Identification and Characterization of a New Latent Transforming Growth Factor-β-binding Protein, LTBP-4*

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Transforming growth factor βs (TGF-βs) are secreted by most cell types as latent high molecular weight complexes consisting of TGF-β and its latency associated peptide (LAP) propeptide dimers, covalently linked to latent TGF-β-binding proteins (LTBPs). Currently, three different LTBPs are known (LTBPs 1, 2, and 3), all with highly similar protein domain structure consisting of epidermal growth factor-like and 8-Cys repeats. The 3rd 8-Cys repeat of LTBP-1 mediates its association with LAP. By using an expressed sequence tag homologous to the 3rd 8-Cys repeat of human LTBP-1 as a probe, a novel cDNA similar to known LTBP-1S was cloned from human heart cDNA library. This cDNA was named LTBP-4 and found to exist in at least four different forms, generated by alternative splicing at the amino terminus and at the central epidermal growth factor repeat domain. One of the alternative amino-terminal forms contained an RGD sequence, indicating possible cell-surface interactions with integrins. LTBP-4 gene was localized to chromosomal position 19q13.1–19q13.2. The major LTBP-4 mRNA form is about 5.1 kilobase pairs in size and is predominantly expressed in the heart, aorta, uterus, and small intestine. Immunoblotting analysis indicated that LTBP-4 was secreted from cultured human lung fibroblasts both in a free form and in a disulfide bound complex with a TGF-β-LAP-like protein. Both LTBP-4 forms were also found to be deposited in the extracellular matrix. The matrix-associated LTBP-4 was susceptible to proteolytic release with plasmin. LTBP-4 is a new member of the growing LTBP-family of proteins and offers an alternative means for the secretion and targeted matrix deposition of TGF-βs or related proteins.

Transforming growth factor βs (TGF-βs, see Refs. 1–3) be-

long to a large superfamily of growth-modulating polypeptides. Members of the TGF-β superfamily regulate the growth and differentiation of multiple cell types and the homeostasis of extracellular proteolysis. They also have important roles in different stages of development (reviewed in Refs. 1–4).

TGF-βs remain biologically latent after secretion. The activity of the mature TGF-β dimer is blocked by its amino-terminal propeptide (LAP), which is cleaved apart from the mature TGF-β during secretion, but remains associated with TGF-β by non-covalent interactions. TGF-β is activated by its dissociation from LAP, which can be accomplished for example by proteolysis (reviewed in Ref. 3). In most cell types studied, including those of mesenchymal, epithelial, and endothelial origin, TGF-β is secreted in a latent form associated with latent TGF-β-binding protein (LTBP; Ref. 5). LTBP-4s are also needed for the secretion and folding of TGF-β (6–8). The activity of all the members of the TGF-β superfamily is under strict control during developmental and pathological processes. Targeted deposition to extracellular structures via association with binding proteins, like LTBP-4s, would provide means to control the spatial activity of these growth factors.

Currently three different LTBP-4s have been cloned from mammals (LTBP-1–3) and found to form the covalent association with TGF-β1-LAP (9–13). The matrix association functions of LTBP-4s are important characteristics of these proteins. LTBP-1 and -2 are assembled into ECM rapidly after secretion (14, 11), the amino-terminal region being responsible for the ECM association (15, 16). LTBP-4s have a high degree of similarity to ECM microfibril proteins called fibrillins 1 and 2 (17, 18). In immunofluorescence and electron microscopic studies, LTBP-1 and LTBP-2 have been found to associate with microfibrils (13, 19) similar to those previously shown to contain fibrillins. LTBP-1 and -2 are released from the extracellular matrix by various proteases including plasmin, leukocyte elastase, and mast cell chymase (20, 21, 45). TGF-β, thus accumulates in the microfibrillar ECM structures in a latent form, where it can be released. The released large TGF-β complex is still latent, unless a high concentration of plasmin is used (5, 22). The released complex is then most likely activated at the cell surface (23, 24).

