), separated by SDS/PAGE, transferred on to nitrocellulose membrane, and probed with antiserum against mouse fetuin-B. (#, glycosylated fetuin-B protein; ⋆, deglycosylated fetuin-B protein). kDa, molecular mass markers.

that in colon, heart, liver, lung, skeletal muscle, small intestine, spleen and stomach (Figure 3A, top panel).

As described above, Figure 3 (A, middle panel) shows immunoblots of a subset of tissues performed with mouse fetuin-A antiserum. In serum of fetuin-A-deficient mice (Figure 3A, middle panel, lane 1), fetuin-A was not detected, confirming the specificity of this antibody for fetuin-A and lack of cross-reactivity with fetuin-B. In mouse wild-type sera, one strong fetuin-A-immunoreactive band was detected (Figure 3A, middle panel, lanes 2 and 10). In adult brain extract, neither fetuin-B nor fetuin-A was detected (Figure 3A, middle panel, lane 4). In all other tissues analysed, fetuin-A migrated as a single band. The distribution of fetuin-A was also different depending on the tissue. In cecum, heart, kidney, placenta, skin and tongue, fetuin-A was detected in higher amounts than in colon, liver, lung, skeletal muscle, small intestine, spleen and stomach (Figure 3A, middle panel, lanes 3, 6, 7, 11, 13 and 17). This result may simply reflect higher blood perfusion of these organs. In addition, for tongue tissue, it indicates sequestration and/or local synthesis of mouse fetuin-A, which had previously gone unnoticed [29,30]. In the liver, the amount of fetuin-B as well as fetuin-A protein was relatively low (Figure 3A, lane 8), when compared with the high amount of mRNA present in this tissue. This seemingly contradictory result is typical of highly expressed liver-derived

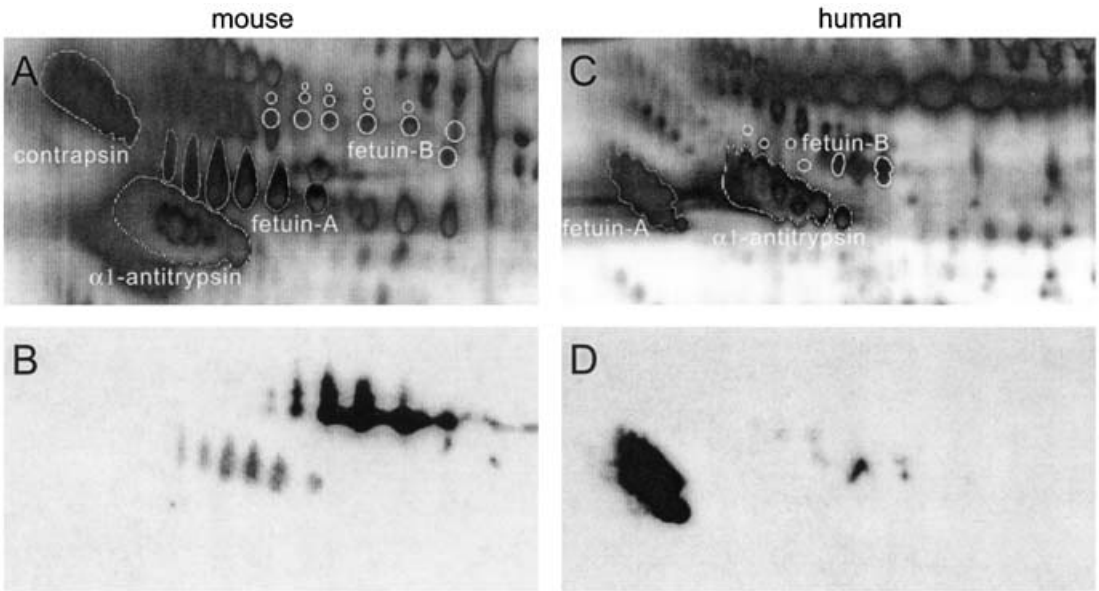

Figure 4 Two-dimensional gel separation of mouse (A, B) and human (C, D) serum proteins

Cut-out of silver-stained two-dimensional PAGE gel showing mouse (**A**) and human (**C**) serum proteins. Proteins were separated in first dimension using a non-linear pH gradient ranging from pH 3 to 10 (IPG-strip) and in the second dimension using SDS/PAGE (10 % gel). Immunoblot analysis of mouse (**B**) and human (**D**) serum proteins separated by two-dimensional PAGE using a mixture of antisera against fetuin-B and fetuin-A. The positions of fetuin-B, fetuin-A, α1-antitrypsin and contrapsin as determined by immunoblot analysis are indicated in **A**, **C**.

serum proteins, which are secreted constitutively, but rarely sequestered by liver parenchyma.

