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Bovine Latent Transforming Growth Factor β1-Binding Protein 2: Molecular Cloning, Identification of Tissue Isoforms, and Immunolocalization to Elastin-Associated Microfibrils

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Monoclonal antibodies to fibrillin 1 (MP340), a component of elastin-associated microfibrils, were used to screen cDNA libraries made from bovine nuchal ligament mRNA. One of the selected clones (cL9; 1.2 kb) hybridized on Northern (RNA) blotting with nuchal ligament mRNA to two abundant mRNAs of 9.0 and 7.5 kb, which were clearly distinct from fibrillin mRNA (10 kb). Further library screening and later reverse transcription PCR by the rapid amplification of cDNA ends (RACE) technique resulted in the isolation of additional overlapping cDNAs corresponding to about 6.7 kb of the mRNA. The encoded protein exhibited sequence similarity of around 80% with a recently identified human protein named latent transforming growth factor β1 (TGF-β1)-binding protein 2 (LTBP-2), indicating that the new protein was bovine LTBP-2. This was confirmed by the specific localization of bovine LTBP-2 cDNA probes to human chromosome 14q24.3, which is the locus of the human LTBP-2 gene. The domain structure of bovine LTBP-2 is very similar to that of the human LTBP-2, containing 20 examples of 6-cysteine epidermal growth factor-like repeats, 16 of which have the consensus sequence for calcium binding, together with 4 examples of 8-cysteine motifs characteristic of fibrillins and LTBP-1. A 4-cysteine sequence which is unique to bovine LTBP-2 and which has similarity to the 8-cysteine motifs was also present. Antibodies raised to two unique bovine LTBP-2 peptides specifically localized in tissue sections to the elastin-associated microfibrils, indicating that LTBP-2 is closely associated with these structures. Immunoblotting experiments identified putative LTBP-2 isoforms as a 260-kDa species released into the medium by cultured elastic tissue cells and as larger 290- and 310-kDa species in tissue extracts. A major proportion of tissue-derived LTBP-2 required treatment with 6 M guanidine for solubilization, indicating that the protein was strongly bound to the microfibrils. Most of the guanidine-solubilized LTBP-2 appeared to be monomeric, indicating that it was not involved in disulfide-bonded aggregation either with itself or with latent TGF-β. Additional LTBP-2 was resistant to solubilization with 6 M guanidine but was readily extracted with a reductive saline solution. This treatment is relatively specific for solubilization of microfibrillar constituents including fibrillin 1 and microfibril-associated glycoprotein. Therefore, it can be inferred that some LTBP-2 is bound covalently to the microfibrils by reducible disulfide linkages. The evidence suggests that LTBP-2 has a direct role in elastic fiber structure and assembly which may be independent of its growth factor-binding properties. Thus, LTBP-2 appears to share functional characteristics with both LTBP-1 and fibrillins.

Elastic fibers are important constituents of the extracellular matrix in tissues such as major arteries, lungs, and skin. They consist of a core of the elastic protein, elastin, surrounded by microfibrils, 10 to 12 nm in diameter, which contain a number of glycoproteins (4–6, 16, 33). During tissue development, the microfibrils appear before the elastin, and they are considered to act as a scaffold for the deposition of the elastin precursor, tropoelastin. Morphologically and structurally similar microfibrils also occur as elastin-free bundles in a wide range of diverse tissues such as ocular zonule, kidney, periodontal ligament, and bone (13, 22, 35). Major components of the elastin-associated microfibrils are large (350-kDa), rod-like glycoproteins called fibrillins (35, 36, 41) and a small (31-kDa), elastin-binding glycoprotein called microfibril-associated glycoprotein (MAGP) (2, 15, 17). Two fibrillins (fibrillin 1 and fibrillin 2) have been cloned (7, 23, 25, 30) and linked to the congenital disorders of Marfan syndrome and the related disorder, congenital contractural arachnodactyly, respectively (9, 23). Marfan syndrome is characterized by major abnormalities of the skeletal and cardiovascular systems and is often associated with ocular defects (26). It is evident that fibrillins and thus microfibrils are essential for the normal development, morphology, and function of a wide range of tissues.

Fibrillins are characterized by a primary structure consisting predominantly of cysteine-rich repeat motifs of two distinct types: (i) 6-cysteine repeats, which are similar to motifs found in epidermal growth factor (EGF) precursor and a range of other proteins (EGF-like repeats) (18, 26, 31), and (ii) 8-cysteine repeats, which until very recently had been identified in only one other protein, latent transforming growth factor-β1 (TGF-β1)-binding protein 1 (LTBP-1) (20, 39). LTBP-1 forms soluble disulfide-bonded complexes with latent forms of TGF-βs (27) and is considered important for the efficient secretion of these growth factors (28).

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In this paper, we describe the identification of cDNAs for a protein with structural similarities to fibrillins and LTBP-1, following expression screening of an elastic tissue library with a monoclonal antibody to fibrillin 1 (originally called MP340 in our laboratory [16]). In a preliminary report, the new protein was named fibrillin-like protein (14). However, the protein has about 80% similarity in sequence to the recently cloned human protein, named LTBP-2 (29). The evidence presented here indicates that the new protein is bovine LTBP-2. However, in the elastic tissue nuchal ligament, bovine LTBP-2 occurs in the extracellular matrix as distinct isoforms which generally appear not to be covalently complexed with latent TGF-β. Moreover, bovine LTBP-2 was immunolocalized specifically to the elastin-associated microfibrils and a significant proportion of the protein appears to be disulfide bonded to these structures. The evidence suggests that bovine LTBP-2 has functional as well as structural similarities to fibrillins and that the protein may play a direct role in elastic-fiber biology.

MATERIALS AND METHODS

Production of monoclonal antibodies to fibrillin 1 (MP340). Monoclonal antibodies were produced by standard techniques using hybridoma cultures derived from X-63 myeloma cells and lymph node cells from BALB/c mice immunized with purified, reduced, and alkylated MP340 (16). Two monoclonal antibodies, A2 and A5, were selected for their ability both to react specifically with fibrillin 1 on enzyme-linked immunosorbent assay and immunoblots and to localize strongly on elastic-fiber microfibrils in tissue sections.

Antibody screening of cDNA libraries and analysis of immunoreactive clones. The anti-fibrillin 1 (MP340) monoclonal antibodies were used to screen two bovine nuchal ligament cDNA libraries in lambda gt 11. One library (random primed) was made from 120-day fetuses, and the other [oligo(dT) primed] was derived from a 15-day-old calf (17). The screening was conducted as described previously (17), except that the nitrocellulose plaque lifts were treated, prior to the blocking step, with 25 mM dithiothreitol (DTT) (for 60 min) followed by 25 mM iodoacetamide (for 30 min) to expose reactive epitopes. Immunoreactive clones were purified, and a stable lysogen of each was produced. β-Galactosidase fusion proteins were identified in extracts of stable lysogens by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Clones which gave large fusion proteins were selected, subcloned into pBluescript, and sequenced as described previously (17).

Determination of mRNA sizes. Selected clones, cL9 and cM7, were excised from pBluescript with *Eco*RI, purified with a Qiaex gel extraction kit (Qiagen Inc., Chatsworth, Calif.), and labeled with [^{32}P]dCTP by random-hexanucleotide priming of Klenow DNA polymerase (Prime-a Gene kit; Promega, Madison, Wis.). Also labelled was a 1.5-kb cDNA for fibrillin 1, MF-13 (23), which was a kind gift from F. Ramirez, Brookdale Center for Molecular Biology, New York, N.Y. The radiolabelled clones were then hybridized to fetal nuchal ligament-derived total RNA (10 μg) which had been electrophoresed on a 1% agarose gel and transferred to a nylon membrane (Qiabrane; Qiagen, Inc.) by standard procedures (37). Hybridization was conducted at 42°C for 18 h in 25 mM sodium phosphate buffer (pH 6.5) containing formamide (50%, vol/vol), dextran sulfate (10%, wt/vol), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), Denhardt's solution, SDS (0.1%), and salmon sperm DNA (50 μg/ml). The membranes were washed three times in 0.1× SSC–0.5% SDS at 65°C for 15 min, and mRNA bands were visualized on X-ray film (Kodak X-Omat AR).

