Fetuin-B, a second member of the fetuin family in mammals

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A set of orthologous plasma proteins found in human, sheep, pig, cow and rodents, now collectively designated fetuin-A, constitutes the fetuin family. Fetuin-A has been identified as a major protein during fetal life and is also involved in important functions such as inhibition of the insulin receptor tyrosine kinase activity, protease inhibitory activities and development-associated regulation of calcium metabolism and osteogenesis. Furthermore, fetuin-A is a key partner in the recovery phase of an acute inflammatory response. We now describe a second protein of the fetuin family, called fetuin-B, which is found at least in human and rodents. On grounds of domain homology, overall conservation of cysteine residues and chromosomal assignments of the corresponding genes in these species, fetuin-B is unambiguously a paralogue of fetuin-A. Yet, fetuin-A and fetuin-B exhibit significant differences at the amino acid sequence level, notably including variations with respect to the archetypal fetuin-specific signature. Differences and similarities in terms of gene regulation were also observed. Indeed, studies performed during development in rat and mouse showed for the first time high expression of a member of the fetuin family in adulthood, as shown with the fetuin-B mRNA in rat. However, like its fetuin-A counterpart, the fetuin-B mRNA level is down-regulated during the acute phase of experimentally induced inflammation in rat.

Key words: acute inflammation, cystatin superfamily, development, liver, α2-HS-glycoprotein.

INTRODUCTION

The cystatins are low-molecular-mass cysteine protease inhibitors that typically comprise a domain of approx. 115 amino acids with four conserved cysteine residues known to form two disulphide bonds and further conserved residues required for the protease inhibitory capacity of these proteins [1,2]. Based on similarities of other proteins with this typical cystatin domain, a cystatin superfamily made of several families has been acknowledged [1]. Specifically, the cystatin superfamily mostly comprises the cystatins themselves, the intracellular stefins, the kininogens and the fetuin family, which has lost a cysteine protease inhibitory activity [1,2].

As yet, the fetuin family encompasses a series of tightly related proteins that are synthesized mostly in the liver and have been variably designated fetuin in sheep, pig and cow, α2-HS-glycoprotein (AHSG) in human, phosphoprotein of 63 kDa (pp63) in rat, or countertrypin in mouse and gerbil [2–7]. In fact, their close sequence similarities indicate that fetuin, AHSG, pp63 and countertrypin all originate from a single ancestral gene and are counterparts of a single protein, i.e. are orthologues in different species. Therefore, the name fetuin has been suggested as the universal nomenclature for this protein in every species [3]. As this name suggests, fetuins have been identified as major proteins during fetal life, primarily in blood and brain, although the development-associated changes in fetuin levels are known to significantly vary between species [4]. Indeed, in human, sheep, pig and cow the absolute level of plasma AHSG/fetuin is 5- (human) to 50-fold (cattle) higher in fetus than in adult and decreases soon after birth [3,4]. In contrast, the plasma fetuin level as well as the hepatic transcription of the corresponding gene are low in both fetal and adult rodents, whereas they are highest shortly after birth in a transient fashion [8–10]. Many functions have been ascribed to fetuins, which are now considered key proteins in several metabolic pathways, although a share of these functions within the fetuin family has been strongly debated. Consistent with the importance of fetuins in development, AHSG is involved in osteogenesis and bone resorption [11,12]. These functions have been ascribed to the capacities of AHSG to control calcium-salt precipitation in blood [13] and to accumulate in bone matrix [14] as well as to counteract a transforming growth factor-β activity required for bone mineralization [12,15]. AHSG modulates some insulin-driven and kinase-mediated signal-transduction pathways, possibly in a tissue-specific fashion [16]. AHSG/pp63 inhibits the insulin receptor autophosphorylation mediated by its intrinsic tyrosine kinase activity [5] and the phosphorylation status of AHSG/pp63 is in turn of critical importance for its tyrosine kinase inhibitory activity [5,17,18]. Furthermore, the phosphorylated form of pp63 inhibits the binding of hepatocyte growth factor to its hepatic receptor [19]. A serine protease inhibitory capacity of human AHSG, bovine fetuin and mouse countertrypin has also been reported [20,21].

Abbreviations used: AHSG, α2-HS-glycoprotein; APP, acute-phase protein; APR, acute-phase response; EST, expressed sequence tag; FCA, Freund's complete adjuvant; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSA, *Homo sapiens* chromosome; LPS, bacterial lipopolysaccharides; MMU, *Mus musculus* chromosome; ORF, open reading frame; pp63, phosphoprotein of 63 kDa; RNO, *Rattus norvegicus* chromosome; SCH, somatic cell hybrid; TO, turpentine oil.

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Finally, fetuins opsonize cationic macrophage-deactivating molecules and appear to be a key partner in the recovery step of an acute-phase response (APR) to systemic inflammation [22]. In this respect, it is to be stressed that during such an APR a transient change in levels of blood fetuin and its hepatic mRNA has been reported. Unexpectedly, this change varies in differing directions in different species. Indeed, human AHSG, rat pp63 and guinea pig fetuin belong to the group of so-called negative acute-phase proteins (APPs) that are down-regulated during APR, whereas mouse and cattle fetuins are up-regulated, i.e. are positive APPs [8,23–25].

Given the numerous suggested functions of fetuins, it is important to have a complete view of this protein family. We now describe a second fetuin or fetuin-B in rat, mouse and human. On the grounds of sequence similarity, changes in levels of hepatic mRNA during development or APR, and chromosomal assignment of its gene, this novel protein appears to be homologous to, yet clearly different from, the fetuin/AHSG/pp63/countertrypin described so far. Therefore fetuin-B represents a novel entity in the fetuin family that now comprises at least two paralogues (i.e. two homologous proteins encoded by duplicated genes) within every species studied here. These two paralogues are now designated fetuin-A (formerly fetuin/AHSG/pp63/countertrypin) and fetuin-B, respectively.

MATERIALS AND METHODS

Animals and treatments

All animal manipulations were performed under the supervision of authorized investigators and according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' (National Institutes of Health, Bethesda, MD, U.S.A.). Adult Sprague–Dawley rats weighing 250–300 g were purchased from Charles River (St-Aubin-les-Elbeuf, France). They were kept at rest for at least 1 week prior to any experiment and were given tap water and chow *ad libitum*. Rat pups and young animals were obtained by breeding in our laboratory. Likewise, adult and young (C57BL/6JxSJL/J)F2 mice were obtained as already described in [9]. Day 0 post-coitus and day 0 at birth were determined as described in [9]. Fetuses and young animals were killed by decapitation. Older animals were killed by cervical dislocation (mouse) or by anaesthesia with ethyl ether (rat). An acute systemic inflammation was induced in adult male rats by a single injection of bacterial lipopolysaccharides (LPS) or Freund's complete adjuvant (FCA) given intraperitoneally or turpentine oil (TO) given subcutaneously, as detailed previously [26,27].