LTBP-4s are mainly composed of EGF-like repeats and protein domains with conserved patterns of eight cysteine residues, called 8-cysteine repeats. The association between LTBP-1 and LAP is mediated by a disulfide bond between Cys-33 of LAP and a yet unidentified cysteine in the 3rd 8-Cys repeat of LTBP-1 (15, 25), providing a biological function

protein sequence motif containing eight cysteines found in LTBP-4s and fibrillins, also called LT domain; LTBP-4S, LTBP-4L, LTBP-4ΔEGF different alternatively spliced forms of LTBP-4; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame; EST, expressed sequence tag; Ab, antibody.
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Fig. 1. Cloning of human LTBP-4 and its sequence. A, schematic representation of the obtained cDNA and positions of some restriction endonuclease recognition sequences. Note the 5'-terminal region in clone D1-1, which differs from that of clones 14.1.1, 11.1.1, and 2.2.1, forming
for the 8-Cys protein domains. The 8-Cys repeats have been found only in five proteins, LTBPs 1–3, and fibrillins 1 and 2. The current study was carried out to find novel proteins containing the 8-Cys repeats that would possibly interact with members of the TGF-β superfamily and extracellular microfibrils. Through an EST data bank search we found a sequence for a new 8-Cys repeat containing protein. By using this sequence as a probe, we cloned a novel cDNA for a protein with high similarity to known LTBPs and named it LTBP-4. We report here the cloning of human LTBP-4S as well as three other alternative forms of it. LTBP-4 is deposited to the extracellular matrix and has an ability to form complexes of high molecular weight with heterologous TGF-β-like proteins.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Pfu and AmpliTaq thermostable DNA-polymerases were from Stratagene (La Jolla, CA) and Perkin-Elmer, respectively. All other molecular biology enzymes were from New England Biolabs (Beverly, MA).

Plasmin was purchased from Chromogenix (Mölndal, Sweden). The proteinases porcine pancreatic elastase, bovine spleen cathepsin B and D, human leukocyte elastase, and human cathepsin G were from Sigma. Human mast cell chymase was a kind gift from Drs. Jussi Saarinen and Petri Kovanen (Wihuri Research Institute, Helsinki, Finland). The chymase was purified to 99% homogeneity from human skin as described (26).

**N-Glycosidase F** was from Boehringer Mannheim (Mannheim, Germany).

**Cell Culture—**SV40-transformed human kidney epithelial cells (293-T, American Type Culture Collection, Rockville, MD) and human embryonic lung fibroblasts (CCL-137, ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 50 μg/ml streptomycin. All experiments were carried out under serum-free conditions. For the collection of conditioned medium, the cells were washed twice with serum-free medium, and the subsequently added serum-free medium was collected after specified periods.

CHO cell clones overexpressing human LTBP-1 (15) and LTBP-2 (45) have been described elsewhere.

**Antibodies—**Antibodies against human LTBP-1 and LTBP-2 were kind gifts of Dr. C.-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). Affinity purified rabbit anti-human TGF-β 1-LAP antibodies (number 680) have been described previously (14). For the generation of LTBP-4-specific antibodies, synthetic peptides derived from LTBP-4 sequence at the beginning of the 3rd (YFDTAAPDACDNILARNVTWQE) and 4th (WQEVGADLVCSHPRLDRQATYTE) 8-Cys repeats (see Fig. 2A), respectively, were coupled to keyhole limpet hemocyanin (Pierce). The keyhole limpet hemocyanin-peptide complexes were used to raise antibodies 28-3 (against the 3rd 8-Cys repeat) and 33-4 (against the 4th 8-Cys repeat) in rabbits. Subsequently, the antibodies were affinity purified with the antigenic peptide. Both Ab 28-3 and Ab 33-4 were reactive in immunoblotting assays under both reducing and nonreducing conditions.

