Novel non-TGF-β-binding splice variant of LTBP-4 in human cells and tissues provides means to decrease TGF-β deposition

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SUMMARY

Small latent TGF-β consists of latency associated peptide (LAP) bound to the 25 kDa TGF-β by noncovalent interactions. Small latent TGF-β is secreted from cells and deposited into the extracellular matrix as covalent complexes with its binding proteins, LTBPs. Four LTBPs have been molecularly cloned and their structures contain repetitive sequences. The 3rd 8-Cys repeats of LTBP-1, -3 and -4 are able to associate with small latent TGF-β. We analyzed by RT-PCR the expression of LTBPs 1-4 in a panel of cultured human cell lines including fibroblasts of different origin, endothelial cells and immortalized keratinocytes. LTBPs were expressed in an overlapping manner, but differences in their expression levels were detected. SV-40 transformed human embryonic lung fibroblasts contained less of the mRNAs for the LTBPs, suggesting that malignant transformation leads to decrease in LTBP expression. A novel alternatively spliced form of LTBP-4 lacking the 3rd 8-Cys repeat (LTBP-4Δ8-Cys3rd) was identified. LTBP-4Δ8-Cys3rd does not bind TGF-β and it was found to be expressed in the same tissues as the full length LTBP-4. The exon-intron structure of LTBP-4 around the 3rd 8-Cys repeat was similar to those of LTBP-2 and -3. LTBP-4Δ8-Cys3rd was produced by alternative splicing over two exons. In addition, HL-60 promyelocytic leukemia cells expressed a splice variant lacking only one exon of this region. The expression of the non-TGF-β-binding variant of LTBP-4 may be important for the regulation of TGF-β deposition in tissues. Since LTBPs are a part of the extracellular matrix microfibrils, the LTBP-4Δ8-Cys3rd protein may also be involved in various structural functions not related to TGF-β signaling.

Key words: LTBP, TGF-β, 8-Cys repeat, Alternative splicing

INTRODUCTION

Transforming growth factor-βs (TGF-β) are multifunctional polypeptide growth factors involved in the regulation of cellular growth and differentiation, of immune functions and of the balance between extracellular matrix production and proteolysis (Roberts and Sporn, 1996; Piek et al., 1999; Taipale et al., 1998). One of their major effects is inhibition of cell proliferation (Piek et al., 1999). TGF-βs are produced by cells as latent complexes unable to associate with the TGF-β signaling receptors (Lawrence et al., 1985). The small latent TGF-β complex is composed of the 25 kDa dimer rendered latent by noncovalent association with its N-terminal propeptide dimer (LAP), cleaved apart during the secretion. The large latent TGF-β complex contains, in addition, one of the latent TGF-β binding proteins (LTBPs) covalently linked to LAP. LTBPs are considered important for the folding and secretion of TGF-β (Miyazono et al., 1991). LTBPs also target TGF-β to extracellular structures from where it can be specifically activated, for example by certain proteases (reviewed by Saharinen et al., 1999).

LTBPs belong to the LTBP/fibrillin-family of extracellular matrix (ECM) proteins (reviewed by Saharinen et al., 1999; Öklü and Hesketh, 2000). The family includes fibrillin-1 and -2, in addition to four different LTBPs (Kanzaki et al., 1990; Morén et al., 1994; Yin et al., 1995; Saharinen et al., 1998). The structures of these large ECM glycoproteins are highly repetitive. They are mainly composed of epidermal growth factor (EGF)-like repeats, eight cysteine (8-Cys) repeats unique to this protein family, as well as flanking regions containing proline rich areas, or in some cases glycine rich areas. LTBPs-1, -2 and -4 are known to get assembled into the ECM (Gibson et al., 1995; Taipale et al., 1996; Saharinen et al., 1998; Hyytiäinen et al., 1998). LTBP-1 and -2 localize to microfibrillar structures in the ECM and associate with fibronectin rich fibers (M. Hyytiäinen and J.K.-O., unpublished; Gibson et al., 1995; Taipale et al., 1996; Dallas et al., 2000). The N- and C-terminal parts of LTBPs mediate matrix binding (Saharinen et al., 1996; Unsöld et al., 2001), and the N-terminally extended form of LTBP-1 associates more readily with the ECM (Olofsson et al., 1995). Only LTBPs-1, -3 and -4 can form large latent complexes with TGF-β (Saharinen et al., 1996; Gleizes et al., 1996; Saharinen and Keski-Oja, 2000) and mediate extracellular matrix localization of these complexes. Several proteases including plasmin can release the LTBP•TGF-β complexes from the ECM (Saharinen et al., 1998; Taipale et al., 1992; Taipale et al., 1995). Plasmin can also cleave LTBP-2, which does not bind small latent TGF-

