Secretion of human latent TGF-β-binding protein-3 (LTBP-3) is dependent on co-expression of TGF-β

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Summary

Latent TGF-β-binding proteins (LTBPs) were initially identified through their binding to the growth factor. Three of the four known LTBPs are able to associate covalently with the small latent forms of TGF-β and mediate their efficient secretion. LTBPs have subsequently been found to associate with the extracellular matrix. We report here the cDNA cloning and characterization of the human LTBP-3 protein, which is the smallest LTB. The hLTBP-3 gene consists of 28 exons, including one alternatively spliced exon. The splice variant contains an additional epidermal-growth-factor-like repeat in the C-terminus. The gene is transcribed to produce a ~4.6 kb mRNA, which is expressed at high levels in human heart, skeletal muscle, prostate and ovaries and in certain osteosarcoma and fibroblastic cell lines. Antibodies were generated against recombinant fragment of hLTBP-3 and used to detect the protein and its secretion from cultured COS-7 and osteosarcoma cells. Immunoblotting analysis indicated that efficient secretion of overexpressed hLTBP-3 from COS-7 cells required co-expression of TGF-β1, which resulted in the secretion of high molecular weight complexes of ~240 kDa. hLTBP-3 protein was secreted from cultured osteosarcoma cells as high molecular weight complexes rather than in the free form. Similar complexes were recognized with antibodies specific to β1•LAP. These findings indicate that human LTBP-3 has an essential role in the secretion and targeting of TGF-β1.

Key words: LTB, Latent TGF-β, Alternative splicing

Introduction

Latent transforming-growth-factor-β (TGF-β)-binding proteins belong to the LTB/fibrillin-family of extracellular matrix (ECM) proteins, which includes LTBP 1-4 (Kanzaki et al., 1990; Moren et al., 1994; Giltay et al., 1997; Saharinen et al., 1998) and fibrillins-1 and -2 (Sakai et al., 1986; Zhang et al., 1990; Moren et al., 1994; Giltay et al., 1997; Saharinen et al., 1998). The previously identified LTBP-3 is a mouse homolog (Yin et al., 1995). LTBPs and fibrillins have a similar protein domain structure, consisting of conserved epidermal-growth-factor-like (EGF-like) and eight cysteine (8-Cys) repeats. Fibrillins are structural components of elastic tissue 10 nm microfibrils, and their defects are responsible for matrix fragility observed in patients diagnosed with Marfan syndrome (MFS) and congenital contractural arachnodactyly (CCA) disorders (Lee et al., 1991). LTBP-1 and -2 have been observed to colocalize with fibronectin-rich fibers and microfibrillar ECM structures (Gibson et al., 1995; Taipale et al., 1996; Hyytiäinen et al., 1998; Dallas et al., 2000). Association of LTBP-4 with the ECM has also been observed (Saharinen et al., 1998). However, direct binding of LTBPs to the constituents of fibrillin or elastin-containing fibrils has not been reported. LTBPs have a dual role in the biology of the ECM. Their other function is to facilitate efficient secretion of the latent forms of TGF-βs (Miyazono et al., 1991; Miyazono et al., 1992) and to target the latent growth factors to the ECM (Taipale et al., 1994).

TGF-βs are cytokine growth factors, which have various biological functions, including regulation of cell growth and differentiation and synthesis, degradation and remodeling of the ECM (reviewed in Moses and Serra, 1996; Roberts and Sporn, 1996). Small latent TGF-β complexes contain the N-terminal pro-domain of TGF-β (illustrated in Fig. 2). The pro-domain, called the latency-associated propeptide (LAP), is proteolytically cleaved from mature TGF-β during secretion. It is responsible for the latency of the mature growth factor by remaining non-covalently associated with the complex. Most cell lines secrete TGF-β•LAP in large latent complexes containing LTBPs. The small latent TGF-β complexes are attached by disulfide bonding of LAP-part to the third 8-Cys repeat of LTBP-3, forming large latent complexes (Saharinen et al., 1996; Saharinen and Keski-Oja, 2000). The third 8-Cys repeats of LTBP-1 and -3 are able to associate efficiently with the propeptides of TGF-β1, -2 and -3, whereas LTBP-4 has weaker binding capacity and complex formation of LTBP-2 with any of the TGF-βs is negligible (Saharinen and Keski-Oja, 2000). Interestingly, a novel non-TGF-β•LAP splice variant lacking the third 8-Cys repeat of LTBP-4 has been identified (Koli et al., 2001a).

After secretion, LTBP-1 targets TGF-β1 to the extracellular matrix (Taipale et al., 1994). Both the N-terminal and C-terminal regions of LTBP-1 seem to mediate its association with the ECM (Unsöld et al., 2001). LTBP-1L is an N-terminally extended form of LTBP-1, which is capable of binding more efficiently to the constituents of the ECM (Olofsson et al., 1995). LTBPs can be released from the matrix as truncated forms by various proteinases, including plasmin and elastases (Taipale et al., 1994; Taipale et al., 1995; Hyytiäinen et al., 1998; Saharinen et al., 1998). The protease-
sensitive sites of LTBP-1 and -2 have been localized to the proline-rich ‘hinge’ regions in their N-terminus. ECM is possibly a storage place, from where latent TGF-β can be released and activated rapidly when needed. The importance of LTBPs in the regulation and targeting of TGF-β activity is emphasized by the fact that several transformed cells show decreased production and secretion of LTBPs (Taipale et al., 1996; Dallas et al., 1994; Koski et al., 1999). Immunohistochemical analysis of prostatic carcinoma indicated that TGF-β1 is produced without associating with LTBP-1 (Eklöv et al., 1993). The lack of LTBP-1 expression in human gastrointestinal carcinomas seems to result in the retention of TGF-β1 inside the cells (Mizoi et al., 1993).

