Recombinant Latent Transforming Growth Factor β-binding Protein 2 Assembles to Fibroblast Extracellular Matrix and Is Susceptible to Proteolytic Processing and Release*

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Latent transforming growth factor β-binding protein 2 (LTBP-2) belongs to the fibrillin-LTBP family and is a component of 10-nm microfibrils. LTBP-2 consists mainly of domains of 8-cysteine and EGF-like repeats linked by proline-rich regions. To characterize the biochemical properties of LTBP-2, its assembly to the extracellular matrix, and its proteolytic release from the matrix, LTBP-2 was expressed recombinantly in Chinese hamster ovary cells and purified to homogeneity. Purified LTBP-2 bound calcium and was glycosylated at the central domain of EGF-like repeats. Antibodies made against the recombinant LTBP-2 decorated fibrillar structures in fibroblast extracellular matrix. Treatment of matrices with plasmin or elastase released a soluble ~160-kDa LTBP-2 fragment. Processing of LTBP-2 was studied by treating purified LTBP-2 with plasmin or porcine pancreatic elastase. LTBP-2 was processed with these proteases initially to a ~160-kDa fragment, and with higher concentrations to a protease-resistant ~120-kDa fragment. Processing sites were localized by amino acid sequencing to proline-rich regions at the N-terminal part of LTBP-2, suggesting that the matrix binding sites locate to the N-terminal ~500 amino acids of LTBP-2. Purified and biotinylated LTBP-2 could be assembled to fibrillar structures in fibroblast extracellular matrix during cell cultivation, indicating that LTBP-2 assembly to the matrix is not strictly linked to cells that make it and suggesting that microfibril assembly may involve soluble intermediates.

Elastic tissue 10-nm microfibrils are composed of several proteins, including the gene products of fibrillin/LTBP family, which include fibrillins 1 and 2, and latent TGF-β-binding proteins 1–4 (1–6). Fibrillins are the main components of 10-nm elastic tissue microfibrils, but there are other integral components present in these fibers, including microfibril-associated glycoproteins 1 and 2 and associated microfibril protein (AMP) (7–11). Mutations in fibrillin-1 are responsible for a dominantly inherited connective tissue disease, Marfan syndrome (12–14), whereas those in fibrillin-2 cause a related disorder, congenital contractual arachnodactyly (15). Microfibrils have been suggested to function as a scaffold for elastin deposition to elastic fibers, but they are present also in matrix structures devoid of elastin (1, 10, 16). In fact, gene-targeted mice expressing only mutated fibrillin-1 seem to assemble normal elastin matrix. These mice die of cardiovascular disorders at the age of 3 weeks (17).

Fibrillins and LTBP-2s have similar domain structures. They are composed mainly of EGF and 8-cysteine repeats. EGF-like repeats are present in multiple extracellular proteins, and they presumably stabilize protein structure and mediate protein-protein interactions. The 8-Cys repeats are much more rare, and so far they have been found only in LTBP-2s and fibrillins. The 8-Cys repeats can bind to heterologous proteins via disulfide bridges, and the third 8-Cys repeat of LTBP-1 mediates the binding of LTBP-1 to latent TGF-β (18).

TGF-βs are secreted from most cells as large latent complexes, which contain the homodimeric mature growth factor, TGF-β latency-associated protein, and a LTBP. LTBP-1 augments the secretion of latent TGF-β from cells (19) and mediates its binding to the extracellular matrix (20). Because there appears to be a molar excess of LTBP-1 over TGF-βs, it is likely that LTBP-1s have a structural role in the ECM, in addition to targeting growth factors to the ECM (21, 22). LTBP-1 associates with the 10-nm microfibrils in the ECM. It also associates with thicker, ~50-nm cell surface-associated fibers containing fibronectin (21, 23). Fibrillin-1 and fibronectin can be found in similar structures in the endothelial cell pericellular matrix (16). LTBP-2 is a structural component of the microfibrils of bovine lamintum nuchae (4). The association of LTBP-3 with the ECM has not been analyzed, but LTBP-4 is also a component of the ECM (6).

Extracellular matrix is assembled, in principle, by two different mechanisms, self-assembly, e.g. collagen fibers (24), and cell-mediated assembly. Cell-mediated assembly can be further divided into two types on the basis of whether the cells are assembling soluble monomers diffusing around cells, as in the case of fibronectin (25), or the assembly is coupled directly to the surfaces of cells that make the monomers, which may be the mechanism for elastin matrix assembly (10). It is not clear whether microfibril assembly is a cell-mediated or a self-assembly process. Soluble members of the fibrillin/LTBP family have not been described in blood or tissue fluids. Fibrillins and LTBP-1, 2, and 4 contain an RGD sequence, which mediates the adhesion of cells to the extracellular matrix ligands via integrins (26), and integrin α,β3 mediates the binding of fibril-1 to cells (27). Microfibrils seem to associate with endothelial cell surfaces near structures called “dense plaques” (16, 28). However, it is unclear whether the association of microfibrils to...
intergins mediates cell adhesion to the ECM or is an event of microfibril assembly.