Library screening with digoxigenin-labeled RNA transcripts of clone cL9. Digoxigenin-labeled RNA transcripts of cL9 (200 ng/ml) were prepared by a previously described method (17) and hybridized to nitrocellulose plaque lifts of the nuchal ligament cDNA libraries (plated at a density of 2×10^4 PFU per plate). Hybridization was conducted at 50°C for 18 h with a solution containing formamide (50%), 5× SSC, casein (5%), *N*-lauroylsarcosine (0.1%), and SDS (0.02%). The filters were washed in 0.1× SSC–0.1% SDS at 65°C for 30 min. Positive plaques were identified with antidigoxigenin antibodies conjugated to alkaline phosphatase. Phage DNA was prepared from each positive plaque, and its cDNA was subcloned into pBluescript for sequencing. Both strands of each clone were completely sequenced, by standard techniques, on overlapping series of truncated subclones obtained by nested deletion (17). Additional clones were identified by library screening with RNA transcripts of clone cF8B.

PCR amplification of 5′ cDNA sequences by the RACE technique. The rapid amplification of cDNA ends (RACE) technique was used to amplify 5′ regions of the cDNA which could not be obtained from the available cDNA libraries. The method used is based on that described by Frohman et al. (11). Briefly, first-strand cDNA was generated with an LTBP-2-specific oligonucleotide primer B, GTCCTTTCCTGACCATGATGTAGC, in conjunction with Moloney murine leukemia virus reverse transcriptase and poly(A)$^+$ RNA derived from the nuchal ligament of a 230-day-old bovine fetus. Terminal transferase was then used to add an oligo(dG) tail to the 5′ end of the cDNA. Following the removal of the RNA and primer B, PCR was conducted with Vent DNA polymerase (New England Biolabs, Beverly, Mass.) together with specific primer R2, TA ACTCGAGGGTAGTGGTGTCGCCAC, and (*Xho*I)-oligo(dC) primer, AATC TCGAG$(C)_{14}$, or (*Eco*RI)-oligo(dC) primer, AAGGAATT$(C)_{14}$. A capillary thermal cycler (model FTS-1; Corbett Research, Sydney, Australia) was used as specified by the manufacturer to generate 45 cycles of 96, 65, and 72°C. Specifically amplified cDNA products, identified by agarose gel electrophoresis and Southern blotting, were excised and purified from the gel as above, digested with the appropriate restriction enzyme (*Xho*I or *Eco*RI), and ligated into pBluescript for sequencing. Sequences obtained from cloned PCR products were confirmed by direct sequencing (fmol kit; Promega) of both strands of the 2-kb PCR product obtained from a fresh amplification of the first-strand cDNA with two specific primers F1 (TATGAATTCCGAAGGCTACAGCGACCG) and RA (AAGAATTCACAGGCCCAGCGTCAG).

Fluorescence in situ hybridization to human chromosomes. A cDNA for bovine LTBP-2 (clone cF5C) was nick translated with biotin-dATP and hybridized in situ (at a final concentration of 15 ng/μl) to metaphase cells from two normal human male subjects. Signal was detected by the fluorescence in situ hybridization technique as described previously (3), except that chromosomes were stained before analysis with 4′,6-diamido-2-phenylindole (DAPI) for chromosome identification and propidium iodide as a counterstain.

Synthetic peptide synthesis and antipeptide antibody production. Two synthetic peptides, peptide A (ASDLEEYDAQEGRC) and peptide F (VEAETIPDKGDSQA), were prepared by *tert*-butoxycarbonyl (T-Boc) chemistry on a 431A peptide synthesizer (Applied Biosystems Inc., Foster City, Calif.) as specified by the manufacturer. The peptide sequences were confirmed with a 473 protein sequencer (Applied Biosystems). The synthetic peptides were coupled to rabbit serum albumin with *m*-maleimidobenzoic acid-*N*-hydroxysuccinimide ester (10) at a substitution of 7.5 mg of peptide per mg of rabbit serum albumin. Antibodies were then raised in rabbits by regular subcutaneous injection of the peptide-rabbit serum albumin conjugates (1 to 10 mg/ml in Freund's adjuvant). The immunoglobulin fraction was recovered following treatment of immune serum with caprylic acid (34). Each antibody was then purified by affinity chromatography with peptide coupled to Affi-Gel 10. Bound antibodies were eluted with 0.2 M glycine (pH 2.3), dialyzed against phosphate-buffered saline (PBS), and concentrated to 1 mg/ml prior to storage at −70°C. For immunoblotting work, the antibodies were further purified by a previously described method (13) of affinity chromatography with a recombinant 35-kDa fragment of bovine LTBP-2 bound to nitrocellulose. The recombinant fragment was prepared from cL9 cDNA ligated into the bacterial expression vector pQE-30 (Qiaexpress kit; Qiagen, Inc.) as specified by the manufacturer.

Immunofluorescence microscopy. Indirect immunofluorescence was used to localize affinity-purified LTBP-2 peptide antibodies on frozen sections of a bovine nuchal ligament from a 180-day-old fetus. Tissue was fixed in 0.5% paraformaldehyde in PBS, infiltrated with 20% sucrose in PBS, and frozen in 2 parts 20% sucrose to 1 part OCT embedding medium (1). For immunostaining, frozen sections (3 μm thick) mounted on glass slides were thawed to room temperature and rinsed with PBS for 15 min. To fully expose reactive epitopes, the sections were treated for 15 min as described previously (16) with 6 M guanidine HCl in 20 mM Tris (pH 8.0) containing 50 mM DTT, rinsed in Tris buffer, and then treated with 100 mM iodoacetamide for an additional 15 min. The sections were rinsed in PBS, incubated with 20% normal goat serum in PBS for 1 h to block nonspecific binding, and then treated with antipeptide antibodies diluted 1:50 in 1% goat serum for 2 h. Control sections were incubated with the antibody solution containing the specific peptide (1 mg/ml) to which the antibody was raised. The sections were washed in 1% serum and incubated with fluorescein-conjugated goat anti-rabbit immunoglobulin G (Cappel, Durham, N.C.) for 1 h. After several washes in PBS, the sections were mounted in 60% glycerol in PBS containing *p*-phenylenediamine (1 mg/ml) and visualized with a Zeiss Axioskop microscope. Similar sections, either unfixed or fixed and then reduced and alkylated, were also stained with monoclonal antibody BA-4 raised to bovine α-elastin (40), an antibody to a synthetic peptide (peptide 468) from the bovine MAGP sequence (24), and commercial polyclonal antibodies to TGF-β1, TGF-β2, and TGF-β3 (Santa Cruz Biotechnology, Santa Cruz, Calif.).

Immunoelectron microscopy. Indirect immunogold labeling was used to specifically localize LTBP-2 on sections of 180-day fetal bovine aorta. Tissue pieces were fixed in 4% paraformaldehyde in 0.1 M Sorensen's buffer (pH 7.4) for 4 h at 4°C and then washed in several changes of the buffer. The tissues were then treated with reductive guanidine HCl solution followed by iodoacetamide as described above. Following treatment, tissues were quenched in 50 mM NH_4Cl in Sorensen's buffer, washed in several changes of Sorensen's buffer containing 4% sucrose, and left overnight at 4°C. The tissues were dehydrated in a graded series of increasing concentrations of methanol at progressively lower temperatures to −20°C, infiltrated, and embedded in Lowicryl K4M (SPI Supplies, West Chester, Pa.). The blocks were polymerized by UV illumination for 24 h at −35°C and an additional 48 h at −10°C. Thin sections on Formvar-coated nickel grids were incubated for 15 min with a blocking solution of 1% bovine serum albumin (BSA) in TBS (50 mM Tris HCl, 100 mM NaCl [pH 7.4]) and then for 18 h at 4°C in the same solution containing anti-LTBP-2 peptide antibodies (30 μg/ml). Control sections were incubated with antibody solution containing its

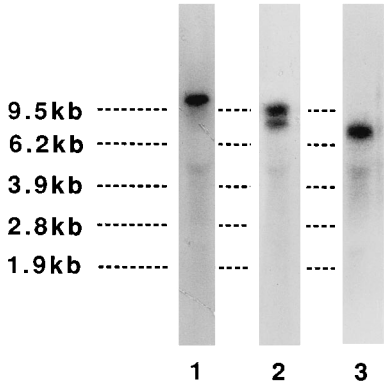

FIG. 1. Sizes of mRNAs for bovine LTBP-2 and LTBP-1. Northern blotting was conducted on total RNA extracted from the nuchal ligaments of fetal calves at 270 days (lanes 1 and 2) and 140 days (lane 3) of gestation as described in the text. Probes used were clone MF13 (fibrillin 1) (lane 1), clone cL9 (LTBP-2) (lane 2), and clone cM7 (LTBP-1) (lane 3). RNA standards (Promega) were coelectrophoresed with the RNA samples, and their sizes and relative mobilities are indicated.