To compare the signal strength of mouse fetuin-B and mouse fetuin-A, among the subset of tissues, a Coomassie-stained gel replica with the same amount of sera and tissue extracts loaded was analysed (Figure 3A, bottom panel). Notice the higher amount of liver protein extract compared with neighbouring sample lanes (Figure 3A, bottom panel, lane 8), excluding the possibility that low sample loading was responsible for the relatively weak signal in the immunoblot.

Analysis of fetuin-B protein heterogeneity

Fetuin-B-immunoreactive bands migrated at approx. 60 kDa (Figures 2A and 3A), which is considerably higher than the theoretical molecular mass of mouse fetuin-B protein at 42.7 kDa. This discrepancy is best explained by post-translational modifications. A 'PROSITE' computer analysis of the fetuin-B preprotein sequence revealed two putative asparagine-glycosylation sites at positions 40 and 139, one cAMP phosphorylation site, five protein kinase C phosphorylation sites, five casein kinase II phosphorylation sites, one tyrosine phosphorylation site and three N-myristoylation sites. Of these secondary modifications, asparagine glycosylation is most typical of secreted serum proteins. As shown in Figure 3(A, top panel, lanes 8 and 17), fetuin-B exists in two forms in liver and tongue, the tissues where fetuin-B mRNA is most highly expressed (see Figure 1). From previous experience with hepatic synthesis of human fetuin-A [23], we assume that the second, smaller fetuin-B form detected in liver and tongue is an immature, cellular precursor form of fetuin-B protein that is not yet fully glycosylated. Interestingly, the mature serum fetuin-B existed only in a single form, supporting this notion. To test if mouse fetuin-B is asparagine glycosylated, we treated protein extracts from kidney and tongue with PNGase F. Immunoblot analysis of mouse fetuin-B protein revealed a shift to lower apparent molecular mass after PNGase F treatment when compared with untreated samples (Figure 3B), indicating that mouse fetuin-B indeed carries asparagine-linked glycan chains. In kidney and tongue protein extracts, the higher molecular mass form of fetuin-B shifted to the lower molecular mass form after PNGase F treatment, indicating that the smaller mouse fetuin-B form represents the deglycosylated form of mouse fetuin-B, which is present in tongue but not in kidney *a priori*, since tongue synthesizes fetuin-B and hence transiently also the non-glycosylated form, but the kidney tissue does not.

To study the micro-heterogeneity of fetuin-B protein, we analysed fetuin-B expression by two-dimensional PAGE and immunoblotting. Mouse and human serum proteins were resolved by two-dimensional PAGE and visualized by silver staining. The entire two-dimensional gels resolved several hundred individual proteins ranging from molecular mass 20 to 200 kDa with pI between 4 and 10. We tentatively identified proteins on the basis of pI and molecular mass with public plasma protein map databases as a reference (Swiss Institute of Bioinformatics proteomics server website http://www.expasy.ch/). The most abundant proteins were albumin and immunoglobulin heavy chains. Many of the spots displayed charge train patterns characteristic of serum glycoproteins. To identify fetuin-B, fetuin-A and the neighbouring proteins α1-antitrypsin and contrapsin, a mouse serum protease inhibitor, we probed immunoblots of serum proteins separated by two-dimensional PAGE using specific antibodies (results not shown). The positive signals obtained by simultaneous immunoblot analysis for fetuin-B and fetuin-A are shown in Figures 4(B) (mouse serum) and 4(D) (human serum). The antibody staining patterns overlapped with the corresponding area of silver-stained replica of two-dimensional gels. Figures 4(A) and 4(C) show that fetuin-B migrated in a similar position relative to α1-antitrypsin in both mouse and human serum samples. In contrast, fetuin-A migrated in an intermediate position relative to these proteins in mouse serum, but migrated at a substantially more acidic position in human serum. As expected, contrapsin was only detected in

mouse serum. In these gels, fetuin-B similar to α1-antitrypsin displayed considerable micro-heterogeneity, which is typical of serum proteins bearing complex asparagine-linked carbohydrate side chains. In addition, fetuin-A was considerably more micro-heterogeneous in mouse serum than in human serum.