Microfibrils are catabolized at least by elastase and certain other serine proteases. Of the integral microfibril proteins, serine proteases can cleave at least fibrillin-1 (29). LTBP-1 can be released from the extracellular matrix as a ~120-kDa fragment together with latent TGF-β, for example, by treatment of the matrices with plasmin or leukocyte elastase (20, 30). The N-terminal part of LTBP-1 mediates its binding to the ECM, whereas an 8-Cys repeat near the C terminus binds latent TGF-β (18). The exact cleavage site(s) of serine proteinasin LTBP-1 has not been determined so far.

To characterize the assembly of LTBP-2 to the matrix and its catabolism, recombinantly produced LTBP-2 was purified from the conditioned medium of an overexpressing Chinese hamster ovary (CHO) cell line. It was found to be a calcium-binding glycoprotein with an apparent molecular mass of 290 kDa. LTBP-2 was susceptible to release from the matrix by proteolysis, and the major proteolytic processing sites were identified by amino acid sequencing. The assembly of soluble LTBP-2 monomers with the sodium deoxycholate-insoluble fibers on the fibroblast extracellular matrix was observed, both with recombinant LTBP-2 from the conditioned medium of overexpressing CHO cells and with purified biotin-labeled LTBP-2.

**EXPERIMENTAL PROCEDURES**

**Materials**—All enzymes used in DNA techniques were from New England Biolabs (Beverly, MA). Purine pancreatic elastase was from Sigma, and plasmin was from Chromogenix (Mölndal, Sweden).

**Cell Culture**—CHO cells (CHO K7, ATCC) were grown in Eagle’s modification of minimal essential medium containing 0.2% bovine serum albumin, supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 U/ml penicillin, and 50 μg/ml streptomycin. Stably transfected neomycin-resistant clones were grown in medium supplemented with 0.4 mg/ml G418 (Life Technologies, Inc.).

For the collection of conditioned medium, the cells were washed twice with serum-free medium. The serum-free-conditioned medium was then collected for two successive 2-day periods. If the conditioned medium was used for LTBP-2 purification, aminomethylbenzenesulfonfluoride (final concentration, 1 mM; Calbiochem) was added to prevent processing by endogenous serine proteases.

**Production of Recombinant LTBP-2 in CHO Cells**—A Sapl-HindIII fragment of LTBP-2 cDNA (base pairs 350–6317; GenBank accession no. Z37976) containing the full-length open reading frame (31) was blunt-end ligated polymerase and ligated to an EcoRV restriction site in pcDNA3 mammalian expression vector (Invitrogen, Oxon, United Kingdom).

CHO cells were transfected using a calcium phosphate transfection kit according to the manufacturer's instructions (Life Technologies, Inc.). One day after transfection, the cells were changed to medium containing 0.8 mg/ml G418, and the selection was maintained for 2 weeks. Expression levels of individual clones obtained by dilution cloning were estimated by immunoblotting.

**Antibodies, Immunoblotting, and Immunoprecipitation**—Rabbit polyclonal antibodies (Ab-178) were raised against a peptide corresponding to amino acids 818–834 of the LTBP-2 sequence (31). The Ab-178 antibodies do not cross-react with LTBP-1 (23). Ab-178 antibodies were used as the IgG fraction purified by protein A-Sepharose according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

Polyclonal antibodies (Ab-222) against full-length recombinant LTBP-2 protein were raised in rabbits. Briefly, rabbits were immunized with ~10 μg of recombinant purified LTBP-2 using complete Freund's adjuvant. Subsequent boosts were carried out at 4-week intervals using ~10 μg of LTBP-2 in incomplete Freund’s adjuvant. For immunoblotting, specific IgG was immunoaffinity purified from the serum as follows. Purified LTBP-2 was coupled to CNBr-activated Sepharose (Amersham Pharmacia Biotech) in PBS (10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl). The antigen was passed three times through the column, and the column was washed with 20 bed volumes of PBS. Bound IgG was eluted first with 1% CH₃COOH and subsequently with 20 mM NaOH. Eluted IgG was immediately neutralized with 1:5 volumes of 1 mM Tris-Cl, pH 7.0. Ab-222 is specific for LTBP-2 in immunoblotting and immunoprecipitation.

Immunoblotting with both anti-LTBP-2 IgGs was performed essentially as described (32), with the exception that biotinylated and horseradish peroxidase-conjugated avidin was used as the last conjugate (Amersham Pharmacia Biotech). SeeBlue molecular mass standard was from Novex (San Diego, CA).

