THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 4, Issue of January 24, pp. 2750–2757, 2003

Printed in U.S.A.

Latent Transforming Growth Factor β-binding Protein 1 Interacts with Fibrillin and Is a Microfibril-associated Protein*

Received for publication, September 10, 2002, and in revised form, November 4, 2002
Published, JBC Papers in Press, November 11, 2002, DOI 10.1074/jbc.M209256200

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Latent transforming growth factor β-binding protein 1 (LTBP-1) targets latent complexes of transforming growth factor β to the extracellular matrix, where the latent cytokine is subsequently activated by several different mechanisms. Fibrillins are extracellular matrix macromolecules whose primary function is architectural: fibrillins assemble into ultrastructurally distinct microfibrils that are ubiquitous in the connective tissue space. LTBPs and fibrillins are highly homologous molecules, and colocalization in the matrix of cultured cells has been reported. To address whether LTBP-1 functions architecturally like fibrillins, microfibrils were extracted from tissues and analyzed immunochemically. In addition, binding studies were conducted to determine whether LTBP-1 interacts with fibrillins. LTBP-1 was not detected in extracted beaded-string microfibrils, suggesting that LTBP-1 is not an integral structural component of microfibrils. However, binding studies demonstrated interactions between LTBP-1 and fibrillins. The binding site was within three domains of the LTBP-1 C terminus, and in fibrillin-1 the site was defined within four domains near the N terminus. Immunolocalization data were consistent with the hypothesis that LTBP-1 is a fibrillin-associated protein present in certain tissues but not in others. In tissues where LTBP-1 is not expressed, LTBP-4 may substitute for LTBP-1, because the C-terminal end of LTBP-4 binds equally well to fibrillin. A model depicting the relationship between LTBP-1 and fibrillin microfibrils is proposed.

The fibrillins and latent transforming growth factor β-binding proteins (LTBPs)[1] are members of a family of homologous molecules. The fibrillins and LTBPs contain multiple calcium-binding epidermal growth factor-like modules interspersed by a domain module (the 8-Cys or TB module), so far found only in these two proteins. Fibrillin-1 (1–4) and fibrillin-2 (5, 6) share a highly similar overall structure. Both molecules are of equivalent size (~350 kDa) and domain organization. In contrast, LTBP-1 (7, 8), LTBP-2 (9), LTBP-3 (10), and LTBP-4 (11, 12) are each smaller than the fibrillins and variable in size.

Extensive immunolocalization data combined with structural analyses of the fibrillin-1 monomer and fibrillin-containing microfibrils (1, 13–15) have established that fibrillin-1 is a major structural component of connective tissue microfibrils. In addition, genetic evidence in humans (16, 17) and mice (18, 19) has confirmed that fibrillin-1 performs a significant role in the maintenance of microfibrils and elastic fibers.

Fibrillin-2, whose structure is predicted to be highly similar to fibrillin-1, has also been immunolocalized to microfibrils (20). However, in contrast to fibrillin-1, the contribution of fibrillin-2 to microfibril structure is temporally and spatially restricted. *In situ* hybridization studies in mice indicated that expression of the *fbn2* gene is most prominent in the early developing fetus (20). Genetic evidence in humans (5, 21) suggests that fibrillin-2 plays a more restricted role in the maintenance of microfibrils and elastic fibers in postnatal connective tissues. Recent immunolocalization studies demonstrate a ubiquitous early distribution of fibrillin-2 in fetal tissues followed by a restricted distribution in postnatal tissues (22). Mice produced by gene targeting experiments recapitulate the features (contractures of large and small joints) of the human disease congenital contractural arachnodactyly caused by mutations in fibrillin-2 (23). In addition, fibrillin-2 null mice revealed an unexpected role for fibrillin-2 in limb patterning, because the mutant animals display syndactyly (23).

LTBP-1 forms a complex with latent TGF-β and targets it to the extracellular matrix (7). Latent TGF-β consists of the mature growth factor plus the TGF-β propeptide, also known as the latency associated peptide (LAP). LAP binds to TGF-β by noncovalent interactions, and the association of LAP with TGF-β prevents the growth factor from binding to its receptor. During the secretory process, CR3, the second 8-Cys module, in LTBP-1 becomes disulfide-linked to the latency associated propeptide (LAP) of TGF-β (24, 25). The processed TGF-β remains noncovalently bound within this complex of LAP and LTBP. Studies of 8-Cys modules from fibrillin-1 indicate that the 8 conserved cysteine residues in this module form 4 intrachain disulfide bonds (15). The solution structure of one 8-Cys module from fibrillin-1 has been determined (26), indicating the position of disulfide bonds. The structure of the 8-Cys module in LTBP-1, which is disulfide-linked and complexed

* This work was supported by grants from the Shriners Hospitals for Children (to L. Y. S. and D. R. K.), the Scleroderma Foundation (to L. Y. S.), the Deutsche Forschungsgemeinschaft (to D. P. R.), and National Institutes of Health Grants CA 23753 and CA 34282 and DE13742 (to D. B. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: LTBP, latent transforming growth factor β-binding protein; LAP, latency associated peptide; TGF-β, transforming growth factor β; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; mAb, monoclonal antibody.

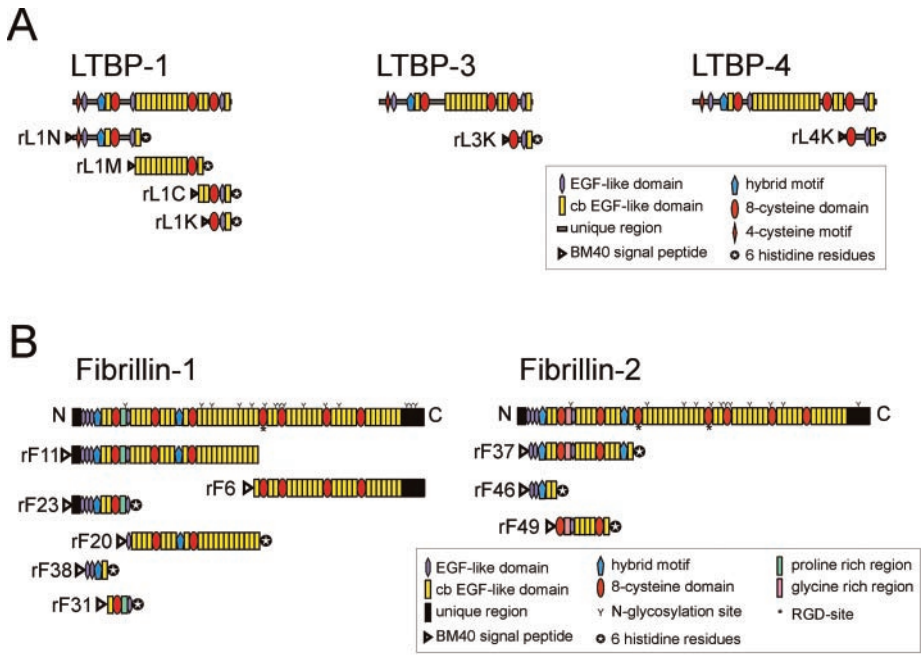

FIG. 1. **Schematic representations of the recombinant polypeptides used in this study.** *A*, LTBP-1, LTBP-3, and LTBP-4 polypeptides. *B*, fibrillin-1 and fibrillin-2 polypeptides.

with latent TGF-β, is not yet known. However, based upon the conformation of the fibrillin 8-Cys motif, binding of LTBP-1 to LAP has been proposed to occur through Cys^4 and Cys^7 or Cys^2 and Cys^6 (26). LTBP-3 and LTBP-4, but not LTBP-2, also interact with LAP (27).