Reagents

Restriction enzymes and DNA-modification enzymes were obtained from Boehringer Mannheim or New England Biolabs. *Taq* polymerase was from Promega-France. [α^{32}P]dCTP (3000 Ci/mmol) was from Amersham. All other chemicals were of analytical grade.

cDNA probes

A rat cDNA for fetuin-A/pp63 and a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were gifts from Professor P. Arnaud (Medical University of South Carolina, Charleston, SC, U.S.A.) and Professor J. M. Blanchard (Centre National de la Recherche Scientifique, Montpellier, France), respectively. A full-length rat cDNA for the so-called IαIH4P precursor of the Inter-α-Inhibitor family of plasma proteins has been described previously [28] and was used in the present study as a probe for the mRNA of a positive APP. Mouse cDNA probes for 18 or 28 S RNAs were kindly provided by Dr Y. Rayssiguier (Institut National de la Recherche Agronomique, Clermont-Ferrand, France) or Dr J. Mallet (Centre National de la Recherche Scientifique, Paris, France), respectively.

Preparation and hybridization of RNA or genomic DNA

Organs were quickly removed from killed animals and immediately frozen in liquid nitrogen prior to extraction of total RNAs. The latter was done according to previously detailed procedures [9,26]. Northern blots were carried out and hybridized with ^{32}P-labelled probes and the resulting signals were quantified and normalized as previously detailed [26,27]. Genomic DNA from rat or mouse was digested with various restriction enzymes and the resulting fragments were resolved by agarose gel electrophoresis, alkali-blotted and hybridized with cDNA probes under stringent conditions following standard procedures. *Bst*EII-restricted λ DNA was used as a size ladder.

DNA sequencing

Sequencing reactions were performed with cDNAs subcloned into a pBluescript phagemid, a ThermoSequenase fluorescent-label primer cycle-sequencing kit (Amersham) and IRD-800-labelled primers (ScienceTec, Les Ulis, France). The reactions were resolved on to an automated LI-COR 4200 sequencer (ScienceTec) that allowed for a reading of 800–1200 nt per run. The resulting DNA sequences obtained on both strands were assembled with the DNASIS software (Pharmacia).

Electronic assembly of expressed sequence tags (ESTs)

Selection of as-yet unrelated nucleotide sequences obtained by single-pass sequencing and stored in a human- or mouse-specific database (dbEST) at the National Center for Biotechnology Information (NCBI) was done with the BLASTN program and the full-length rat fetuin-B cDNA (see the Results section) as a query. The selected ESTs that best matched this query were in turn used as a query for matching further ESTs in the database until no further ESTs were found with a further query. Elsewhere, the largest open reading frame (ORF) of the full-length rat fetuin-B cDNA was used as a query and searched with the TBLASTN program against the six conceptual translations of all ESTs in either of the above-mentioned species-specific databases. The results obtained with both procedures were then compared and a limited set of ESTs was eventually retained from the first panel of selected ESTs with the following general criteria: (i) extent and quality of matches between ESTs, (ii) length of ORF within a given EST and (iii) EST submission date, as the most recent ESTs usually present the highest sequence quality. The ESTs eventually selected were finally gathered by contig assembly with the DNASIS software and the resulting curated cDNA was considered the human or mouse counterpart of the rat fetuin-B cDNA used as the first query.

Protein-sequence comparisons

Matches of ORFs from our full-length cDNAs with the SwissProt and Trembl protein databases were searched with the BLASTP algorithm. The PROSITE [29] and BLOCKS [30] databases were searched with the MOTIFS (Wisconsin package, version 8) and BLIMPS [31] programs for the occurrence of known patterns in our ORFs used as a query. Multiple alignments of DNA or

protein sequences were done with the CLUSTALW (version 1.7) software. The percentage of amino acid residues conserved between two or more sequences was the number of conserved residues over the total number of residues in the largest sequence.

Rat gene assignment with a panel of rat × mouse somatic cell hybrids (SCHs)

A panel of 18 rat × mouse SCHs [32] was kindly made available to us by Professor T. Serikawa (Institute of Laboratory Animals, Kyoto University, Kyoto, Japan). From the rat and mouse fetuin-B cDNAs (see the Results section), an oligonucleotide pair that would favour an amplification of the corresponding rat cDNA was designed with the following conditions: amplified area to be located in the 3′-non-coding sequence of the cDNAs; a 100 % match between the oligonucleotides and the rat cDNA; a high number of mismatches in each oligonucleotides versus the mouse fetuin-B cDNA; amplicon size in the 200-bp range; and T_m in the 56–60 °C range. Computer-aided selection of these features was done according to the program OLIGO [33] and resulted in selection of an oligonucleotide pair (5′-CCCTGAGCAGGAGGAGCC-3′ and 5′-CATCAGGGGGTTCTTTGCTTT-3′) that allowed for a 219-bp amplicon (nt 1030–1248 in the rat fetuin-B cDNA sequence) to be obtained from the rat *fetuin-b* gene. A PCR was done in 25 μl with 100 ng of genomic DNA from a given SCH, 50 pmol of each oligo, 2.5 μl of buffer (× 10 stock buffer, Promega), 1.5 mM $MgSO_4$, 200 μM of each dNTP and 1.25 units of *Taq* polymerase (Promega). After a first denaturation step at 94 °C for 4 min, the PCR was carried out for 30 cycles (94 °C for 30 s, 60 °C for 60 s and 72 °C for 60 s) followed by a final step at 72 °C for 5 min. The resulting amplicons were resolved on a 8 % polyacrylamide gel followed by ethidium bromide staining.

RESULTS

A fetuin-B cDNA from rat liver

In a recent study that aimed at a systematic cloning of hepatic mRNAs corresponding to inflammation-driven genes in rat, we isolated a large set of clones coding for as-yet undescribed APPs, including a clone designated IRL685A [27]. Preliminary sequence data indicated that this clone could represent a novel fetuin, which was the reason for the present study. The IRL685A cDNA cloned in a pBluescript phagemid was completely sequenced by various procedures of cDNA fragmentation and subcloning or primer walking (strategies not detailed). The final cDNA sequence (EMBL accession number AJ242926) is 1388 nt in length [poly(A)$^+$ tail excluded], which fits with the size (1.4 kb) of the corresponding mRNA in rat liver as determined in our previous study [27]. This cDNA contains a 5′-non-coding sequence, a major ORF (nt 110–1243), a TGA stop codon, a 3′-non-coding sequence, an AATAAA polyadenylation signal (nt 1355–1360) and a poly(A)$^+$ tail. The major ORF codes for a polypeptide that is 378 residues in length. Next, this polypeptide was searched for known domains or family signatures with the BLOCKS and PROSITE databases (see the Materials and methods section). First, the best hit with the BLOCKS database (version 10.1, as of April 1998) was a significant similarity with pig fetuin. Second, two fetuin signatures, as stored in the PROSITE database, were found in our polypeptide. These signatures included (i) a set of 12 cysteine residues located at fixed distances that are perfectly conserved in our polypeptide and (ii) a LETXCHXLDPTP motif that is present in part (LETGCHVL) in our polypeptide. Therefore, this polypeptide encoded by our clone IRL685A was considered a novel fetuin and accordingly called rat fetuin-B, as shown in Figure 1.