**Purification and Quantitation of LTBP-2**—Conditioned medium from CHO-L2 cells (400 ml in each purification) was first precipitated with 30% (w/v) (NH₄)₂SO₄ at room temperature. The precipitates were resuspended in resuspension buffer and centrifuged and resuspended in 50 mM Tris-Cl buffer, pH 7.0, followed by dialysis overnight against the same buffer.

The dialysate was applied to MonoQ HR 5/5 column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-Cl buffer, pH 7.0. The column was washed with 5 bed volumes of 50 mM Bis-Tris-Cl buffer, pH 6.0, followed by 20 bed volumes of the same buffer containing 210 mM NaCl. The bound proteins were then eluted with a NaCl gradient (210–700 mM). Finally, LTBP-2 containing fractions were fractionated on a Superdex 200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with PBS.

The amounts of LTBP-2 in the various fractions obtained during the purification were quantitated by dot immunoblotting and chemiluminescence detection of several dilutions of the samples from each fraction with the anti LTBP-2 antibodies (Ab-178). The dot blots were quantitated by scanning x-ray films and measuring relative intensities of dots by the NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/). The amount of LTBP-2 present in the conditioned medium was considered as 1 unit/ml. Total protein concentrations of the conditioned medium and redissolved ammonium sulfate precipitations were quantitated by the Bradford assay (Bio-Rad) using bovine serum albumin as a standard. After this point, the protein amounts were estimated by measuring A₂₈₀. Protein concentrations were estimated by calculating the areas of the LTBP-2 containing peaks ( Aktka Explorer/Unicorn software; Amersham Pharmacia Biotech) in anion exchange or gel filtration chromatogram and assuming A₂₈₀ = 1 to correspond to 0.82 mg/ml (value calculated from the amino acid sequence of LTBP-2).

**Calcium Binding**—Calcium binding was performed essentially as described earlier (33). Briefly, partially purified LTBP-2 (LTBP-2 containing fraction after anion exchange chromatography; see Fig. 2, ~2 μg of total protein) was concentrated with a Microcon concentrator (Amicon, Beverly, MA). Ca²⁺ ions were chelated by addition of EDTA (2 mM, final concentration). The proteins were separated using 4–15% SDS–PAGE, nonreducing conditions and then transferred onto nitrocellulose filters. The filters were washed three times with 60 mM KCl, 5 mM MgCl₂, 10 mM imidazole-Cl, pH 6.8. The filters were then incubated with 1 μCi/ml ⁴⁵CaCl₂ (30 Ci/mg Ca; Amersham Pharmacia Biotech) for 22 h in the same buffer, rinsed several times with distilled water, dried, and subjected to PhosphorImager (Fuji BAS-1500, Fuji Co. Ltd., Tokyo, Japan) analysis.

**Chemical Deglycosylation**—Chemical deglycosylation of LTBP-2 was performed with trifluoromethanesulfonic acid according to the manufacturer’s instructions (Oxford Glycosciences, Oxford, United Kingdom). Carbohydrate structures in proteolytically processed fragments of LTBP-2 were detected using Glycotrak carbohydrate detection kit (Oxford Glycosciences). A fraction of purified LTBP-2 treated with plasmin or elastase was transferred onto nitrocellulose filter after separation by SDS-PAGE. Carbohydrates were then detected based on selective biotinylation of the carbohydrates, and subsequent streptavidin–alkaline phosphatase and nitroblue tetrazolium/bromo-4-chloro-3-indolyl phosphate-based detection.

**Amino Acid Sequencing**—Aliquots of purified LTBP-2 were digested with plasmin (70 μg/ml) or elastase (2 μg/ml) at 37 °C for 1 h. The samples were concentrated on a Microcon 10 concentrator (Amicon), and separated using 4–15% gradient SDS-PAGE. Samples were transferred to Immobilon-PSQ membranes with the same blotting buffer as in immunoblotting procedure. The membranes were thoroughly washed with 50% methanol, after which the protein bands were visualized with Coomassie Blue staining. Protein containing bands were cut out with a razor blade and N-terminally sequenced (Institute of Biotechnology, University of Helsinki, Finland). Separated fragments were subjected to PhosphorImager (Fuji BAS-1500, Fuji Co. Ltd., Tokyo, Japan) analysis.

**Biotinylation of LTBP-2, Incorporation to the Extracellular Matrix, and Immunofluorescence Analysis**—Purified LTBP-2 was biotinylated as follows. Sulfosucinimido biotin (10 μg) (Pierce) was reacted with ~2 μg of purified LTBP-2 in 0.5 ml of PBS. The reaction was allowed to proceed at 22 °C for 1 h, after which 20 μl Tris-Cl (final concentration), pH 7.0, was added to block unreacted sulfosucinimido biotin. After

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² M. Hyytiäinen, unpublished observations.
that, sulfo-NHS-Tris was removed with a Microcon 10 concentrator (Amicon).