The purpose of this study was to analyze the expression and secretion of LTBP-3. For this reason we isolated the cDNA of human LTBP-3 and generated polyclonal antibodies against a large recombinant fragment of the protein. Human LTBP-3 is expressed in various human tissues, and its secretion is connected with the expression of TGF-β1. Immunoblotting analysis indicated that overexpressed hLTBP-3 was secreted efficiently only if TGF-β1 was simultaneously co-expressed. Moreover, in human osteosarcoma cell lines, the large latent complexes of hLTBP-3 and TGF-β1•LAP appeared to be the most prominent secreted forms of hLTBP-3.

Materials and Methods
Reagents
Thermostable AmpliTaq Gold polymerase was purchased from Perkin Elmer Life Sciences Inc. (Boston, MA), and all the DNA modifying enzymes were from New England Biolabs (Beverly, MA). Radioactive labels were from Amersham Pharmacia Biotech (Uppsala, Sweden) and Perkin Elmer Life Sciences Inc (Shelton, CT). Oligonucleotides were custom made by TAG Copenhagen A/S (Copenhagen, Denmark) except for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers, which were from Clontech (Palo Alto, CA). SeeBlue protein molecular mass standard was from Novex (San Diego, CA). Western Blot Recycling Kit was purchased from Alpha Diagnostic International (San Antonio, TX). Complete, EDTA-free protease inhibitor cocktail tablets were from Roche (Mannheim, Germany).

DNA cloning and sequence analysis
cDNA for human LTBP-3 was cloned from λgt10 human heart cDNA library (Clontech). The phage library was screened using [α-32P]dCTP-labeled EST clone 49899 (GenBank accession number H15208) as an initial probe. cDNA from positive, overlapping phage clones (Fig. 1A) was extracted and cloned into pBluescript II KS (+) cloning vector (Stratagene, La Jolla, CA) was extended by Superscript II reverse transcriptase (Invitrogen) from overlapping cDNA clones CL44 and CL52 (Fig. 1A). Oligonucleotides were custom made by TAG Copenhagen A/S (Copenhagen, Denmark) except for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers, which were from Clontech (Palo Alto, CA). SeeBlue protein molecular mass standard was from Novex (San Diego, CA). Western Blot Recycling Kit was purchased from Alpha Diagnostic International (San Antonio, TX). Complete, EDTA-free protease inhibitor cocktail tablets were from Roche (Mannheim, Germany).

The genomic clones containing human LTBP-3 gene were obtained by screening genomic PAC (Genome Systems, St. Louis, MO) and lambda FIX II (Stratagene) libraries with PCR-derived probes corresponding to nucleotides 1951 to 1284 and from 385 to 539 of LTBP-3 cDNA, respectively. The exon-intron organization of hLTBP-3 gene was determined by cycle sequencing of the clones with primers specific for hLTBP-3. The sizes of the larger introns were determined by PCR and verified by sequencing.

To generate a phylogenetic tree, the amino-acid sequences of different LTBPs were derived from GenBank and aligned using the multiple alignment program ClustalX using Gonsnet PAM 250 comparison matrix. The generated alignment was displayed by TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

cDNA expression constructs
A full-length human LTBP-3 cDNA expression construct named ‘phL3’ was constructed in a pCDNA3 eukaryotic expression vector (Invitrogen) from overlapping cDNA clones CL44 and CL52 (Fig. 1A). The 5‘ coding region of hLTBP-3 was obtained from the previously mentioned RT-PCR product (see DNA cloning and sequence analysis). The cloning was accomplished in three individual steps using internal recognition sites of Apal, PshAI and XcmI restriction endonucleases (Fig. 1A) and endonucleases that recognize the vector polylinkers.

The ‘phL3+ATG’ cDNA expression construct contains an additional N-terminal extension of 42 bp compared with phL3 (Fig. 1B). It was constructed using the recognition site of AscI restriction endonuclease, which digests 34 bp upstream of the translation initiation site of phL3+ATG.

For baculoviral expression, a cDNA fragment corresponding to nucleotides 2097-3459 of human LTBP-3 was amplified by PCR and cloned into a eukaryotic secretory expression vector pSignal (Saharinen et al., 1996). The construct contained a HA-epitope tag of pSignal and an additional six-histidine tag for purification and immunoblotting of the recombinant protein, respectively. The cDNA construct was finally cloned from pSignal into the pACGP67A baculovirus transfer vector (Pharmingen, San Diego, CA) as a whole BamHI-XbaI restriction fragment and named ‘phL3/699-1153’. The protein domains coded by phL3/699-1153 cDNA are shown in Fig. 2A.

cDNA construct containing the full-length human pTGF-β1 has been described previously (Saharinen et al., 1996).