**cDNA Cloning and Sequence Analysis—**cDNA for human LTBP-4 was cloned from two human heart cDNA libraries, obtained from CLONTECH (Palo Alto, CA). Library HL3005q is poly(T)-primed pCDM-8 plasmid library, and library HL3026a is both poly(T)- and random-primed λgt10 phage library. Libraries were first screened using EST clone 302831 as a probe (see Fig. 1A). Cloning was carried the LTBP-4S form. Note also the alternative splicing in clones EST 302831 and 14.1.1. kb, kilobase pairs. B, the sequence of the human LTBP-4S cDNA. Protein translation is shown below the DNA sequence. Nucleotide numbering starts at the ATG translation initiation sequence. Potential N-glycosylation sites are shown by ●. The EGF-like repeats are shown with solid lines, and the 8-Cys repeats are shown with dashed lines. A jagged line shows the potential glycosaminoglycan attachment site. The hybrid domain is shown with a dotted line. The RGD sequence is boxed and shaded.
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Cloning of Human Latent TGF-β-binding Protein-4 (LTBP-4)—To determine whether there are yet unknown 8-Cys repeat containing proteins, we searched an expressed sequence tag (EST) subsection of the GenBank data bank (release 100.0, January 1997) against the 3rd 8-Cys repeat of human LTBP-1 protein sequence using TBLAST program (30). We obtained several overlapping clones with identical sequences of various lengths, whose deduced amino acid sequence contained an 8-Cys-like motif of an unknown protein. We used an EST clone 302831 (GenBank accession number W19505) as an initial probe to clone a homologous cDNA from human heart cDNA library. A large number of overlapping cDNA fragments were obtained, which were assembled to the full-length ORF sequence. Some of these cDNA fragments are depicted in Fig. 1A.

The new cDNA was named LTBP-4S. The LTBP-4S ORF is 4536 base pairs long, having a methionine codon in favorable context toward translational initiation (31) at position 137. This initiator methionine is followed by a deduced signal peptide. The translated open reading frame codes for a protein of 1511 amino acids with a predicted molecular mass of 161 kDa and a pI of 4.76 containing five potential N-glycosylation sites, an integrin-mediated cell attachment motif (RGD), and a putative glycosaminoglycan attachment site. In the LTBP-4S sequence, a total of 20 EGF-like and 3 8-Cys repeat sequences were found as well as a hybrid domain between an EGF-like and an 8-Cys repeat. Of the 20 EGF-like repeats, 17 contain the consensus sequence (D/N)X(D/N)E/QXn(D/N)Xn(Y/V), characteristic of a calcium binding type of an EGF-like repeat (see Fig. 2A). LTBP-4S has 39–52% similarity to previously cloned human LTBP-1 and -2 and mouse LTBP-3 (Fig. 2B). In domain structure, LTBP-4S, as all other LTBP-4s, is structurally related to fibrillin-1 and -2 (Fig. 2A).

Identification of Alternatively Spliced Forms of LTBP-4—In addition to LTBP-4S, we also obtained three different forms of LTBP-4 (see Fig. 1A), which we named as LTBP-4L, LTBP-4S2E, and LTBP-4AE. LTBP-4S and LTBP-4L have different amino-terminal coding sequences, whereas LTBP-4S2E and LTBP-4AE code for LTBP-4S forms lacking one or two EGF-like repeats from the long stretch of EGF-like repeats (Fig. 2A).

LTBP-4L is a putative amino-terminal extended form of LTBP-4S. Only one of the four 5’-terminal clones encoded for LTBP-4L (clone D1-1 in Fig. 1A). However, the obtained LTBP-4L ORF was not of full length, and therefore we were unable to clone the full-length LTBP-4L. The extended 5’ sequence of LTBP-4L obtained from clone D1-1 codes for eight cysteine residues, which are, however, not in the characteristic order C—C—CCC—C—C as the other 8-Cys repeats in LTBP-4s and fibrillins (Fig. 3A). After this cysteine-rich region, there is a high local concentration of proline and arginine sequences, implying potential protease-sensitive hinge region (see “Processing of LTBP-4 by Proteinases,” below). Also a motif PSPIPS is repeated twice in the LTBP-4L coding D1-1 fragment.