Table 1. PCR primer sequences, locations in LTBP cDNA sequences and sizes of expected PCR products*

Primer pair name	Forward primer	Reverse primer	Product size (bp)
LTBP-1.1	*4037*5′-ACACCTGCGATTGCTTGG-3′*4054*	*4409*5′-GAGGACTTGCAACTCCATGC-3′*4390*	373
LTBP-1.2	*2980*5′-GAACTGCTCAGTGGGGTGTGT-3′*3000*	*3504*5′-GCACTGCAGTTTCACAGGATCATA-3′*3481*	525
LTBP-2.1	*5603*5′-ATCCTGAACGGCTGTGAGAA-3′*5622*	*6338*5′-TCTGGAGTCAGTCCCCAAGC-3′*6319*	736
LTBP-2.2	*4435*5′-TGAGCTTATGCTGGCGGTATG-3′*4455*	*4917*5′-ACATAACCAGGCACGGTGTTG-3′*4897*	483
LTBP-3.1	*3287*5′-TTCCTTCTGGGACACAAGCC-3′*3306*	*3672*5′-CGCGGAAATCCCTCAGTG-3′*3655*	386
LTBP-3.2	*2522*5′-TGTCTGCGATGAGGGCTT-3′*2539*	*2902*5′-CACTCGTAGCCAGGCTGC-3′*2885*	381
LTBP-4.1	*4371*5′-TGGCTGAGCCCTACGAGG-3′*4388*	*4750*5′-CAGACTAAGCGGGCTGCAG-3′*4732*	380
LTBP-4.2	*3102*5′-GTGGAGCTGCCCTGTGTG-3′*3119*	*3587*5′-CCTGGTCTCGGAAGAGCTG-3′*3569*	486
‘RPA’	*3449*5′-TGCCGCATCCAGCAGTGC-3′*3466*	*3779*5′-CCAGTGGGGGCTCGCAGGTA-3′*3760*	331
‘RPA-2’	*2943*5′-ATGTGGACGAATGCCGGA-3′*2960*	*3381*5′-TTGTCACATGCATCCGGG-3′*3364*	439
‘3rd 8-Cys’	*3216*5′-GCATGTTCCCAGGCTCGCAG-3′*3235*	*3554*5′-CTCTCCGGAGGCTCAGGTCTCC-3′*3533*	339

*LTBP sequences were retrieved from GenBank and the accession numbers were M34057 (LTBP-1), Z37976 (LTBP-2), LTBP-3 (J.S. et al., unpublished), and AF051344 (LTBP-4). ‘RPA’ and ‘RPA-2’ denote here the probes used for northern analysis and in the RNase protection assay.

β. The specific cleavage sites have been located to the hinge region of LTBP-2, before a proteolysis resistant core consisting mainly of EGF-like repeats (Hyytiäinen et al., 1998).