Northern hybridization analysis
Total RNA was extracted from different cell lines by commercial RNeasy kit (Qiagen, Hilden, Germany). Total RNA (7 μg) was electrophoresed on a 0.8% formaldehyde-agarose gel and transferred to Hybond-N nylon filter (Amersham Pharmacia Biotech). Commercial Human Multitissue Northern Blots were purchased from Clontech. The SeaBlot-RsmI restriction fragment corresponding to nucleotides from 2783 to 3103 of CL44 (see Fig. 1A) was used as a cDNA probe for hLTBP-3. The probe was labeled with [α-32P]dCTP by random priming. The hybridizations were performed using Express Hyb hybridization solution (Clontech) according to the manufacturer’s instructions, and the filters were washed under high stringency conditions followed by autoradiography. Loadings were controlled by hybridizations with radioactively labeled β-actin or G3PDH cDNA probes.
Fig. 1. Cloning and sequencing of human LTBP-3. (A) A schematic presentation of the obtained cDNA and of some of the recognition sites of the restriction enzymes, which were used in the process of cloning. (B) The nucleotide and deduced amino-acid sequence of human LTBP-3. Nucleotide numbering starts from the proposed upstream translation initiation codon. Exon-intron boundaries are marked with vertical lines and EGF-like repeats are shaded with gray. 8-Cys repeats and the hybrid domains are indicated by patterns of eight circled cysteines. An alternative splice site of hL3+EGF is marked with an asterisk (*). Specified arrows are used to show the 5’ coding ends of phL3 and phL3+ATG cDNA expression constructs.
RT-PCR products were subjected to Southern hybridization analysis. An oligonucleotide (5'-GAA GAG ATG GGA CGT GGA CGT GGA CGA GTG CCA G-3') covering a part of the additional exon of the splice variant and the 3'-CTG CAC-3' was used to amplify a 983 bp product in order to control the normalization of the cDNA panel.  

PCR products, which were immobilized to a Hybond-N nylon filter (Amersham Pharmacia Biotech). Hybridizations were carried out using Express Hyb hybridization solution, and the signals were detected by autoradiography.

Table 1. Exon-intron structure and splice junction sites of human LTBP-3 gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Size (bp)</th>
<th>Coding DNA (bp)</th>
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<th>Intron</th>
<th>Size (bp)</th>
<th>3' splice acceptor</th>
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<td>27</td>
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</tr>
</tbody>
</table>

The human LTBP-3 gene covers a genomic region of about 19.8 kb and consists of 28 exons. Exons are shown in upper case and introns in lower case letters. The size of the 28th exon corresponds to the coding sequence of the gene. An alternative splice form hLTBP-3+EGF is marked with an asterisk. The nucleotide sequences surrounding the splice donor and acceptor sites of the additional 141 bp exon of hLTBP-3+EGF are shown.
Preparation of antibodies and immunoblotting
Polyclonal antibodies (Ab-hL3/2925) against recombinant hL3/699-1153 protein were raised in rabbits (Sigma-Genosys, Cambridge, UK). The rabbits were immunized with ~20 mg of purified keyhole-limpet-hemocyanin-coupled hL3/699-1153 recombinant fragment in Freund’s complete adjuvant. Seven subsequent boosters in Freund’s incomplete adjuvant were given at 2 week intervals. Antibodies were purified by affinity chromatography from whole serum. Recombinant hL3/699-1153 fragment was coupled to HiTrap NHS-activated affinity columns (Amersham Pharmacia Biotech). The antiserum was passed through the column for 1 hour and washed with PBS. Bound proteins were eluted with 1 mM CH₃ COOH, 140 mM NaCl and neutralized immediately with 1:10 volume of 1 M Tris-HCl buffer, pH 8.0. Affinity-purified antibodies were finally subjected to buffer change with Fast Desalting Column HR10/10 (Amersham Pharmacia Biotech) equilibrated with PBS. The resulting antibody recognized LTBP-3 specifically, as determined by immunoblotting assays using samples containing high amounts of LTBP-1, which Ab-hL3/2925 did not recognize.

Proteins were separated by SDS-PAGE under non-reducing conditions using commercial 4-15% gradient or 7.5% Tris-HCl gels (BioRad, Hercules, CA). When necessary, conditioned cell culture medium was concentrated five-fold using Microcon YM-30 centrifugal filter devices (Millipore, Bedford, MASS). Electrophoretically separated proteins were transferred to nitrocellulose membranes by semi-dry blotting, and subsequently treated with 5% nonfat milk in PBS/Triton X-100 to saturate non-specific protein-binding sites. The saturated membranes were incubated with affinity-purified Ab-hL3/2925 and subsequently, after washing, with biotinylated anti-rabbit IgG antibodies (DAKO A/S, Glostrup, Denmark) in TBS (Tris-buffered saline) buffer containing 0.05% Tween-20 and 5% bovine serum albumin (BSA). The bound antibodies were finally detected using peroxidase-conjugated streptavidin and enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech). Immunodetection using affinity-purified rabbit antihuman TGF-β1•LAP (680) and anti-LTBP-1 (ab39) antibodies was carried out as described previously (Taipale et al., 1994).