LTBP-1 becomes immobilized into the extracellular matrix of tissue culture cells in a covalent manner involving tissue transglutaminase-mediated cross-linking of a region in the N-terminal sequence of LTBP to an undefined matrix protein (28). In tissue culture, LTBP-1 colocalizes with both fibronectin and fibrillin-1 (29, 30). LTBP-1 has been immunolocalized to fibrillin-containing microfibrils in the skin (31) and bone (30, 32) and to microfibrillar structures in the heart (33). LTBP-2 has also been immunolocalized to fibrillin-containing microfibrils (34) and exogenous LTBP-2 can be incorporated into the extracellular matrix by fibroblasts (35). Studies of mRNA have indicated that LTBPs are differentially expressed in various tissues. However, surveys of the distribution of LTBP-1 protein in tissues have not been conducted. Moreover, the relationship between LTBPs and fibrillins within microfibril structures is not understood. The investigations presented here were undertaken to determine whether LTBP-1 is present in extracted fibrillin microfibrils, whether LTBP-1 and fibrillin bind to each other, and if so, which regions of the two proteins interact.

EXPERIMENTAL PROCEDURES

Production of Recombinant Polypeptides—Recombinant human fibrillin polypeptides used in these investigations have been previously described and characterized (15, 22, 36–38). These are depicted schematically in Fig. 1*B*. All recombinant fibrillin polypeptides were expressed using 293 human embryonic kidney cells.

Full-length recombinant human LTBP-1 was expressed in insect cells as previously described (25). To make the expression construct rL1N, coding for Asn^{21} to Ile^{629} of LTBP-1, clone pACUW-51 was amplified with sense primer LTBP-1-1S (5′-CTGCTAGCAAACCACACTGGCCGCATCAAG-3′) introducing a *Nhe*I restriction site at the 5′ end, and antisense primer LTBP-1-1AS (5′-CTCGAGTCAATGATGATGATGATGATGTATGCAGTTAGTACCCTCCTC-3′), introducing the sequence for 6 histidine residues, a stop codon, and a *Xho*I restriction site at the 3′ end. The resulting *Nhe*I-*Xho*I fragment was subcloned into the expression vector pCEP/γ2III4, which contains the sequence for the BM40/SPARC signal peptide (39). To make the expression construct rL1M, coding for Asp^{588} to Phe^{1139} of LTBP-1, template was amplified with LTBP-1-2S (5′-CTGCTAGCAGATATTGATGAGTGTACTCAGCAGGTC-3′) and LTBP-1-2AS (5′-CTCTCGAGTCAATGATGATGATGATGATGAAAGCACTGCAGTTTCACAGG-3′). For rL1C, coding for Asp^{1097} to Glu^{1394}, the primer set LTBP-1-3S, (5′-CTGCTAGCAGATGCAGATGAATGCCTACTTTTTG-3′) and LTBP-1-3AS (5′-CTCTCGAGTCAATGATGATGATGATGCTCCAGGTCACTACTGTCTTTCTC-3′) was used. rL1K, coding for Arg^{1181} to Glu^{1394}, was amplified with LTBP-1-29S (5′-AGCTGCTAGCACGACCGGCTGAGTCAAACGAAC-3′) and LTBP-1-3AS. The correct in-frame insertion of all constructs and the sequence of PCR amplified products were confirmed by sequence analysis using a DNA sequencer (Applied Biosystems 373A).

The cDNA that was used to obtain expression construct rL4K (Ser^{1301}-Ala^{1587} of LTBP-4) was derived from normal human dermal fibroblast RNA by reverse transcriptase-PCR. PCR amplification of the cDNA used sense primer rL4K-S (5′-GATCGCTAGCATCCAACGAGAGCCAGAGCC-3′) and antisense primer rL4K-AS (5′-GAGTCTCGAGCTCAGTGATGGTGATGGTGATGGGCCCGGGGCCGTGCGG-3′), which introduced, respectively, a 5′ *Nhe*I restriction site and a 3′ sequence for 6 histidine residues, a stop codon, and an *Xho*I site. An 894-bp *Nhe*I-*Xho*I-restricted insert was subcloned into the expression vector pCEP4/γ2III4. The entire insert of the resulting construct, designated pCEPSP-rL4K, was then verified by DNA sequencing.

Mouse LTBP-3 specific sense primer L3-4F (5′-CCAGAAGGAGAGTCTGTGGC-3′) and antisense primer L3-4R (5′-TGTGGGCACTTGTGACACTT-3′) were designed based on the published sequence (10) and used in reverse transcriptase-PCR to amplify a fragment of mouse LTBP-3 cDNA (nucleotide 460–902, with the A in the translation start codon ATG designated as +1) from RNA extracted from 2T3 mouse osteoblast cells. This fragment was used as a probe for screening of a mouse heart cDNA library (Clontech). Several full-length LTBP-3 cDNA clones were isolated and cloned as *Eco*RI-*Eco*RI fragments into pBluescript SK (Stratagene) vector. Several errors were found in the original sequence by analyzing these cDNA clones.[2] To make the expression vector rL3K, encoding the region from the beginning of CR4 to the COOH terminus, a fragment of the cDNA was amplified by PCR using sense primer L3CR4C-S (5′-CGGCTAGCCCCAAAGAGACGTGAAGTG-3′) and antisense primer L3CR4CAS (5′-CCGCTCGAGTCAGTGGTGGTGGTGGTGG-CGGCGGCGCTGAGGCAC-3′), introducing an *Nhe*I site at the 5′ end and the sequence for 6 histidine residues, a stop codon, and an *Xho*I site at the 3′ end. The *Nhe*I-*Xho*I fragment was subcloned into the expression vector pCEP4/γ2III4. The correct orientation of the insert and the sequence of the PCR amplified fragment were verified by DNA sequencing.

Schematic representations of the LTBP constructs used for these studies are shown in Fig. 1*A*. For stable episomal expression, 293 EBNA cells (Invitrogen) were transfected with the expression plasmids by a calcium phosphate precipitation method as described previously (40). Purification of the recombinant peptides was accomplished using che-

[2] Y. Chen, unpublished result.

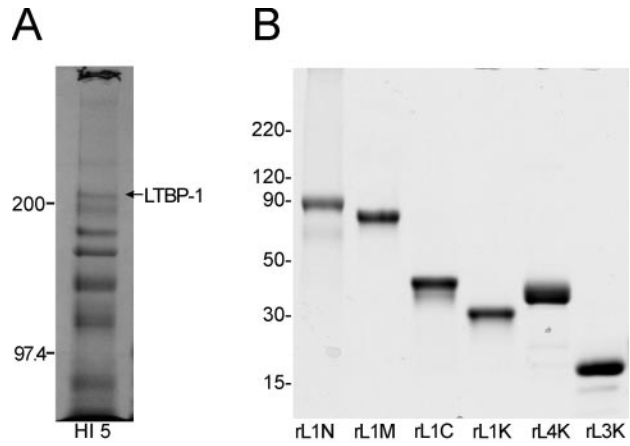

FIG. 2. **Coomassie Blue-stained gels of recombinant LTBP-1 polypeptides used in these investigations.** *A*, full-length LTBP-1 protein present in conditioned insect cell medium. LTBP-1 was identified by immunoblotting (data not shown). This sample, run without disulfide bond reducing agent, was used for blot overlay experiments. *B*, purified recombinant LTBP-1, LTBP-3, and LTBP-4 polypeptides. Samples were run on SDS-PAGE without reducing agent.

lating chromatography (HiTrap chelating, Amersham Biosciences) (15) followed by molecular sieve chromatography, using Superose 6 (Amersham Biosciences) in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl (TBS) for rL1M and rL1C or 50 mM Tris-HCl, pH 7.5, 1 M NaCl for rL1N.