After full sequencing of the clones 14.1.1 and EST 302831, the two other alternative splices in the central part of LTBP-4 cDNA were found. The form of LTBP-4 cDNA coded by the clone 14.1.1, and named as LTBP-4AEGF, lacked the 14th EGF-like repeat, which is 3rd from the carboxyl-terminal end of the long stretch of EGF-like repeats (amino acids 892–935, cence microscope equipped with a ISIS digital image analysis system (Metasystems, Altusheim, Germany). The chromosome identity was verified by painting with a chromosome-specific probe according to manufacturer’s instructions (Cambio, Cambridge, UK).
corresponding to LTBP-4S) (Fig. 3B). LTBP-4Δ2EGF clone coded by EST 302831 lacked the 14th and 15th EGF-like repeats (amino acids 892–977) (Fig. 3B).

Chromosomal Localization of the Human Gene for LTBP-4—By using a NotI fragment of LTBP-4 cDNA as a probe, the corresponding genomic fragment was obtained from a human PAC library. The clone was verified to contain the LTBP-4 gene by partial sequencing. This PAC clone was used to analyze the chromosomal localization of the LTBP-4 gene. Human metaphase leukocyte chromosomes were hybridized with biotinylated LTBP-4 PAC probe and analyzed with a fluorescence microscope equipped with a digital imaging system. LTBP-4 gene was localized to chromosome 19, at the region of 19q13.1–19q13.2 (Fig. 4A). The localization to chromosome 19 was verified with painting using chromosome 19-specific probe.

Conservation of the LTBP-4 Gene in Other Species—To analyze whether the LTBP-4 gene is also present in other species, we probed the EcoRI-digested genomic DNA from different species using the LTBP-4 cDNA clone 1.1.1A (see Fig. 1A). The results of the Southern hybridization indicated that a gene homologous to human LTBP-4 exists in all mammalian species analyzed, including human, monkey, mouse, and rat (Fig. 4B). In addition, we have identified murine EST clones, representing mouse LTBP-4.3

Analysis of the Expression Levels of LTBP-4 in Different Tissues—A NotI fragment of LTBP-4 was used as a probe to elucidate the expression levels of LTBP-4 in different tissues. Based on the hybridization results under high stringency conditions in Northern blots, the size of LTBP-4 mRNA was found to be approximately 5.1 kilobase pairs (Fig. 5A). This correlates well with the cloned LTBP-4S sequence of 4944 base pairs, implying that the cloned LTBP-4S represents the full-length ORF of this mRNA. The highest expression levels of LTBP-4 were found in the heart, uterus, and small intestine (Fig. 5A and B). LTBP-4 mRNA from all tissues studied was of the same size, suggesting that the different forms of LTBP-4 are not detectable, because their small size differences are beyond the resolution of the agarose gels used for Northern hybridization analysis. Essentially identical results were obtained also from another Northern analysis using an AgeI-NotI fragment of LTBP-4 (data not shown).

LTBP-4 expression levels were studied next by using a dot blot of poly(A) mRNAs from 50 different human tissues. The amounts of total mRNA in the dots have been equalized by using the probes of mRNA levels for 8 different housekeeping genes. The same two fragments of LTBP-4 that were used in Northern blotting were used as probes in two independent analyses. After high stringency washes, the dots representing different tissues on the filter were quantitated by Phosphor Imager analysis. The highest expression levels of LTBP-4 were seen in the same organs as in Northern blots (Fig. 5B). In addition, relatively high hybridization signals were detected in

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aorta, ovary, and adrenal gland. Tissues in which the expression levels were found to be below average of all tissues quantitated were omitted from Fig. 5B.

**Secretion of LTBP-4 by Human Lung Fibroblasts**—As fibroblastic cells are known to produce LTBP-1 and -2, we used the serum-free conditioned medium from human lung fibroblasts (CCL-137) to analyze the expression and secretion of the LTBP-4 protein. Polyclonal antibodies were raised in rabbits against two synthetic peptides of LTBP-4. These peptides were from the 3rd (epitope for the Ab 28-3) and 4th (epitope for the Ab 33-4) 8-Cys repeats of LTBP-4. Immunoblotting analysis of the conditioned medium from confluent fibroblasts under reducing conditions indicated that both Ab 28-3 and 33-4 detected LTBP-4 protein, which had a molecular mass of approximately 250 kDa (Fig. 6A).