Cloning '*in silico*' of human and mouse fetuin-B cDNAs

Given the current richness of human and mouse dbEST databases, we searched for counterparts of rat fetuin-B in these resources. A strategy of EST match and assembly was done with the rat fetuin-B cDNA as a query and the human or mouse dbEST (as of April 1999), as detailed in the Materials and methods section. These searches and the resulting EST contigs eventually provided two human or mouse cDNAs (GenBank accession numbers AJ242928 and AJ242927), the sizes of which [human, 1623 nt; mouse, 1481 nt; poly(A)$^+$ tail excluded] were similar to that of rat fetuin-B cDNA. Within each novel human or mouse cDNA, a major ORF codes for a polypeptide that is 382 (human) or 388 (mouse) residues in length. The corresponding amino acid sequences are aligned with that of rat fetuin-B in Figure 1 (last three lines). The close similarities between all three sequences (61 % strictly conserved residues between them) as well as the conservation of the twelve critical Cs and of the truncated LETXCHVL fetuin motif (see above) in these human and mouse sequences led us to consider them as fetuin family members and orthologues of rat fetuin-B. Accordingly, they are now named human or mouse fetuin-B.

Similarities and differences between fetuin-A and fetuin-B

The rat, mouse and human fetuin-B sequences were aligned with a series of mammalian fetuin-As as shown in Figure 1. The overall amino acid sequence similarity between rat, mouse and human fetuin-As and -Bs is significant (17 % strictly conserved and 24 % similar residues between all six sequences) but much lower than the similarity within fetuin-As (58 % strictly conserved residues between rat, mouse and human and 37 % between all species shown in Figure 1) or within fetuin-Bs (61 %, as already mentioned). These figures again point to a paralogous relationship between fetuin-A and fetuin-B. As recalled in Figure 1, three major domains have been previously identified within fetuin-As and include two homologous, tandemly arranged cystatin-like domains D1 and D2 followed by a unique C-terminal domain D3 [3,34]. D3 is loosely conserved between species and can itself be divided into a hydrophobic, proline-rich, N-terminal half (D3a) and a C-terminal half (D3b) that includes a so-called connecting peptide involved in protein processing, at least in humans [3,34]. This overall arrangement of domains and motifs is obviously retained in fetuin-B, as indicated by the overall sequence similarity between fetuin-As and fetuin-Bs which, for instance, includes the twelve Cs already mentioned as well as a proline-rich D3a domain (see legend to Figure 1). Also, two dibasic motifs, db1 and db2, that are respectively found in D1 and D2 of most fetuin-A sequences and account for a trypsin susceptibility of human and bovine fetuin-As [3,34,35] are loosely retained in fetuin-B (Figure 1). Indeed, db1 is present in human fetuin-B and db2 is replaced by a neighbouring basic tripeptide K(K/R)K in all fetuin-B sequences. However, some important differences between fetuin-A and fetuin-B can also be noticed in Figure 1. Notably, a motif within D1 that has been proposed as a calcium-binding site for fetuin-A [34] is absent in fetuin-B. Also, the connecting peptide that is well conserved in the centre of D3 in fetuin-A and is released at least in human fetuin-A [3,34] is absent in fetuin-B. Finally, the proven or potential enzyme inhibitory sites found in fetuin-A and fetuin-B exhibit sequence differences, as discussed below.

Tissue-specific expression of fetuin-B mRNA

The tissue-specific expression of the fetuin-B mRNA investigated in adult male rats by Northern-blot experiments has previously indicated that the *fetuin-B* gene is transcribed (mRNA size in rat, 1.4 kb) in liver but not in total brain, heart, intestine, kidney, lung and muscle [27]. A further analysis made by searching matches of our rat, mouse or human cDNA sequences with, respectively, the rat, mouse or human dbEST databases (an 'electronic Northern') confirmed the liver-specific expression of

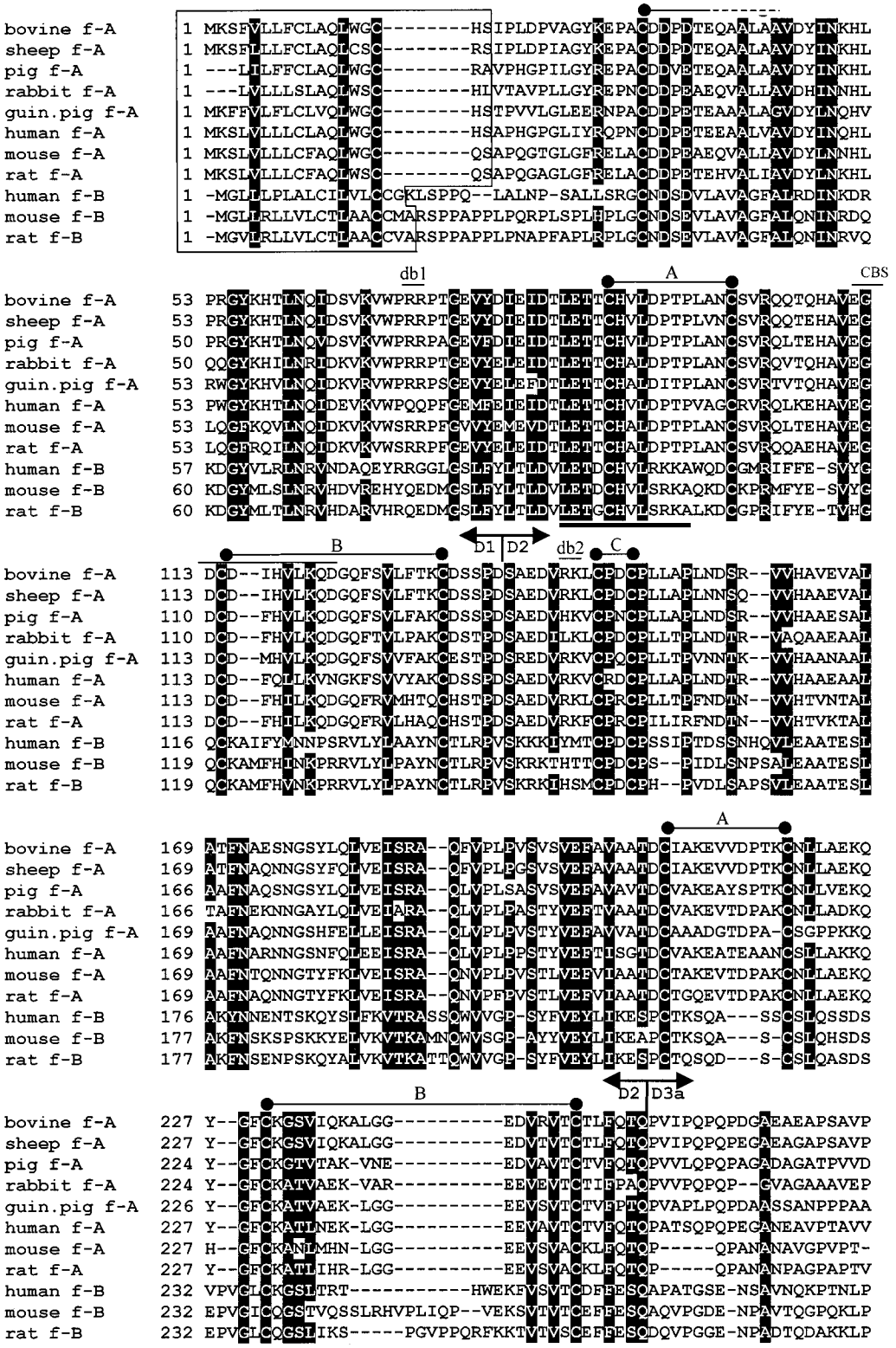

Figure 1 For legend see opposite.