Each of the expressed polypeptides was secreted into the medium resulting in yields of more than 0.5 μg/ml. N-terminal sequence analysis of the purified peptides using Edman degradation and amino acid analysis confirmed the expected polypeptide sequence and also demonstrated the purity of the peptides. SDS-PAGE analysis under nonreducing and reducing conditions revealed that the LTBP-1, LTBP-3, and LTBP-4 recombinant polypeptides were secreted as monomers from 293 cells (Fig. 2).

Antibodies and Immunoassays—Mouse monoclonal antibodies 201 and 69 to fibrillin-1 have been characterized previously (1, 13, 15, 38). Polyclonal anti-fibrillin-1 9543 was also characterized (18, 22). Monoclonal antibodies, 75G, and 42E, were generated using full-length LTBP-1 expressed by Sf9 insect cells (25). Mouse monoclonal antibody 246 against the TGF-β1 propeptide, known as the LAP, was purchased from R & D Systems (Minneapolis, MN), and rabbit polyclonal antibody 39 against human LTBP-1 was purchased from BD Pharmingen.

The specificity of monoclonal antibodies was determined by ELISA, as described (41). Recombinant fibrillin-1 subdomains, rF11 and rF6, and LTBP-1 subdomains, rL1N, rL1M, and rL1C, were used to coat microtiter plates at 10 μg/ml. The antibodies were diluted in TBS. For Western blot analysis, serum-free conditioned medium from normal skin fibroblasts was collected for 48 h (13). Proteins in the medium were precipitated, subjected to 7.5% SDS-PAGE, and analyzed by immunoblotting as previously described (42).

Extraction and Size Fractionation of LTBP-1 from Tissues—Extracts of normal adult human skin, bovine calf tendon, and human fetal membranes were prepared as follows. Nonexposed human skin (~1 g) was obtained as excess tissue from a skin grafting procedure. Bovine tendon (~1.5 g) was dissected from an 80-cm crown to rump fetal calf (~245 days gestation, almost full-term) obtained from a local slaughterhouse. A 40-ml suspension of fetal membranes, washed and homogenized as described (42), was also extracted as follows.

Tissue samples were minced, and the pellets were washed briefly with 50 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$, and 1 mM phenylmethylsulfonyl fluoride. The buffer was removed after centrifugation, and the pellet was extracted in 6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride for 72 h at 4 °C with vigorous shaking. The supernatant was collected after centrifugation, and the pellet was extracted in the same buffer for 48 h followed by centrifugation and another 24 h of extraction. The supernatants were pooled and concentrated to 5 ml using an Amicon concentrator (cut-off M_r = 30,000). Sieve chromatography under dissociative conditions was performed using a Sepharose CL-2B (Amersham Biosciences) molecular sizing column (90-ml total volume), equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl, pH 7.5, at a flow rate of 0.1 ml/min. The fractions were collected every 2 ml. Protein concentrations were determined using a BCA protein assay kit (Pierce) with bovine serum albumin as the standard. Dot blot analysis was performed using 2.5 μl of spotted fractions and either polyclonal antibody 39 or monoclonal antibodies 69, 75G, and 42E, as described (42). Western blot analysis was performed using two combined consecutive fractions. Guanidine hydrochloride was eliminated by ethanol precipitation as described (43).

Microfibrils were also isolated from tissues using crude collagenase (Sigma) digestion. Procedures used were the same as those we have previously detailed (42).

Immunolocalization Studies—Light and electron microscopic immunohistochemical procedures were the same as those we have previously described (44). Tissues were frozen in hexanes for light and confocal microscopy. Fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Sigma) was used for immunofluorescence microscopy, using 8-μm sections. For confocal microscopy, 25-μm sections were incubated with primary antibodies, followed by Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Stained sections were viewed with a Leica TCS SP2 confocal microscope and merged images were generated using Leica software. For electron microscopic immunolocalization, fresh tissue blocks were first incubated with dilutions of primary antibody, followed by a gold-conjugated second antibody, and then embedded and prepared for electron microscopy.

Binding Studies—Interactions between LTBP and fibrillin were investigated by solid phase ELISA binding or blot overlay assays. For ELISA binding assays, multiwell plates were coated with purified LTBP-1 peptides (50 nM, 100 μl/well) in 15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.2, at 4 °C overnight. Coated wells were blocked with 5% nonfat dry milk in TBS at room temperature for 1 h. Recombinant fibrillin-1 polypeptides were serially diluted 1:2 in 2% milk, TBS, containing 2 mM $CaCl_2$ or 5 mM EDTA, and incubated in the wells for 3 h. Monoclonal antibodies against soluble ligands were diluted in 2% milk, TBS and used to detect the bound ligands, after a final incubation with enzyme-conjugated secondary antibodies. Color reaction of the enzyme immunoassay was achieved using *p*-nitrophenyl phosphate (Sigma tablets) or 1 mg/ml 5-aminosalicylic acid. Absorbance was determined at 405 nm using a Titertek Multiskan.

For blot overlay assays, serum-free conditioned media was collected from High Five™ cells that were transfected with recombinant LTBP-1. 1 ml of media was precipitated using trichloroacetic acid, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk in TBS at room temperature for 1 h, the membrane was incubated with recombinant fibrillin peptides (50 μg/ml or 1 μM) in 2% nonfat milk in TBS at 4 °C overnight or room temperature for 3 h. Monoclonal antibodies diluted in 2% milk, TBS were used to detect the bound ligands, after a final incubation with enzyme-conjugated secondary antibodies. The blots were developed by color reaction using 4-chloro-1-naphthol (Bio-Rad).

RESULTS

LTBP-1 and Fibrillin-1 Are Co-distributed in Some Tissues—Although immunolocalization studies of LTBP-1 have been performed using cultured cells and tissues, surveys of tissue distribution have not been published. In addition, most published results have relied upon polyclonal antiserum 39. To immunolocalize LTBP-1 with greater confidence and to establish tissue distribution patterns, monoclonal antibodies were generated using purified full-length recombinant human LTBP-1 expressed in insect cells. Two monoclonal antibodies (mAb 75G and mAb 42E), which immunoblotted and immunoprecipitated LTBP-1 produced by insect cells (data not shown), were selected for further characterization. Epitopes for these antibodies were mapped by immunoblotting using four LTBP-1 recombinant polypeptides (Fig. 1*A*) expressed in 293 human embryonic kidney cells. MAb 75G recognized an epitope in rL1M, but did not bind to rL1N or rL1C (data not shown). mAb 42E bound to a site close to the C-terminal region in rL1C, not in rL1K (data not shown). In addition, mAb 75G and mAb 42E recognized authentic LTBP-1 present in the medium of cultured human fibroblasts (*NSF lane*, in Fig. 7). 75G and 42E displayed no reactivity with authentic fibrillin in fibroblast-conditioned medium, nor with rF11 and rF6, the two recombinant halves of fibrillin-1 (data not shown).