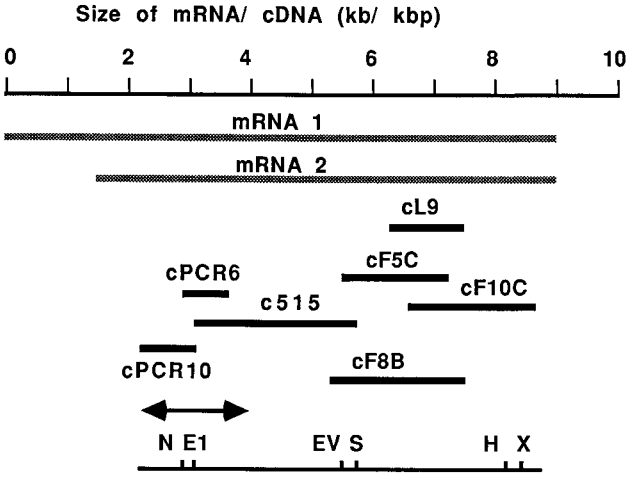

FIG. 2. Diagrammatic representation of overlapping bovine LTBP-2 cDNAs. The grey lines represent the two mRNA sizes for LTBP-2 (9.0 and 7.5kb). The black lines represent the bovine LTBP-2 cDNA clones sequenced to date. Clones which were obtained by PCR are labelled appropriately. The remaining clones were obtained from cDNA libraries. The double arrow corresponds to sequence confirmed by direct cycle sequencing of PCR-amplified product. Also shown is a map which denotes cleavage sites of restriction enzymes *Not*I (N), *Eco*RI (E1), *Eco*RV (EV), *Sal*I (S), *Hin*dIII (H), and *Xba*I (X).

specific peptide (1 mg/ml). Other negative controls used included omission of primary antibody or its replacement with nonimmunized rabbit immunoglobulin G at the same concentration. The sections were then rinsed in TBS containing Tween 20 (0.1%), incubated in blocking solution for 15 min, and treated with $F(ab')_2$ fragments of goat anti-rabbit immunoglobulin G antibodies conjugated to 10-nm-diameter colloidal gold particles (BioCell Research Laboratories, Cardiff, United Kingdom) diluted 1:40 in blocking solution. After 1 h, the grids were washed and counterstained with methanolic uranyl acetate followed by lead citrate as previously described (8).

Cell culture and immunoblotting. Fibroblasts from the nuchal ligament and chondrocytes from the ear cartilage of a 230-day-old fetal calf were cultured by the explant technique. Medium was conditioned over confluent cultures for 40 h and fractionated by addition of $(NH_4)_2SO_4$ to 30% saturation. Precipitated proteins were analyzed by SDS-PAGE on 6.5% gels followed by immunoblotting with antipeptide antibodies by previously described methods (15).

Extraction of LTBP-2 from nuchal ligament tissue. Nuchal ligament tissue from a 210-day-old fetal calf was extracted as described previously for the preparation of microfibrillar proteins (13). Briefly, homogenized tissue (5 g) was treated sequentially with TBS–Nonidet P-40 (twice with 20 ml), 1 M NaCl (twice with 20 ml), and 6 M guanidine HCl (twice with 40 ml). The residue was washed exhaustively with TBS (four times with 50 ml) and extracted with TBS–25 mM DTT (twice with 10 ml) and then 6 M guanidine HCl–DTT (10 ml). All extractions were performed at 4°C in the presence of proteinase inhibitors. The compositions of the extraction solutions are as described previously (13). A proportion of each extract (1/500) was analyzed by SDS-PAGE and immunoblotting with anti-peptide A antibodies.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL data bank under accession number U35363.

RESULTS

Analysis of cDNA clones identified by anti-fibrillin 1 (MP340) antibodies. Approximately 20 immunoreactive clones were isolated from the nuchal ligament cDNA libraries following screening with antifibrillin 1 monoclonal antibody A2. Northern (RNA) blotting with nuchal ligament RNA revealed that two clones, cL9 (1.2 kb) and cM7 (1.9 kb), had mRNAs which were distinct from fibrillin mRNA, indicating that they represented distinct proteins (Fig. 1). In the 270-day fetal nuchal ligament, fibrillin mRNA was detected as a single prominent band of 10 kb (Fig. 1, lane 1). In contrast, clone cL9 hybridized to two strongly labelling mRNAs of 9.0 and 7.5 kb (lane 2). Clone cM7 hybridized to an mRNA of 6.4 kb, which was much more prominent at 140 days (lane 3) than at 270 days (not shown) of fetal development. Clones cL9 and cM7 were sequenced and were both shown to encode proteins containing EGF-like repeats and 8-cysteine motifs characteristic of fibrillins (data not shown). A search of the GenBank database indicated that clone cM7 had over 90% similarity with human LTBP-1 and that it could be precisely aligned between bases 195 and 2153 of the published sequence (20). This suggested that clone cM7 was a partial cDNA for bovine LTBP-1 which has not previously been cloned. The mRNA for the putative bovine LTBP-1 appeared to be closer in size to LTBP-1 mRNA from rats (6.2 kb) than to that from humans (5.1 kb) (20, 39). The DNA sequence of clone cL9 was distinct from any present in the database at the time. A combination of further library screening, using cL9 as a probe and RACE-PCR, led to the isolation of additional cDNAs corresponding to over 6.7 kb of the 3′ end of the mRNA containing an incomplete open reading frame encoding 1,997 amino acids (Fig. 2). The encoded protein sequence was later found to have around 80% similarity to a recently cloned human protein called LTBP-2 (29), suggesting that the new protein is bovine LTBP-2.

Structural comparison of bovine LTBP-2 with LTBP-1 and fibrillins. The domain structure of bovine LTBP-2 is almost identical to that of the human form of the protein, with 20 examples of EGF-like 6-cysteine repeats and 4 examples of 8-cysteine repeats which had previously been found only in LTBP-1 and fibrillins (Fig. 3 and 4). Two of the EGF-like repeats found toward the amino terminus of the molecule are significantly shorter (33 residues) than the others (39 to 45 residues). Seventeen of the longer repeats contain the consensus sequence [C X N X_4 (F/Y) X C X C] for β-hydroxylation of asparagine, and 16 have the additional consensus sequence [D X (D/N) E C] for calcium binding found in blood coagulation factor IX (18). This suggests that LTBP-2 is a calcium-binding protein like fibrillins (7, 21). The 8-cysteine repeats include one example of a hybrid motif which contains the sequence CC corresponding to cysteines 3 and 4 and three examples containing the sequence CCC corresponding to cysteines 3, 4, and 5. Also present in LTBP-2 from both species are two 4-cysteine motifs. One motif is similar to a motif found close to the amino terminus of fibrillins and LTBP-1, whereas the other occurs only in LTBP-1 and LTBP-2. An RGD motif, which may represent an integrin-binding site for interaction

