Characterization of the Bovine Tenascin-X*

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The primary structure of flexilin, an extracellular matrix glycoprotein previously identified in bovine tissues (Lethias, C., Descollonges, Y., Boutillon, M.-M., and Garonne, R. (1996) Matrix Biol. 15, 11–19) was determined by cDNA cloning. The deduced amino acid sequence (4135 residues) reveals that this protein is composed of a succession of peptide motifs characteristic of the tenascin family: an amino-terminal domain containing cysteine residues and heptads of hydrophobic amino acids, 18.5 epidermal growth factor-like repeats, 30 fibronectin type III-like (FNIII) domains, and a carboxyl-terminal fibrinogen-like motif. Sequence analysis indicated that this protein is the bovine orthologue of human tenascin-X. By rotary shadowing, bovine tenascin-X was identified as monomers with a flexible aspect, which are ended by a globule. More FNIII motifs were characterized in the bovine protein than in human tenascin-X. The main difference between the human and bovine tenascin-X is found in the arrangement of the three classes of highly similar FNIII repeat types in the central region of tenascin-X. The bovine FNIII motif b10 exhibits an RGD putative cell attachment site. The functional role of this sequence is corroborated by cell adhesion on purified tenascin-X, which is inhibited by RGD peptides. Moreover, we demonstrate that this RGD site is conserved at the same location in the human molecule.

The tenascins are a family of extracellular matrix glycoproteins with a typical multidomain structure. These proteins are widely encountered in the animal kingdom (1, 2). In invertebrates, tenasin-like molecules were demonstrated in leech (3) and in Porifera, the most primitive phylum of multicellular organisms (4). In Drosophila, two molecules related to tenascins, coded by the genes tena and tenm, were characterized (5, 6). Four members of the tenascin family have been identified in vertebrates so far: tenascin-C (also called cytotactin), tenascin-R (for restrictin), tenascin-X, and the recently characterized tenascin-Y. Tenascin-C consists of an amino terminus, which is involved in polymerization into oligomers, a series of epidermal growth factor (EGF)-like repeats, a variable number of fibronectin type III (FNIII)-like repeats, and a carboxyl-terminal globular fibrinogen-like domain (1, 2). In contrast to other known tenascins, tenascin-Y harbors a domain containing repeated serine-proline-Xaa motifs, which interrupts the series of FNIII domains (7). The numbers of FNIII and EGF repeats differ among the tenascins and among species. Different isoforms of tenascin-C, tenascin-R, and tenascin-Y arise from various splicing events in the region coding for FNIII repeats (7–9). An isoform lacking part of the NH2-terminal cysteine-rich region was reported in chicken tenasin-R (10).

Tenascin-X was initially reported as a partial sequence coded by gene X, found on the opposite strand of the human 21-hydroxylase gene (P450c21B) and located in the human major histocompatibility complex class III region (11–13). By extending genomic sequences, Bristow et al. (14) obtained enough data to predict the modular structure of the tenasin-X protein, which comprises an amino-terminal cysteine-rich region with four heptad repeats, 18.5 EGF-like repeats, at least 29 FNIII repeats, and a fibrinogen-like domain. The carboxyl termini of mouse (15), rat (16), and pig tenascin-X (17) have been characterized by cDNA cloning. Northern blot analysis showed that the major species of tenasin-X mRNA was about 13 kb (15, 17, 18). Other, smaller bands were detected, suggesting the possibility of alternative splicing (15).

Tenascin-X is expressed much more widely than tenascin-C or tenasin-R (2). Most tissues express detectable levels of tenasin-X mRNA, but some fetal tissues (skeletal and heart muscles, dermis, testes, nerves, and digestive tract) express high levels (14–17), which are maintained in the adult tissues (except testes) and are also found in tendons, ligaments, and peripheral nerves (17). Several cell lines of various origins, including normal and transformed fibroblasts, carcinoma, and glioma cells, also express tenasin-X (15, 18). Biochemical analysis indicated that the molecular mass of the tenasin-X monomer is 450–500 kDa (15, 18). A smaller variant of 220 kDa has been described in cultured fibroblasts (18).