For ECM incorporation analysis, human fetal lung fibroblasts (CCL-137) were cultured on glass coverslips to ~50% confluence in serum containing medium, after which the serum-free medium was changed. The cells were then cultivated with 0.1 μg/ml of biotinylated LTBP-2 in serum-free medium for 5 days. For the detection of biotinylated LTBP-2, the cells were washed twice with PBS and either fixed with 3% paraformaldehyde for 15 min or the extracellular matrices were isolated by sodium deoxycholate as follows. The cells were treated 3 times with 0.5% sodium deoxycholate, 10 mM Tris-Cl, pH 8.0, on ice for 20 min each (20, 34). To prevent nonspecific binding, the cells were incubated with 5% bovine serum albumin in PBS for 30 min at 22 °C. Cells or isolated matrices were then washed twice with PBS containing 0.5% bovine serum albumin, and incubated with Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. The coverslips were washed next three times with PBS containing 0.5% bovine serum albumin, flushed with distilled water, and mounted on glass slides using Vectashield (Vector Laboratories, Burlingame, CA).

To analyze the incorporation of nonpurified LTBP-2 present in the cell-conditioned medium, the cells were cultured in CHO-L2 or control CHO cell-conditioned medium for 4 days. Immunofluorescence analysis was then carried out with paraformaldehyde-fixed cells using a protein A-purified IgG fraction of Ab-L22 antiserum and fluorescein isothiocyanate-conjugated anti-rabbit antibodies (Jackson ImmunoResearch Laboratories).

RESULTS

Production of Recombinant LTBP-2 in CHO Cells—CHO cell lines overexpressing human LTBP-2 were established as described under “Experimental Procedures.” Immunoblotting analysis with polyclonal anti LTBP-2 antibodies (Ab-178) of the conditioned media produced by three independent LTBP-2-expressing clones revealed an immunoreactive protein of 240 kDa, as detected by SDS-PAGE under nonreducing conditions (Fig. 1), which is consistent with the previously published molecular mass of the protein (31). The immunoreactive bands of molecular masses 180 and 160 kDa (Fig. 1, lane 1) represent most likely degradation products of expressed LTBP-2. From 60 clones screened, CHO clone 1 produced the highest amount of LTBP-2 among the clones analyzed and was chosen for further studies (called CHO-L2 below). Immunoblotting analysis indicated that the conditioned medium of untransfected CHO cells did not contain detectable amounts of Ab-178 immunoreactive LTBP-2 (Fig. 1, ctrl).

Purification of LTBP-2—LTBP-2 was purified from the conditioned medium of CHO-L2 cells with successive steps of ammonium sulfate precipitation, anion exchange, and gel filtration chromatography. Fractions from each step of purification were analyzed by dot blotting with anti-LTBP-2 antibodies (Ab-178). Dot blot positive fractions were further analyzed by SDS-PAGE for the presence of the 290-kDa LTBP-2 species in the presence of reducing agent (Fig. 2). The amounts of total protein and LTBP-2 were quantitated from column fractions containing LTBP-2. The total amount of protein was decreased from 200 mg to 26 μg during the purification process (Table I). LTBP-2 was purified about 110-fold, and it was the only protein band detected in Coomassie Blue staining of SDS-polyacrylamide gels (Fig. 2).

LTBP-2 was first precipitated with 30% ammonium sulfate, yielding approximately 6-fold purification. LTBP-2 was further purified ~12-fold by anion exchange chromatography, in which the major LTBP-2 peak eluted at 550 mM NaCl (Fig. 2, left panel, LTBP-2), but it was also present in the fractions preceding this peak (data not shown). In gel filtration, LTBP-2 eluted

![Fig. 1. Immunoblotting analysis of LTBP-2 from the conditioned medium of overexpressing cells.](image)

![Fig. 2. Purification of LTBP-2.](image)
as a protein of ~600–700 kDa (standards not shown), which suggests that it formed either dimeric or trimeric complexes under these conditions, or it eluted anomalously due to its shape. The first peak was a high molecular mass complex of a contaminating protein (Fig. 2, middle panel, 1) that migrated in SDS-PAGE as an ~80-kDa protein and was visible only by silver staining of the gels (data not shown). LTBP-2 eluted as the second major peak (Fig. 2, LTBP-2). The third peak (Fig. 2, c) was an impurity of ~150 kDa that was present in the LTBP-2 fractions after anion exchange chromatography (Fig. 2, right panel, anion exchange).