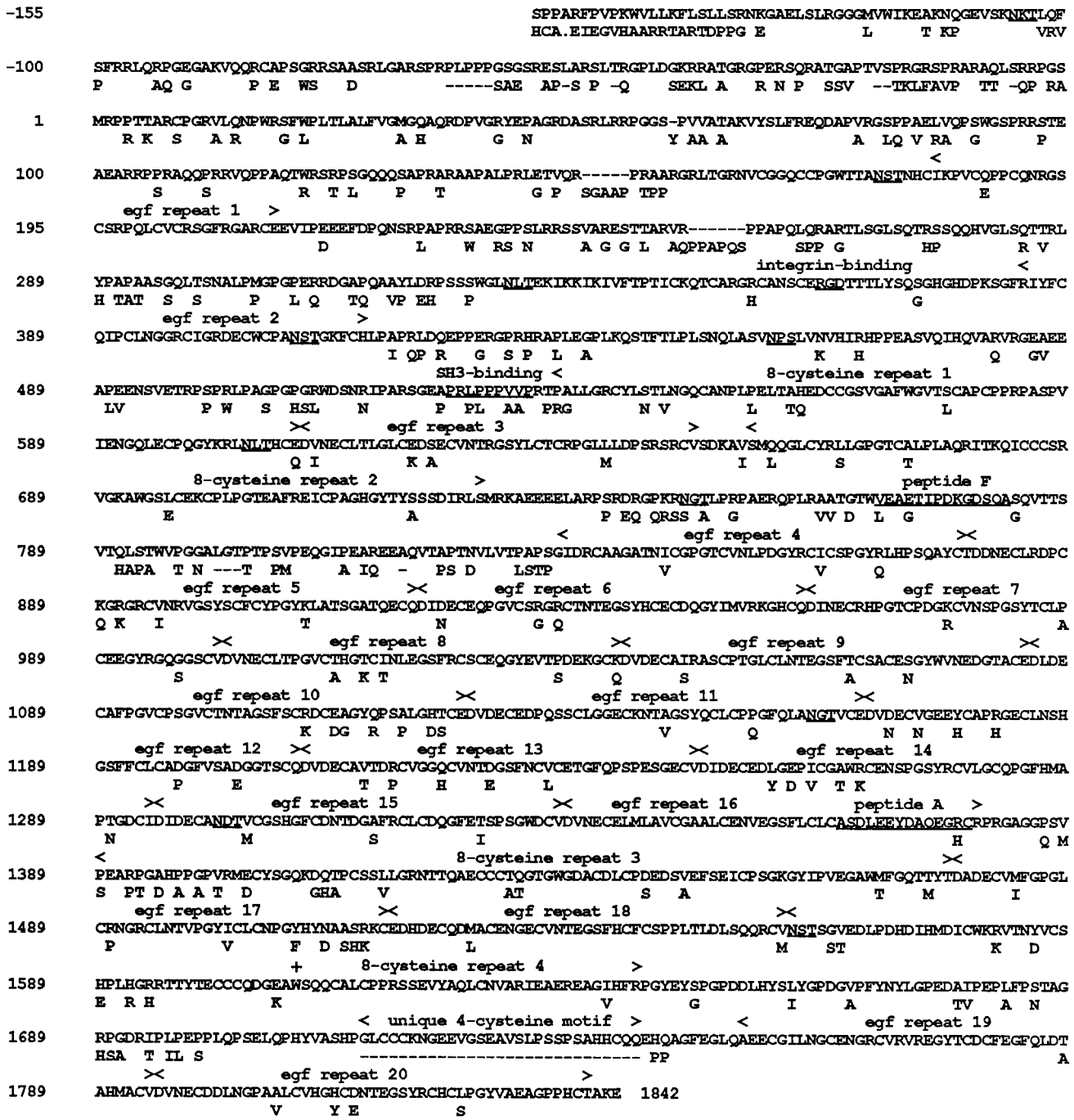

FIG. 3. Amino acid sequence of bovine LTBP-2. The amino acid sequence of bovine LTBP-2 translated from the 6.7 kbp of cDNA sequence is shown. Underneath are assignments from the human LTBP-2 sequence determined by Moren et al. (29) which differ from the bovine sequence. The amino acids are numbered from the Met residue (Met-1) suggested by Moren et al. to be the translation initiation point. Note that the open reading frame of the mRNA from both species extends at least 465 bases (155 amino acids) upstream of that point. The positions of the EGF-like and the 8-cysteine repeat structures are shown, as is a 4-cysteine sequence found only in the bovine sequence. The boundaries of each of these motifs are indicated (< and >). Consensus sequences for N glycosylation, a putative RGD cell-binding sequence, and a proline-rich SH3-domain-binding motif are underlined (32). Also underlined are the sequences of synthetic peptides A and F. The peptide A sequence forms part of EGF repeat 16. This includes part of a sequence which differs markedly from the human sequence published by Moren et al. (29). We have sequenced human LTBP-2 cDNA in this region and have included the sequence (amino acids 1385 to 1401) which we consider correct. This corrected human sequence more closely resembles the bovine sequence and contains a cysteine distribution pattern similar to that of the other EGF-like repeats.

with the cell surface, also occurs within a hydrophilic sequence likely to be present at the surface of the LTBP-2 molecule. However, the proximity of a cysteine residue involved in disulfide bonding suggests that steric hindrance may preclude the RGD motif from functioning as a cell-binding motif.

The distribution of the cysteine repeat motifs in LTBP-2 is very similar to the patterns found in fibrillin 1 and LTBP-1, suggesting that LTBP-2 is also a rod-like molecule. The arrangement of two 8-cysteine repeats separated from each other by two EGF-like repeats is common to all three proteins and

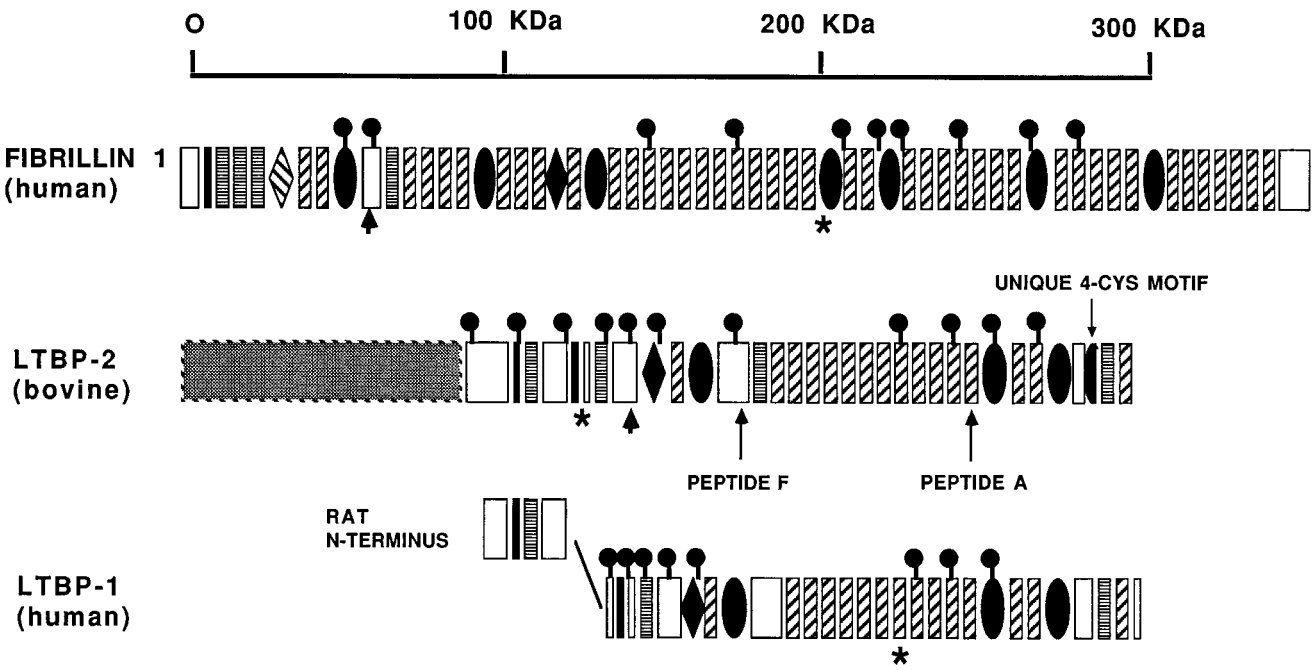

FIG. 4. Diagrammatic representation of the domain structure of bovine LTBP-2. The arrangement of the cysteine repeat motifs in bovine LTBP-2 is compared with that in human TGF-BP (20) and human fibrillin 1 (30). Fibrillin 2 (41) has a domain structure almost identical to that of fibrillin 1 (not shown). Human LTBP-2 (not shown) lacks the 4-cysteine motif indicated by the arrow. Also illustrated is the more extensive amino-terminal region of rat LTBP-1 (39), which has not yet been described for the human protein. Horizontally hatched rectangles, EGF-like motifs lacking calcium-binding consensus; cross-hatched rectangles, EGF-like motifs with calcium-binding consensus; black ovoids, 8-cysteine repeats; black diamonds, hybrid 8-cysteine motifs intermediate in structure between the EGF-like repeats and the 8-cysteine repeats; hatched diamond, 9-cysteine motif; black rectangles, 4-cysteine motifs; half-ovoid, unique 4-cysteine sequence; white boxes, cysteine-free regions; shaded box, region of bovine LTBP-2 with unknown structure; black circles, potential N-glycosylation sites; stars, RGD potential cell-binding sequences; bold arrows, possible SH3 domain-binding sites (32). The positions of sequences corresponding to synthetic peptides A and B are also indicated. The scale allows an estimate of the molecular size of each protein.

may be of particular functional significance. However, it is clear that LTBP-2 more closely resembles LTBP-1 than it resembles fibrillin 1 in overall structure. The amino-terminal half of LTBP-2 shows little similarity to fibrillin 1, whereas the carboxyl-terminal half of LTBP-2 exhibits about 35% similarity to the central region of fibrillin. Similarity in the primary structures of bovine LTBP-2 and human fibrillin 1 is confined almost entirely to the conserved sequences present in the cysteine-rich motifs (not shown). Conserved sequences represent approximately 40% of EGF-like repeats and 20% of 8-cysteine repeats. In contrast, LTBP-2 has much more similarity to human LTBP-1, 35% to the amino-terminal half and 45% to the carboxyl-terminal half of the molecule (not shown). This similarity includes sequences from the more variable parts of cysteine-rich motifs and from cysteine-free regions. Interestingly, LTBP-2 contains an amino-terminal region which is analogous to that of rat LTBP-1 but is lacking in the cloned form of human LTBP-1 (Fig. 4). The order of the domains in LTBP-2 is almost identical to the arrangement found in rat LTBP-1 except for the presence in LTBP-2 of a larger, cysteine-poor, amino-terminal domain, the unique 4-cysteine motif, and two additional EGF-like repeats. Alignment analysis with LTBP-1 suggests that the additional EGF-like motifs found in LTBP-2 are those numbered 13 and 14 in Fig. 3. Interestingly, the region of greatest similarity (about 80%) between LTBP-2 and LTBP-1 includes the penultimate EGF-like repeat at the carboxyl terminus of both proteins, designated repeat 19 in LTBP-2 and repeat 15 in LTBP-1. This suggests that the region, which curiously lacks consensus for calcium bonding and β-hydroxylation of asparagine, performs some function common to LTBP-2 and LTBP-1 but absent from fibrillins.