Organization of the fetuin-B gene

To study the fetuin-B gene organization, we used the mouse fetuin-B cDNA obtained by PCR cloning to identify cloned mouse genomic DNA vectors containing the fetuin-B gene. An 834 bp cDNA fragment, flanked by a 5′-artificial *Bam*HI site and an endogenous *Bam*HI site, was used as a probe for screening a genomic mouse PAC library at RZPD (Germany). The probe covered exons 2–8 of mouse fetuin-B. The PAC clone isolated using a fetuin-A probe was confirmed by Southern blotting to contain the entire mouse fetuin-A gene (Figure 5, lower panel). Interestingly, this PAC clone also contained the mouse fetuin-B gene. Figure 5 shows a Southern-blot analysis using mouse fetuin-B and mouse fetuin-A probes and DNA from PAC clone RPCIP711A0548Q2 and mouse genomic DNA respectively. Identical restriction patterns were obtained with both DNA samples indicating that the PAC clone contained most, if not all, of the mouse fetuin-A and mouse fetuin-B genes. Identical fragment patterns were detected with probes specific for fetuin-B and fetuin-A respectively [*Xho*I–fetuin-B probe, lanes 2 and 7: 6, 1.8 and 0.9 kb (#); *Xho*I–fetuin-A probe: 25 kb (#); *Hin*dIII–fetuin-B, lanes 3 and 8: 6.6, 3.0 and 2.2 kb (×); *Hin*dIII–fetuin-A: 7.0 kb (×); *Eco*RI–fetuin-B, lanes 4 and 9: 22.0 kb (⋆); *Eco*RI–fetuin-A: 1.2kb (⋆); *Bam*HI–fetuin-B, lanes 5 and 10: 6.6 and 4.8 kb (<); *Bam*HI–fetuin-A: 3.5 kb (<)]. This result indicates that the mouse fetuin-B and the mouse fetuin-A genes are located within approx. 50 kb of a contiguous DNA segment on the same chromosome of the mouse genome. Recently published genomic sequences confirm our own map derived from genomic Southern analysis and show that the mouse fetuin-B and fetuin-A genes are separated by 18.8 kb and are both on chromosome region 16 B1 in the mouse genome [31].

A computer analysis using the similarity search tool 'BLAST' demonstrated identity with draft sequences from the mouse genome project located on chromosome 16 (accession no. NW_000107). Alignment of the mouse fetuin-B cDNA to the genomic sequence revealed that the fetuin-B gene contains eight exons, in contrast with fetuin-A, which is encoded by seven exons. A putative TATA-box is localized at positions −34 to −18 relative to the first nucleotide of exon 1. From the 5′-sequence of fetuin-B cDNA, it appears that not the first but the second exon contains the ATG start codon at position 19691314 of accession number NW_000107.

The finding that fetuin-A and fetuin-B are next neighbours in the genome prompted us to analyse if both genes are co-regulated and if the genetic deletion of fetuin-A influenced the expression of fetuin-B. Fetuin-A-deficient mice had been previously generated by our group [32].