When tested using a panel of human tissues, mAb 75G and

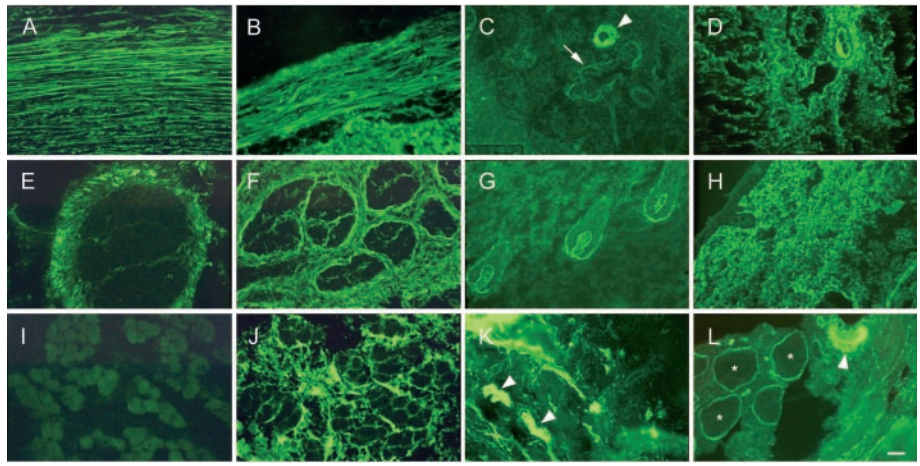

FIG. 3. **Immunofluorescence staining of tissues using antibodies specific for LTBP-1 (*A*, *C*, *E*, *G*, *I*, *K*, and *L*) and fibrillin-1 (*B*, *D*, *F*, *H*, and *J*).** Sections in *C* and *D* were 20-week human fetal lung, and sections in *K* and *L* were from an 8-year-old human toe. *A* (tendon), *B* (perichondrium), *E* and *F* (peripheral nerve), *G* and *H* (skin), and *I* and *J* (skeletal muscle) were 16-week human fetal tissues. The *arrow* in *C* indicates LTBP-1 staining around an airway. *Arrowheads* in *C*, *K*, and *L* point to blood vessels, and *asterisks* in *L* designate peripheral nerves. *Bar* = 50 μm.

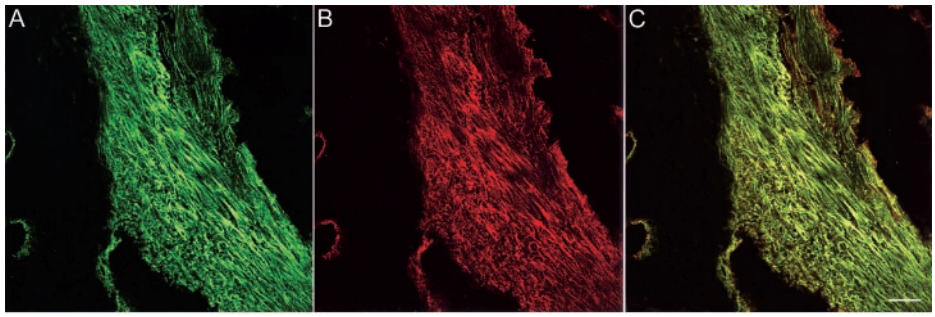

FIG. 4. **Colocalization of LTBP-1 and fibrillin-1 in tendon.** Confocal microscopy using LTBP-1 mAb 75G (*A*) and fibrillin-1 pAb 9543 (*B*) demonstrated colocalization of LTBP-1 and fibrillin-1 (*C*) in tendon of a 15-week fetal human foot specimen. *Bar* = 80 μm.

mAb 42E yielded similar immunohistochemical results. In tissues such as tendon, perichondrium, and blood vessels, the staining patterns for LTBP-1 and fibrillin-1 were apparently identical. In tendon and perichondrium, long fluorescent fibrils were evident after staining with anti-LTBP-1 (Fig. 3, *A* and *L*) or with anti-fibrillin-1 (Fig. 3*B*). Matrix around blood vessels was densely stained with antibodies to LTBP-1 (*arrowheads* in Fig. 3, *C*, *K*, and *L*) and fibrillin-1 (Fig. 3*D*). However, in other tissues such as skeletal muscle (Fig. 3, *I* compared with *J*) and lung (Fig. 3, *C* compared with *D*) where fibrillin-1 was abundant, LTBP-1 appeared to be absent or more limited in spatial distribution. In lung, bright staining for LTBP-1 was found around blood vessels but faint staining outlined the airways (Fig. 3*C*, *arrow*). In peripheral nerves, LTBP-1 appeared to be present primarily in the outer nerve sheath (Fig. 3, *E* and *L*, *asterisks*), whereas fibrillin-1 was found in all three connective tissue sheaths of the nerve (Fig. 3*F*). In skin, fibrillin-1 was present throughout the dermis (Fig. 3*H*), whereas LTBP-1 was much less abundant and was concentrated primarily in hair follicles (Fig. 3*G*) and scattered fibers (Fig. 3*K*).

These studies demonstrated that LTBP-1 is not present in all tissues containing fibrillin microfibrils. Co-distribution of LTBP-1 and fibrillin was prominent in tendon, perichondrium, cartilage, and all blood vessels. Partial co-distribution was found in tissues like skin, lung, and peripheral nerve.

Confocal and Electron Microscopic Immunolocalization Demonstrate Colocalization of Fibrillin-1 and LTBP-1—Tissues demonstrating strong LTBP-1 immunofluorescence were chosen for additional analyses to determine whether LTBP-1 and fibrillin-1 are colocalized. In the developing fetal foot, tendons were well labeled with LTBP-1 antibodies (Fig. 4*A*) and fibrillin-1 antibodies (Fig. 4*B*). The merged image (Fig. 4*C*) indicated that in the tendon most of the fibrillar staining directed by LTBP-1 antibodies was labeling the same fibrillar structures that were stained by fibrillin-1 antibodies.

In addition, neonatal foreskin, fetal bovine tendon, and fetal bovine aorta were immunolabeled with anti-LTBP-1 mAb 75G and examined by electron microscopy. As has been reported, immunogold labeling was observed on microfibrils (Fig. 5, *A* and *C*). However, in contrast to the periodic labeling obtained with antibodies to fibrillin-1 (1, 15) (Fig. 5*B*) and with polyclonal antibodies to LTBP-1 (30), LTBP-1 antibody-directed gold labeling was sparse and irregular (Fig. 5, *A* and *C*).

Microfibril Extracts from Tissues Do Not Contain LTBP-1—To determine whether LTBP-1 is contained within microfibrils or is a microfibril-associated molecule, extraction studies were performed. Bovine calf tendon, human fetal membranes (data not shown), and human skin, tissues in which LTBP-1 co-localizes with fibrillin, were used. Microfibrils were extracted either by crude collagenase digestion or denaturation in guanidine HCl. After fractionation using Sepharose CL-2B chromatography, fibrillin microfibrils were eluted in the void volume fraction both from the collagenase digests (human fetal membranes and human skin) and the guanidine HCl extracts (human fetal membranes and fetal bovine tendon), as shown by dot blot analysis with several anti-fibrillin-1 antibodies and rotary shadowing (Fig. 6 and data not shown). Interestingly, no immunoreactivity with LTBP-1 antibodies (mAb 75G, mAb 42E, or pAb 39) was detected in the microfibril fractions from any of the tissues examined, including collagenase and guanidine extracts of human skin and bovine tendon (Figs. 6 and 7) and collagenase and guanidine extracts of human fetal membranes (data not shown). These results suggested that LTBP-1 is not an integral structural component of the beaded string microfibril.

When LTBP-1 was extracted from tendon and skin using guanidine HCl, Western and dot blots using mAb 75G (data not shown), mAb 42E and pAb 39 (data not shown) detected LTBP-1 in the included fractions of the Sepharose CL-2B column with a molecular mass close to 200 kDa (Fig. 7). This molecular mass, similar to that of authentic LTBP-1 found in normal skin fibroblasts (*NSF lane*, in Fig. 7), corresponds to the

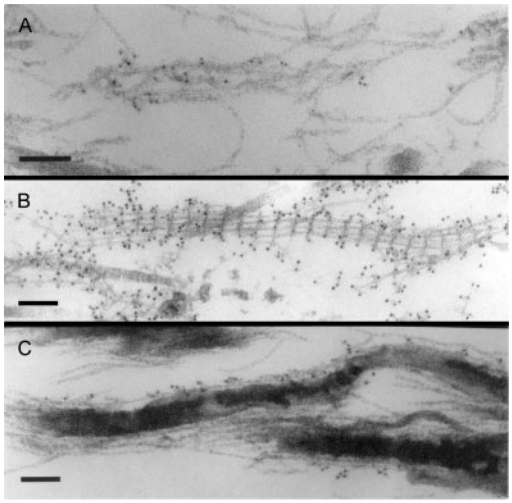

FIG. 5. **Immunolocalization of LTBP-1.** MAb 75G, followed by immunogold-labeled microfibril bundles in neonate skin (*A*) as well as elastic fiber microfibrils in fetal bovine aorta (*C*). The *middle panel* (*B*) demonstrates typical periodic labeling of microfibrils in neonate skin using anti-fibrillin-1 mAb 69. *Bars* = 100 nm.