LTBP molecules not containing small latent TGF-β can also be detected from the ECM (Taipale et al., 1994), and it appears that LTBPs are produced in molar excess compared with TGF-βs. LTBPs are known as structural components of the ECM, in addition to targeting TGF-β. Structural variation in LTBPs have been described and found to be caused by various mechanisms, including proteolytic processing, alternative splicing and the use of different promoters (Koski et al., 1999). Splice variants lacking a specific domain or with additional repeats have been characterized from all four LTBPs (reviewed by Saharinen et al., 1999). N-terminally extended forms may associate with the ECM with differing affinity, while deletions of part of the hinge region may provide protease resistance (Olofsson et al., 1995; Michel et al., 1998). The biological functions of the numerous alternatively spliced forms are under keen investigation at present.

The expression patterns of the four different LTBP isoforms have not been well characterized so far. We analyzed here by RT-PCR the expression of LTBPs in a panel of cultured cell lines including fibroblasts of different origin, endothelial cells and immortalized keratinocytes. LTBPs were found to be expressed in an overlapping manner. However, our semi-quantitative analyses suggested differences in the expression levels of the different isoforms. A novel LTBP-4 splice variant lacking the small latent TGF-β binding domain (LTBP-4Δ8-Cys^{3rd}), was identified and found to be expressed in the same tissues as intact LTBP-4. The targeting and activation of TGF-βs have numerous levels of regulation. LTBPs are necessary for the correct folding and secretion of TGF-β (Miyazono et al., 1991; Miyazono et al., 1992; Eklöv et al., 1993). In the current work we have identified a novel way to regulate this complex network, namely to decrease the ECM-associated small latent complexes in certain cells expressing LTBP-4. Regulation of the ratio of the two LTBP-4 variants may have an effect on the cellular phenotype and be involved in the developmental control and in the pathogenesis of diseases including cancers.

MATERIALS AND METHODS

Cell culture and RNA isolation

Human embryonic lung fibroblasts WI-38 (American Type Culture Collection, Rockville, MD), SV-40 virus-transformed WI-38/VA-13 fibroblasts (CCL-75.1, ATCC), adenovirus transformed human kidney epithelial cells (293-T, ATCC), locally established human skin fibroblasts (provided by Olli Saksela, Helsinki, Finland) and HaCat human keratinocytes (Boukamp et al., 1998) were cultured in Dulbeccos’s modified Eagle’s medium (DMEM). Human embryonic lung fibroblasts (CCL-137, ATCC) and HT-1080 human osteosarcoma cells (CCL-121, ATCC) were cultured in Eagle’s minimal essential medium. Human umbilical vein endothelial cells (HUVEC) (provided by Ari Ristimäki, Helsinki, Finland) as well as microvascular endothelial cells (PromoCell, Heidelberg, Germany) were cultured in Endothelial Cell Growth Medium MV (PromoCell). Culture media were supplemented with 10% fetal calf serum (Life Technologies, Gaithersburg, MD), 100 IU/ml penicillin and 50 μg/ml streptomycin.

Total cellular RNA was isolated using RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. RNA concentrations and purities were determined spectrophotometrically (Ultrospec 3000, Amersham Pharmacia Biotech, Uppsala, Sweden) as well as by agarose gel electrophoresis followed by ethidium bromide staining.

Antibodies

Rabbit polyclonal LTBP-4-specific antibodies #28-3 (against the 3rd 8-Cys repeat) and #33-4 (against the 4th 8-Cys repeat) have been described previously (Saharinen et al., 1998). The antibodies have been affinity purified with the immunogenic peptide and are reactive in immunoblotting assays under both reducing and nonreducing conditions. Mouse monoclonal antihemagglutinin (α-HA) antibody 12CA5 was from BabCO (Richmond, CA).