Immunofluorescence analysis
For immunofluorescence analysis, the cells were plated on glass coverslips and transfected the next day as described before (Cell culture and transient transfection). After three days the cells were fixed with 3% paraformaldehyde in PBS, permeabilized with 0.5% Nonidet P-40 in PBS and subsequently treated with 3%
BSA in PBS for 10 minutes. Pre-tested dilutions of affinity-purified anti-hLTBP-3 (Ab-hL3/2925) were then applied to the cells in PBS containing 3% BSA and incubated at room temperature for 1 hour. Binding of the primary antibody was detected with biotinylated anti-rabbit IgG antibodies (DAKO A/S) and lissamine rhodamine-conjugated streptavidin (Jackson Immuno Research Laboratories, West Groove, PA). The coverslips were finally washed with PBS and mounted on glass slides using Vectashield anti-fading reagent (Vector Laboratories, Inc., Burlingame, CA).

Metabolic labeling and immunoprecipitation
For immunoprecipitation, the cells were seeded on 100 mm diameter dishes and transiently transfected. The next day they were changed to serum-free medium depleted of cysteine and methionine for 2 hours. Subsequently, 50 µCi of Easy Taq Express-[35S] protein labeling mix was added in the same medium, and the cells were cultured for 24 hours.

After radiolabeling, the medium was collected, and the cells were lysed with 20 mM Tris-HCl buffer, pH 8.0, containing 120 mM NaCl, 0.5% Nonidet P-40 and protease inhibitors. The samples were incubated with anti-hLTBP-3 (Ab-hL3/2925) antibodies followed by precipitation of immune complexes with protein A sepharose beads (Sigma). Pre-adsorption with rabbit pre-immune serum was used in order to reduce non-specific background. The immune complexes were separated by SDS-PAGE using 4-15% gradient gels under reducing conditions and detected by fluorography.

Proteinase digestion of hLTBP-3
COS-7 cells were transiently transfected with pL3 and pTGF-β1 expression constructs (Cell culture and transient transfection). The serum-free culture medium was collected and subjected to proteinase treatment with plasmin (3, 10 and 30 μg/ml) or elastase (0.1, 0.5 and 1.0 μg/ml) at 37°C for 1 hour. The samples were subsequently analyzed by immunoblotting using the Ab-hL3/2955 antibody.

Results

cDNA and genomic organization of human latent TGF-β-binding protein-3 (hLTBP-3)
A databank search using the TBLASTN program of the Human Expressed Sequence Taq (EST) subsection of GenBank against the third 8-Cys repeat of human LTBP-1 resulted in the identification of an EST clone that was highly homologous to the known mouse Ltbp-3 cDNA (Yin et al., 1995; GenBank accession number L40459). Screening of lgt10 human heart cDNA library by walking strategy using EST clone 49899 as an initial probe resulted in several overlapping clones (Fig. 1A). The individual clones were assembled into a full-length ORF of 3771 nucleotides (Fig. 1B). The repetitive GC-rich 5′ coding end was amplified by RT-PCR, which was optimized for GC-rich templates. The 5′ part of hLTBP-3 open reading frame (ORF) contains two possible initiating methionine codons separated by 42 bp of GC-rich sequence. The adenine of the upstream AUG codon is referred to as nucleotide +1 in this report. Sequence analysis of the entire 4064 bp cDNA

![Fig. 3. Analyses of the mRNA expression levels of hLTBP-3 and hL3+EGF splice variant in various human tissues and cell lines.](image-url)
Northern hybridization analysis of various human tissues identified a transcript of about 4.6 kb (see Fig. 3A below), suggesting that the 5' untranslated region may exceed 300-400 bp.

The genomic structure of the hLTBP-3 gene was determined by direct sequencing of human genomic PAC and lambda FIXII clones and by PCR amplification using gene-specific primers. The human LTBP-3 gene spans a genomic region of about 19.8 kb and consists of 28 exons including an exon that is alternatively spliced (Table 1). All the internal exon-intron junctions of hLTBP-3 are defined by canonical 5' splice donor and 3' splice acceptor sequences (Table 1). Chromosomal localization of human LTBP-3 gene has been determined to be 11q12 (Li et al., 1995).

The translated open reading frame of hLTBP-3 encodes a polypeptide of 1256 amino-acid residues with a calculated molecular weight of 134 kDa and estimated pI of 5.71. The signal peptidase cleavage site exists probably between glycines at position 43-44, as was predicted by SignalP (Nielsen et al., 1997). Human LTBP-3 has 5 potential N-glycosylation sites. It consists structurally mainly of two types of cysteine-rich motifs, EGF-like and 8-Cys repeats, which are characteristic of members of the LTBP family (Fig. 2A). Also another type of 8-Cys repeat, often known as a hybrid domain, is present in hLTBP-3. Most of the EGF-like repeats of hLTBP-3 are of the calcium-binding type and contain a characteristic consensus sequence [D/N][D/N][E/Q][Xa][D/N][Xa][Y/F]. Comparison of the translated protein sequence with that of mouse Ltbp-3 confirmed that the cloned ORF codes for a human homologue of LTBP-3 protein. It is 87% similar to mouse Ltbp-3, but only 47-32% similar to human LTBP-1, LTBP-4 or LTBP-2 (Fig. 2B).