Little is known about the function of tenascin-X, but indirect evidence suggests that it is vital. Numerous mutations in the gene coding for steroid 21-hydroxylase, which cause adrenal hyperplasia, are observed in humans. The fact that none of these deletions extends to the region coding for tenasin-X suggests that this protein has an essential function (11). The pattern of tenasin-X expression in heart, skeletal muscle, and limbs during development is consistent with this hypothesis (16).

In a previous study, we characterized flexilin, a bovine extracellular matrix glycoprotein (19). This high molecular mass molecule, which appears to be a flexible monomer, is located on collagen fibrils. To characterize flexilin further, specific antibodies were used to screen a fetal calf skin expression library. We report here the complete cDNA structure of flexilin. The

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‡ The abbreviations used are: EGF, epidermal growth factor; bp, base pair(s); DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; FNIII, fibronectin type III; kb, kilobase pair(s); PCR, polymerase chain reaction; RT, reverse transcriptase; PAGE, polyacrylamide gel electrophoresis.

8 This paper is available on line at http://www.jbc.org
deduced amino acid sequence indicates that the overall domain organization of flexilin is similar to that of tenascins and that it corresponds to the bovine orthologue of human tenascin-X. Thirty FNIII repeats were found in the bovine cDNA, some of which were unusually long (up to 134 amino acids), compared with typical FNIII repeats identified previously (90–100 amino acids). These results, biochemical, cell attachment, rotary shadowing data, and immunofluorescence localizations of flexilin are discussed in relation to the available data on tenascin-X.

EXPERIMENTAL PROCEDURES

Antibodies—Mouse monoclonal antibodies were produced according to previously described procedures (20). After immunization with a complex antigenic mixture (containing flexilin and types XII and XIV collagens) and immunofluorescence screening, nine monoclonal antibodies specific for flexilin were selected. They were characterized by comparing their properties with those of the 4E7 clone, which was described previously (19), by tests that included enzyme-linked immunosorbent assay (ELISA), Western blotting against purified flexilin, and immunofluorescence assays in various organs. The 8F2 clone was chosen for this study. Polyvalent antisera were obtained by immunizing mice with three injections (10 µg each) of immunopurified flexilin.

cDNA Cloning—Skin from fetal calf (15 weeks old) was dissected, frozen, and powdered in liquid nitrogen. Total RNA was prepared with RNA-B™ (Bioprobe Systems), and poly(A)-rich RNAs were selected by chromatography on oligo(dT)-cellulose. A cDNA library was prepared from 5 µg of poly(A)-rich RNA, using random hexamer primers and reagents from a cDNA synthesis kit and a AMOSELlox cloning kit (Amersham Corp., according to the manufacturer’s protocol. This expression cDNA library was screened with monoclonal antibodies 8F2 and 4E7. About 200,000 recombinant AMOSELlox clones were plated with Escherichia coli strain BL21 DE3 and grown for 6 h at 37 °C. Nitrocellulose filters (Hybond C, Amersham), which had previously been treated with 10 µg isopropl-1-thio-galactoynpranoside, were placed on the plates and incubated at 37 °C for 4 h. The filters were washed in 25 mM Tris-HCl, pH 8.0, 140 mM NaCl, and 2.7 mM KCl, and proteins were visualized with an antibody (see above). Insert cDNAs of the three clones were therefore purified from amosel exoning gel slices, cloned into pBluescript II SK (+) (Stratagene), and sequenced.

To obtain information about human sequences coding for the FNIII motifs VII to IX (numbering from Bristow et al., Ref. 14), RT-PCR experiment was carried out as above using poly(A)-rich RNAs purified from MG63 cells. The RT-PCR product coded for amino acids 1284–1350 of the human tenascin-X (14). Using the poly(A)-rich RNAs, a MG63 cDNA library was prepared in the AMOSELlox cloning kit (see above) and screened with the RT-PCR product. This allowed the purification of one cDNA clone (HFXXI). For genomic analyses, PCR experiment was performed using human genomic DNA (Promega). The amplified human genomic region corresponds to nucleotides 6486–6797 of the human tenascin-X gene (numbering from Bristow et al., Ref. 14).