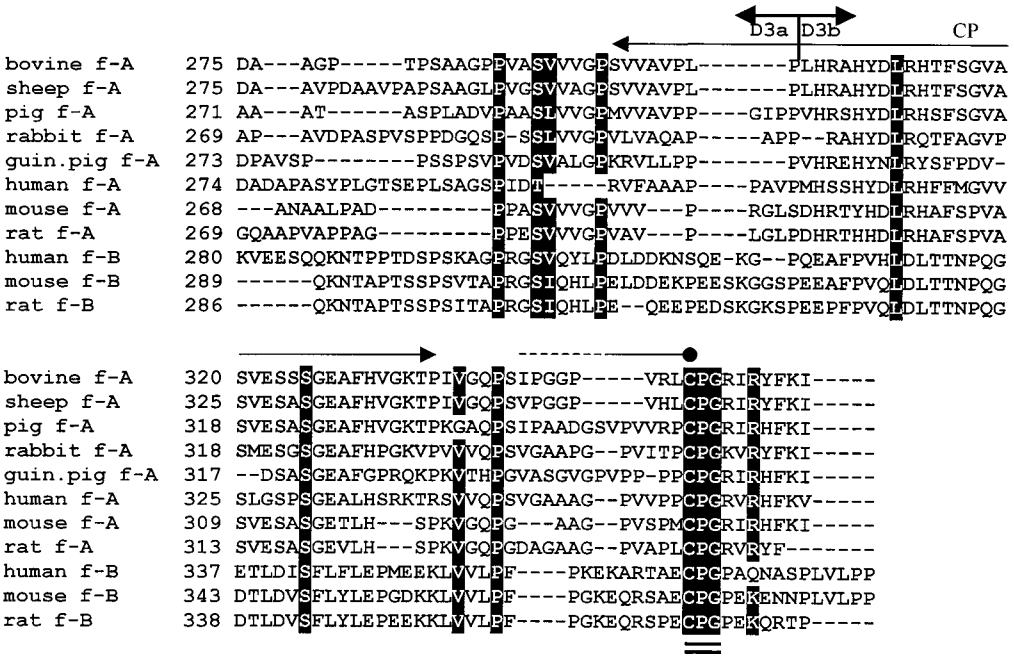

Figure 1 Multiple alignment of rat, mouse and human fetuin-B (f-B) with fetuin-A (f-A) from various mammals

Species are noted on the left. Identical or similar residues conserved in at least 10 out of 11 sequences are printed as white on a black background. A minimum number of gaps, indicated by hyphens, has been introduced to maximize alignments. The signal peptides for fetuin-A, as proposed in [4,7], or fetuin-B, as inferred from [45], are boxed. The LETXCHXLDPTP fetuin signature [7,34] found in the PROSITE database [29] is underlined. Likewise, the 12 critical Cs used as a fetuin signature [2,34] are marked with ●. A CPG tripeptide conserved in the fetuin, kininogen and histidin-rich glycoprotein families that all belong to the cystatin superfamily [4] is double underlined. The type-A, -B or -C disulphide bonds that are proven (human) or proposed (all other species) in fetuin-A domains [2,3,35] are indicated by a horizontal connecting line between Cs whereas the bond connecting the first and ultimate Cs [2,3,35] is noted with a dashed line. Two cystatin-like domains D1, D2 and a third domain D3 sub-divided into two D3a and D3b domains previously identified in fetuin-A [3,34] are marked with broken arrows. A so-called connecting peptide and a potential calcium-binding site found in fetuin-As [3,34,35] are overlined and marked CP and CBS, respectively. Two dibasic peptides db1 and db2 that are prone to enzymic cleavage in at least some fetuin-As [3,34,35] are overlined. Note that D3a contains 16–24% P residues in fetuin-As (rat, mouse and human) or 15–17% in fetuin-Bs.

the *fetuin-B* gene in adult rat and further identified an expression of this gene in adult mouse liver, lung and tongue and in adult human liver and testis.

Development-associated changes in hepatic level of fetuin-B mRNA

Electronic Northern in dbEST databases detected *fetuin-B* gene expression in mouse liver at 8 days post-coitus and in human liver at 20 weeks post-coitus, which indicates some level of gene expression at mid-gestation in both rodents and primates. This was more precisely investigated by Northern blots carried out with RNAs from rat and mouse liver and both fetuin-A and fetuin-B cDNA probes. The results are presented in Figure 2 and show significant differences in the developmental regulation of *fetuin-A* and *-B* genes in liver within and between rodent species. First, a very different pattern of expression for the *fetuin-A* versus *-B* genes was seen in rat. Indeed, the *fetuin-A* gene displayed a transient up-regulated expression during the post-natal period and next returned to a low level, which is in agreement with a former study [10], whereas the *fetuin-B* gene exhibited a developmentally regulated expression that plateaued at adulthood. Secondly, the developmental pattern for the *fetuin-A* gene was similar in mouse as in rat whereas the *fetuin-B* gene pattern was clearly different between species. Indeed, in mouse the fetuin-A mRNA level exhibited a perinatal and transient increase during the early post-natal life, which is in agreement with former studies [8,9], whereas the changes in levels of fetuin-B mRNA (size in mouse, 1.5 kb) were similar to those of fetuin-A mRNA. Hence, the transient increase in fetuin-B mRNA level in the developing mouse clearly departs from the high fetuin-B mRNA level retained in adulthood in rat.

Inflammation-associated changes in hepatic level of fetuin-B mRNA

The hepatic level of fetuin-A mRNA is down-regulated during an APR in rat [23,26] and our preliminary data indicated that the hepatic level of fetuin-B mRNA also varies following an inflammatory challenge in rat [27]. Therefore, we compared in more detail the changes of both mRNA levels of both genes in liver during an APR in rat. A time-course study of these hepatic mRNA levels following a TO challenge is shown in Figure 3. The fetuin-A mRNA level was transiently down-regulated as expected from former reports [23,36] while the mRNA level for a positive APP used as a control, namely the IαIH4P protein of the Inter-α-Inhibitor family, was transiently up-regulated as described previously [37]. The level of fetuin-B mRNA was down-regulated as early as 9 h post-TO, reached a 2-fold decrease at days 1–2 and returned to a normal value beyond 1 week, which on the whole is similar to the variations seen for the fetuin-A mRNA level. We further investigated in rat whether this down-regulated level of fetuin-B mRNA in liver depends on the nature of the inflammatory stimulus. As shown in Table 1, three different stimuli, namely TO, LPS or FCA, were all able to promote a down-regulation of both fetuin-A and fetuin-B mRNA levels in rat liver, albeit to a variable extent. Indeed, at day 1 post-challenge