Structural comparison of bovine LTBP-2 with human LTBP-2. There are significant differences between the bovine and human LTBP-2 amino acid sequences. In general, the sequences are more divergent in the cysteine-free regions of the molecule, where several apparent insertions and deletions are evident. This suggests that the conservation of sequences within the cysteine-rich regions of the molecule is most important for the function of the molecule. Of particular interest, however, is the presence in bovine LTBP-2 of an additional 29-amino-acid sequence containing four cysteine residues which has not been described in the human protein (Fig. 3). This motif occurs toward the carboxyl-terminal region of the molecule and has significant structural resemblance to the 8-cysteine repeat motifs (Fig. 5). Not only does the 4-cysteine motif contain the characteristic tripeptide CCC sequence but also it has similarities to the more variable regions of the 8-cysteine motifs. It seems likely that this unique 4-cysteine motif is encoded in the bovine gene as a distinct exon which is either absent or alternatively spliced from the human gene.

It is interesting that the close sequence similarities between bovine and human LTBP-2 extend to a point close to the 5′ end of the partial bovine cDNA. This point is 399 bases (133 amino acids) upstream of the putative ATG translation initiation site designated for human LTBP-2 by Moren et al. (29), which corresponds to Met 1 in Fig. 3. No stop codons in the same reading frame are present in this region of either bovine or human LTBP-2 cDNA.

Hybridization of bovine LTBP-2 cDNA to human chromosome preparations. Since bovine and human LTBP-2 showed amino acid sequence similarity of only around 80%, it was necessary to confirm that they are species-specific variants of

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8-cys repeat                  1        2             345          6  7              8
consensus                ------CY------T CS-PL GR-TT--ECCC-- G--WG-- C-LCP--- S--F---   CP-G- GY---------- amino acid
                                                                                          number
Repeat 1                 PALLGRCYLSTLNGQ CANPL PELTAHEDCCGSV GAFWGVTSCAPCPPRPASPVIENGQLECPQGYKRLNLTHCE      536- 609
Repeat 2                 SMQQGLCYRLLGPGT CALPL AQRITKQICCCSRVGKAWGSL CEKCPLPG TEAFREI   CPAGH GYTYSSSDIRLS  656- 728
Repeat 3    VPEARPGAHPPGPVRM ECYSGQKDQTPCSSLL GRNTTQAECCCTQ GTGWGDA CDLCPDED SVEFSEI   CPSGK GYIPVEGAWMFG 1388-1471
Repeat 4    STSG VEDLPDHDIHMDICWKRV TNYVCSHPLHGRRTTYTECCCQD GEAWSQQ CALCPPRS SEVYAQL   CNVARIEAEREAGIHFRP 1561-1645
4-cys motif STAGRPGDRIPLPEPP LQPSELQPHYVASHP        GLCCCKN GEEVGSE  AVS LPS SPSAHH    CQQ      EHQAGFEGL  1685-1753

8-cys/4-cys ST-GRPGD--P-P--- L--S-L-P-YV-SHP        --CCC-- GE--GS-  A-- LPS S-----    C       E--AG---L
Consensus                                             123                              4
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FIG. 5. Similarity of the unique 4-cysteine motif and the 8-cysteine motifs of bovine LTBP-2. Shown is the alignment of the amino acid sequences of the two types of 8-cysteine motifs and a related 4-cysteine motif found in bovine LTBP-2. The sequences are aligned (with gaps) to highlight similarities between the motifs. The consensus sequences between the 8-cysteine motifs (above) and between the 8-cysteine motifs and the 4-cysteine motif (below) are shown.

the same protein and not closely related but distinct gene products. A biotin-labeled cDNA for bovine LTBP-2 was hybridized to 25 metaphase cells from a normal human male subject. Twenty-one of the metaphases showed signal on one or both chromatids of chromosome 14 in the region of 14q24.2 to 14q24.3 (Fig. 6). More than 80% of the signal was at 14q24.3. No background signal was found at any other chromosome location in more than one metaphase. A similar result was obtained with a human LTBP-2 cDNA probe (not shown). This result confirms that the protein cloned in this study is indeed the bovine equivalent of human LTBP-2.

Tissue localization of bovine LTBP-2. Two synthetic peptides containing unique amino acid sequences encoded in LTBP-2 cDNA were made. Peptide A corresponded to a sequence in an EGF-like domain, whereas peptide F corresponded to a sequence in a 8-cysteine domain (Fig. 3). Antibodies to both peptides were raised in rabbits and affinity purified on the peptide bound to Sepharose. Neither antibody reacted with purified fibrillin 1 or MAGP by Western blot (immunoblot) analysis (data not shown). Specific staining of elastic fibers is illustrated by immunofluorescence staining of nuchal ligament tissue with antitropoelastin antibodies (Fig. 7A) and with antibodies to the microfibrillar protein, MAGP (Fig. 7B). Antibodies raised to LTBP-2 peptide A (Fig. 7C) and peptide F (Fig. 7D) also showed strong specific localization to elastic fibers. Other elements of the extracellular matrix, such as collagen fibers, were not stained. Studies have shown that with antibodies raised to molecules such as fibronectin and type VI collagen, all structures of the extracellular matrix are accessible to antibodies in frozen-thawed sections of this tissue (12). No immunoreactivity was detected on control sections incubated with the anti-LTBP-2 peptide antibodies absorbed with their respective antigenic peptide (Fig. 7E and F). Similar sections were also stained with antibodies specific to TGF-β1, TGF-β2, or TGF-β3. Antibodies to TGF-β1 and TGF-β3 both gave faint, diffuse staining of the extracellular matrix, whereas anti-TGF-β2 antibodies localized only to the cells of small blood vessels (data not shown). None of the anti-TGF-β antibodies localized to the elastic fibers in either unfixed or pretreated sections, indicating that these growth factors were not extensively associated with these structures.

Immunoelectron microscopy of fetal bovine aorta was used to determine the precise location of LTBP-2 on the elastic fibers. The pattern of staining with anti-LTBP-2 peptide F