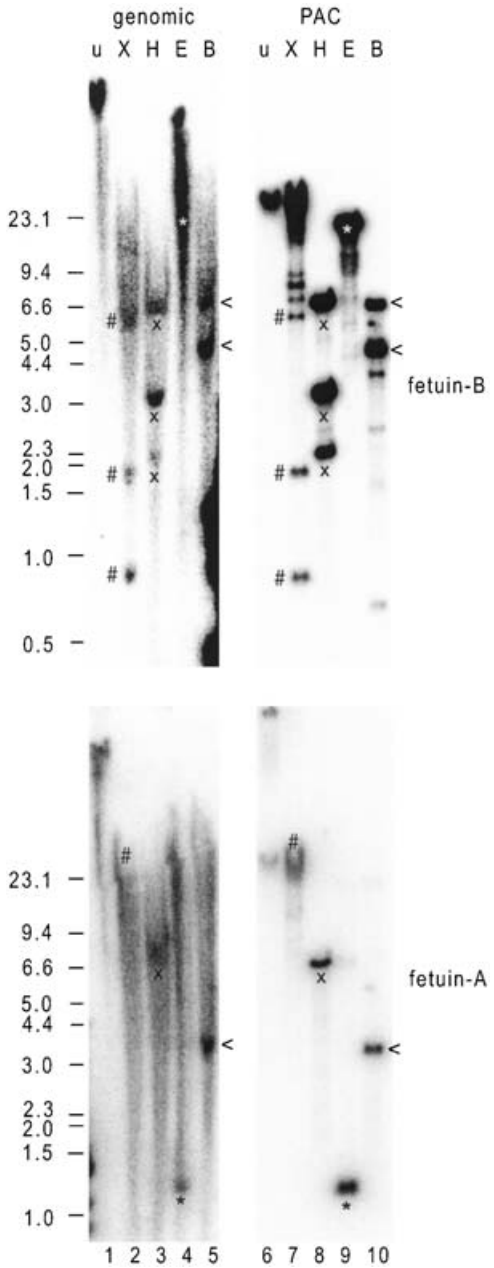

Figure 5 Southern-blot analysis of genomic mouse DNA and cloned mouse DNA (PAC clone)

Genomic DNA and the PAC clone DNA were left undigested (u; lanes 1 and 6) or digested with *Xba*I (X), *Hin*dIII (H), *Eco*RI (E) or *Bam*HI (B), separated by gel electrophoresis (0.8 % agarose), blotted on to nitrocellulose and hybridized with a ^{32}P-labelled cDNA probe. Fetuin-B-specific cDNA probe (fetuin-B, upper panel), and fetuin-A-specific cDNA probe (fetuin-A, lower panel). Note that the major labelled fragments (#, ×, ⋆ and <) are identical in each separation of genomic and cloned DNA. Left-hand side, marker sizes (kb).

Down-regulation of hepatic fetuin-B mRNAs and protein expressions in fetuin-A-deficient mice

Using Northern-blot analysis, we determined a significantly decreased amount of fetuin-B mRNA in the liver of fetuin-A-deficient mice when compared with wild-type mice. Figures 6(A) and 6(B) illustrate the fetuin-B mRNA expression in liver for wild-type mice (+/+) and fetuin-A-deficient mice (−/−) respectively. Overall, we determined a reduction of fetuin-B mRNA expression in fetuin-A-deficient mice by approx. 30 % ($n = 6$, $P = 0.0041$). We examined if this would likewise affect the level of fetuin-B protein circulating in the serum. To this end, we analysed the sera from fetuin-A-deficient and wild-type mice maintained on two separate genetic backgrounds, C57BL/6 and DBA/2. As expected, no fetuin-A protein was detected in fetuin-A-deficient mice (Figure 6C, middle panel, lanes 5–7 and 11–13). In contrast, fetuin-B was expressed both in wild-type and in fetuin-A-deficient mice (Figure 6C, top panel). However, in the fetuin-A-deficient mice, the amount of fetuin-B protein was