DNA Sequencing and Computer Analysis—Sequencing was performed with the TT sequencing kit (Pharmac Biotech Inc.) using the dideoxyucleotide chain termination procedure on double-stranded DNA. Both DNA strands were sequenced using universal primers and synthetic oligonucleotides (Isoprim, Toulouse, France) corresponding to the appropriate cDNA sequences. For G- and C-rich regions, the sequencing reactions were carried out with dITP, an analogue of dGTP. The nucleotide sequences were analyzed by the DNAid computer program (23). Multiple alignments and identity studies were performed via the IBCP site server2 with the Antheprot (24) and Multalin (25) programs. The osteosarcoma cell line MG63 was obtained from the IBCP site server as described elsewhere (22). Briefly, 10 µg of total RNA were ethanol precipitated on a 0.8% agarose, 0.66 M formaldehyde gel. The gel was soaked in 50 mM NaOH and 10 mM NaCl for 20 min and neutralized twice in 0.1 M Tris-HCl, pH 7.4, for 20 min. The RNAs were transferred overnight onto nylon membranes (Hybond N+, Amersham). The filters were hybridized with a 32P-labeled cDNA probe (5′ Petl fragment of clone Flex-12) overnight at 65 °C in 6 × SSC, 5 × Denhardt’s solution, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA. The final washing was done in 0.1 × SSC and 0.1% SDS at 65 °C. The 0.28–6.58-kb RNA ladder from Promega was used as a size marker.

Biochemical Methods, Rotary Shadowing, and Immunocalization—Flexilin was extracted from bovine fetal skin with 0.5 M NaCl in the presence of proteases inhibitors and immunopurified on a column prepared with the 8F2 antibody. The purification procedures, SDS-polyacrylamide gel electrophoresis (PAGE), immunoblotting, rotary shadowing, and immunofluorescence were performed as described previously (19).

Cell Adhesion Assays—The osteosarcoma cell line MG63 was obtained from American type culture collection (Rockville, MD) and was maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and 50 µg/ml gentamicin. Adhesion substrates were prepared by adding 50 µl of immunopurified bovine tenascin-X (at 5 µg/ml) to 96-well ELISA plates (Nunc) overnight at 4 °C. The plates were blocked with 1% bovine serum albumin for 2 h. Cell suspension was obtained by a brief treatment of the cell layer with 0.25% trypsin and 0.05% EDTA, centrifugation with medium containing 1 mg/ml

2 The IBCP site server is accessible via the World Wide Web (http://www.ibcp.fr).
RESULTS

Cloning of Flexilin cDNA—Poly(A)⁺ RNAs extracted from early embryonic calf skin, a flexilin-rich tissue (19), were used to construct an expression cDNA library. Screening of this library with monoclonal antibodies specific for flexilin resulted in the detection of three cDNA clones (Flex-1, Flex-2, and Flex-3; Fig. 1), which were also immunoreactive with polyclonal anti-flexilin serum. The 5’-ends of these clones were sequenced according to the expression vector. The sequence NLYGFHDR, previously obtained by amino acid sequencing of flexilin tryptic peptides (19), was identified in two of these cDNA clones (Flex-1, Flex-2; Fig. 2). Moreover, complete characterization of the three immunoreactive clones shows that none of them overlap. Analysis of their sequences reveals that they potentially code for FNIII repeats, the highest score being shared with the central FNIII repeats of human tenascin-X (14). Owing to large gaps between the bovine and human sequences, the percentage of identity could not be determined precisely.