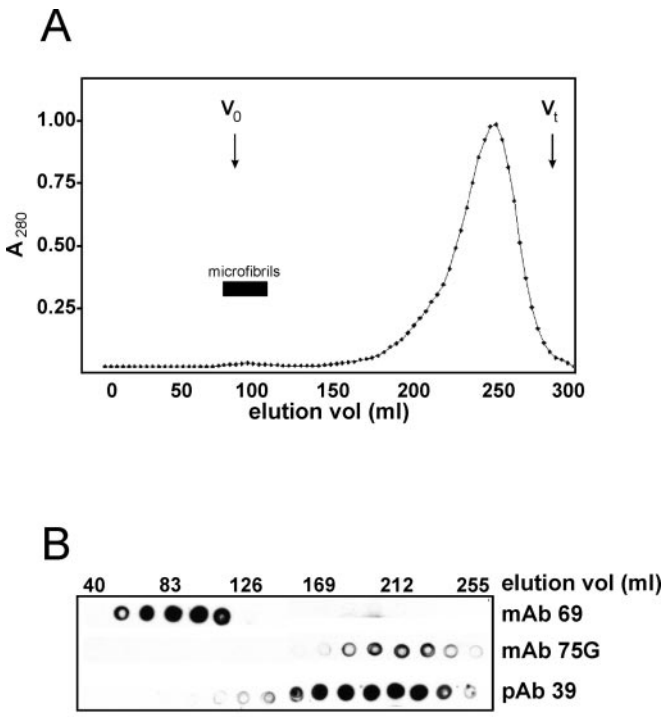

FIG. 6. **Analyses of collagenase-digested microfibrils from human skin.** *A*, Sepharose CL-2B chromatograph, indicating the elution position of beaded strings of microfibrils, identified by rotary shadowing electron microscopy. *B*, dot blot analysis demonstrated the presence of fibrillin-1 (mAb 69) in the microfibril fractions but not in subsequent fractions; dot blot analyses of the same fractions with mAb 75G and pAb 39 failed to detect LTBP-1 in the microfibril fractions, but revealed LTBP-1 in subsequent fractions. Each row contains replicate dotted samples from the microfibril fractions through the later eluting peak.

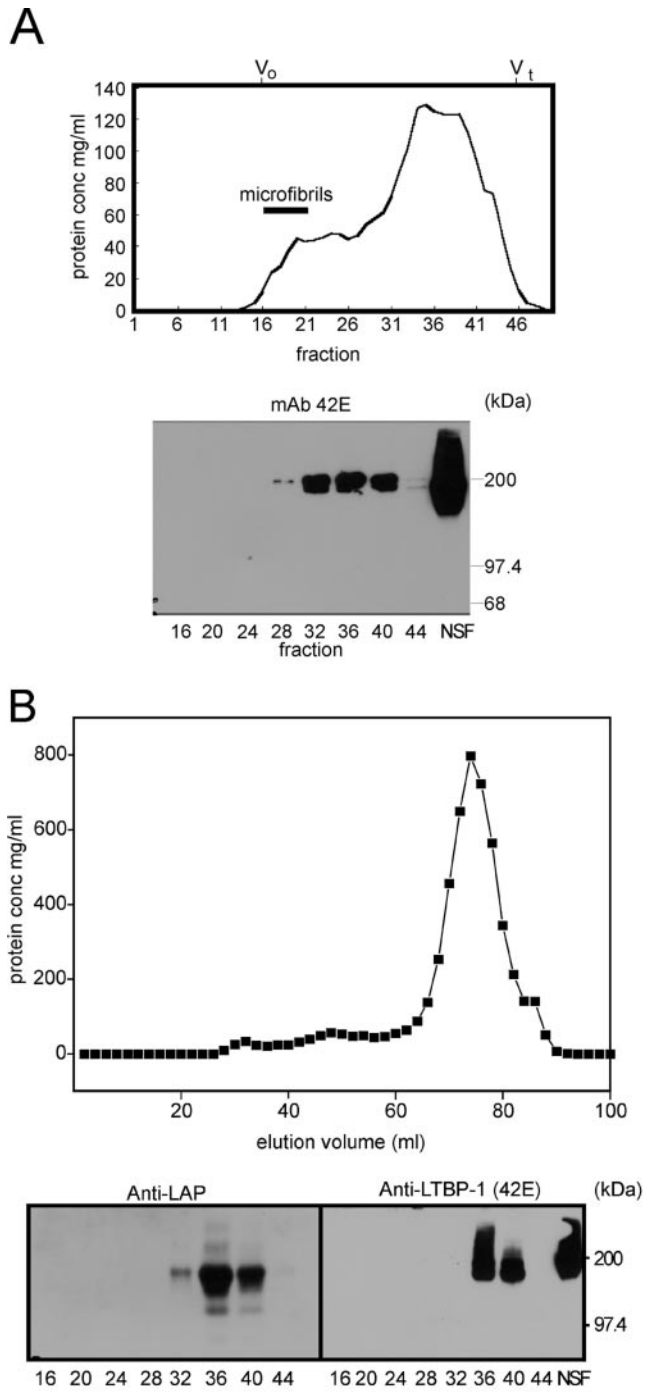

FIG. 7. **Analyses of guanidine HCl extracted tissues.** *A*, Sepharose CL-2B chromatography of extract from fetal bovine tendon followed by Western blotting of fractions revealed LTBP-1 in fractions 28–44 but not in the earlier microfibril containing fractions (16–20). *B*, Sepharose CL-2B chromatography of human skin followed by Western blotting of fractions revealed both LAP and LTBP-1 in fractions 32–40. Each fraction contains 2 ml (fractions 32–40 are equivalent to 64–80 ml of elution volume). Gels were run without reducing agent. Partially purified authentic LTBP-1 from fibroblast cell culture medium (*NSF*) was applied as a control and to demonstrate molecular mobility in these gels. V_0 and V_t are the same for *A* and *B*.

complex of LTBP and LAP (25). Indeed, in human skin extracts, LTBP-1 was detected as a complex with LAP (Fig. 7*B*). The small apparent differences in molecular masses of the LTBP-1 complexes from these two tissues may reflect either the presence of differentially spliced isoforms or proteolytic degradation in the case of skin.