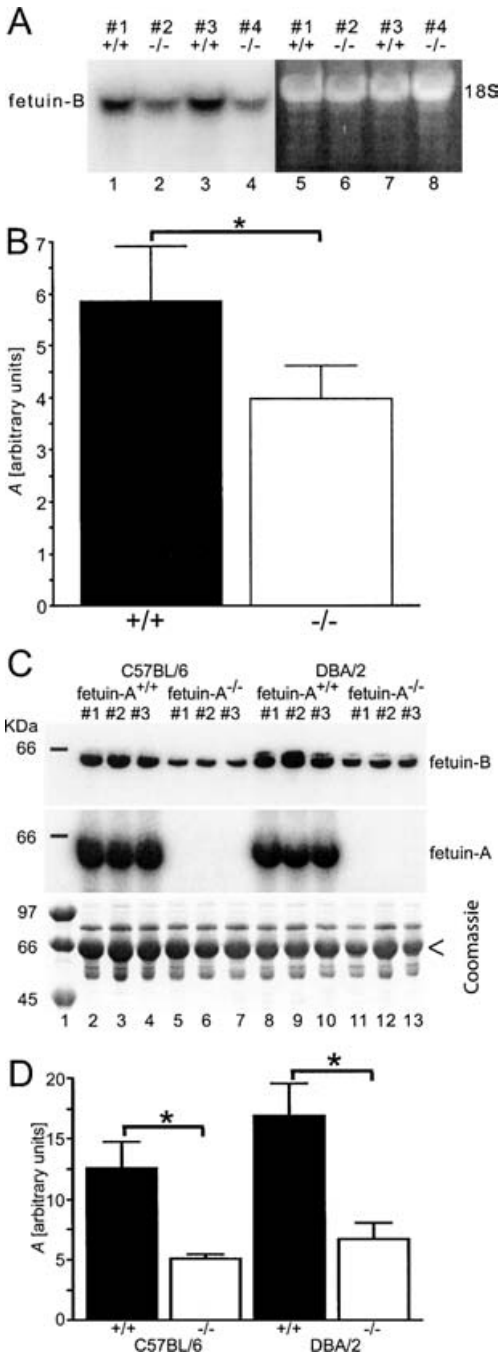

Figure 6 Expression analysis of fetuin-B in wild-type and fetuin-A-deficient mice

(**A**) Northern-blot analysis of liver RNA for fetuin-B in wild-type (+/+) and fetuin-A-deficient (−/−) mice. Liver RNA from wild-type (+/+) and fetuin-A-deficient (−/−) mice was separated by gel electrophoresis on a 1 % formamide/agarose gel, blotted on to Hybond N membrane, and analysed by hybridization with a ^{32}P-labelled mouse fetuin-B-specific cDNA probe (lanes 1–4). The ethidium-bromide-stained 18 S rRNA pattern shown in the right panel served as a loading control (lanes 5–8). (**B**) Quantification of fetuin-B mRNA amount. Fetuin-B-specific bands from multiple Northern blots were analysed by densitometric scanning and were normalized to the amount of the 18 S rRNA band. Results were analysed using the unpaired Student's *t* test. The bars indicate means ± S.D. ($n = 6$, $^*P = 0.0041$). (**C**) Immunoblot analysis of fetuin-B and fetuin-A in mouse sera. Sera from C57BL/6 wild-type mice (C57BL/6, fetuin-A$^{+/+}$), C57BL/6 fetuin-A-deficient mice (C57BL/6, fetuin-A$^{-/-}$), DBA/2 wild-type mice (DBA/2, fetuin-A$^{+/+}$) and DBA/2 fetuin-A-deficient mice (DBA/2, fetuin-A$^{-/-}$) were separated by SDS/PAGE, blotted on to nitrocellulose membrane and analysed with antisera against mouse fetuin-B (top panel) and against mouse fetuin-A (middle panel). The corresponding Coomassie-stained gel is shown in the bottom panel; <, albumin band. (**D**) Quantification of the amount of fetuin-B protein. The fetuin-B-immunoreactive bands (**C**, top panel) were analysed densitometrically and normalized to the Coomassie-stained albumin band (**C**, <, bottom panel). Results were analysed using the unpaired Student's *t* test. The bars indicate the means ± S.D. ($n = 3$, $^*P = 0.0042$ for C57BL/6 and $n = 3$, $^*P = 0.0041$ for DBA/2).

reduced. Coomassie-stained gels served to estimate total protein loading in each lane (Figure 6C, bottom panel). Densitometric quantification of fetuin-B protein signal strength after normalizing to the respective Coomassie-stained albumin band is shown in Figure 6(D). The amount of fetuin-B protein in the serum of the fetuin-A-deficient mice was significantly reduced by approx. 60 % compared with wild-type mice, independent of the genetic background (Figure 6D; $n = 3$, $P = 0.0042$ for C57BL/6 mice and $n = 3$, $P = 0.0041$ for DBA/2 mice). Thus the reduction of fetuin-B expression at the protein level was 2-fold higher than the reduction determined at the level of mRNA (Figures 6A and 6B). The reduced expression of fetuin-B in fetuin-A-deficient mice may be best explained by the fact that the fetuin-A and fetuin-B genes are located side by side on chromosome 16 and that manipulating the fetuin-A gene locus also affected regulatory sequences of the fetuin-B gene.