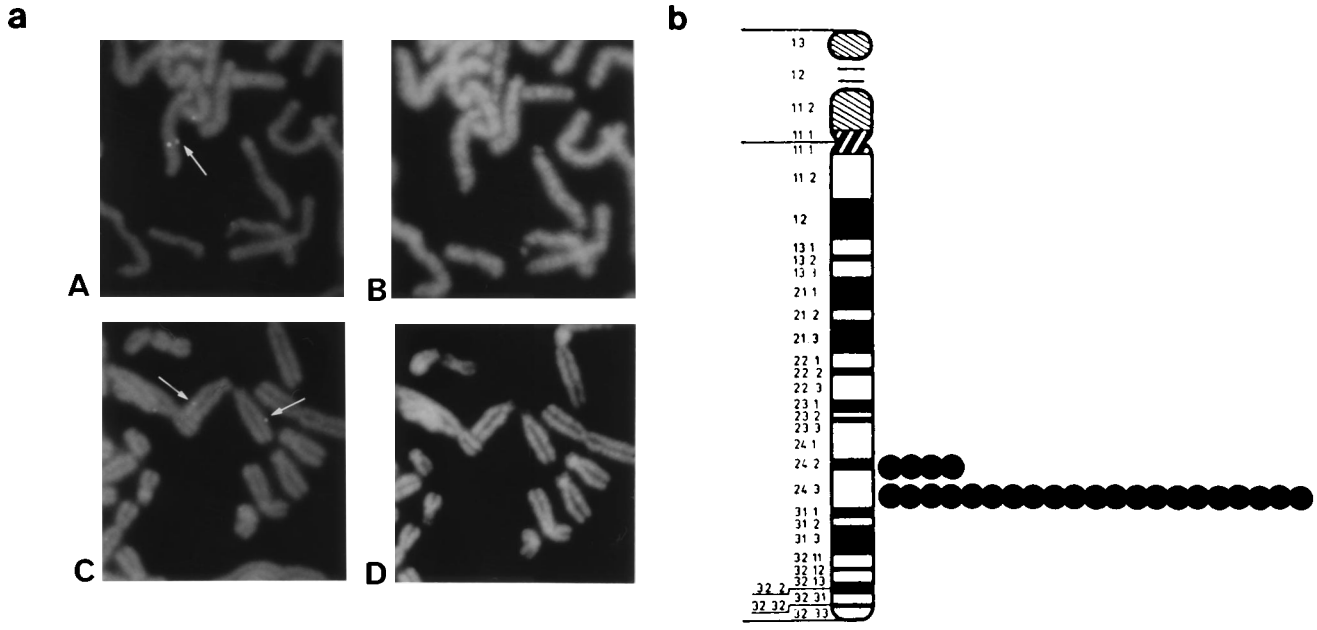

FIG. 6. Specific hybridization of bovine LTBP-2 cDNA to the human gene locus 14q24.3. (a) Partial metaphases of normal human chromosomes. (A and C) Fluorescence in situ hybridization with bovine LTBP-2 cDNA (clone cF5C) counterstained with propidium iodide. Hybridization sites are indicated by arrows. (B and D) The same metaphases stained with DAPI for chromosome identification. (b) Idiogram showing the total number of fluorescence signals detected on chromosome 14 from 25 metaphases with the bovine LTBP-2 cDNA probe. Each black circle represents an individual signal.

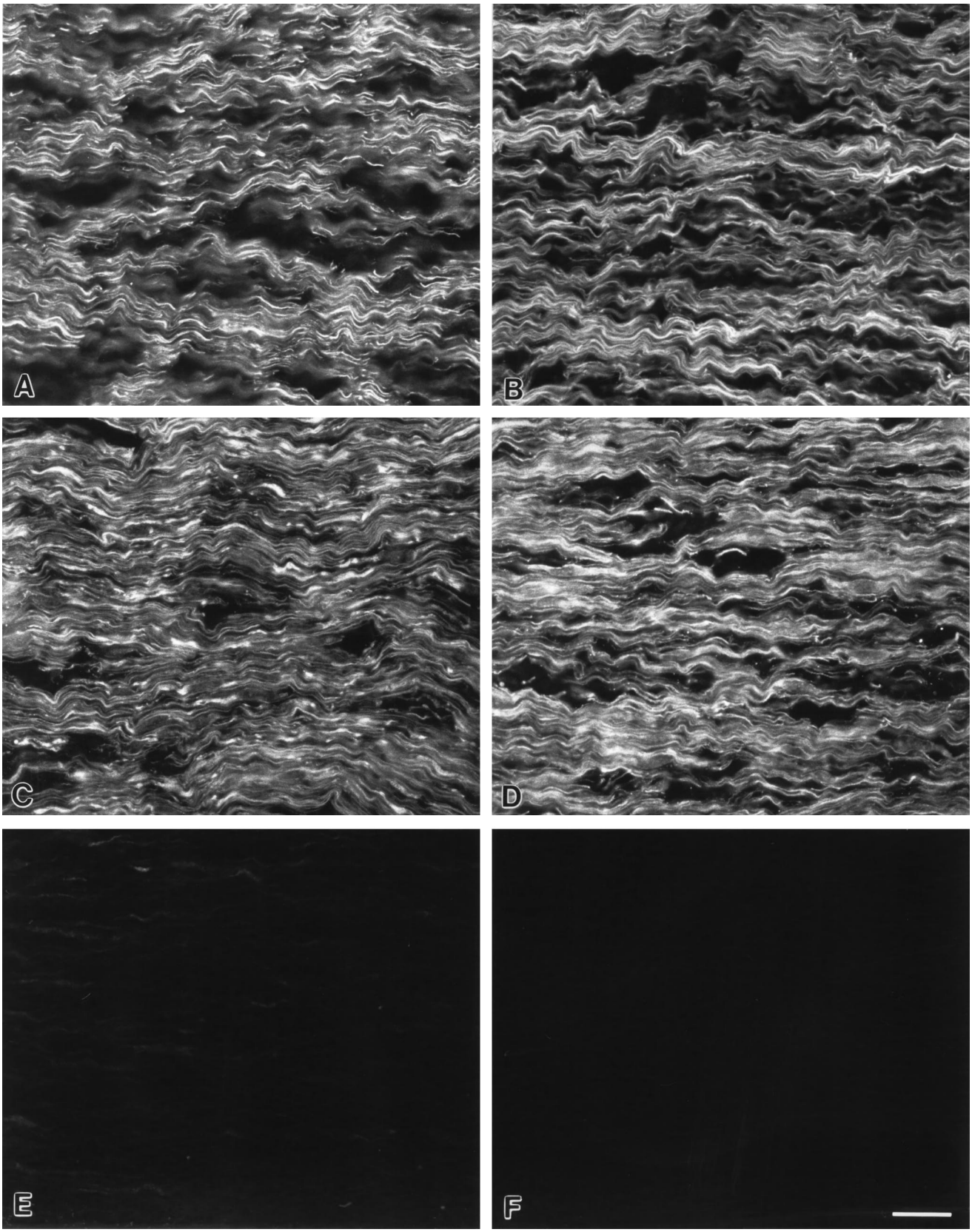

FIG. 7. Immunofluorescence localization of tropoelastin, MAGP, and LTBP-2 on developing elastic fibers in 180-day fetal bovine nuchal ligament. Elastic fibers in the ligament stain strongly for tropoelastin (A) and MAGP (B). Identical immunostaining was observed with antibodies to LTBP-2 peptide A (C) and to LTBP-2 peptide F (D). Addition of peptide A or F to the respective antibody solution completely blocked immunostaining of the elastic fibers (E and F). Magnification, ×430. Bar, 25 μm.