Primers

Primers were custom made by TAG Copenhagen A/S (Copenhagen, Denmark) except for G3DPH primers, which were from Clontech (Palo Alto, CA). Primer sequences and the sizes of expected PCR products are summarized in Table 1.

cDNA constructs

Construct pFull codes for the 3rd 8-Cys repeat, the following two EGF-like repeats and the 4th 8-Cys repeat of LTBP-4 and is cloned in pSignal (Saharinen et al., 1996), a eukaryotic secretory expression vector derived from pcDNA3 (InVitrogen, Carlsbad, CA). Constructs pΔ8-Cys^{3rd} and pΔ24 8-Cys are similar, but lack the 3rd 8-Cys repeat or the last 24 amino acids of the repeat, respectively.

Reverse transcription and PCR

Reverse transcription was carried out with Oligo $(dT)_{12-18}$ primer (Life Technologies) and Superscript II reverse transcriptase (Life Technologies) using 5 μg of total RNA or 0.5 μg of polyA$^+$ RNA (Clontech) according to manufacturer’s instructions. The cDNAs

were amplified using AmpliTaq Gold (Perkin Elmer, Branschburg, NJ) in a 35 cycle PCR reaction. For G3DPH (glyceraldehyde 3-phosphate dehydrogenase) only 25 cycles of amplification was needed to obtain quantifiable results. The number of cycles was optimized so that the PCR reaction did not reach a plateau. Semi-quantitation of the RT-PCR products was carried out after equalizing the amounts of the specific products to the amounts of G3DPH in a particular cell line.

The primers were designed to yield products spanning exon-intron boundaries so that a possible genomic contamination in total RNA preparation would result in a higher molecular weight product. Another test for genomic contamination was PCR performed directly from RNA samples that had not been reverse transcribed. In the 3rd negative control, the cDNA template was substituted with water. All these controls were negative.

Cloning and sequencing

PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI) and sequenced using an ABI 310 automatic DNA sequencer (Perkin-Elmer). Several individual clones from separate PCR reactions were sequenced.

Ribonuclease protection assay

Ribonuclease (RNase) protection analysis was carried out following the instructions of the commercial Direct Protect Kit (Ambion Inc., Austin, TX). PCR products of size 331 ('RPA', nucleotides 3449-3779) or 439 bp ('RPA-2', nucleotides 2943-3381) from LTBP-4 were cloned into pGEM-T Easy vector (Promega), which contains the recognition site of T7 polymerase. The plasmids were further linearized and antisense RNA probes were synthesized with T7 RNA-polymerase in the presence of [α-^{32}P]UTP (Amersham Pharmacia Biotech) using a MAXIscript Kit (Ambion). Total RNA (5 μg) or 0.5 μg of polyA$^+$ RNA (Clontech) were hybridized with the appropriate antisense RNA probe. Following hybridization overnight, unpaired single-stranded probes were digested with RNase A/Rnase T1 mix and the samples were subsequently precipitated. Protected fragments were analyzed in a denaturing 6% polyacrylamide gel containing 8 M urea, and their sizes were determined by comparison with a Century Marker Template Plus (Ambion).

Northern hybridization analysis

For northern analysis 10 μg of total RNA was fractionated on 1.2% agarose gels containing formaldehyde and transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech) by capillary transfer. Pre-hybridization and hybridization were performed at 68°C in ExpressHyb hybridization solution (Clontech). PCR products from LTBP-4 spanning nucleotides 3216-3554 ('3rd 8-Cys', which detects only LTBP-4 containing the 3rd 8-Cys repeat) and 3448-3779 ('RPA', which detects total LTBP-4, whether or not it contains the 3rd 8-Cys repeat) were labeled with [^{32}P]-dCTP (>3000 Ci/mmol, Amersham Pharmacia Biotech) using a Rediprime Labeling Kit (Amersham Pharmacia Biotech). Washes were carried out under high stringency conditions, first with 2×SSC containing 0.05% SDS and then with 0.1×SSC containing 0.1% SDS at 50°C for 40 minutes each.