Inhibition of BCP (basic calcium phosphate) precipitation by fetuin-B protein

Genetic evidence from mutant mice suggested that fetuin-A is a systemic inhibitor of precipitation of BCP, preventing unwanted calcification. Owing to the fact that fetuin-B, similar to fetuin-A, contains two cystatin-like domains typical for members of the cystatin superfamily, we analysed how fetuin-B inhibits the formation of BCP. We produced mouse fetuin-B and mouse fetuin-A fusion proteins, with GST and MBP respectively and measured their ability to inhibit BCP formation from a super-saturated salt solution as described in [15,16]. Confirming previous reports, native serum bovine fetuin-A was most active in this assay ($IC_{50} = 1.8\ \mu$M), whereas BSA did not significantly inhibit BCP precipitation under identical conditions up to a concentration of 10 μM. In comparison with bovine serum fetuin-A, recombinant mouse fetuin-A fused to GST or MBP was only a slightly less active inhibitor of BCP precipitation ($IC_{50} = 2.1\ \mu$M). Extending our earlier results, we now studied the concentration-dependence of BCP precipitation inhibition using all three species homologues of fetuin-B proteins at concentrations ranging from 0 to 10 μM. Recombinant mouse fetuin-B fused to GST ($IC_{50} = 7.4\ \mu$M) or MBP ($IC_{50} = 6.1\ \mu$M) was a weaker inhibitor of BCP precipitation. Similar IC_{50} values were obtained with recombinant rat and human fetuin-B fused to GST (results not shown). We conclude that fetuin-B, similar to fetuin-A, is an inhibitor of BCP precipitation, although less active compared with fetuin-A under the specific conditions of our precipitation assay.

DISCUSSION

Fetuin-B is a recently discovered member of the fetuin protein family within the cystatin superfamily of proteins [2]. The first fetuin family member described was bovine fetuin, now known as fetuin-A [3]. Later it was established that the human serum protein α_2-HS glycoprotein [33] is the human species homologue of bovine fetuin-A. Additional homologous proteins of α_2-HS glycoprotein/fetuin-A (genetic symbol AHSG/FETUA in humans and Ahsg in mice) were identified in mammals and marsupials [29]. These proteins are now termed fetuin-A to distinguish them

from fetuin-B (genetic symbol FETUB in humans and Fetub in mice). We present experimental data to establish that fetuin-B is expressed both at the mRNA level and at the protein level in human, rat and mouse. Fetuins are highly expressed foetal serum α-globulins and are therefore often confused with α-fetoprotein (genetic symbol AFP/FETA in humans and Afp in mice). However, α-fetoproteins belong to the albumin protein family and are structurally and genetically not related to fetuins.