Immunoblotting results obtained using both Ab 28-3 and 33-4 under reducing conditions indicated that free LTBP-4 exists in two forms of slightly different molecular mass. Since the sequence for LTBP-4 codes for 5 N-glycosylation sites, we analyzed whether the observed heterogeneity of free LTBP-4 forms could be a consequence of N-glycosylation. Fibroblast-conditioned medium was subjected to removal of N-linked oligosaccharides by N-glycosidase F, followed by SDS-PAGE and immunoblotting analyses (data not shown). We found no detectable changes in the observed molecular mass of LTBP-4 after deglycosylation.

In order to control the specificity of Ab 28-3 and 33-4, we analyzed the conditioned medium from Chinese hamster ovary (CHO) cells, as well as from two CHO cell clones overexpressing human LTBP-1 and LTBP-2. Neither Ab 28-3 nor Ab 33-4 recognized the overexpressed LTBP-1 or LTBP-2 proteins under reducing or nonreducing conditions (Fig. 6, A and B), indicating that they are specific for LTBP-4. Immunoblotting of the conditioned media from the overexpressing cell clones using antibodies against human LTBP-1 or LTBP-2 detected the respective proteins (Fig. 6A).

**LTBP-4 Is Expressed Both in Free Form and in a Form Associated with a Heterologous Protein That Binds Most Likely**

**Fig. 3. Alternative forms of LTBP-4.** A, alternative forms of the 5' end. The 5' end for LTBP-4 exists in at least two different forms. The full-length LTBP-4S sequence (upper sequence in the figure) was obtained from three independent cDNA clones (see Fig. 1A). In addition, a clone coding for another form of the 5' end of LTBP-4 (LTBP-4L, clone D1-1, see Fig. 1A) was found (lower sequence in the figure). The common region starts at nucleotide 83 of the LTBP-4S, and the inferred amino acid sequence is boxed. The RGD motif (boxed and shaded in the figure) found in the shorter form is missing from the LTBP-4L. The cysteine residues in the 5' end of LTBP-4L form are circled and shaded.

B, deletions of EGF-like domains. The 14th and 154th EGF-like domains were missing in one cDNA clone, EST 302831, and the 14th EGF domain was found to be missing also in clone 14.1.1. The sequences were aligned by neighboring regions. The translated amino acid sequence is shown above the nucleotide sequences, and the lines above the amino acid sequence indicate the boundaries of individual EGF-like repeats. Since the sequence GATGTC exists on both sides of both splice entry points, the alignment presented in the figure is not necessarily the right one around the GATGTC region. However, the most frequent prevalence of different nucleotides in intron-exon branch point suggests the presented alignment (44).
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LTBP-4 Associates Covalently with TGF-β1—The interaction between LTBP-4 and TGF-β1 was studied using a mammalian cell co-expression system. We have found earlier that the 3rd 8-Cys repeats of LTBP-1 and LTBP-3 (15) bind TGF-β1–3 in a covalent manner. We constructed an expression vector pLTBP-4, containing the full-length cDNA of LTBP-4S. The construct was co-expressed with TGF-β1 cDNA in 293T cells, and the cell-conditioned medium was analyzed for complex formation between TGF-β1 and LTBP-4S. Immunoblotting analysis under nonreducing conditions using Ab 680 against the LAP region of TGF-β1 indicated that co-expressed LTBP-4 formed a covalent complex with TGF-β1–LAP (Fig. 7, lane 1). As a positive control, the cells were transfected with full-length human LTBP-1S (Fig. 7, lane 2). When the cells were transfected with only pTGF-β, the large complex was not seen (Fig. 7, lane 3).

LTBP-4 Is a Protease-sensitive Extracellular Matrix Component—LTBP-1 and -2 associate covalently with extracellular structures rapidly after secretion (11, 14). The possible association of LTBP-4 with the extracellular matrices was studied using confluent human lung fibroblast cultures. The sodium deoxycholate-insoluble extracellular matrices of fibroblasts were partially solubilized with plasmin. Immunoblotting analysis of the plasmin-digested solubilized extracellular matrices using Ab 28-3 and Ab 33-4 revealed that LTBP-4 was present in these matrix fractions in a proteolytically processed form (Fig. 8). Under reducing conditions, LTBP-4 was detected as a single 200-kDa band (Fig. 8, two left lanes), whereas under nonreducing conditions, a major and 2–3 minor forms of LTBP-4 with apparent molecular masses of about 80–220 kDa were observed (Fig. 8, Non-reduced). The 220-kDa form detected with only Ab 33-4 most likely represent similar disulfide bonded LTBP-4 complexes, as seen from samples of conditioned medium (Fig. 7). With Ab 28-3, only the lower molecular weight forms were seen. As a control, the immunoblotting for plasmin-digested LTBP-1 detected a 120–140-kDa band (Fig. 8, right lane).