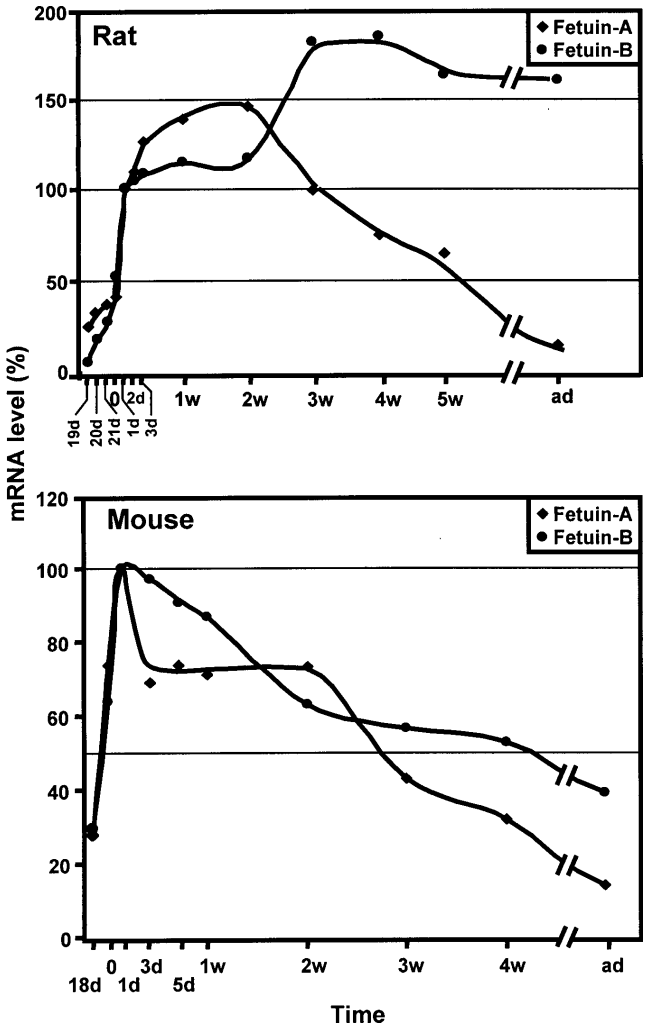

Figure 2 Development-associated changes in hepatic levels of fetuin-A and fetuin-B mRNAs

Abcissa: time scale (3 animals per time point). Zero corresponds to birth and the time after birth is given as days (d) or weeks (w). Days before birth are given as the number of days post-coitus (rat, 19–21 days; mouse, 18 days). Ordinate: relative mRNA levels expressed as a percentage of the level measured at day 1 after birth (100%), after normalization with the 18 S (rat) or 28 S (mouse) RNA level. Note that the absolute levels of fetuin-A versus fetuin-B mRNA cannot be compared within or between species in this figure. ad, adult.

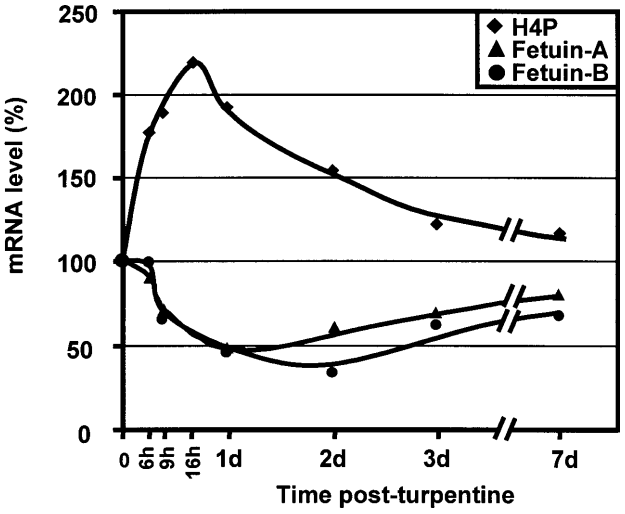

Figure 3 Inflammation-associated changes in hepatic levels of fetuin-A and fetuin-B mRNAs in rat

Inflammatory stimulus: turpentine. Abcissa: time scale (d, day) post-turpentine challenge (5 animals per time point). Ordinate: relative mRNA levels expressed as a percentage of the level measured in healthy animals (100%) after normalization with the 18 S RNA level. The results obtained with an hepatic mRNA whose level is up-regulated by a turpentine challenge, namely the IαIH4P mRNA [28,37], are shown as a control for a positive APP mRNA. Note that the absolute levels of fetuin-A versus fetuin-B mRNA cannot be compared in this figure.

Table 1 Hepatic levels of fetuin-A and fetuin-B mRNAs as a function of the inflammatory stimulus

In all groups, total hepatic RNAs from five animals were pooled, separated by electrophoresis, blotted and successively hybridized with four rat cDNA probes, namely those for (i) fetuin-A, (ii) fetuin-B, (iii) IαIH4P used herein as a control for a positive APP mRNA [28,37] and (iv) GAPDH used as a control for an mRNA whose level does not vary in liver during APR [36,37]. A single blot was used throughout the study. mRNA levels were measured 24 h post-challenge. All values are given as percentages of the control group, set at 100%.

Probe	Control	TO	LPS	FCA
Fetuin-A	100	30.5	46.5	49
Fetuin-B	100	51.5	58.5	60.5
IαIH4P	100	368	141	212
GAPDH	100	101	140	84

their effects upon both fetuin-A and fetuin-B mRNA levels could be ranked as follows: TO > LPS = FCA.

The *fetuin-B* gene is a single-copy gene

The rat fetuin-B cDNA was used as a probe to identify the number and size of fragments generated by restriction enzyme digest of genomic DNA from rat or mouse. As seen in Table 2, a very limited number of fragments was detected in all instances, which indicates a single-copy *fetuin-B* gene in rodent genomes.

Chromosomal assignment of the *fetuin-B* gene in rat and human

The rat and mouse fetuin-B cDNA sequences identified above allowed us to predict that a 219-nt-long amplicon would be produced by PCR of rat genomic DNA with a pair of oligonucleotides located at the 3′ end of the corresponding cDNA, whereas no homologous amplicon would be obtained from the

Table 2 Restriction-enzyme-generated fragments detected in the rat and mouse genomes with a rat fetuin-B cDNA

	Fragment size (kb)	
Restriction enzyme	Rat	Mouse
*Bam*HI	> 10	6.8
*Eco*RI	8.0 + 7.0	3.5 + 3.1
*Xba*I	10 + 8.0 + 5.3	7.0 + 4.6 + 1.8

Table 3 Chromosomal assignment of the rat *fetuin-B* gene

The number of SCHs that showed segregation discordance between the presence/absence of given rat chromosomes and the presence/absence of a rat *fetuin-B* amplicon are listed, and the smallest number of discordances is underlined.

Rat chromosome number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	X	Y
Number of discordants	10	11	7	2	8	11	10	9	11	8	9	9	9	8	9	9	8	10	9	8	9	7

mouse genome with these oligos (see the Materials and methods). This allowed for chromosomal assignment of the rat *fetuin-B* gene from a panel of 18 rat × mouse SCHs. Indeed, the distributions of rat chromosomes in these 18 hybrids [32] versus the distribution of a rat *fetuin-B* gene amplicon in the same hybrids (Table 3) allowed us to assign the *fetuin-B* gene to *Rattus norvegicus* chromosome 4 (RNO4).

We have further searched a match of the human fetuin-B cDNA sequence with a very large set of human ESTs that have been previously assigned to *Homo sapiens* chromosomes (HSAs) [38]. We found that several ESTs (e.g. N76638) that perfectly match the human fetuin-B cDNA all map to the HSA3q27 area. Therefore the human *fetuin-B* gene is located at HSA3q27. This novel gene has been officially designated *FETUB* and *fetub* (in human and rodent, respectively) and its chromosomal assignments in human and rat genomes have been deposited in the appropriate data libraries. Likewise, the gene for fetuin/AHSG/pp63/countertrypin is now designated *FETUA*/*fetua*.