LTBP-1 Binds to Fibrillins—Blot overlay assays were utilized to screen for regions of fibrillins that might interact with LTBP-1. Insect cell-conditioned medium containing full-length recombinant human LTBP-1 (and multiple other proteins) (Fig. 2*A*) was subjected to SDS-PAGE, transferred to nitrocellulose, and used as substrate for the binding assays. Recombinant human fibrillin-1 and fibrillin-2 polypeptides (Fig. 1*B*) were purified and used as ligands for these assays. Detection of bound ligands was accomplished by using specific mAbs to fibrillins or by using a monoclonal antibody that reacts with the

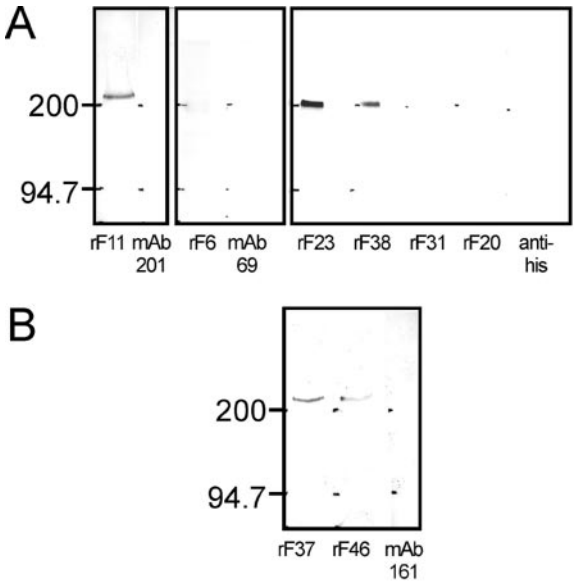

FIG. 8. **Blot overlay assays of immobilized recombinant full-length LTBP-1 in insect cell medium (Fig. 2*A*) incubated with fibrillin ligands and antibody probes.** Control lanes, where soluble ligand was omitted but antibody probe was incubated with the blot, are identified with the antibody used for detection. *A*, recombinant fibrillin-1 ligands that bound to LTBP-1 included rF11 and subdomains rF23 and rF38. rF11 was detected with mAb 201. Detection of rF6 was with mAb 69. Antihistidine was used to detect rF23, rF38, rF31, and rF20. *B*, recombinant fibrillin-2 polypeptides rF37 and rF46, detected by mAb 161, also bound to LTBP-1.

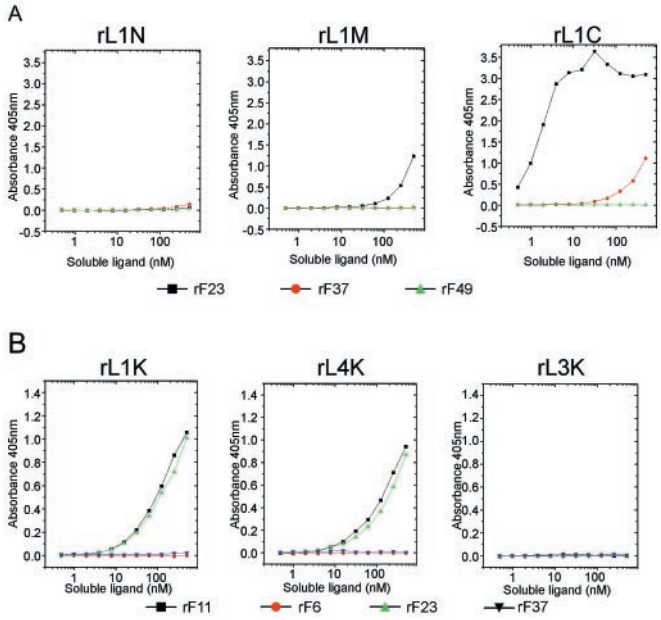

FIG. 9. **ELISA binding assays using recombinant LTBP-1 polypeptides and recombinant fibrillin ligands.** *A*, fibrillin ligands bound preferentially to rL1C and demonstrated higher affinity of fibrillin-1 (rF23) compared with fibrillin-2 (rF37). *B*, fibrillin-1 ligands (rF11 and rF23, but not rF6) bound equally well to rL1K and rL4K, but did not bind to rL3K.

histidine tag present at the C-terminal end of some fibrillin recombinant polypeptides. When the large N- and C-terminal halves of fibrillin-1 (rF11 and rF6, respectively) were tested, rF11 bound to LTBP-1 but rF6 did not (Fig. 8*A*). When subregions of rF11 were tested, rF23 and rF38 were positive, whereas other regions of rF11 (rF20 and rF31) were negative (Fig. 8*A*). These data localized the binding site in fibrillin-1 to the four domains contained in rF38 (see Fig. 1*B*). When fibrillin-2 recombinant polypeptides were used as ligands, rF37 and rF46 interacted with LTBP-1 (Fig. 8*B*), suggesting that the homologous region of fibrillin-2 was also capable of binding to LTBP-1.

To define the binding site in LTBP-1, recombinant LTBP-1 polypeptides, rL1N, rL1M, rL1C, and rL1K were tested in both blot overlay (data not shown) and ELISA. Results indicated that the major binding site for fibrillin-1 is contained in the C-terminal region of LTBP-1, rL1C (Fig. 9*A*). A small subdomain of rL1C, rL1K, contained the fibrillin-1 binding site (Fig. 9*B*). When ELISAs were performed to compare the binding of fibrillin-1 and fibrillin-2 with LTBP-1, fibrillin-1 polypeptides displayed much higher affinities for LTBP-1 than fibrillin-2 polypeptides (Fig. 9*A*). Incubation of ligands in the presence of EDTA did not affect the LTBP-1/fibrillin interaction (data not shown).

To test whether homologous regions of other LTBPs might perform similar functions, recombinant LTBP-3 and LTBP-4 polypeptides were expressed and purified. In ELISA binding assays, fibrillin ligands interacted equally well with rL1K and rL4K, but not with rL3K (Fig. 9*B*). As observed for the binding to rL1K, the same region of fibrillin-1 (rF23 but not rF31) contained the binding site for rL4K (Fig. 9*B*). Additional studies that precisely define the binding site in LTBP-1 and LTBP-4 are required to understand why these bind to fibrillin and LTBP-3 does not.

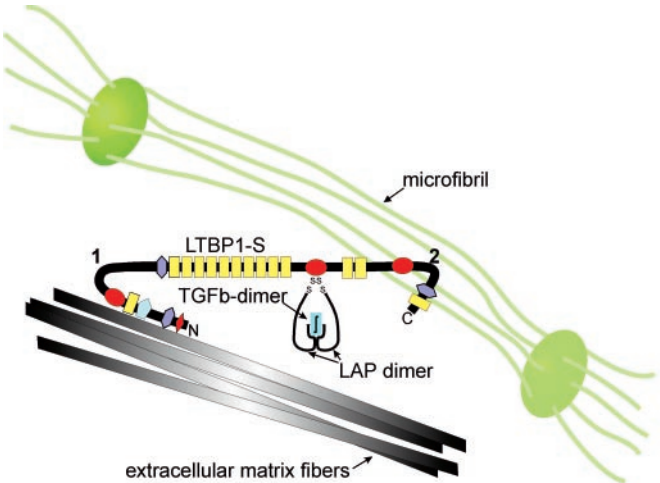

FIG. 10. **Proposed model of LTBP-1 in relation to fibrillin microfibrils and other extracellular matrix components.** This model is adapted from Ref. 24. The N-terminal end of LTBP-1 is transglutaminase cross-linked to the extracellular matrix. The small latent TGF-β complex is bound covalently to CR3 of LTBP-1. The numbers, *1* and *2*, mark protease-sensitive sites in LTBP-1. Interaction between LTBP-1 and fibrillin takes place between the C-terminal region of LTBP-1 and the N-terminal region of fibrillin. Latent TGF-β may be targeted and sequestered by the interactions occurring at both the N-terminal and C-terminal ends of LTBP-1.

DISCUSSION

Beaded string structures have been extracted from various tissues and shown by immunolabeling to contain periodically spaced fibrillin molecules (14, 15). Together with the known extended structure of fibrillin (13), these studies have suggested that the strings connecting the globular beads in the beaded microfibrils are fibrillin molecules. However, molecules similar in shape to fibrillins may also constitute the string-like connecting filaments in microfibrils. LTBPs are candidate molecules to be considered, because they have been immunolocalized to microfibrils and because they are structurally homologous to fibrillins.