Expression of hLTBP-3 mRNA in different human tissues and cell lines

Expression of hLTBP-3 was analyzed using northern blots containing polyA+ and total RNA from various human tissues and cells. A BseRI-BsmI restriction fragment or a cDNA fragment coding for CL44 was used as a gene-specific probe. A single transcript species of about 4.6 kb was detectable in various human tissues (Fig. 3A). The transcript fits quite well in size to the ORF of hLTBP-3. On the basis of the hybridization results of the tissue northern blots, human LTBP-3 is expressed prominently in heart, skeletal muscle, prostate and ovaries (Fig. 3A). Significant expression was detected also in testis and the small intestine.
The result was confirmed by another hybridization analysis using a similar Northern blot and the same BseRI-BsmI restriction fragment (nt 2738-3103) as a radioactively labeled probe.

Since the expression of mouse Ltbp-3 mRNA is induced during mouse osteoblast differentiation (Yin et al., 1995), the mRNA pattern of human LTBP-3 was determined from some osteosarcoma cell lines and in osteoblasts. Of the analyzed cell lines, human primary osteoblasts and G-292 and MG-63 human osteosarcoma cells showed the highest expression of hLTBP-3 mRNA (Fig. 3B). WI-38 human embryonic fibroblasts were found to express hLTBP-3 mRNA at high levels, whereas WI-38/VA13 fibroblasts, which are SV-40-virus-transformed counterparts of normal WI-38 fibroblasts, showed very little expression (Fig. 3B). Similar results of the expression levels of hLTBP-3 in normal and transformed osteoblasts have been obtained by semi-quantitative RT-PCR (Koli et al., 2001a). In general, malignant transformation has been observed to result in downregulation of the transcription, mRNA expression and protein levels of the different LTBP proteins (Koli et al., 2001a). Furthermore, an additional, slightly larger transcript was detected in osteoblasts, MG-63 osteosarcoma cells and in WI-38 lung fibroblasts. The size of this transcript is similar, but distinct from that of LTBP-1, and its general mRNA expression pattern in those cells is clearly distinct from that of LTBP-1 (P. Vehviläinen, M.H. and J.K-O., unpublished). For example, in WI-38 fibroblasts the expression of LTBP-1 was very strong, whereas we detected only a trace of the large hLTBP-3 transcript. By contrast, osteoblasts expressed slightly lower levels of LTBP-1 than WI-38 fibroblasts but much higher levels of the large hLTBP-3 transcript than WI-38 cells. In summary, the migration of these two transcripts is different, and the differences in their expression levels further indicate that they are distinct.

Northern blot hybridization analyses of LTBP-1 and -2 revealed the presence of two transcripts (Moren et al., 1994; Kanzaki et al., 1990). The different transcripts of LTBP-1 possess N-terminally distinct regions (Olofsson et al., 1995). Molecular cloning of LTBP-4 also revealed an N-terminally extended cDNA clone (Saharinen et al., 1998). The larger hLTBP-3 transcript could thus correspond to an N-terminally extended transcript or some other alternative splice variant of hLTBP-3.

Identification of an alternative splice variant of hLTBP-3
Screening of the cDNA library revealed a diverse cDNA clone CL21, which contains an additional coding sequence compared with hLTBP-3. A similar extension was detected in EST 49899 cDNA clone (Fig. 1A). The variant splice form named hL3+EGF contains an additional 141 bp exon, which codes for an additional EGF-like repeat of the calcium-binding type (Fig. 2A). hL3+EGF is alternatively spliced between exons, which code for the 13th and 14th EGF-like repeats of hLTBP-3 (Fig. 2A). Inspection of the genomic sequence surrounding the alternative splice site revealed the presence of characteristic splice donor and acceptor sites (Table 1).

RT-PCR using cDNA prepared from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas was performed to characterize the expression of hL3+EGF. Oligonucleotides for RT-PCR were designed to amplify the region encompassing the 13th EGF-like repeat and the novel EGF-like repeat of hL3+EGF. RT-PCR generated a 154 bp product, which was confirmed as hL3+EGF by Southern hybridization analysis using an internal oligonucleotide as a probe. hL3+EGF was found to be expressed in other studied tissues except skeletal muscle (Fig. 3C). Strong expression of the splice variant was detected in the pancreas and liver. In order to verify the normalization of the cDNA panel, the samples were amplified with oligonucleotides specific for abundantly expressed glyceraldehyde-3-phosphate dehydrogenase (G3PDH; Fig. 3C). In order to determine whether this splice variant is expressed in other species as well, we carried out a Blast search (http://www.ncbi.nlm.nih.gov/BLAST/) on the mouse EST database against the mouse cDNA sequence coding for the 13th EGF-like repeat and the subsequent 8-Cys repeat. With this approach, we were able to identify two EST clones (Genbank accession numbers B1904087 and B1409427) containing sequences homologous to hL3+EGF, showing that the alternative splice variant is also expressed in other species.

Secretion of LTBP-3 requires co-expression of TGF-β in COS-7 cells
To analyze the protein expression of hLTBP-3, rabbit polyclonal antibodies were generated against human hL3/699-1153 recombinant protein coding for the amino acids 699-1153 (Fig. 2A). Specific antibodies were isolated from rabbit antiserum by affinity columns containing the antigenic protein.
The specificity of the isolated antibodies was subsequently tested by immunofluorescence staining of transfected COS-7 cells and immunoblotting. The fluorescence signal of the translation product of the transfected human LTBP-3 expression construct was detected mainly inside the cells, probably to some extent in the Golgi (Fig. 4A).