DISCUSSION

This is the first report on the structure and regulation of the fetuin-B protein and its gene. We have now brought evidence for the inclusion of fetuin-B in the fetuin family, itself a member of the cystatin superfamily. On a structural basis, fetuin-B is clearly homologous to fetuin-A, as shown by a shared arrangement in three domains, including two cystatin-like domains D1 and D2 and a domain D3 that is itself divided into the proline-rich D3a and C-terminal D3b subdomains. All these features suggest that the exon/intron arrangement described previously for the human, rat and mouse *FETUA* genes, namely three exons each for D1 and D2 and a single large exon for D3 [13,39,40], is likely to be conserved in the *FETUB* gene. The *FETUB* gene assignments also argue for this view. Indeed, we have mapped the human *FETUB* gene to HSA3q27, where the human *FETUA* gene has already been located [41] along with other cystatin superfamily members, namely stefins and kininogens [42]. We have further

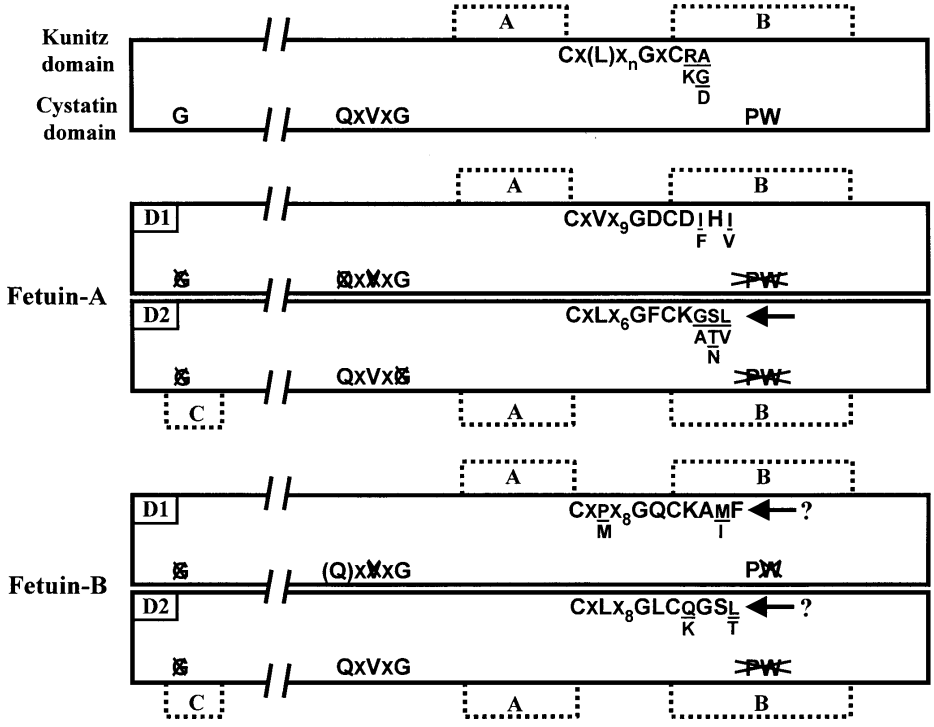

Figure 4 Proven or potential protease inhibitory sites in fetuin-As and fetuin-Bs

The archetypal Kunitz-type inhibitory domain [21,46,47] and cystatin domain [2] are shown as a box with critical disulphide bonds (of type A or B in the cystatin domain) symbolized by dotted lines outside. The residues that are critical for the inhibitory activity are written in upper (Kunitz motif, $n = 4$–6) or lower (cystatin motif) positions inside the box. The D1 and D2 domains in fetuin-A or fetuin-B are depicted as paired boxes with their type-A, -B or -C disulphide bonds shown outside. A proven serine protease inhibitory site found in human, bovine and mouse fetuin-As [20,21] is so indicated with an horizontal arrow whereas potential sites in fetuin-B are noted with a question mark. A residue written in parentheses indicates its occurrence in some but not all relevant sequences. Within fetuin-As or fetuin-Bs, a crossed-out residue indicates its absence in all D1 or D2 sequences. Detailed amino acid sequences are taken from Figure 1.

mapped the rat *fetub* gene to RNO4 whereas its *fetua* paralogue maps to RNO11 [39]. Syntenies between HSA3q and RNO4 and between HSA3q and RNO11 have been identified (http://ratmap.ims.u-tokyo.ac.jp/cgi-bin/comparative_home.pl), which is in agreement with all above assignments. Overall, we conclude that the *FETUA* and *FETUB* genes most probably resulted from a duplication and are still co-localized at a single chromosomal area in human whereas they have separated in the rat genome. This fits with the current view that the rodent chromosomes have rearranged and diverged from the ancestral mammalian genome to a larger extent than the human chromosomes [43].

Beyond their D1–D3 domain arrangements, two critical features are conserved between fetuin-A and fetuin-B. First, the number and location of all C residues are identical in both sequences. It remains to be clarified whether the pattern of resulting disulphide bonds in fetuin-B is similar to that found in fetuin-A [2,3,35]. Secondly, the presence and location of a short LETXCHXL motif is identical in fetuin-A and fetuin-B. This motif used as a query finds a match with all fetuin-As and fetuin-Bs as well as with a few other, apparently unrelated, proteins in databanks, whereas a N-terminally extended DXLETXCHXL motif only matches with fetuin-As and fetuin-Bs (results not shown). The short LETXCHXL motif is part of the larger LETXCHXLDPTP, itself considered a fetuin signature [29], but the DPTP tetrapeptide in this signature is absent in fetuin-Bs (see above). Therefore, we now propose that the DXLETXCHXL motif represents the genuine signature of the fetuin family whereas the LETXCHXLDPTP motif should be considered as a more restricted, fetuin-A-specific signature.

Given the fetuin family/cystatin superfamily relationship mentioned above as well as the trypsin inhibitory activity displayed by a Kunitz-type domain within D2 in human, mouse and bovine fetuin-As [20,21], we have sought for similarities or differences in the amino acid residues that are located at potential enzyme inhibitory sites in fetuin-As versus fetuin-Bs, as summarized in Figure 4. The typical cystatin inhibitory domain mostly requires a critical G residue, a QXVXG motif and a PW dipeptide to be active [2,44]. These residues have been partly lost in fetuin-A, which accounts for its lack of cystatin activity [4]. Likewise, fetuin-B did not fully retain these residues and hence a cystatin activity for this protein is most unlikely. Elsewhere, the PROSITE pattern associated with Kunitz-type inhibitors [29] is found in none of the fetuin-A or fetuin-B sequences, although fetuin-A D2 harbours an active Kunitz-type inhibitory site [21]. Therefore, we relied on another Kunitz inhibitor-associated motif that is located around the active site (Figure 4, top). This pattern is present in the active Kunitz domain found in fetuin-A D2 whereas it is clearly abrogated in fetuin-A D1. In fetuin-B, both D1 and D2 sequences are in agreement with the archetypal Kunitz-type motif, apart from the spacing between critical cysteine residues (compare various values of *n* in Figure 4). Therefore, a biochemical approach is now required to clarify whether these potential inhibitory domains in fetuin-B proteins are indeed active.