The physiological function of fetuin-A has been determined by genetic experimentation using targeted deletion of the Ahsg gene in mice [32]. Fetuin-A deficiency was associated with a mild phenotype affecting the transforming growth factor-β signalling [11], insulin-dependent glucose metabolism [12] and sporadic soft-tissue calcification [32]. Combining the fetuin-A deficiency with the calcification-sensitive mouse strain DBA/2 dramatically exacerbated this mild calcification phenotype into a severe systemic soft-tissue calcification phenotype affecting virtually all animals of this genotype [17]. We concluded that the major physiological function of fetuin-A is the inhibition of unwanted calcification. This was confirmed independently by a series of biochemical studies [16,34–36] and we could show that fetuin-A deficiency is associated with increased mortality in patients with uremia [37]. To test whether fetuin-B was likewise a potent inhibitor of BCP precipitation, we performed precipitation-inhibition assays and determined that fetuin-B is indeed an inhibitor of BCP precipitation. Comparing the respective serum concentrations in adult mice of fetuin-A (approx. 58 kDa and 14 μM [32]) and fetuin-B (approx. 60 kDa and 3 μM) and their IC_{50} for the inhibition of BCP precipitation (approx. 2 μM versus approx. 6 μM), one can calculate that fetuin-A will contribute to the overall inhibition of BCP precipitation in serum roughly 14 times more than fetuin-B. In humans, the contribution of fetuin-B to the overall inhibition of BCP precipitation is even lower, because the serum concentration is only approximately 1/30th of the serum concentration in mice. The relative lack of fetuin-B may be one of the reasons why humans are less well protected against ectopic calcification than mice [17]. In summary, our results suggest that fetuin-B can potentially inhibit BCP precipitation *in vitro* and *in vivo*, but is unlikely to contribute as a major systemic inhibitor in circulation. Yet, fetuin-B may attain much higher concentration, and hence activity, when locally sequestered. Such a situation exists in the most potent inhibitor of aortic calcification, matrix GLA protein [38], which is a major tissue-bound inhibitor, but contributes little to systemic inhibition [36]. We conclude that fetuin-B may have partly overlapping but not identical functions of fetuin-A.

We studied the mRNA and protein expression of fetuin-B and fetuin-A to identify common features and possible differences in their expression profiles. At the mRNA level in human and mouse tissues, both genes were most highly expressed in liver, as would be expected for major hepatic serum proteins (Figure 1). The high expression in embryo is probably due to hepatic expression, as we have shown previously for fetuin-A in the mouse [15] and rat [30] embryos. However, several extra hepatic expression sites were also detected. The expression of mRNA for both fetuin-B and fetuin-A in placenta has not been reported before and may indicate a role for these proteins in placental transport. Similarly, the expression of mRNA for both fetuin-B and fetuin-A in kidney and tongue, affected tissues with secreting epithelia, which may express high amounts of fetuins to aid solute transport across tissue boundaries. In addition to the tissues already mentioned, which expressed both fetuin-B and fetuin-A mRNA, we detected fetuin-B mRNA alone in mouse lung and ovary (Figure 1B), again two tissues, which are highly secretory. In summary, our results indicate that the mRNA expression patterns of fetuin-B and fetuin-A are overlapping, but not identical, which suggests that both genes share regulatory elements. This conclusion is strengthened by the unexpected finding that the deletion of the gene for fetuin-A (Ahsg) in mice caused a marked down-regulation of fetuin-B expression both at the level of mRNA and of serum protein (Figure 6). Regarding complex genetic loci (gene clusters), several mechanisms of independent regulation are known: (i) promoter competition as a result of the preferential interaction between an enhancer and a promoter reducing the interaction with neighbouring promoters [39] and (ii) DNA methylation-dependent chromatin boundaries or insulators, i.e. DNA elements, which can block transcriptional influences such as enhancer–promoter interactions and chromatin-mediated effects on gene expression [40]. It is described in serial deletions that within gene clusters, the 'function' of a given gene cannot be considered on its own, but instead must be integrated into a larger functional context, that of the cluster itself. Similar to the deregulation of fetuin-B caused by the deletion of fetuin-A described in the present study, deleting the Hoxd13 locus in mice resulted in a deregulation of the 3′-neighbouring HoxD12 gene [41]. An alternative explanation for the down-regulation of fetuin-B in fetuin-A-deficient mice may be that a strong phosphoglycerate kinase promoter driving the constitutive expression of a neomycin resistance gene was artificially replacing the Ahsg gene to generate fetuin-A-deficient mice [32]. This genomic rearrangement could also negatively influence the fetuin-B gene transcription in *trans*. The phenomenon requires a detailed analysis of fetuin-A and fetuin-B promoter structure and regulation.