**LTBP-2 Is a Calcium-binding Glycoprotein**—Because LTBP-2 contains 20 EGF-like repeats, 16 of which contain the consensus sequence for calcium binding (DXXD/N/E), we determined whether LTBP-2 is able to bind Ca$$^{2+}$$

A aliquots of LTBP-2 containing fraction from anion exchange chromatography (2 μg of total protein; see Fig. 2) were transferred onto a nitrocellulose filters after SDS-PAGE, and the filters were incubated with $$^{45}$$CaCl$_2$. After extensive washing, the filters were subjected to PhosphorImager analysis. The 240-kDa LTBP-2, which could be visualized by Coomassie Blue staining, was found to bind $$^{45}$$Ca (Fig. 3a). Impurities present in this preparation did not bind detectable amounts of $$^{45}$$Ca, suggesting that Ca$$^{2+}$$ binding was specific to LTBP-2.

To determine whether LTBP-2 is glycosylated, the proteins present in the conditioned medium produced by CHO-L2 cells were chemically deglycosylated. The proteins were then separated by 7.5% SDS-PAGE and transferred onto nitrocellulose filters. LTBP-2 was then detected by immunoblotting with Ab-L22. Molecular masses of the highest molecular mass species of untreated (−) and deglycosylated (+) LTBP-2 are indicated by arrowheads on the right. c, detection of carbohydrates in LTBP-2 proteolytically processed fragments. Aliquots of purified LTBP-2 were treated with plasmin (70 μg/ml) or elastase (2.0 μg/ml) at 37 °C for 1 h. The digested fragments were separated by SDS-PAGE using 4–15% gel under nonreducing conditions and transferred onto a nitrocellulose filter. Carbohydrates present in LTBP-2 were detected by a method based on selective biotinylation of carbohydrates and subsequent streptavidin-alkaline phosphatase detection (see under “Experimental Procedures” for details). The detected LTBP-2 fragments are indicated by arrowheads on the right. Migration of molecular mass markers is indicated on the left.

![Fig. 3](image3)

**FIG. 3. Biochemical characterization of recombinant LTBP-2.** a, calcium binding of LTBP-2. Proteins present in peak fraction of LTBP-2 after anion exchange chromatography (see Fig. 2, right panel) were separated by SDS-PAGE using 4–15% gels under nonreducing conditions, after which the proteins were transferred to a nitrocellulose filter. The filter was then incubated with $$^{45}$$Ca, washed, and subjected to PhosphorImager analysis ($^{45}$Ca binding). An aliquot of the same sample was analyzed in parallel by separating the proteins in SDS-PAGE and staining them with Coomassie Blue (Protein). LTBP-2 is indicated by an arrowhead on the right side of each lane. b, deglycosylation of LTBP-2. Conditioned medium from CHO-L2 cells was chemically deglycosylated as described under “Experimental Procedures.” The polypeptides were separated by SDS-PAGE using 7.5% gels under nonreducing conditions and transferred to nitrocellulose. LTBP-2 was detected by immunoblotting with Ab-L22. Molecular masses of the highest molecular mass species of untreated (−) and deglycosylated (+) LTBP-2 are indicated by arrowheads on the right. c, detection of carbohydrates in LTBP-2 proteolytically processed fragments. Aliquots of purified LTBP-2 were treated with plasmin (70 μg/ml) or elastase (2.0 μg/ml) at 37 °C for 1 h. The digested fragments were separated by SDS-PAGE using 4–15% gel under nonreducing conditions and transferred onto a nitrocellulose filter. Carbohydrates present in LTBP-2 were detected by a method based on selective biotinylation of carbohydrates and subsequent streptavidin-alkaline phosphatase detection (see under “Experimental Procedures” for details). The detected LTBP-2 fragments are indicated by arrowheads on the right. Migration of molecular mass markers is indicated on the left.

![Fig. 4](image4)

**FIG. 4. Proteolytic processing of LTBP-2 by plasmin and elastase.** Conditioned medium from CHO-L2 cells was treated with the indicated concentrations of porcine pancreatic elastase or plasmin at 37 °C for 1 h. The digested polypeptides were separated by SDS-PAGE using 4–15% gels under nonreducing conditions. LTBP-2 was subsequently detected by immunoblotting with Ab-178. Molecular masses of the digested LTBP-2 fragments are indicated by arrowheads on the right. Migration of molecular mass markers is indicated on the left.
the central domain were used to detect the major proteolytically cleaved fragment of LTBP-2. The majority of LTBP-2 processed by plasmin and porcine pancreatic elastase migrated as a 160-kDa fragment (Fig. 4), which corresponds to a minor immunoreactive fragment detected in the conditioned medium of CHO-L2 cells (see Fig. 1). The presence of this size fragment in untreated conditioned medium suggests that LTBP-2 is processed by endogenous proteinases during cell cultivation. Higher concentrations of both enzymes cleaved LTBP-2 into fragments of 120–140 kDa that appeared not to be processed further (Fig. 4). Leukocyte elastase and mast cell chymase cleaved LTBP-2 in a similar manner (data not shown). Cathepsins G, B, and D did not appreciably digest LTBP-2 (data not shown).