The tissue-specific and developmental pattern of expression of the *fetub* gene in rodents is reminiscent of what has been described previously for the *fetua* gene. Notably, both *fetua* and *fetub* genes exhibit a major expression in liver. In this organ, the relative level of fetuin-A to fetuin-B mRNA is approx. 8:1 in adult rat (results not shown). Both genes exhibit the same sharp and transient up-regulation of their hepatic expression around birth, at least in mouse. In mouse embryo, transient and low-level transcription of the *fetua* gene further takes place in brain as well as in limb buds at early stages of cartilage/bone formation [11]. Analysing the expression of the *fetub* gene in various tissues during rodent development will indicate whether the extra-hepatic transcription of this second gene in the fetuin family is also critical at an early stage of life. Remarkably, the behaviour of the *fetub* gene transcription in rat liver departs from that of the *fetua* gene as only the former displays sustained expression in adulthood. Therefore, and given the relatively low expression of the *fetua* gene in adulthood compared with its expression during development in all species studied so far [3,4,8–10], we report for the first time that a fetuin-family gene can be expressed to a higher extent in adulthood compared with its expression in the young. This feature could point to some specific functions of the *fetub* gene product, at least in rat.

Similar regulation of *fetua* and *fetub* genes was also observed during an experimentally induced APR in rat. Indeed, both mRNAs were down-regulated 2-fold or more after a TO challenge, whereas they were both modulated to a lower extent by an LPS or FCA stimulus. Therefore, both *fetua* and *fetub* belong to the set of negative acute-phase genes, at least in rodents. It will remain to be seen whether in some other mammalian species the *fetub* gene is up-regulated during APR, as described previously for the *fetua* gene in cattle [24].

Overall, the fetuin family now encompasses at least two paralogues. It remains to be investigated whether such a fetuin redundancy provides mammals with protection against the occurrence of a detrimental gene mutation or deletion and/or the production of a defective protein, as is currently envisioned as a possible reason for conservation of redundant proteins within other gene families. However, fetuin-A and fetuin-B display not only similarities but also differences, at the levels of both protein structure and gene expression. These differences along with the conservation of both genes from rodent to human now suggest discrete rather than fully overlapping functions for the corresponding fetuin-A and fetuin-B proteins.

We are indebted to T. Serikawa (Kyoto, Japan) for providing us with a set of rat × mouse SCHs. E.S. and P.R. are the recipients of doctoral fellowships from Region Haute-Normandie and Section de l'Eure Ligue Nationale Française contre le Cancer, respectively. This work was supported in part by the University of Rouen.

REFERENCES

1 Rawling, N. D. and Barrett, A. J. (1990) Evolution of proteins of the cystatin superfamily. J. Mol. Evol. **30**, 60–71
2 Brown, W. M. and Dziegielewska, K. M. (1997) Friends and relations of the cystatin superfamily – new members and their evolution. Protein Sci. **6**, 5–12
3 Brown, W. M., Saunders, N. R., Møllgård, K. and Dziegielewska, K. M. (1992) Fetuin. An old friend revisited. BioEssays **14**, 749–755
4 Dziegielewska, K. M. and Brown, W. M. (1995) Fetuin, Molecular Biology Intelligence Unit, pp. 1–178, R. G. Landes Company, Austin
5 Auberger, P., Falquerho, L., Contreres, J. O., Pages, G. L. E., Le Cam, G., Rossi, B. L. E. and Le Cam, A. (1989) Characterization of a natural inhibitor of the insulin receptor tyrosine kinase: cDNA cloning, purification, and anti-mitogenic activity. Cell **58**, 631–640
6 Rauth, G., Pöschke, O., Fink, E., Eulitz, M., Tippmer, S., Kellerer, M., Häring, H. U., Nawratil, P., Haasemann, M., Jahnen-Dechen, W. and Müller-Esterl, W. (1992) The nucleotide and partial amino acid sequences of rat fetuin. Identity with the natural tyrosine kinase inhibitor of the rat insulin receptor. Eur. J. Biochem. **204**, 523–529
7 Goto, K., Yoshida, K., Suzuki, Y., Yamamoto, K. and Sinohara, H. (1997) Molecular cloning and sequencing of cDNA encoding plasma countertrypin, a member of mammalian fetuin family, from the mongolian gerbil, *Meriones unguiculatus*. J. Biochem. **121**, 619–625
8 Yang, F., Chen, Z. L., Bergeron, J. M., Cupples, R. L. and Friedrichs, W. E. (1992) Human alpha2-HS-glycoprotein/bovine fetuin homologue in mice: identification and developmental regulation of the gene. Biochim. Biophys. Acta **1130**, 149–156
9 Salier, J. P., Chan, P., Raguenez, G., Zwingman, T. and Erickson, R. P. (1993) Developmentally-regulated transcription of the four liver-specific genes for Inter-α-Inhibitor family in mouse. Biochem. J. **296**, 85–91