Multiple screenings of the cDNA library with specific cDNA oligonucleotide probes (see “Experimental Procedures”), allowed us to purify eight new clones (Flex-11–Flex-13, Flex-21–Flex-23, Flex-31, and Flex-41; Fig. 1). These overlapping cDNA clones cover 9253 bp, with an open reading frame of 3045 codons ended by a stop codon TAG, and 113 bp of 3’-untranslazed region. The open reading frame could be divided into two parts (Fig. 1), comprising a succession of 27 FNIII repeats and a fibrinogen-like carboxyl-terminal motif. The last FNIII repeats (numbered 26–29, Fig. 1) and the fibrinogen-like motif share more than 80% of identity with comparable domains of human, mouse, and pig tenascin-X (14, 15, 17). Moreover, the 3’ end of flexilin cDNA is 100% identical to the 3’ end of the bovine cytochrome P450c21 gene (data not shown; Ref. 28). The same overlap was identified in human, rodents, and pig genes (11, 15–17). Taken together, these findings indicate that bovine flexilin and human tenascin-X are orthologous proteins.

Since even extensive screening was insufficient to obtain the full cDNA sequence, PCR products coding for either the amino terminus or the furthest amino-terminal FNIII repeats of human tenascin-X were generated and used to screen our cDNA library again. With this procedure, we obtained two clones, TX1 and TX2 (Fig. 1). The two last gaps, of 69 and 135 bp, were filled by sequence analysis of RT-PCR products. The complete cDNA sequence is 12,706 bp long and contains an open reading frame of 4135 codons. The deduced amino acid sequence is presented in Fig. 2. The 5’-untranslated region (185 bp long) is comparable to the counterpart sequence of the major mRNA of human tenascin-X (29). Northern blot analysis of total RNAs from fetal skin showed that bovine tenascin-X transcript is about 13 kb (Fig. 1).

Complete Primary Structure of Bovine Tenascin-X—Bovine tenascin-X harbors a typical tenasin structure, which is presented in Figs. 1 and 2. The first 22 amino acids may correspond to a typical signal peptide (30). Thus, the predicted molecular size of mature bovine tenasin-X monomer was calculated to be 445 kDa. The signal peptide is followed by a cysteine-rich region (amino acids 23–152). Next is found the region responsible for the trimerization of tenascin-C and tenasin-R monomers and is composed of four heptad repeats flanked by seven cysteine residues. Next is found the carboxyl-terminal half of an EGF-like domain, separated from 18 complete EGF-like domains by a proline- and serine-rich spacer of 15 amino acids (amino acids 153–744). The cysteine residues in these EGF-like repeats are organized in the same way as in other tenasins, i.e. X₃Cₓ₁₋₃CXₓ₂₋₄CXₓ₃₋₄CXₓ₅₋₆C (26). As in human tenascin-X, the series of 18 EGF-like modules is interrupted by
FIG. 2. Primary structure of bovine tenascin-X. The sequence is arranged in groups of related regions, as suggested by Spring et al. (27). The amino acids are numbered on the right. EGF- and FNIII-like repeats are numbered from 1 to 18 and b0 to b29, respectively. The FNIII repeats were aligned by the Multalin program (25). All cysteine residues, the completely conserved tryptophan and tyrosine residues of the FNIII repeats, and the RGD sequence are indicated in bold. Potential N-linked glycosylation sites and sequences identified by protein sequencing (19) are underlined. Nine amino acid motifs (nonades) located at the amino termini of the FNIII repeats are identified by horizontal brackets. Gaps are indicated by dashes.
a four-amino acid insertion between repeats 16 and 17. The sequence following the EGF-like motifs is composed of 30 FNIII repeats (amino acids 745–3910). The size of each FNIII domain varies from 88 to 134 residues. Finally, a fibrinogen-like domain is found at the carboxyl terminus (amino acids 3911–4135). The predicted amino acid sequence also reveals five N-linked oligosaccharide acceptor sites in the amino and carboxyl termini of the molecule (Figs. 1 and 2). A single RGD sequence, suggesting a cell adhesive site, is found in the b10 FNIII motif (Figs. 1 and 2).