Immunoblot analysis was used to assess whether human LTBP-3 is released into conditioned cell culture medium of COS-7 cells. Two expression vectors containing hLTBP-3 cDNAs with the different translation initiation codons (Fig. 1B) were transiently transfected into COS-7 cells. A pH3/699-1153 cDNA construct in an eukaryotic expression vector was used as a positive control. A recombinant pH3/699-1153 cDNA construct was translated into a protein of ~82 kDa. Some higher molecular weight forms, which most probably correspond to multimeric forms of it, were also detected (Fig. 4B). Immunoblot analysis did not reveal any specific signal for the full-length human LTBP-3 protein in the conditioned medium of COS-7 cells unless an expression vector encoding for human TGF-β1 cDNA was co-transfected. Co-expression of hLTBP-3 and TGF-β1 in COS-7 cells resulted in the secretion of high molecular weight complexes of ~240 kDa (Fig. 4B). No such complexes were detected if COS-7 cells were transfected with pTGF-β1 only (Fig. 4B) or if the cells overexpressing human LTBP-3 were treated with 5 ng/ml of TGF-β1 (data not shown). In addition, co-transfection of pTGF-β1 cDNA increased the secretion of pH3/699-1153 protein as well as its complexed forms in COS-7 cells. Both hLTBP-3 expression constructs containing the different translation initiation codons were translated into hLTBP-3 (Fig. 4B). Therefore, no definite proof of the functional translation initiation site of human LTBP-3 was found.

The high molecular weight forms of the transfected COS-7 cells were subsequently characterized by immunoblotting using an affinity-purified human TGF-β1•LAP antibody, which recognizes the LAP part of TGF-β1 (Taipale et al., 1995). An immunoreactive signal corresponding to the ~240 kDa band was detected in the cell-conditioned medium of COS-7 cells co-expressing hLTBP-3 and TGF-β1 (Fig. 4C; arrow). The high molecular weight forms were thus identified as large complexes of human LTBP-3 and β1•LAP. Complex formation was also detected in cells overexpressing the shorter pH3/699-1153 control plasmid and pTGF-β1 (Fig. 4C; arrowhead).

Immunoprecipitation was used to confirm the composition of the large ~240 kDa complex. COS-7 cells overexpressing hLTBP-3 and TGF-β1 were metabolically labeled, and the conditioned medium was collected for 24 hours. The samples were immunoprecipitated with Ab-hL3/2925 antibody and analyzed by 4-15% gradient SDS-PAGE under reducing conditions. hLTBP-3 migrated at ~180 kDa. Two lower molecular weight bands corresponding to β1•LAP and unprocessed TGF-β1•LAP monomers were detected. Their mobility was comparable to that observed by immunoblotting. The unprocessed TGF-β1•LAP form results from overexpression, when the furin cleavage required for converting TGF-β1•LAP precursor into a mature growth factor does occur efficiently (Dubois et al., 1995). Together, these findings indicate that overexpressed human LTBP-3 mRNA is translated into a protein, whose secretion seems to depend strictly on the expression of and complex formation with TGF-β1.

Secretion of hLTBP-3 from human osteosarcoma cells
As various human osteosarcoma cell lines express human LTBP-3 mRNA (Fig. 3B), we assessed whether hLTBP-3 protein is expressed and secreted by the cells at similar levels. The conditioned medium of MG-63, U-2OS and Saos-2 human osteosarcoma cell lines was subjected to immunoblotting analysis for hLTBP-3. Untransfected COS-7 cells and COS-7 cells overexpressing hLTBP-3 and TGF-β1 were used as negative and positive controls, respectively. A single, strongly immunoreactive band of about 240 kDa was detected in the medium of MG-63 and U-2OS human osteosarcoma cells, whereas Saos-2 osteosarcoma cells showed no secretion of hLTBP-3 protein (Fig. 5A). The 240 kDa band co-migrated with the positive control. The expression levels of hLTBP-3 protein corresponded well with its mRNA expression pattern in the analyzed osteosarcoma cells (Fig. 3B).

To determine whether the anti-β1•LAP antibody recognizes similar endogenously expressed secreted complexes, we carried out an immunoblot analysis of the serum-free conditioned medium from MG-63, U-2OS and Saos-2 osteosarcoma cells on the same, recycled filter. Secretion of two complexes of slightly different molecular weights was detected in U-2OS osteosarcoma cells (Fig. 5B; two arrows). The slightly larger complex ~260 kDa, which was more prominent in U-2OS cells, was not detectable in the medium of MG-63 osteosarcoma cells. MG-63 cells secreted only a complex of ~240 kDa. Saos-2 cells did not secrete such complexes. Instead, a smaller ~90 kDa band corresponding to the migration of β1•LAP dimer was detected with long exposure (data not shown).

Since LTBP-1 associates with TGF-β1•LAP, we carried out
a similar immunoblotting analysis using an antibody against human LTBP-1 (Ab39). Distinct forms of LTBP-1 were detected in U-2OS cells (Fig. 5C). The smaller forms most probably represent the free forms of LTBP-1 (Kanzaki et al., 1990), whereas the larger form, which corresponds to the size of the larger ~260 kDa band detected with anti-β1•LAP antibody (Fig. 5B), represents the large latent complex of LTBP-1 and TGF-β1•LAP. The molecular size of the complex is similar to that secreted by human erythroleukemia cells (Miyazono et al., 1991). No such complex was detected in MG-63 osteosarcoma cells. The secreted TGF-β1 seems to be exclusively complexed with LTBP-3 in MG-63 osteosarcoma cells, even though LTBP-1 is produced by these cells. Therefore, the 240 kDa immunoreactive band detected in MG-63- and U-2OS-conditioned media is likely to represent the large latent complex of human LTBP-3 and TGF-β1•LAP, which seems to be the most prominent secreted form of LTBP-3 in these osteosarcoma cells.