Processing of LTBP-4 by Proteinases—The sequence of LTBP-4 reveals plausible proteinase-sensitive regions, also called hinge domains, having high local concentrations of both basic amino acids and proline. These regions are found between the hybrid domain and the long stretch of EGF-like repeats and near the carboxyl terminus after the last 8-Cys repeat (see Fig. 2A). LTBP-1 has been found to be susceptible to proteinase digestion at similar sites resulting in the release of large latent TGF-β complexes from the extracellular matrix. An immunoblotting analysis from fibroblast-conditioned medium was therefore carried out under reducing conditions with Ab 33-4 to detect the susceptibility of LTBP-4 for digestion by various proteinases. Of the proteinases used, plasmin, human mast cell chymase, and leukocyte and pancreatic elastases processed LTBP-4 (Fig. 9). Digestion with plasmin, chymase, and pancreatic elastase resulted in the formation of proteinase-resistant fragments with apparent molecular mass of about 230–220

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Leukocyte elastase treatment resulted in the loss of the high molecular weight form (Fig. 9), most likely due to a cleavage between the long stretch of EGF-like repeats and the 3rd 8-Cys repeat (45) leading to the loss of the peptide epitope of the antibodies used. Unlike the other LTBPs, the amino acid sequence of LTBP-4 is rich with proline, and basic residues in this region are indicative of an additional protease-sensitive site. Digestion of the cell-conditioned medium with cathepsins B, D, or G produced no detectable cleavage(s) in LTBP-4.

**DISCUSSION**

The present report describes the cloning of a new member of the LTBP family, LTBP-4, and several of its alternatively spliced forms. Its structure is very similar to that of the other previously cloned LTBPs, and, like the other ones, LTBP-4 also associates with the extracellular matrix. LTBP-4 is mainly composed of EGF-like and 8-Cys repeats, as the previously identified LTBPs and fibrillins. EGF like repeats are found in multiple ECM and cell-surface proteins. The 8-Cys repeats have been found thus far only in LTBP4s and fibrillins, and the only known function for these domains is the formation of a covalent association between TGF-β1 and the 3rd 8-Cys repeat of LTBP-1 (15, 25).

The amino termini of all LTBPs, including LTBP-4, contain two copies of EGF-like repeats, one copy of 8-Cys repeat, and another 8-Cys repeat, which is often also called a hybrid domain, since its sequence resembles both EGF-like and 8-Cys repeats (Fig. 2A). The amino termini of LTBP4s are responsible for their association with the extracellular matrix (Ref. 15 and also see Ref. 16). Following the repetitive amino-terminal domain, there is a proteinase-sensitive region, rich in proline and basic amino acid residues. The large latent TGF-β complex is supposedly released from the extracellular matrix by proteolytic digestion at this region (14). LTBP-1 deposited to the extracellular matrix can be released by plasmin without its amino-terminal matrix binding domain (15).