Comparison of Human and Bovine Tenascin-X—For this analysis, the human and bovine tenascin-X sequences were separated into three parts. Two of them, the amino-terminal region (comprising the probable oligomerization region and the EGF-like domains) and the fibrinogen-like domain, could be aligned perfectly in the two species, with an identity greater than 80%. The third region, which includes the FNIII repeats, is more difficult to analyze since the human tenascin-X genomic sequence has not been fully characterized, especially in the 5′ region.

The 30 bovine (b0–b29) and the 28 human (h1–h28) FNIII repeats were also compared; the main results are presented in Fig. 3. The farthest amino-terminal bovine FNIII repeat (b0, Fig. 3A) has not yet been characterized in human tenascin-X, but the third bovine FNIII repeat (b2) may represent the human FNIII-2 repeat (h2), which has been identified only by Southern blotting (13). The b0 and b2 repeats showed strongest identity with b1 and b28 (31%) and with b26 (28.4%), respectively. As shown in Fig. 3 (A and B), three regions of bovine and human tenasin-X appear to be colinear. The first contains FNIII repeats 1–5 in both species (Fig. 3A). The second corresponds to FNIII motifs b9–b13 and h7–h11 (Fig. 3A). The third encompasses FNIII repeats 22–29 in both species (Fig. 3B).

The remaining FNIII repeats are essentially located in the central part of both bovine and human tenasin-X. Three classes of repeats could be identified (Fig. 3C). The first, X1, comprises highly homologous FNIII repeats (greater than 80% identity), and the prototypes of this class are FNIII motifs b7 and h6. The major characteristic of these FNIII motifs is the presence of a variable number of nonade (nine amino acids in length) motifs at the amino terminus. These nonade motifs, which have the consensus sequence T(E/A)XXETPSP, are generally followed by the PEEPPEP sequence (Fig. 2). Two of the human X1 FNIII domains (h6 and h15) lack the nonade motif. In one of them, h15, the choice of an upstream AG splicing acceptor site from the published data (14) leads to the addition of 12 amino acids at the amino terminus, including a nonade motif. The second class of FNIII repeats, X2, also represents strongly identical repeats with prototype domains, b13 and h11. The third class of FNIII repeats, observed only in bovine, is called type X1–2, as the repeats are related to both of the other FNIII types (70–80% identity). The bovine FNIII repeats b6 and b14, which are included in the X1–2 class, harbor nonade motifs at their amino termini, like type X1.

Biochemical Properties, Rotary Shadowing, and Tissue Expression of Bovine Tenascin-X—SDS-PAGE analysis under reducing conditions and Coomassie Blue staining show freshly

FIG. 3. Sequence analyses of individual amino-terminal (A), carboxyl-terminal (B), and central (C) FNIII repeats of human and bovine tenasin-X. The percentages of identity were calculated with the Antheprot software (24). Owing to the variable size of the amino terminus of FNIII repeats, the sequences used for this analysis were limited to the shortest b29 or h28 repeats defined from alignments with the Multalin program (25). In the amino-terminal region, the partial colinearity between human and bovine sequences is shown by shading the corresponding scores. Perfect colinearity between the two sequences in the carboxyl-terminal part is shown by shading the greatest values. Three types of FNIII repeats (X1, X2, and X1–2 types) were characterized after analysis of the sequences of the central region. Values characterizing the X1 and X2 type repeats are represented by bold and open boxes, respectively. The X1–2 type is shown by a triangle. Asterisk indicates the human FNIII repeats that have not been completely characterized.
isolated, immunopurified tenasin-X as three high molecular mass bands (Fig. 5A, lane 1). The faster migrating bands (380–400 kDa) correspond to those described previously (19). The molecular mass of the more slowly migrating band (420 kDa) was estimated by comparison with the migration of unreduced bovine plasma fibronectin. This value is in correct agreement with the mass calculated from the deduced amino acid sequence. The latter band disappears rapidly during storage, whatever the conditions.