Procedures for extraction of microfibrils are now routinely utilized (45, 46). These procedures rely upon crude collagenase digestion of tissues to release microfibrils from the insoluble

extracellular matrix environment. We have recently found that highly purified bacterial collagenase does not release fibrillin microfibrils from tissues, suggesting that microfibrils are not simply trapped in the matrix by collagen fibers (42). Further digestion with crude collagenase, which contains other proteases, is required to release microfibrils. These results indicate that molecules linking microfibrils to the insoluble extracellular matrix must be degraded to release microfibrils. We have demonstrated that versican is one of these molecules (42).

In the current investigations, we have shown that LTBP-1 is not a component of crude collagenase-digested microfibrils. Therefore, LTBP-1 is likely not to be a connecting string in these microfibrils. Because LTBP-1, unlike fibrillins, contains several regions of protein sequences that may provide sensitive sites for proteolysis (47), LTBP-1 may have been degraded in the digestion protocol. Hence, it is possible that LTBP-1 was present in the collagenase-digested beaded microfibrils as degraded stubs but undetected with the available antibodies. To address this possibility, microfibrils were extracted from tissues using nondegradative procedures and fractionated by sieve chromatography. Denaturing guanidine HCl extractions released small amounts of microfibrils found in fractions that failed to react with antibodies specific for LTBP-1. However, LTBP-1 immunoreactive materials close to the size of complexes containing full-length LTBP-1 and LAP were detected in the guanidine extracts. Taken together, these data suggest that LTBP-1 is not substantially cross-linked into the beaded string microfibril structure and may not be an integral component of microfibrils.

Results from immunoelectron microscopic analyses of tissues are consistent with the conclusion that LTBP-1 may not be an integral component of microfibrils. In the present studies, labeling of tissues with anti-LTBP-1 was always sparse and never periodic. In contrast, immunolabeling of calvarial cell cultures revealed some periodic labeling of microfibrils using an anti-LTBP-1 serum (30). It may be that cells in culture can secrete and deposit larger amounts of LTBP-1 into the extracellular matrix than was observed in the tissues we sampled.

Because immunolocalization experiments indicated that LTBP-1 is associated with microfibrils, binding studies were conducted to determine whether LTBP-1 interacts with fibrillin. Binding was detected using well characterized recombinant fibrillin polypeptides in blot overlay assays with either full-length recombinant LTBP-1 or authentic LTBP-1 present in fibroblast cell culture medium. ELISA binding studies demonstrated that the interaction was not calcium-dependent and that the interaction between fibrillin-1 and LTBP-1 appeared to be much stronger than the interaction between fibrillin-2 and LTBP-1. These studies mapped the binding site for fibrillin to the C-terminal region of LTBP-1, a region corresponding to the domains contained in peptide rL1K. This result is consistent with a study showing that the C-terminal region of LTBP-1 can associate with extracellular matrix in cell culture experiments (48). The binding site for LTBP-1 in fibrillin-1 is contained within four domains (rF38) close to the N-terminal end of fibrillin-1. The homologous region of fibrillin-2 (rF46) may also mediate binding to LTBP-1, but with lower affinity than fibrillin-1.

Because LTBP-1 is similar in structure and function to other LTBP family members, it seemed plausible that other LTBPs might interact with fibrillins, using binding sites homologous to rL1K. To test this hypothesis, recombinant polypeptides were constructed for mouse LTBP-3 (rL3K) and human LTBP-4 (rL4K). Binding by fibrillin to the LTBP-4 polypeptide was strong, equivalent to the interaction with LTBP-1, and specifically mediated by the same region in fibrillin. However, the comparable C-terminal region of LTBP-3 did not appear to bind to the N-terminal region of fibrillin-1 (rF23), suggesting that LTBP-3 may specifically bind to other sites in fibrillins or to another fibrillin, that it uses other domains to mediate interactions with fibrillins, or that it does not interact with fibrillins. Immunolocalization of LTBP-3 and LTBP-4 to microfibrils in tissues has not yet been performed.

Based on these findings, we propose a model of LTBP-1 in association with the extracellular matrix and with fibrillin networks (Fig. 10). The N-terminal region of LTBP-1 is cross-linked to the extracellular matrix (28). In our investigations, the N-terminal third of LTBP-1 (rL1N) did not display any binding to fibrillin-1 or fibrillin-2. Therefore, it is likely that the N-terminal region of LTBP-1 is cross-linked to components other than fibrillins. We cannot, however, exclude that the N-terminal region of LTBP-1 interacts with components of microfibrils other than the fibrillins. Because our studies of extracted microfibrils did not demonstrate the presence of LTBP-1 in the beaded strings, we suggest that the N-terminal region of LTBP-1 is associated with matrix molecules close to microfibrils, but not present within the beaded microfibril.

Networks such as the one depicted in Fig. 10 can be modified *in vivo* in a tissue-specific manner. LTBPs may be present in different tissues, as shown for LTBP-1 in this investigation. Fibrillins are also present in tissues in differential temporal and spatial patterns. Additional information regarding tissue distributions of the fibrillins and the LTBPs is required. However, we can speculate that in skeletal muscle, for example, fibrillin-1 may interact with LTBP-4, because LTBP-1 is not present in this tissue and LTBP-4 is highly expressed in skeletal muscle (11). In the developing and postnatal lung, fibrillin-1 may interact with LTBP-1 in blood vessels and cartilage and with LTBP-1 and LTBP-4 in the airways.

Based upon our findings, we propose that interactions between LTBP-1 and fibrillin-1 may stabilize latent TGF-β complexes in the extracellular matrix. It is possible that loss of fibrillin-1 would abolish this stabilization and lead to activation of TGF-β. Activation of TGF-β was recently demonstrated in the lungs of fibrillin-1-deficient mice.[3] In Marfan syndrome, caused by mutations in fibrillin-1, loss of fibrillin-1 microfibrils leads to multiple phenotypic features that may result in part from activation of TGF-β. This hypothesis will be tested in future investigations.

Acknowledgment—We thank Sara Tufa for excellent technical assistance.

REFERENCES

1. Sakai, L. Y., Keene, D. R., and Engvall, E. (1986) *J. Cell Biol.* **103,** 2499–2509
2. Maslen, C. L., Corson, G. M., Maddox, B. K., Glanville, R. W., and Sakai, L. Y. (1991) *Nature* **352,** 334–337
3. Corson, G. M., Chalberg, S. C., Dietz, H. C., Charbonneau, N. L., and Sakai, L. Y. (1993) *Genomics* **17,** 476–484
4. Pereira, L., D'Alessio, M., Ramirez, F., Lynch, J. R., Sykes, B., Pangilinan, T., and Bonadio, J. (1993) *Hum. Mol. Genet.* **2,** 961–968
5. Lee, B., Godfrey, M., Vitale, E., Hori, H., Mattei, M. G., Sarfarazi, M., Tsipouras, P., Ramirez, F., and Hollister, D. W. (1991) *Nature* **352,** 330–334
6. Zhang, H., Apfelroth, S. D., Hu, W., Davis, E. C., Sanguineti, C., Bonadio, J., Mecham, R. P., and Ramirez, F. (1994) *J. Cell Biol.* **124,** 855–863
7. Miyazono, K., Hellman, U., Wernstedt, C., and Heldin, C. H. (1988) *J. Biol. Chem.* **263,** 6407–6415
8. Kanzaki, T., Olofsson, A, Morén, A., Wernstedt, C., Hellman, U., Miyazono, K., Claesson-Welsh, L., and Heldin, C. H. (1990) *Cell* **61,** 1051–1061
9. Morén, A., Olofsson, A., Stenman, G., Sahlin, P., Kanzaki, T., Claesson-Welsh, L., ten Dijke, P., Miyazono, K., and Heldin, C. H. (1994) *J. Biol. Chem.* **269,** 32469–32478
10. Yin, W., Smiley, E., Germiller, J., Mecham, R. P., Florer, J. B., Wenstrup, R. J., and Bonadio, J. (1995) *J. Biol. Chem.* **270,** 10147–10160
11. Giltay, R., Kostka, G., and Timpl, R. (1997) *FEBS Lett.* **411,** 164–168
12. Saharinen, J., Taipale, J., Monni, O., and Keski-Oja, J. (1998) *J. Biol. Chem.*

[3] E. R. Neptune, P. A. Frischmeyer, D. A. Arking, L. Myers, T. E. Bunton, B. Gayraud, F., Ramirez, D. Rifkin, R. Ono, L. Sakai, and H. C. Dietz, submitted for publication.