**Proteolytic processing of hLTBP-3**

LTBP-2 is proteolytically cleaved by plasmin and elastases from a proline-rich, N-terminal hinge region located between the hybrid domain and the long stretch of EGF-like repeats (Hytyiäinen et al., 1998) (Fig. 2A). LTBP-1 from fibroblast-conditioned medium is processed into similar 120-140 kDa fragments with serine proteases (Taipale et al., 1994; Taipale et al., 1995). Proteinase processing of LTBP-4 is also evident but it is different from that of LTBP-1 and -2. LTBP-4 is processed to 230-220 kDa fragments (Saharinen et al., 1998). To assess the susceptibility of hLTBP-3 for proteolytic processing, the conditioned medium of COS-7 cells overexpressing the large latent complex of hLTBP-3 and TGF-β1•LAP was subjected to digestion with plasmin and leukocyte elastase. The molecular weight of the complex was reduced from ~240 kDa to ~230-220 kDa when increasing concentrations of plasmin were used (Fig. 6). Leukocyte elastase was unexpectedly not able to cleave hLTBP-3 (Fig. 6). It therefore seems that hLTBP-3 is not proteolytically cleaved at the proline-rich region analogous to LTBP-1 and -2. The reduction of the molecular mass of the large latent complex of hLTBP-3, instead, resembles that of LTBP-4.

**Discussion**

We report here the cloning and characterization of the human LTBP-3. Human LTBP-3 is composed primarily of EGF-like and 8-Cys repeats, which are characteristic of LTBPs and fibrillins. Eleven out of 14 EGF-like repeats of hLTBP-3 are of the calcium-binding type, which are coded by individual exons. Whereas hLTBP-3 resembles other LTBPs in overall domain structure, the central region of hLTBP-3 contains a continuum of only eight EGF-like repeats, which makes it the smallest of all the human LTBPs known so far. Human LTBP-3 shares 87% sequence similarity with the mouse Ltbp-3, and their structural domains are alike.

The 5’ coding end of the human isoform has very high GC content, and it contains two putative translation initiation codons separated by a 42 bp region of GC-rich sequence. According to Kozak there are two positions around the AUG initiator codon that are critical for function: 97% of vertebrate mRNAs have a purine in position –3, and 46% have a G in position +4 (Kozak, 1991). The nucleotide sequence CCTGAGUGC surrounding the putative downstream initiator codon of human LTBP-3 agrees with Kozak’s GCCA/GCCAUGG consensus sequence for initiation of translation, whereas the upstream sequence CCCCCGAUGC does not. However, in most vertebrate mRNAs, the first upstream AUG is commonly used as the ultimate initiation site. Translation initiation efficiency of human LTBP-3 was studied in COS-7 cells co-expressing hLTBP-3 and TGF-β1. Both the upstream and downstream AUG codons served as translation start codons when hLTBP-3 was overexpressed. Therefore, either one of them may drive the initiation of the translation of hLTBP-3 protein.

Structural variations have been described for all known LTBPs (reviewed by Koli et al., 2001b). The splice variants generally either contain additional or lack conserved protein domains. Independent promoters regulate N-terminally distinct forms of LTBP-1, which provides a means for their cell-type specific expression (Koski et al., 1999). N-terminally extended forms may associate more efficiently with the ECM, as has been shown for LTBP-IL (Olofsson et al., 1995). A splice variant lacking the third 8-Cys repeat is unique to human LTBP-4 and may affect TGF-β deposition into tissues (Koli et al., 2001a). Splice variants of the hinge region may, in turn, provide protease resistance (Gong et al., 1998; Michel et al., 1998). We identified here two cDNA clones, which contain an additional EGF-like repeat in the C-terminus between the two C-terminal 8-Cys repeats of hLTBP-3. Tissue distribution of hL3+EGF mRNA is different from that of hLTBP-3, suggesting its functional specificity. In contrast to hLTBP-3, hL3+EGF is not expressed in skeletal muscle but is highly expressed in the pancreas. EGF-like repeats have been found in many extracellular and cell-surface proteins, and they are considered to be important in mediating protein-protein interactions (reviewed in Davis, 1990). The additional EGF-like repeat of the hL3+EGF variant is located next to the homologous region of LTBP-1S, which has recently been proposed to interact with the extracellular matrix of human fibroblasts (Unsöld et al., 2001) (Fig. 2A). Therefore, an alternative hL3+EGF form may have altered affinity for the ECM.