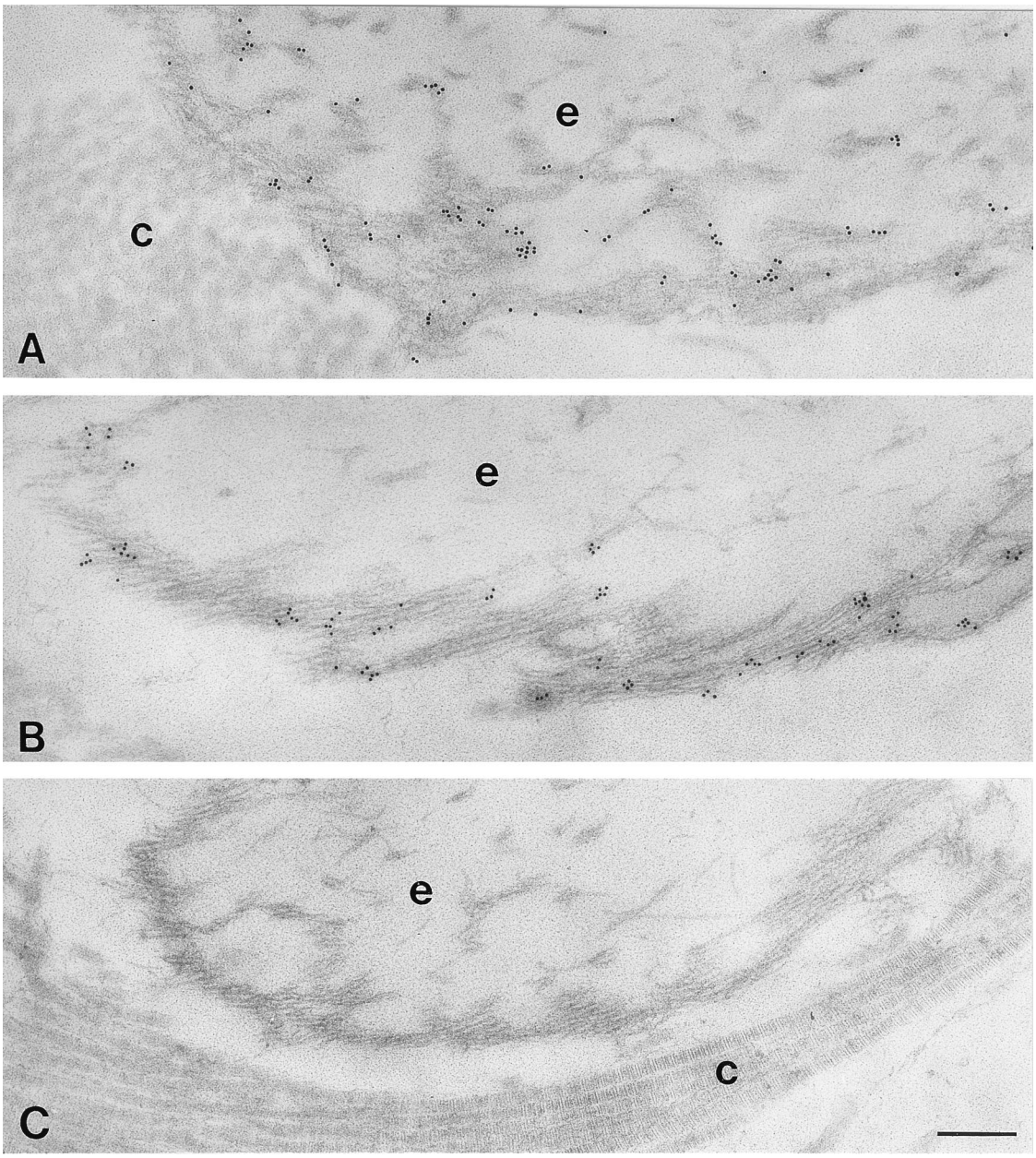

FIG. 8. Immunogold labeling of MAGP and LTBP-2 on a developing elastic fiber in 180-day fetal bovine aorta. (A) Immunolabeling with an antibody to the microfibrillar protein, MAGP, showed gold particles localized to the peripheral mantle of microfibrils of the elastic fiber. (B) Identical immunolabeling was observed with anti-LTBP-2 peptide F antibody, thus demonstrating the presence of LTBP-2 on the microfibrils. The central core of amorphous elastin (e) is devoid of label. Adjacent collagen fibers are also identified (c). (C) Peptide F added to anti-LTBP-2 peptide F antibody was able to completely block immunogold labeling of the microfibrils. Magnification, ×44,800. Bar, 0.25 μm.

antibody was compared with that of the microfibrillar protein, MAGP (Fig. 8A). Immunolabeling with the anti-LTBP-2 antibody revealed that the protein is specifically associated with the mantle of microfibrils (Fig. 8B). No immunostaining of the amorphous elastin core was observed. The staining pattern observed for LTBP-2 was indistinguishable from that of MAGP. Control sections incubated with anti-LTBP-2 peptide F antibody containing peptide F showed no immunogold staining of any structure (Fig. 8C), confirming the specificity of the LTBP-2 immunolocalization to the microfibrils.

Detection of LTBP-2 in cultures of cells from elastic tissues. Proteins were precipitated with $(NH_4)_2SO_4$ from cultures of ear cartilage chondrocytes and nuchal ligament fibroblasts. The precipitated proteins were reduced and alkylated and then immunoblotted with affinity-purified antibodies to the LTBP-2 peptide A and with monoclonal anti-MP340 (fibrillin 1) antibody A2. Antibody A2, which cross-reacts with LTBP-2, identified two prominent bands with apparent molecular weights of 340,000 and 260,000 in culture medium of both cell types (Fig. 9A). In contrast, the anti-LTBP-2 peptide A antibody identified a single 260-kDa protein (Fig. 9B). The results indicated that the 340-kDa species was fibrillin 1 since it comigrated with the fibrillin 1 (MP340) standard and that the 260-kDa species was a soluble form of LTBP-2.

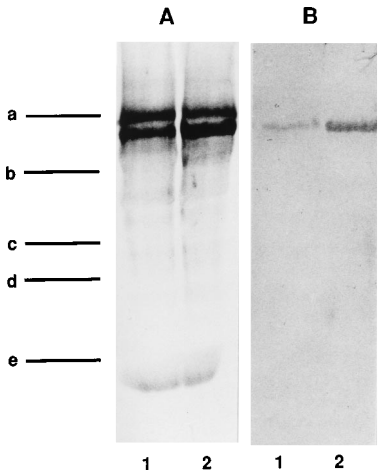

FIG. 9. Identification of LTBP-2 released into the medium by elastic tissue cells in culture. Medium was conditioned over cultures of confluent cells as described in the text. Proteins were precipitated with $(NH_4)_2SO_4$, reduced and alkylated, and analyzed by SDS-PAGE and immunoblotting with different antibodies, i.e., monoclonal anti-MP340 (fibrillin 1) antibody A2 (A) and affinity-purified anti-LTBP-2 peptide A antibody (B). Proteins from cultures of nuchal ligament and ear cartilage cells were analyzed in lanes 1 and 2, respectively. The mobilities of protein standards run concurrently are indicated: a, fibrillin 1 (340,000); b, myosin (200,000); c, β-galactosidase (116,000); d, phosphorylase *b* (97,000); e, BSA (66,000).

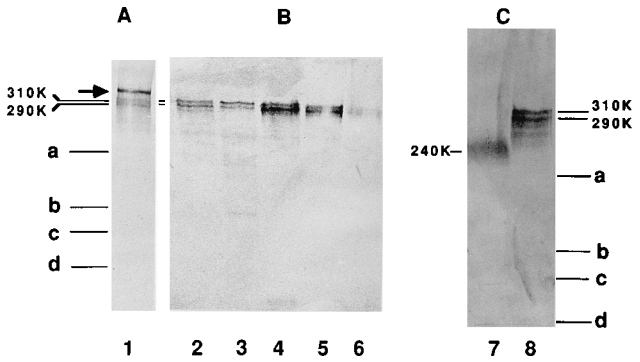

FIG. 10. Extraction of LTBP-2 isoforms from nuchal ligament tissue. Tissue homogenates were sequentially extracted with TBS plus detergent, 1 M NaCl, 6 M guanidine HCl, TBS plus DTT (reductive saline), and 6 M guanidine HCl plus DTT. Each extract was analyzed by SDS-PAGE on a 6.5% acrylamide gel (A and B) or a 5% acrylamide gel (C) followed by immunoblotting with monoclonal anti-MP340 (fibrillin 1) antibody A2 (A) or anti-LTBP-2 peptide A antibody (B and C). Lanes: 1 and 5, TBS-DTT extract; 2, TBS-detergent extract; 3, 1 M NaCl extract; 4, 7, and 8, 6 M guanidine HCl extract; 6, 6 M guanidine HCl–DTT extract. All samples were reduced and alkylated prior to electrophoresis, except for lane 7. The sample in lane 7 was electrophoresed under nonreducing conditions, transferred to nylon, reduced and alkylated on the membrane, and reacted with antibody. The apparent molecular weights of the major LTBP-2 polypeptides are indicated in thousands. The large arrow indicates fibrillin 1 (340 kDa) detected by the A2 monoclonal antibody. The molecular weights of protein standards run concurrently are indicated: a, myosin (200,000); b, β-galactosidase (116,000); c, phosphorylase *b* (97,000); d, BSA (66,000).

Detection of bovine LTBP-2 in extracts of an elastic tissue. Sequential extracts of nuchal ligament tissue were immunoblotted with anti-LTBP-2 peptide A antibody. When samples were reduced and alkylated prior to electrophoresis, two strongly staining polypeptides with apparent molecular weights of 310,000 and 290,000 and two minor species that were slightly smaller (260,000 and 280,000) were identified (Fig. 10B). Similar immunoblots with anti-LTBP-2 peptide F antibody identified bands of the same sizes, indicating that the two antibodies identified the same polypeptides (not shown). No bands were detected on control immunoblots in which the antipeptide antibodies were preabsorbed with their respective peptides (not shown). It is noteworthy that monoclonal antibody A2, which cross-reacts with LTBP-2, also identified 310- and 290-kDa species as well as fibrillin 1 (340 kDa) in the reductive saline extract (Fig. 10A).

Interestingly, LTBP-2 was present in the 6 M guanidine HCl and saline-DTT extracts and to a lesser extent in the guanidine HCl-DTT extract, suggesting that the protein is strongly associated with the extracellular matrix. In particular, a significant proportion of the LTBP-2 was resistant to extraction with the strong chaotrope, 6 M guanidine HCl, but was subsequently solubilized by the reductive saline treatment (Fig. 10B, lane 5). This indicates that some LTBP-2 was bound to an insoluble element of the matrix by disulfide bridges which are disrupted by this treatment. A much smaller proportion of LTBP-2 was solubilized by the final treatment with 6 M guanidine HCl-DTT (lane 6). This treatment is considered to solubilize any remaining protein which is not cross-linked to insoluble material by nonreducible covalent bonds (4, 5). Assuming that LTBP-2 does not form such bonds, it appears that almost all of this protein had been extracted from the tissue homogenate by the preceding treatments. When the 6 M guanidine HCl extract was analyzed under nonreducing conditions, LTBP-2 was identified as a single, diffuse band, corresponding to an apparent molecular weight of 240,000 (Fig. 10C, lane 7), which is smaller than reduced and alkylated forms of the protein (lane 8). This indicates that most of the LTBP-2 in the extract was not disulfide bonded into aggregates either with itself or with other proteins.