To determine the actual cleavage sites hydrolyzed by high concentrations of elastase and plasmin in LTBP-2, purified LTBP-2 was digested with these proteases. The digestion products were separated by SDS-PAGE and transferred to PVDF membranes, and the N-terminal amino acid sequences of the fragments were determined by automated Edman degradation. High concentrations of both enzymes produced major fragments of molecular masses of ~120 and ~30 kDa (see Figs. 4 and 6). The sequences obtained from the larger ~120-kDa fragment of plasmin cleavage (70 μg/ml) was XWGALPGPAERQ. The sequence obtained from the ~30-kDa elastase-cleaved fragment was XXGEPPRPLP. The N-terminal sequence of the larger fragment (120 kDa) locates to the region preceding the fourth EGF-like repeat in LTBP-2, before the stretch of 13 EGF-like repeats. The N-terminal sequence of the elastase-cleaved 30-kDa fragment is located to the region preceding the hybrid domain (see Fig. 8). In addition, elastase produced a fragment of ~15 kDa, which had an N-terminal sequence KRLPLTHCQD. This sequence locates between the hybrid domain and the third EGF-like repeat. The sequence obtained was identical to LTBP-2 except for the proline (underlined), which is asparagine in the published LTBP-2 sequence. This may be due to a modification of the asparagine by N-linked modification and subsequent anomalous migration in amino acid analysis.

**LTBP-2 Is Released from the Extracellular Matrix by Proteinases**—Of the four LTBPs, at least LTBP-1 and LTBP-2 are known to be components of the extracellular matrix, localizing to elastic tissue 10-nm microfibrils (4, 21). LTBP-1 and LTBP-4 are covalently associated with the ECM as high molecular mass complexes and can be released from the matrix by various proteinases (6, 20, 30). To answer the question of whether LTBP-2 is susceptible to proteolytic release from the matrix, isolated extracellular matrices of CHO-L2 cells were treated with increasing concentrations of leukocyte elastase or plasmin. The released, soluble proteins from the supernatant fluids were analyzed by immunoblotting with antibodies to LTBP-2 (Ab-L22). Both enzymes released proteolytically cleaved fragments of LTBP-2 from the matrix (Fig. 5). Intact 240-kDa LTBP-2, present as a major form of LTBP-2 in the conditioned medium of CHO-L2 cells, was not detected in the solubilized fraction. Both plasmin and elastase released LTBP-2 from the matrices initially as 160-kDa species, whereas higher concentrations of elastase yielded also fragments of ~120 kDa, similar to those found in protease-treated CHO-L2 cell-conditioned medium (Fig. 4).

**Human Fibroblasts Secrete LTBP-2**—To detect the expression levels of LTBP-2 in various cells, Ab-L22 against the recombinant LTBP-2 was used in immunoblotting. Conditioned media secreted by CHO-L2 cells, CCL-137 human embryonic lung fibroblasts, and CHO cells producing LTBP-1 (18) were separated by SDS-PAGE and transferred to a nitrocellulose filter. The affinity purified Ab-L22 antibodies recognized 240-, 180-, and 160-kDa forms of LTBP-2 from the conditioned medium of CHO-L2 cells (Fig. 6), which are of molecular masses similar to the fragments recognized by Ab-178 (Fig. 1). LTBP-2 was also detected in the conditioned medium of human lung fibroblasts (CCL-137). Antibodies did not recognize any polypeptides from the conditioned medium of the CHO clone overexpressing LTBP-1 (Fig. 6, LTBP-1), indicating that Ab-L22 does not cross-react with LTBP-1. Fragments of molecular masses of ~120 and ~30 kDa were detected after treatment of CHO-L2 conditioned medium either with elastase (2 μg/ml) or plasmin (70 μg/ml; compare with immunoblotting with Ab-178, which is anti-peptide antibody, Fig. 4).

Immunoprecipitation of the conditioned medium of human fibroblasts and CHO-L2 cells indicated that Ab-L22 was also of sufficient affinity for immunoprecipitation analysis (data not shown).

**LTBP-2 Assembles to Fibrillar Structures in Fibroblast Extracellular Matrix**—To analyze the distribution of LTBP-2 in cultured fibroblasts, immunofluorescence assays were carried out using Ab-L22 (purified IgG fraction). Subconfluent cultures of CCL-137 fetal lung fibroblasts were washed with serum-free medium and subsequently cultured in the serum-free conditioned medium of CHO-L2 or untransfected CHO cells for 4 days. After the incubation, the cells were fixed with paraformaldehyde. The cells were then subjected to immunofluorescence analysis. In cells that were cultured in control CHO cell-conditioned medium, the antibodies decorated fibrillar structures in the extracellular matrix (Fig. 7a), which, in accordance with the immunoblotting analysis (Fig. 6), confirms that fetal lung fibroblasts produce LTBP-2. In cells that were cultured in the CHO-L2 medium, fibrillar staining was remarkably more intense (Fig. 7b), indicating that exogenous LTBP-2 from CHO-L2-conditioned medium is able to assemble to the fibroblast ECM. Cells incubated with the preimmune serum showed only faint background staining (Fig. 7c).