LTBPs are essential for the efficient secretion and correct folding of TGF-βs (Miyazono et al., 1991; Miyazono et al., 1992). In a recent study we found that overexpressed third 8-Cys repeats of LTBP-1 and LTBP-3 associate covalently with all TGF-β•LAP isoforms, whereas LTBP-4 has a binding capacity for TGF-β1•LAP only, and LTBP-2 or fibrillins do not bind to any of the TGF-βs (Saharinen and Keski-Oja, 2000). Different LTBPs may function and mediate the effects of TGF-βs in a tissue-specific manner (reviewed by Koli et al., 2001b). This finding is supported by the fact that in all the osteosarcoma cells we studied, the major fraction of human LTBP-3 was secreted into the large latent complexes. Bone tissue is known to be a rich source of TGF-βs, which are involved in bone remodeling (reviewed in Bonewald and Mundy, 1990). A recent report describes bone abnormalities in LTBP-3-null mice, suggesting that hLTBP-3 has an essential role in regulating TGF-β growth factor deposition and availability in bone (Dabovic et al., 2002). Earlier studies have, however, shown that certain bone cells and human
glioblastoma cells secrete TGF-βs exclusively as free forms lacking LTBPs (Olofsson et al., 1992; Dallas et al., 1994). We found that Saos-2 osteosarcoma cells secrete minor amounts of the growth factor in free form without any LTBP. Therefore, LTBP may not be vital for efficient secretion of TGF-βs in all cell types. In this report we found that efficient secretion of overexpressed hLTBP-3 in COS-7 cells required co-expression of TGF-β1. Overexpressed hLTBP-3 was not secreted unless TGF-β1 was co-expressed simultaneously. This finding demonstrates the importance of hLTBP-3 as a binding protein for TGF-β1. Dallas and co-workers (Dallas et al., 1994) have reported earlier that MG-63 osteosarcoma cells secrete TGF-β1 in a 290 kDa complex containing LTBP-1. Our results favor the idea that the large latent TGF-β1 complex secreted by MG-63 cells contains hLTBP-3 rather than LTBP-1. The major fraction of the secreted LTBP-1 was found to be in free form in MG-63 cells. The discrepancy between the results may be caused by dissimilar detection methods, which were not able to distinguish slight size differences and to the fact that hLTBP-3 protein was not known at that time.

LTBP-1,-2 and -4 associate with the extracellular matrix, from where they can be released by treatment with serine proteases such as plasmin, elastases and chymase (Taipale et al., 1994; Hyytiäinen et al., 1998; Saharinen et al., 1998). We analyzed the cellular localization of hLTBP-3 by immunofluorescence analysis. A fluorescence signal from the translation product of the transfected hLTBP-3 cDNA was detected inside COS-7 cells, whereas no staining was observed in the extracellular matrix. Co-transfection of hLTBP-3 and TGF-β1 cDNA expression vectors resulted in the secretion of high molecular weight complexes when assessed by immunoblotting of the conditioned medium of COS-7 cells. By immunofluorescence analysis, co-transfection appeared to have no effect in possible matrix deposition of hLTBP-3 in COS-7 cells or in G292 osteosarcoma cells, which produce high amounts of ECM (data not shown). Our preliminary immunoblotting data from the extracellular matrices of CCL-137 embryonic lung fibroblasts suggests, however, that a fraction of the large latent complexes of hLTBP-3 and TGF-β1 exists in SDS-soluble and plasmin-digested matrices. Faint signals corresponding to the sizes of plasmin-digested complexes of overexpressed hLTBP-3 and TGF-β1 (Fig. 6) were detectable with long exposures of the ECM immunoblots (data not shown), suggesting that hLTBP-3 may be able to associate with the ECM to some extent. Immunofluorescence analyses of transfected cells suggest that the faint signal in matrix preparations is caused by inefficient matrix deposition of hLTBP-3.

Characterization of the third human LTBP revealed that even though the different LTBP share structural and functional characteristics, they all are unique. Their abilities to bind to latent TGF-βs vary as do their capacities for ECM association. For instance, analysis of hypomorphic LTBP-4–/– mice indicated that LTBP-4 has several important functions in the regulation of TGF-β1 bioavailability in the heart, lung and colon (Sterner-Kock et al., 2002). Although LTBP-2 seems to function more as a structural component of the microfibrillar network and resembles fibrillins, hLTBP-3 appears to be an essential binding protein for TGF-β1. Novel functions for LTBP may include the regulation of cell adhesion as described for LTBP-2 (Hyytiäinen and Keski-Oja, unpublished data). The vital importance of LTBP-2 for development has been observed in knockout mice, which are embryonically lethal (Shipley et al., 2000). Mice lacking the alleles of LTBP-3 survive but suffer from premature obliteration of synchondroses, osteosclerosis and osteoarthritis (Dabovic et al., 2002). The finding that LTBP-3 does not get secreted from the cells without co-expression of TGF-β, and the fact that TGF-β1 forms complexes only with LTBP-3 in MG-63 cells, suggest very specific functions for LTBP-3. These results indicate a clear and tight interaction between TGF-β1 and hLTBP-3 and support the observation that bone anomalies in LTBP-3 knockout mice may result from deficient TGF-β-β1 growth factor availability. Further studies directed at determining the fate of secreted large latent complexes of hLTBP-3 and TGF-β1-LAP will provide more information on the role of hLTBP-3 in storage and activation of the latent growth factor.

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Note added in proof

After submission of this work we learned of similar, accordant results that demonstrated the need for TGF-β and LTBP-3 co-expression for secretion of human LTBP-3 [Chen, Y., Dabovic, B., Annes, J. P. and Rifkin, D. B. (2002). FEBS Lett. 517, 277-280].

References