DISCUSSION

In this paper, we describe the cloning and partial characterization of a large, cysteine-rich protein from a bovine elastic tissue; the protein has structural similarities to the fibrillin family of matrix proteins (26, 31, 36) and to LTBP-1 (20, 39), a protein which forms covalent complexes with the latent form of TGF-β1 (27). Alignment of cDNA and encoded amino acid sequences indicated that the new protein has levels of similarity to human LTBP-1 of around 40% and to human fibrillins 1 and 2 of 20%. Recently, a related protein from human sources, which was named LTBP-2 because of its structural similarity to LTBP-1 and from evidence that it could also form covalent complexes with TGF-β1 in cotransfection experiments, has been identified (29). Human LTBP-2 exhibits around 80% homology with the protein described herein, suggesting that the latter is bovine LTBP-2. This has been confirmed by fluorescence in situ hybridization studies, which showed that a cDNA probe for the bovine protein specifically hybridized only to the LTBP-2 gene locus at 14q24.3 in human chromosome preparations. Bovine LTBP-2 exhibits many similarities to its human homolog, including an almost identical domain structure of 20 EGF-like, 6-cysteine repeats and four examples of the rare 8-cysteine motifs interspersed with cysteine-free regions. Also, mRNA sizes of 9.0 and 7.5 kb were identified in both bovine and human cells (29), suggesting that alternate splicing of the mRNA may occur, resulting in at least two LTBP-2 isoforms of significantly different sizes. It is interesting that distinct species and tissue isoforms of the related protein LTBP-1 have been identified which appear to differ mainly in the size of their amino-terminal domains (20, 39). Thus, it can be predicted that LTBP-2 isoforms with different amino-ter-

minal regions as the result of alternate splicing in the 5′ region of the mRNA also occur.

Differential splicing of the 5′ end of LTBP-2 mRNA may explain the different sizes of LTBP-2 found in the medium of bovine cells in culture (260 kDa) and tissue extracts (260 to 310 kDa) and the larger size of these isoforms compared with the human protein (240 kDa) identified in cell culture by Moren et al. (29). The observed molecular sizes of these LTBP-2 isoforms are much larger than the molecular weight of bovine LTBP-2, calculated as 198,000 from the translated amino acid sequence from 1 to 1842 shown in Fig. 3. This finding may be explained by possible extensive glycosylation of the molecule. However, it also suggests that the bovine LTBP-2 sequence may be incomplete even though it corresponds to the human LTBP-2 sequence described as complete by Moren et al. (29), in which the Met residue labeled as number 1 is considered the first amino acid. There is evidence that additional coding sequence exists upstream of this point in both the bovine and human cDNAs (Fig. 3). First, the amino acid sequence translated from bovine LTBP-2 cDNA contains an open reading frame from the 5′ end of the clone which is around 400 bases upstream of the putative translation initiation site. Second, there are strong similarities in the cDNA and translated amino acid sequences of bovine and human LTBP-2 which extend upstream of the putative translation initiation site until they diverge suddenly at a point about 1,420 bases from the 5′ end of the published human LTBP-2 cDNA sequence. This raises the possibility that the first 1,420 bases of the published human LTBP-2 cDNA are not from the LTBP-2 mRNA. It is possible that this is intronic sequence, but it seems more likely that it is the result of the ligation of a distinct cDNA with an LTBP-2 cDNA during library construction to give a chimeric clone. Third, the Met residue corresponding to the putative initiation site (Met-1) identified by Moren et al. (29) does not precede a typical signal sequence for secretion of the protein, suggesting that the true initiation site(s) has not yet been identified and that it occurs in the uncharacterized 5′ region of the mRNA. Obviously, determination of the complete structures of the two (or more) bovine LTBP-2 mRNAs is necessary before all translation initiation sites can be identified.

The structural similarity of LTBP-2 to both fibrillin and LTBP-1 raised the interesting question of whether LTBP-2 is a structural component of the extracellular matrix and/or a TGF-β-binding protein. We have raised antibodies to two synthetic bovine LTBP-2 peptides and used them to determine the ultrastructural location of the protein in tissues. Both antibodies specifically localized in nuchal ligament and aorta to the fibrillin-containing, microfibrillar component of elastic fibers, indicating that LTBP-2 was associated with these structures. Sequential extraction of tissues indicated that LTBP-2 is strongly bound to the microfibrils. The strong denaturant 6 M guanidine HCl was required to solubilize a significant proportion of LTBP-2, and a further proportion could be solubilized only by subsequent reductive saline treatment. Reductive saline treatment, after exhaustive extraction with 6 M guanidine HCl, has been shown to be relatively specific for the extraction of proteins, including fibrillin 1 and MAGP, which are covalently linked to the microfibrils by disulfide bonding (16). Thus, it appears that LTBP-2 is also disulfide bonded to these structures. This finding suggests that the protein may play a integral structural role in elastic-fiber architectural organization and/or assembly, as is the case for fibrillins (36). Additional evidence that LTBP-2 plays a direct role in elastic-fiber formation has been obtained from the analysis of steady-state mRNA levels in nuchal ligament and aorta at several stages during fetal development. The results indicate that expression of LTBP-2 is greatly and coordinately increased with that of elastin and fibrillins during the period of rapid elastinogenesis in these tissues (unpublished data).

Although Moren et al. (29) have demonstrated that human LTBP-2 can form covalent complexes with latent TGF-β1 in cells transfected with both proteins, our studies indicate that most of the bovine LTBP-2 identified in tissues is not covalently bound to the growth factor. When analyzed under nonreducing conditions, LTBP-2 extracted from tissue with a strong guanidine HCl solution was found to electrophorese on gels as a monomer which migrated more quickly than did reduced and alkylated LTBP-2. Presumably, this is due to stabilization of the protein in a folded conformation by extensive intramolecular disulfide bonding within the cysteine repeat structures. Monomers of fibrillin and LTBP-1 also migrate on gels more quickly under nonreducing conditions (28, 35). The above finding indicated that the LTBP-2 was not disulfide bonded to latent TGF-β, which has an apparent molecular mass of 90 to 100 kDa under nonreducing conditions, because a complex of the two proteins would migrate more slowly than would reduced and alkylated LTBP-2 (29). This is consistent with immunoprecipitation experiments with commercial antibodies to a range of TGF-βs, which failed to precipitate detectable LTBP-2 from conditioned medium of nuchal ligament fibroblast cultures or from nondenatured tissue extracts (unpublished observations). It is interesting that Moren et al. (29) found that most of the human LTBP-2 released into the medium by skin fibroblasts in culture was not covalently bound to latent TGF-β but migrated on SDS-PAGE as a monomer under nonreducing conditions. These authors also suggest that human LTBP-2 is present in the extracellular matrix of transfected cells in culture, although in their system the protein appears to be easily extracted without the need of a denaturant or reducing agent. Overall, the above observations support the idea that a major role of LTBP-2 in elastic tissues may be that of a structural component strongly bound to the extracellular matrix and not solely that of a chaperone protein for the secretion of TGF-βs. An intriguing possibility is that different isoforms of LTBP-2 perform distinct functions. The larger isoforms identified in tissues may act predominantly as extracellular matrix components, whereas smaller isoform(s) may form soluble complexes with latent TGF-β.

The possibility that LTBP-2 acts as a repository for TGF-βs as integral components of, or attached to, the surface of the microfibrils cannot be ruled out. TGF-βs are known to become bound within the extracellular matrix of a variety of tissues and cell lines, and there is evidence that this interaction may be mediated by LTBP-1 in some instances (38). Such a role has already been suggested for fibrillins because of their structural similarity to LTBPs (27), although fibrillin binding to TGF-βs has not been demonstrated. However, there has been only one report of immunolocalization of TGF-β to the elastin-associated microfibrils, and that was during regeneration of synovial tissue in a rat model of experimental arthritis (19). Our own immunofluorescence experiments failed to detect an association of TGF-β1, TGF-β2, or TGF-β3 with elastic fibers. Thus, if there is widespread association of TGF-βs with the microfibrils, these molecules appear to be well masked.

In conclusion, LTBP-2 appears to have a structure and function intermediate between those of fibrillins and LTBP-1. In terms of size and tissue location, it more closely resembles the fibrillins, whereas in domain structure and TGF-β binding properties, it is more similar to LTBP-1. Obviously it will be necessary to isolate and further characterize LTBP-2 from tissues and cultured cells before the precise range of functions of its various isoforms can be elucidated. This in turn will lead

to greater understanding of the purpose for the structural similarities between fibrillins and LTBPs.

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