**Purified LTBP-2 Is Assembled to the Fibroblast ECM**—To analyze whether the purified LTBP-2 was functionally active and to further confirm that exogenous LTBP-2 is able to interact with the extracellular matrices of cultured cells, purified LTBP-2 was biotinylated, and its ability to assemble to the extracellular matrix was analyzed. Subconfluent cultures of...
CCL-137 fibroblasts were cultured in serum-free medium containing biotinylated LTBP-2 for 5 days. Biotinylated LTBP-2 was visualized either from the paraformaldehyde-fixed cells (Fig. 7d) or from the deoxycholate-insoluble extracellular matrix (Fig. 7e) by Cy3-conjugated streptavidin and fluorescence microscopy. Staining revealed extracellular matrix fibers (Fig. 7d) that were similar to fibrils stained by anti-fibrillin-1 or LTBP-1 antibodies (1, 21). Similar fibrillar staining was seen in the isolated extracellular matrices (Fig. 7e). This indicates that biotinylated LTBP-2 associates with the matrix in a sodium deoxycholate-resistant form. The matrices of cells that were cultured in the absence of biotin labeled LTBP-2 were not stained by Cy3-streptavidin (Fig. 7f).

**DISCUSSION**

Extracellular matrix microfibrils have several structural components, including fibrillins, LTBP’s, and microfibril-associated glycoproteins (10). LTBP’s have a dual role in the biology of ECM. They are components of microfibrils, and they also target latent forms of TGF-β growth factors to the ECM. In this work, we have purified recombinantly produced LTBP-2 and characterized its properties as an extracellular matrix protein. LTBP-2 was produced by LTBP-2 transfected CHO cell clone as a 290-kDa protein, as estimated by migration in SDS-PAGE under reducing conditions. It was purified to homogeneity under non-denaturing conditions to characterize its biochemical properties and to study its proteolytic processing and assembly to the extracellular matrix. So far, only fibrillin-1 has been purified of integral microfibril components (35), but, to our knowledge, this is the first time that a full-length LTBP/fibrillin family microfibril component has been successfully purified under non-denaturing conditions in a functionally active form.

The purified LTBP-2 was found to bind calcium. Calcium binding stabilizes the structure of protein against proteolytic degradation, for example in fibrillin (36). Calcium binding may also stabilize protein-protein interactions (37, 38). LTBP-2 contains 20 EGF-like repeats, 16 of which contain the consensus sequence for calcium binding (4, 39). It is thus apparent that these EGF-like repeats perform this function in LTBP-2 also.

Of the integral microfibril proteins, at least fibrillin-1 has been shown to be a glycoprotein (40), but the domains that are glycosylated have not been identified. Chemical deglycosylation decreased the molecular mass of LTBP-2 by ~20 kDa, as estimated by the migration in SDS-PAGE, indicating that LTBP-2 is glycosylated. Carbohydrates were detected in the central, proteolytically stable core fragment of LTBP-2, consisting mainly of EGF-like repeats (Fig. 8). This domain has been suggested to form a rigid rod-like structure, and in this model, multiple monomers associating laterally would form the 10-nm microfibrils (41). The glycans in this region would probably point outward to give a hydrophilic character to the microfibril. Other glycosylated fragments were not detected, although potential N-glycosylation sites are present also in the N terminus of LTBP-2 (Fig. 8). This may be due to the absence of carbohydrates or to extensive degradation of N-terminal matrix binding domain by plasmin and elastase and subsequent migration out of the gel in SDS-PAGE.

Microfibrils have been shown to be catabolized by, for example, neutrophil elastase (29), and LTBP-1 and LTBP-4 are processed by multiple serine proteases (6, 30). The peptide bonds hydrolyzed by these enzymes could not be identified, because purified proteins have not been previously available in sufficient quantities. When proteolytic processing of LTBP-2 was studied by using recombinantly produced LTBP-2 and immunodetection, it was found to resemble that of LTBP-1. Purified LTBP-2 was processed directly with elastase and plasmin. By using purified material, we were able to exclude the possibility that these proteases would activate another protease present in cell-conditioned medium or in the ECM. LTBP-2

**FIG. 6.** LTBP-2 is produced by human fetal lung fibroblasts. The secreted proteins of the conditioned medium of CHO cells, CHO-L2 cells treated with elastase (2 μg/ml) or plasmin (70 μg/ml) at 37 °C for 1 h, CCL-137 fetal lung fibroblasts, and CHO clone overexpressing LTBP-1 were separated by 4–15% SDS-PAGE under nonreducing conditions. LTBP-2 was then detected by immunoblotting using Ab-L22. Arrowheads (240, 120, and ~30) on the right indicate the migration of the unprocessed LTBP-2 and processed LTBP-2 fragments. Migration of molecular mass markers is indicated on the left.