The central parts of all LTBPs and fibrillin family members are composed of a long stretch of EGF-like repeats. In LTBP-4 this part consists of 12–14 repeats and is over half of the total
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FIG. 6. Human fibroblasts secrete LTBP-4 in free form as well as in a complex with heterologous proteins associated with the 3rd 8-Cys repeat. CCL-137 human lung fibroblast-conditioned medium was collected for 7 days under serum-free conditions. The secreted proteins were separated by 4–15% gradient SDS-PAGE under reducing or nonreducing conditions followed by immunoblotting with Ab 28-3 or Ab 33-4. A. LTBP-4 forms a covalent complex via its 3rd 8-Cys repeat with a heterologous protein. Under nonreducing conditions Ab 28-3 (raised against 3rd 8-Cys repeat) recognizes only one form of LTBP-4, whereas Ab 33-4 (against the 4th 8-Cys repeat) recognizes both the free and complexed forms of LTBP-4. A two-headed arrow indicates the free LTBP-4 form, and the additional arrows on the Ab 33-4 blot indicate the LTBP-4 complexes. As a control the medium samples from CHO cell clones overexpressing LTBP-1 or LTBP-2 were analyzed by immunoblotting for human LTBP-1 and LTBP-2, respectively. Note the different sizes of LTBP-1, -2, and -4. Arrows indicate the LTBP-1 and LTBP-2 proteins. B. Association to heterologous protein is mediated by disulfide bonding. Under reducing conditions both Ab 28-3 and Ab 33-4 recognize only one form of LTBP-4, which is released from the heterologous protein. The two-headed arrow indicates the uncomplexed LTBP-4.

FIG. 7. LTBP-4 associates covalently with TGF-β1 in transfected cells. The 293T cells were co-transfected with pLTBP-4 or pLTBP-1 as indicated on the figure, together with pTGF-β. Subsequently, the cells were washed with serum-free medium. Cell-conditioned medium was harvested at 72 h and analyzed by 7% SDS-PAGE under nonreducing conditions followed by immunoblotting for TGF-β1-LAP. Arrows on the right indicate the covalent complexes formed between the protein encoded by pLTBP-4 or pLTBP-1 construct and TGF-β1. The brackets indicates the free TGF-β1-LAP.

FIG. 8. Release of the large LTBP-4 containing complex from fibroblast extracellular matrix by plasmin. Confluent human fibroblast cultures were lysed with sodium deoxycholate, and the remaining extracellular matrix fraction was partly solubilized by treatment with plasmin. The proteins released from the ECM were analyzed by SDS-PAGE both under reducing and nonreducing conditions. The released forms of LTBP-4 were detected by immunoblotting using Ab 28-3 and Ab 33-4, as indicated on the figure. As a control, the nonreduced samples were stained for LTBP-1 (Ab-39). The two-headed arrows indicate the LTBP-4 containing bands recognized with both LTBP-4 antibodies under reducing and nonreducing conditions. The upper arrow on the Ab 33-4 blot indicates the LTBP-4 complexes.

protein size. The region of EGF-like repeats has been suggested to form a helical rod-like structure, like similar regions in fibrillins (33). In LTBP-1 it has been found out that TGF-β1-binding region is near its carboxyl terminus, where a typical structure of 8-Cys—EGF—EGF—8-Cys repeats is found. TGF-β1 is covalently associated with the first 8-Cys repeat of LTBP-1 in this domain (Fig. 2A, Refs. 15 and 25).

In LTBP-1 and -2 and in fibrillin-1, there exist at least two alternative forms of the amino-terminal region (16, 34). We found two alternative forms of the amino terminus of LTBP-4. The longer form extends further to the 5′ direction than was obtained from clone D1-1 (see Figs. 1A, 2A, and 3A). The fragment of the longer form obtained in clone D1-1 does not encode for any EGF-like or 8-Cys-like repeats. Instead, it encodes a nonrepetitive amino acid sequence, like those found in the amino termini of other LTBPs. An RGD sequence, a known target for integrin-mediated cell adhesion (reviewed in Ref. 35), is present in the amino terminus of the shorter LTBP-4 form (LTBP-4S) at amino acid position 30. In addition to LTBP-4, the RGD sequence is also found in LTBP-2, human isoform of LTBP-1, and in both fibrillin-1 and -2. Recombinant protein fragments containing the RGD sequence of fibrillin-1 have been found to interact with purified integrin αvβ3 (36, 37). The existence of RGD sequences in LTBPs could thus be involved in the targeting of the latent TGF-β complexes to cell-surface activation. Another possibility is that the RGD motif is functional in the microfibril assembly process. Whether the alternative amino-terminal forms of LTBP-4 are due to alternative splicing or the use of different promoter regions is not known at present. Since the amino termini of two other LTBPs, namely LTBP-1 and LTBP-2, have been found to be responsible for the extracellular matrix interactions, the different amino-terminal regions of LTBP-4 may confer to different affinities to various extracellular fibrillar structures.