To verify that some of these bands do not correspond to proteins that interact with tenasin-X, we performed immunoblotting using the 8F2 antibody on purified protein. All three bands were clearly revealed (Fig. 5A, lane 3), demonstrating that monomers of different sizes were present in our purified preparation. Only two bands were observed after SDS-PAGE under nonreducing conditions, the band with intermediate migration being stronger than under reducing conditions (Fig. 5A, lane 2). Moreover, very faint bands are found on the top of the gel under nonreducing conditions. When examined by rotary shadowing, the immunopurified preparations were found to be highly homogeneous, consisting of flexible units of 150–180 nm ending with a globule (Fig. 5B).

The expression of tenasin-X has been tested in various bovine organs. It has been shown to be present in skin, tendon, and kidney glomeruli and under endothelia (19). In this study, we extended our observations to muscle and intestine. Connective tissue associated with skeletal muscle (epimysium and perimysium) was intensely labeled with anti-flexilin antibodies (Fig. 6A), and similar results were obtained within the striated perimysium (Fig. 6B). In cardiac tissue, typical extracellular matrix staining is observed along muscle cells (Fig. 6B). In gut sections, thin fibers of the lamina propria are stained (Fig. 6C). In gut sections, thin fibers of the lamina propria are stained (Fig. 6D).

**Functional Role of the RGD Site**—In a previous study (14), no RGD site was characterized in human tenasin-X. From Fig. 4, the domain comparable to bovine FNIII b10, which harbors the RGD sequence, is the human h8 motif. To detect the presence of an RGD site in the uncharacterized amino-terminal part of the h8 motif, we performed RT-PCR experiments covering part of the sequence coding for the h7 and h8 domains (Fig. 7). As shown in Fig. 7B, the amino-terminal side of the FNIII motif h8 contains an RGD sequence. This result was confirmed by analysis of a human cDNA clone, although some discrepancies with the published human sequence were noted (Fig. 7). Several sequence conflicts are clustered just after a gap of 500 bp, including the 5′ part of the exon coding for h8 repeat. With our data, an improved identity score was obtained between the FNIII motifs b10 and h8 (87.5% instead of 72.9%). Moreover, we found that the sequence of the amino-terminal part of the human FNIII motif h9 also differs from the published data. At the nucleotide level, the divergence is located upstream of a PstI site. Analysis of a genomic PCR product reveals that a 327-bp PstI fragment was previously omitted (Fig. 7B and Ref. 19). From this PstI fragment, it appears that the human FNIII motifs h8 and h9 are coded by two exons but not by an exon fusion as described previously (14). After these corrections, the identity between the h9 and b11 repeat sequences increases from 80.2 to 84.6%.

The function of the RGD site in bovine tenasin-X was tested by cell adhesion studies using the osteosarcoma cell line MG63. These cells were shown to adhere on tenasin-X-coated wells (Fig. 8). Moreover, they exhibited a round morphology and did not spread on tenasin-X (Fig. 8B), in contrast to the results obtained on immobilized fibronectin (Fig. 8A). Inhibition studies conducted with a synthetic peptide containing the RGD sequence clearly demonstrate that this adhesion is RGD-dependent (Fig. 8C), and that inhibition increases with peptide concentration (Fig. 8D).

**DISCUSSION**

In this study, we have characterized the primary structure of bovine flexilin. Sequence analysis indicates that this large extracellular matrix glycoprotein is the bovine orthologue of human tenasin-X. Moreover, the immunofluorescence localizations are in good agreement with those observed in other species (15, 16). Our results therefore represent the first complete sequencing of the nucleotides and deduced amino acid for the full length of the cDNA of a vertebrate tenasin-X.