10 Kazi, J. A., Nakamura, O., Ohnishi, T., Arakaki, N., Kajihara, T., Nakagawa, S. and Daikuhara, Y. (1998) Changes with age of the rat fetuin concentration in serum and its mRNA expression. J. Biochem. (Tokyo) **124**, 179–186
11 Yang, F., Schwartz, Z., Swain, L. D., Lee, C. C., Bowman, B. H. and Boyan, B. D. (1991) Alpha2-HS-glycoprotein: expression in chondrocytes and augmentation of alkaline phosphatase and phospholipase A2 activity. Bone **12**, 7–15
12 Binkert, C., Demetriou, M., Sukhu, B., Szweras, M., Tenenbaum, H. C. and Dennis, J. W. (1999) Regulation of osteogenesis by fetuin. J. Biol. Chem. **274**, 28514–28520
13 Jahnen-Dechent, W., Schinke, T., Trindl, A., Müller-Esterl, W., Sablitzky, F., Kaiser, S. and Blessing, M. (1997) Cloning and targeted deletion of the mouse fetuin gene. J. Biol. Chem. **272**, 31496–31503
14 Schinke, T., Amendt, C., Trindl, A., Pöschke, O., Müller-Esterl, W. and Jahnen-Dechen, W. (1996) The serum protein α2-HS glycoprotein/fetuin inhibits apatite formation in vitro and in mineralizing calvaria cells. A possible role in mineralization and calcium homeostasis. J. Biol. Chem. **271**, 20789–20796
15 Demetriou, M., Binker, C., Sukhu, B., Tenenbaum, H. C. and Dennis, J. W. (1996) Fetuin/α2-HS glycoprotein is a transforming growth factor-β type II receptor mimic and cytokine antagonist. J. Biol. Chem. **271**, 12755–12761
16 Chen, H., Srinivas, P. R., Cong, L. N., Li, Y., Grunberger, G. and Quon, M. J. (1998) α2-Heremans Schmid glycoprotein inhibits insulin-stimulated Elk-1 phosphorylation, but not glucose transport, in rat adipose cells. Endocrinology **139**, 4147–4154
17 Akhoundi, C., Amiot, M., Auberger, P., Le Cam, A. and Rossi, B. (1994) Insulin and interleukin-1 differentially regulate pp63, an acute phase phosphoprotein in hepatoma cell line. J. Biol. Chem. **269**, 15925–15930
18 Jahnen-Dechent, W., Trindl, A., Godovac-Zimmermann, J. and Müller-Esterl, W. (1994) Post-translational processing of human alpha(2)-HS glycoprotein (human fetuin). Evidence for the production of a phosphorylated single-chain form by hepatoma cells. Eur. J. Biochem. **226**, 59–69
19 Ohnishi, T., Nakamura, O., Arakaki, N. and Daikuhara, Y. (1997) Effect of phosphorylated rat fetuin on the growth of hepatocytes in primary culture in the presence of human hepatocyte-growth factor. Evidence that phosphorylated fetuin is a natural modulator of hepatocyte-growth factor. Eur. J. Biochem. **243**, 753–761
20 Yamamoto, K. and Sinohara, H. (1993) Isolation and characterization of mouse countertrypin, a new trypsin inhibitor belonging to the mammalian fetuin family. J. Biol. Chem. **268**, 17750–17753
21 Yoshida, K., Suzuki, Y., Yamamoto, K. and Sinohara, H. (1996) Cystatin-like domain of mouse countertrypin, a member of mammalian fetuin family, is responsible for the inhibition of trypsin. Evidence from site-directed mutagenesis. Biochem. Mol. Biol. Int. **39**, 1023–1028
22 Wang, H., Zhang, M., Bianchi, M., Sherry, B., Sama, A. and Tracey, K. J. (1998) Fetuin (α2-HS-glycoprotein) opsonizes cationic macrophage-deactivating molecules. Proc. Natl. Acad. Sci. U.S.A. **95**, 14429–14434
23 Daveau, M., Davrinche, C., Djelassi, N., Lemetayer, J., Julen, N., Hiron, M., Arnaud, P. and Lebreton, J. P. (1990) Partial hepatectomy and mediators of inflammation decrease the expression of liver α2-HS glycoprotein gene in rats. FEBS Lett. **273**, 79–81
24 Dziegielewska, K. M., Brown, W. M., Gould, C. C., Matthews, N., Sedgwick, J. E. and Saunders, N. R. (1992) Fetuin: an acute phase protein in cattle. J. Comp. Physiol. B **162**, 168–171
25 Yoshida, K., Suzuki, Y., Yamamoto, K. and Sinohara, H. (1999) cDNA sequencing of guinea pig alpha2-HS glycoprotein, its expression in various tissues and acute-phase expression. Biol. Chem. **380**, 95–99
26 Daveau, M., Rouet, P., Scotte, M., Faye, L., Hiron, M., Lebreton, J. P. and Salier, J. P. (1993) Human Inter-α-Inhibitor family in inflammation: simultaneous synthesis of positive and negative acute-phase proteins. Biochem. J. **292**, 485–492
27 Olivier, E., Soury, E., Risler, J. L., Smih, F., Schneider, K., Lochner, K., Jouzeau, J. Y., Fey, G. H. and Salier, J. P. (1999) A novel set of hepatic mRNAs preferentially expressed during an acute inflammation in rat represents mostly intracellular proteins. Genomics **57**, 352–364
28 Soury, E., Olivier, E., Daveau, M., Hiron, M., Claeyssens, S., Risler, J. L. and Salier, J. P. (1998) The H4P heavy chain of Inter-α-Inhibitor family largely differs in the structure and synthesis of its proline-rich region from rat to human. Biochem. Biophys. Res. Commun. **243**, 522–530
29 Hofmann, A., Bucher, P., Falquet, L. and Bairoch, A. (1999) The PROSITE database, its status in 1999. Nucleic Acids Res. **27**, 215–219
30 Henikoff, S., Pietrokovski, S. and Henikoff, J. G. (1998) Superior performance in protein homology detection with the Blocks database servers. Nucleic Acids Res. **26**, 309–312
31 Henikoff, J. G. and Henikoff, S. (1996) BLOCKS database and its applications. Methods Enzymol. **266**, 88–105
32 Yasue, M., Serikawa, T. and Yamada, J. (1991) Chromosomal assignments of 23 biochemical loci of the rat by using rat x mouse somatic cell hybrids. Cytogenet. Cell Genet. **57**, 142–148
33 Rychlik, W. and Rhoads, R. E. (1989) A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and *in vitro* amplification of DNA. Nucleic Acids Res. **17**, 8543–8551
34 Brown, W. M., Dziegielewska, K. M., Saunders, N. R., Christie, D. L., Nawratil, P. and Müller-Esterl, W. (1992) The nucleotide and deduced amino acid structures of sheep and pig fetuin. Common structural features of the mammalian fetuin family. Eur. J. Biochem. **205**, 321–331
35 Kellermann, J., Haupt, H., Auerswald, E. A. and Müller-Esterl, W. (1989) The arrangement of disulfide loops in human alpha2-HS glycoprotein. Similarity to the disulfide bridge structures of cystatins and kininogens. J. Biol. Chem. **264**, 14121–14128
36 Milland, J., Tsykin, A., Thomas, T., Aldred, A. R., Cole, T. and Schreiber, G. (1990) Gene expression in regenerating and acute-phase rat liver. Am. J. Physiol. **259**, G340–G347
37 Daveau, M., Jean, L., Soury, E., Olivier, E., Masson, S., Lyoumi, S., Chan, P., Hiron, M., Lebreton, J. P., Husson, A. et al. (1998) Hepatic and extra-hepatic transcription of Inter-α-Inhibitor family genes under normal or acute inflammatory conditions in rat. Arch. Biochem. Biophys. **350**, 315–323
38 DeLoukas, P., Schuler, G. D., Gyapay, G., Beasley, E. M., Soderlund, C., Rodriguez-Tome, P., Hui, L., Matise, T. C., McKusick, K. B., Beckmann, J. S. et al. (1998) A physical map of 30000 human genes. Science **282**, 744–746
39 Falquerho, L., Patey, G., Paquereau, L., Rossi, V., Lahuna, O., Szpirer, J., Szpirer, C., Levan, G. L. E. and Le Cam, A. (1991) Primary structure of the rat gene encoding an inhibitor of the insulin receptor tyrosine kinase. Gene **98**, 209–216
40 Osawa, M., Umetsu, K., Sato, M., Ohki, T., Yukawa, N., Suzuki, T. and Takeichi, S. (1997) Structure of the gene encoding human alpha2-HS glycoprotein (AHSG). Gene **196**, 121–125
41 Magnuson, V. L., McCombs, J. L., Lee, C. C., Yang, F., Bowman, B. H. and McGill, J. R. (1988) Human α2-HS-glycoprotein localized to 3q27-q29 by *in situ* hybridization. Cytogenet. Cell. Genet. **47**, 72–74
42 Schnittger, S., Gopal Rao, V. V. N., Abrahamson, M. and Hansmann, I. (1993) Cystatin C (CST3), the candidate gene for hereditary cystatin C amyloid angiopathy (HCCAA), and other members of the cystatin gene family are clustered on chromosome 20p11.2. Genomics **16**, 50–55
43 Lundin, L. G. (1993) Evolution of the vertebrate genome as reflected in paralogous chromosomal regions in man and the house mouse. Genomics **16**, 1–19
44 Elzanowski, A., Barker, W. C., Hunt, L. T. and Seibel-Ross, E. (1988) Cystatin domains in alpha-2-HS-glycoprotein and fetuin. FEBS Lett. **227**, 167–170
45 Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10**, 1–6
46 Broze, Jr, J. G., Girard, T. J. and Novotny, W. F. (1990) Regulation of coagulation by a multivalent Kunitz-type inhibitor. Biochemistry **29**, 7539–7546
47 Salier, J. P. (1990) Inter-α-trypsin Inhibitor: emergence of a family within the Kunitz-type protease inhibitor superfamily. Trends Biochem. Sci. **15**, 435–439

Received 10 December 2000/9 May 2000; accepted 8 June 2000