In addition to the varying amino-terminal region of LTBP-4, we found alternatively spliced regions in the long stretch of EGF-like repeats (Fig. 2A). This region is neither required for extracellular matrix deposition nor for the observed binding to TGF-β-like molecules. We identified two independent cDNA clones, named LTBP-4ΔE and LTBP-4Δ2E, in which the EGF-
like repeat 14 or both 14 and 15 were missing. There are two propositions for the function for these EGF-like repeats and the long stretch of EGF-like repeats found in some matrix proteins, including LTBP-1 and fibrillins. One possibility is that the long stretch of EGF-like repeats in fibrillins forms a helical rigid rod-like structure, which acts as a “fiber forming unit” of the fibrillin-containing microfibrils (33). The alternative splicing found in the central parts of LTBP-4 is likely to be involved in the regulation of the extracellular structures, most likely fibrillin-containing microfibrils, that associate with LTBP-4. The regulation of the number of EGF-like repeats in the central, rod-forming regions of LTBP-1 and fibrillins may affect the length of the rod or change the angle between the amino and carboxyl termini of the monomers. The deletion of a dimer of Ca²⁺-binding EGF-like repeats would reduce the length of an LTBP-4 monomer by about 5.7 nm (33). The alternative splicing of the microfibrillar proteins, fibrillins and LTBP-1s, could give an explanation to the observed variation of the length of monomers in these fibrils (38). On the other hand, the EGF-like repeats are known to mediate protein-protein interactions (39, 40), including those between cell-surface receptors and their ligands (41). As LTBP-1 released from ECM has been suggested to interact with the cell surface in the supposed TGF-β activation process (24), the observed variability in the number of EGF-like repeats in LTBP-4 may have a role in its interactions with molecules at the cell surface.

The structural similarity between LTBP-4 and other LTBP-1s suggests similar functions for all of them. We found by immunoblotting analyses from normal human skin fibroblasts that the processing of the Ab 33-4 after leukocyte (Leu) elastase digestion to be conserved in different mammalian species by an interspecies Southern blot and further supported by the existence of mouse LTBP-4 cDNA ESTs in GenBank™. From the eukaryotic species studied, only yeast was found not to have a related gene in its genome. This is well in accordance with the results of another microfibrillar protein, fibrillin, which is found to exist in a broad variety of species, even as distant to human as the jellyfish. The conservation of LTBP-4 gene speaks for the important role for LTBP-4 both in the microfibril composition as well as a vehicle for the deposition of latent growth factors to extracellular structures.

The presence of four members of LTBP-1s and the shared structural and functional similarities raises questions of the differences between these proteins. The expression patterns of different LTBP-1s in different tissues are only partially overlapping. LTBP-1 is mainly expressed in heart, placenta, and lung (10, 11) and LTBP-2 in lung and skeletal muscle, liver, and placenta (11). LTBP-4 is predominantly expressed in aorta, heart, small intestine, ovaries, and uterus. However, the expression levels of LTBP-4 in most fetal tissues were significantly lower than in adult tissues. This might indicate that LTBP-4 is not required for the initial formation of microfibrillar structures but provides a way to store latent TGF-β or related molecules in extracellular fibrils. “Switching” of isoform expression during development may be typical of the LTBP fibrillin family, since fibrillin-2 has been shown to appear earlier and in a more transient manner in the mammalian development than fibrillin-1, which is expressed at later stages of development (43). As there exist at least four members in the LTBP family, it is of interest to compare the expression levels of different LTBP-1s in different stages of development and thus to provide possible explanations and plausible biological functions for the existence of multiple LTBP-1s.

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Note Added in Proof—During the preparation of this manuscript a report was published describing the cloning of an identical cDNA that was also named LTBP-4. Its 5′ end was different from those described here, further indicating that there are numerous alternatively spliced forms of the protein (Giltay, R., Kostka, G., and Timpl, R. (1997) FEBS Lett. 411, 164–168).

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