**FIG. 7.** LTBP-2 associates with fibrillar structures in fibroblast extracellular matrix. a–c, immunofluorescence analysis. Human fetal lung fibroblasts (CCL-137) were cultured in the conditioned medium of control CHO cells (a) or CHO-L2 cells (b) for 4 days. Subsequently, the cells were fixed with paraformaldehyde, and LTBP-2 was detected by indirect immunofluorescence with Ab-L22 and fluorescein isothiocyanate-conjugated anti-rabbit IgG. c, cells stained with preimmune serum. d–f, labeling with biotinylated LTBP-2. The cells were cultured in serum-free medium in the presence or absence of biotinylated LTBP-2 for 5 days. Subsequently, either the cells cultured in the presence of biotinylated LTBP-2 were fixed with paraformaldehyde (d) or the extracellular matrix was prepared by removal of cells with sodium deoxycholate (e). Biotinylated LTBP-2 was then detected by Cy3-conjugated streptavidin. f, cells cultivated in the absence of biotinylated LTBP-2.
the hybrid and third EGF-like domain, and it probably repre-
3
area where they were detected (see Fig. 8). The sequences between LTBP-1 and LTBP-2 are similar in the sense that they contain also an additional ECM binding site in the N-terminal part of LTBP-2, before the hybrid domain. Accordingly, the large splice variant of LTBP-1, which is extended by −350 amino acids in its N terminus, associates more efficiently with the ECM than the shorter form (43). This suggests that an additional ECM binding site is present in the N-terminal part of LTBP-1L. However, in the short splice variant of LTBP-1 the matrix binding site has been mapped to an area that is homol-
ogous to the region containing the third EGF-like repeat and the hybrid domain (Fig. 8). Subsequent processing generates the major protease-resistant fragments of ~120 and ~30 kDa. The ~30-kDa fragment thus probably comprises the hybrid do-
350 amino acids in its N terminus, associates more efficiently with the ECM than the shorter form (42). Interestingly, the corresponding region in fibrillin-2 is not proline-rich but contains instead multiple glycines (2), which may affect the sensitivity of different fibrillin containing microfibrils to proteolytic degradation.

Like LTBP-1 (20), LTBP-2 was released from the extracellu-
lar matrix by proteolysis. A major protease released fragment had a molecular mass of 160 kDa as estimated by SDS-PAGE under nonreducing conditions. These results do not directly show the molecular mass of LTBP-2 present in the ECM. How-

ever, it is likely that only full-length LTBP-2 can assemble to the ECM, as is the case with LTBP-1 (20, 30), and that the cleavage of LTBP-2 is needed to release it from the extracellu-
lar matrix. LTBP-2 fragments of 160 kDa were generated also in solution, and the cleavage yielding the 160-kDa fragment occurred N-terminally to the hybrid domain. Thus, the only site(s) responsible for covalent ECM binding appear to be in the N-terminal part of LTBP-2, before the hybrid domain. Accordingly, the large splice variant of LTBP-1, which is extended by −350 amino acids in its N terminus, associates more efficiently with the ECM than the shorter form (43). This suggests that an additional ECM binding site is present in the N-terminal part of LTBP-1L. However, in the short splice variant of LTBP-1 the matrix binding site has been mapped to an area that is homol-
ogous to the region containing the third EGF-like repeat and the first 8-Cys domain in LTBP-2 (see Fig. 8) (44). Different LTBP-3 and their splice forms may thus have different mecha-
nisms for ECM association. The amino acid sequences in this area are, however, quite similar between LTBP-1 and 2 (not shown), and the discrepancy between the results is probably due to different experimental systems.

At present, it is not clear whether cells are needed for microfibril assembly or microfibrils are capable of self assembly from monomers under suitable conditions. Human fetal lung fibroblasts were found to express LTBP-2 and assemble it to fibrous structures in the ECM that resembled those observed using anti-LTBP-1 or anti-fibrillin antibodies (1, 21). The staining was significantly more intense in cells cultured in the presence of exogenous LTBP-2. The association of exogenous LTBP-2 with ECM was confirmed by using biotinylated LTBP-2. Purified LTBP-2 could be assembled to the ECM of human fibroblasts in a sodium deoxycholate-resistant form. These findings show that exogenous LTBP-2 is able to assemble to the ECM. However, endogenous production of LTBP-2 or some other LTBP may still be needed for the assembly, but
these results suggest that the incorporation of LTBP-2 to the matrix and possibly the assembly of other microfibril components may involve soluble intermediates.

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