Electron micrographs of purified bovine tenasin-X (Fig. 5B and Ref. 19) show the presence of monomeric molecules appearing as 150–180-nm flexible structures with a knob at one end, which is probably the fibrinogen-like module. The tenasin-X molecule looks like the tenasin-C, -R, and -Y monomers, ex-
Bovine Tenascin-X

FIG. 6. Immunofluorescence localizations of flexilin in fetal bovine tissues. Figure shows detection with the 8F2 monoclonal antibody. In skeletal (A) and hypodermal (B) muscles, labeling is found within the filamentous network of perimysium and epimysium. In cardiac muscle (C), the thin connective tissue layer embedding muscular cells is specifically stained. In intestine (D), muscularis mucosae fibers are intensely labeled.

cept for its size and its flexible aspect. The length of the molecule is due to the large number of FNIII repeats (30, whereas tenascin-C has only 8–16); its flexible aspect may reflect the unusual size (up to 134 amino acids) of some FNIII repeats in bovine tenasin-X, in comparison with 90–100 amino acids in these repeats in other proteins (31). The additional amino acids are located at the amino terminus and are mostly multiple nonade motifs with the canonical sequence T(E/A)EETPSP. It is unknown whether these conserved regions are an integral part of the FNIII repeats or act as a link between them. The amino-terminal extensions observed in bovine tenasin-X FNIII repeats, which are rich in glutamic acid and proline, are also present in chicken tenasin-Y but in a shorter form. Other similarities between tenasin-Y and tenasin-X were described by Hagios et al. (7), who indicated that the FNIII domains YB, YC, YD, and YE of tenasin-Y are similar to the 23 central FNIII repeats of human tenasin-X. Our comparisons between bovine tenasin-X and chicken tenasin-Y led us to the same conclusion.

As suggested by Bristow et al. (14), the four heptad repeats located at the amino terminus of human tenasin-X could provide the structural basis for the assembly of three monomers, which might form a triple-stranded, coiled helix analogous to TN-C and TN-R trimers. These heptads are flanked by seven cysteine residues that may stabilize the trimer by forming disulfide bonds. Human and bovine tenasin-X lack the amino-terminal cysteine found in tenasin-C and -R, which is involved in the polymerization process in tenasin-C, by which two trimers form an hexamer. The production of monomeric tenasin-X molecules from fetal bovine skin may be due to proteolysis in a sensitive site at the amino terminus. In some reports, tenasin-C and -R were also shown to be extracted from tissues as monomers (10, 32). This hypothesis is likely since the 420-kDa band tends to disappear during storage. We postulate that other bands observed in SDS-PAGE (Fig. 5A) arise from either another protease-sensitive site or from alternatively spliced isoforms.

Bovine tenasin-X contains five putative sites of N-glycosylation (NXS/T), which are observed in same locations as in the human tenasin-X molecule. Another interesting sequence, a putative RGD cell attachment site, is located in the FNIII repeat b10 (Fig. 2). By RT-PCR and cDNA analysis, we have shown that the corresponding human FNIII domain (h8) contains this RGD site. It is noteworthy that the tenasin-X RGD site is located at the amino terminus of the FNIII b10 and h8 modules (Figs. 2 and 7A), whereas in human and chicken tenasin-C and in fibronectin the RGD site is located at the carboxyl terminus of FNIII repeats (25, 33, 34). It is tempting to speculate that its location (between two folded FNIII repeats) might promote its interactions. Cell adhesion experiments using purified bovine tenasin-X and inhibition with RGD peptides are consistent with this hypothesis. Other adhesive sites may also be present in the tenasin-X molecule, such as the fibrinogen-like motif, which was demonstrated to be active in tenasin-C; adhesion to this domain was inhibited by RGD peptides, even though it has no RGD sequence (35). It is likely that both RGD site and fibrinogen-like domain are involved in cell adhesion to tenasin-X.