273, 18459–18469
13. Sakai, L. Y., Keene, D. R., Glanville, R. W., and Bächinger, H. P. (1991) *J. Biol. Chem.* **266,** 14763–14770
14. Keene, D. R., Maddox, B. K., Kuo, H. J., Sakai, L. Y., and Glanville, R. W. (1991) *J. Histochem. Cytochem.* **39,** 441–449
15. Reinhardt, D. P., Keene, D. R., Corson, G. M., Pöschl, E., Bächinger, H. P., Gambee, J. E., and Sakai, L. Y. (1996) *J. Mol. Biol.* **258,** 104–116
16. Hollister, D. W., Godfrey, M., Sakai, L. Y., and Pyeritz, R. E. (1990) *N. Engl. J. Med.* **323,** 152–159
17. Dietz, H. C., Cutting, G. R., Pyeritz, R. E., Maslen, C. L., Sakai, L. Y., Corson, G. M., Puffenberger, E. G., Hamosh, A., Nanthakumar, E. J., Curristin, S. M., Stetten, G., Meyers, D. A., and Francomano, C. A. (1991) *Nature* **352,** 337–339
18. Pereira, L., Andrikopoulos, K., Tian, J., Lee, S. Y., Keene, D. R., Ono, R. N., Reinhardt, D. P., Sakai, L. Y., Jensen-Biery, N., Bunton, T., Dietz, H. C., and Ramirez, F. (1997) *Nat. Genet.* **17,** 218–222
19. Pereira, L., Lee, S. Y., Gayraud, B., Andrikopoulos, K., Shapiro, S. D., Bunton, T., Biery, N. J., Dietz, H. C., Sakai, L. Y., and Ramirez, F. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3819–3823
20. Zhang, H., Hu, W., and Ramirez, F. (1995) *J. Cell Biol.* **129,** 1165–1176
21. Putnam, E. A., Zhang, H., Ramirez, F., and Milewicz, D. M. (1995) *Nat. Genet.* **11,** 456–458
22. Charbonneau, N. L., Dzamba, B. J., Ono, R. N., Keene, D. R., Reinhardt, D. P., and Sakai, L. Y. (November 11, 2002) *J. Biol. Chem.* 10.1074/jbc.M209201200
23. Arteaga-Solis, E., Gayraud, B., Lee, S. Y., Shum, L., Sakai, L. Y., and Ramirez, F. (2001) *J. Cell Biol.* **154,** 275–281
24. Saharinen, J., Taipale, J., and Keski-Oja, J. (1996) *EMBO J.* **15,** 245–253
25. Gleizes, P. E., Beavis, R. C., Mazzieri, R., Shen, B., and Rifkin, D. B. (1996) *J. Biol. Chem.* **271,** 29891–29896
26. Yuan, X., Downing, A. K., Knott, V., and Handford, P. A. (1997) *EMBO J.* **16,** 6659–6666
27. Saharinen, J., and Keski-Oja, J. (2000) *Mol. Biol. Cell* **11,** 2691–2704
28. Nunes, I., Gleizes, P. E., Metz, C. N., and Rifkin, D. B. (1997) *J. Cell Biol.* **136,** 1151–1163
29. Taipale, J., Saharinen, J., Hedman, K., and Keski-Oja, J. (1996) *J. Histochem. Cytochem.* **44,** 875–889
30. Dallas, S. L., Keene, D. R., Bruder, S. P., Saharinen, J., Sakai, L. Y., Mundy, G. R., and Bonewald, L. F. (2000) *J. Bone Miner. Res.* **15,** 68–81
31. Raghunath, M., Unsold, C., Kubitscheck, U., Bruckner-Tuderman, L., Peters, R., and Meuli, M. (1998) *J. Invest. Dermatol.* **111,** 559–564
32. Dallas, S. L., Miyazono, K., Skerry, T. M., Mundy, G. R., and Bonewald, L. F. (1995) *J. Cell Biol.* **131,** 539–549
33. Nakajima, Y., Miyazono, K., Kato, M., Takase, M., Yamagishi, T., and Nakamura, H. (1997) *J. Cell Biol.* **136,** 193–204
34. Gibson, M. A., Hatzinikolas, G., Davis, E. C., Baker, E., Sutherland, G. R., and Mecham, R. P. (1995) *Mol. Cell. Biol.* **15,** 6932–6942
35. Hyytiäinen, M., Taipale, J., Heldin, C. H., and Keski-Oja, J. (1998) *J. Biol. Chem.* **273,** 20669–20676
36. Reinhardt, D. P., Sasaki, T., Dzamba, B. J., Keene, D. R., Chu, M. L., Göhring, W., Timpl, R., and Sakai, L. Y. (1996) *J. Biol. Chem.* **271,** 19489–19496
37. Reinhardt, D. P., Gambee, J. E., Ono, R. N., Bächinger, H. P., and Sakai, L. Y. (2000) *J. Biol. Chem.* **275,** 2205–2210
38. Keene, D. R., Jordan, C. D., Reinhardt, D. P., Ridgway, C. C., Ono, R. N., Corson, G. M., Fairhurst, M., Sussman, M. D., Memoli, V. A., and Sakai, L. Y. (1997) *J. Histochem. Cytochem.* **45,** 1069–1082
39. Mayer, U., Pöschl, E., Gerecke, D. R., Wagman, D. W., Burgeson, R. E., and Timpl, R. (1995) *FEBS Lett.* **365,** 129–132
40. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752
41. Sakai, L. Y., Engvall, E., Hollister, D. W., and Burgeson, R. E. (1982) *Am. J. Pathol.* **108,** 310–318
42. Isogai, Z., Aspberg, A., Keene, D. R., Ono, R. N., Reinhardt, D. P., and Sakai, L. Y. (2002) *J. Biol. Chem.* **277,** 4565–4572
43. Oike, Y., Kimata, K., Shinomura, T., Nakazawa, K., and Suzuki, S. (1980) *Biochem. J.* **191,** 193–207
44. Sakai, L. Y., and Keene, D. R. (1994) *Methods Enzymol.* **245,** 29–52
45. Kielty, C. M., Cummings, C., Whittaker, S. P., Shuttleworth, C. A., and Grant, M. E. (1991) *J. Cell Sci.* **99,** 797–807
46. Baldock, C., Koster, A. J., Ziese, U., Rock, M. J., Sherratt, M. J., Kadler, K. E., Shuttleworth, C. A., and Kielty, C. M. (2001) *J. Cell Biol.* **152,** 1045–1056
47. Taipale, J., Lohi, J., Saharinen, J., Kovanen, P. T., and Keski-Oja, J. (1995) *J. Biol. Chem.* **270,** 4689–4696
48. Unsold, C., Hyytiainen, M., Bruckner-Tuderman, L., and Keski-Oja, J. (2001) *J. Cell Sci.* **114,** 187–197

Latent Transforming Growth Factor β-binding Protein 1 Interacts with Fibrillin and Is a Microfibril-associated Protein

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J. Biol. Chem. 2003, 278:2750-2757.
doi: 10.1074/jbc.M209256200 originally published online November 11, 2002