Interestingly, unlike fetuin-A, fetuin-B was expressed at a higher level in females compared with males (Figures 2B and 2C) in humans. This hints to a steroid dependence of the maximum expression, as for leptin [42]. Leptin is an adipocyte-derived hormone secreted into the bloodstream. In humans, serum leptin levels are 2- to 3-fold higher in females than in males [43]. Sex steroids are regulators of leptin expression, but the exact mechanism or interaction is not yet known. A suppressive effect of androgens in men has been suggested, but not formally proven [44], whereas oestrogen and/or progesterone may have a stimulatory effect. Whether or not sex steroids regulate the fetuin-B promoter activity remains yet to be determined. We have isolated genomic DNA clones containing both fetuin-A and fetuin-B genes in one contiguous sequence, allowing the study of interdependence of their genomic regulation by reporter gene constructs.

Recently, draft versions of the human [45,46] and mouse genome [31] became available to the general public. In full agreement with the published organization of the genomic loci in the human and mouse genomes, we determined that the fetuin-A gene (Ahsg) and the fetuin-B gene (Fetub) are localized side by side on chromosome region 16 B1 in the mouse genome. The corresponding region in the human genome is chromosome 3q27. In the mouse genome, the 3′-end of exon 7 of the fetuin-A gene and the 5′-end of exon 1 of the fetuin-B gene are separated by 18.8 kb. It is probable that this region harbours the basic promoter of the fetuin-B gene. A computer search using the sequence analysis software 'MatInspector V 2.2' for putative promoter elements in this stretch of DNA returned a calculated transcription start site with the highest theoretical likelihood ($P = 0.95$) at position -41 relative to the 5′-end of the published cDNA (accession no. NM_021564; **TAAGCGTTCGTTTTCTGAGCTTTGGGAACGTGTTCAAAGCA**CACCAGACTT, boldface letters represent nucleotides not included in the published cDNA). A putative TATA-signal (TCTGTAAAGGCGCCA) was detected at positions -73 to -59 relative to the first nucleotide of cDNA sequence, corresponding to positions -32 to -18 relative to the calculated

transcription start point, which is in good agreement with the preferred position (−35) of a TATA-box relative to the transcription start point.

In the mouse chromosome region 16 B1, the genes for fetuin-A and fetuin-B are located side by side and, in addition, two further members of the cystatin gene family, HRG and Kng (kininogen) are also located. This organization probably reflects the evolution of the cystatin family and could be important in the co-ordinated control of cystatin family member gene expression. The four genes located in the cystatin family cluster belong to three subfamilies, fetuins A and B, HRG, and kininogen. Correcting earlier reports [6,8], recent gene maps show that the orthologous locus in humans is 3q27 (http://www.ensembl.org/), suggesting a strong overall conservation of this locus between species. The observation that the order and the number of cystatin family genes are conserved in mice and humans suggests that this cluster is probably formed before the divergence of the human and the mouse genome. It is probable that the members of the cystatin family, and in particular fetuin-A and fetuin-B, originated by gene duplication. A recent paper reported the duplication of one of the two genes for human α-1 acid glycoprotein by genomic rearrangement [47], and a similar mechanism may have caused the fetuin gene duplication. The fact that the sequence identity of the fetuin-B coding sequences in mice and humans (56.3%) is higher than the sequence identity of fetuin-B and fetuin-A in mice (47.4%) and humans (49.0%) suggests that the duplication of the fetuin gene occurred before speciation.

We have established that fetuin-B is expressed at the protein level and that it circulates in the serum as an acidic glycoprotein carrying asparagine-linked carbohydrate side chains. A computer search for putative post-translational modifications of the fetuin-B protein sequence using the analysis software 'PROSITE' revealed two putative asparagine-glycosylation sites at positions 40 and 139 of the mouse and rat, and three putative asparagine-glycosylation sites at positions 37, 136 and 182 in the human fetuin-B pro-protein sequence. Deglycosylation of mouse fetuin-B using PNGase F confirmed that at least one of the two possible N-glycosylation sites was occupied. Further heterogeneity may be due to phosphorylation, as shown for fetuin-A [23,48]. In conclusion, we confirm fetuin-B expression at the protein level as a hepatic serum glycoprotein like fetuin-A. Genetic studies using gene knockout mice will be able to determine whether the functions of fetuin-B and fetuin-A are completely different or are similarly overlapping as the mRNA expression pattern.

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