An overall comparison of bovine and human tenasin-X sequences shows good correlation between the two termini of these proteins. Greater than 80% identity is observed for the cysteine-rich potential polymerization region including the four heptad repeats, the 18.5 EGF-like domains, and the fibrinogen-like domain. Within the FNIII region, this score is reached for the first five FNIII repeats, bovine b7 and human b6 FNIII domains, bovine b9–b12 and human b7–b10 FNIII modules, and the eight carboxyl-terminal FNIII repeats (Figs. 3 and 4). Large uncharacterized sequences between the exons coding for the human FNIII repeats h5 and h6 and for h6 and h7, of 1.8 and 6.5 kb (14), respectively, may contain genetic information coding for repeats corresponding to bovine FNIII b6 and b8. Following the same argument, bovine repeats b0 and h2 may have their counterparts in human tenasin-X.

The central parts of human and bovine tenasin-X seem to be organized differently (Figs. 3 and 4). They are made of three highly homologous types of FNIII repeats, which we have named X1, X2, and X1–2. The last group, characterized only in bovine, is related similarly to types X1 and X2 and may represent a common ancestor. The absence of colinearity in the central part of bovine (deduced from cDNA data) and human (deduced from genomic analysis) tenasin-X arises from a distinct number and a different organization of the three types of FNIII repeats. The first hypothesis to explain this organization is that the genomic region coding for the central part of the tenasin-X has evolved differently in the two species. Similarly, the available data indicate that human tenasin-C harbors two more FNIII repeats than mouse and chicken proteins. In the second hypothesis, bovine and human tenasin-X genes have the same genetic information, and the discrepancy observed between the two species arises from alternative splicing events. Several lines of evidence are consistent with the latter hypothesis. In the region coding for the human FNIII repeats h11–
there are five gaps of 0.5, 1, 3.2, 1, and 0.8 kb (Fig. 4). Although the 11 human FNIII repeats may reflect the entire genetic information of this region, the possibility that one or more additional FNIII repeats are contained within these gaps cannot be excluded. In bovine tenascin-X, only nine FNIII repeats (b13–b21) are present in this region, and one of them, FNIII b14 (type X1–2), has not been identified in the human sequence. It might be suggested that the exons coding for the central FNIII repeats of tenascin-X are involved in alternative splicing events. It is noteworthy that these repeats have the strongest identity with the FNIII repeats of tenascin-Y, -C, and -R, which undergo alternative splicing. This possibility does not contradict the results of Speek et al. (29), who showed that human tenascin-X transcripts always contain the sequence coding for the last FNIII repeats (h23–h29). An alternate explanation for the differences between bovine and human tenascin-X could be a combination of the two hypotheses.

The present study has elucidated the primary structure of bovine tenascin-X by cDNA cloning and has correlated these results with biochemical data. Morphology of cells in contact with tenascin-X suggests a function similar to that of tenascin-C, i.e. the modulation of cell-matrix interactions, and the RGD sequence found in tenascin-X seems of particular importance for its biological properties.

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REFERENCES

**FIG. 7. RGD site in the human tenascin-X sequence.** Part of the FNIII motif h7 is presented in A. The complete FNIII motifs h8 and h9 are presented in B. Our human nucleotide sequence (HumTX) was aligned with the corresponding sequence (14) published previously. Encoded amino acids are indicated below the second base of the corresponding human nucleotide sequence codons. Identical amino acid between human h7 or h9 and bovine b9 or b10 and b11 FNIII modules are bold. Different amino acids are circled. The deduced amino acids from the published human tenascin-X sequence (14) are indicated at top only when they are in conflict with our data. Numbering of the nucleotide sequence labeled Ref. 14 is as in Bristow et al. (14). The PstI site flanking the omitted fragment of the human tenascin-X gene and the RGD site are underlined. Lowercase letters indicate intronic sequences.

**FIG. 8. Adhesion of MG63 cells to bovine tenascin-X.** Morphology of cells plated on substrates coated with fibronectin (A) and tenascin-X (B and C) is shown. Cells were previously incubated with control peptide (B) and RGD peptide (C). In D, cell adhesion on bovine tenascin-X was quantified using a colorimetric method, and a significant inhibition was observed in the presence of RGD peptide.
Bovine Tenascin-X

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