Multiple tissue expression array

Multiple tissue expression (MTE) arrays were carried out according to the manufacturer's instructions (Clontech). The amounts of RNA in MTE array have been equalized by the manufacturer by comparing the expression levels of eight different housekeeping mRNAs. The blot was hybridized with cDNA probes as in Northern blot. The MTE arrays have several controls including RNA and DNA from yeast, *E. coli* and humans. The two probes used gave negative results for all these controls and showed no nonspecific binding. The radioactivity levels were quantified with a BAS-2500 bio-imaging analyzer (Fuji, Tokyo, Japan).

Transfection of cell lines and immunoblotting

Approximately 7.5×10^5 293T cells were seeded per well in six-well plates and transfected the following day with 2 μg of the plasmids indicated using FuGENE6 liposome mediated transfection system (Roche Molecular Biochemicals). Six hours after transfection the cells were washed, fed with serum-free medium and the conditioned medium was collected after 48 hours. Aliquots of medium samples were electrophoresed under reducing conditions in 4-15% gradient polyacrylamide gels in the presence of SDS and the proteins were electrophoretically transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Immunodetection using anti-HA antibody or LTBP-4 specific antibodies was performed as described (Saharinen et al., 1996).

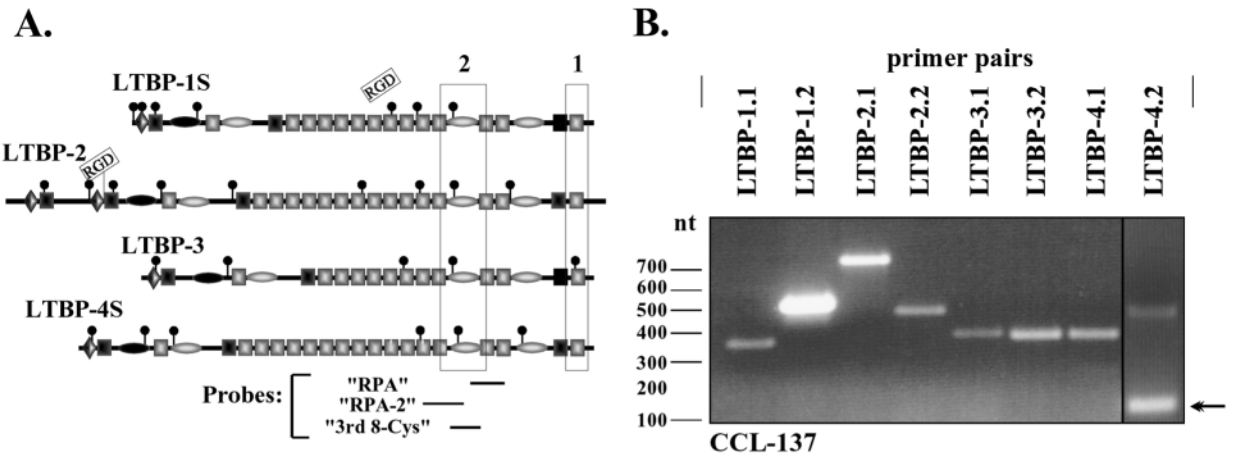
RESULTS

RT-PCR analysis of LTBP expression in cell lines

It has been observed that CCL-137 lung fibroblasts express at least LTBPs-1, -2 and -4 (Saharinen et al., 1998). To address the question whether this is a general feature of fibroblasts and other cell types to express several or all LTBPs, we analyzed by semi-quantitative RT-PCR the LTBP expression profiles in a panel of cell lines. These cell lines included two human embryonic lung fibroblast (CCL-137, WI-38), SV-40 transformed lung fibroblast (WI-38/VA-13), human skin fibroblast and fibrosarcoma (HT-1080) cell lines, as well as human umbilical vein endothelial cells (HUVEC) and immortalized human epidermal keratinocytes (HaCat).

Two sets of primers (see Materials and Methods) were used in the detection of LTBP isoforms. Because of the different splice variants described (see Saharinen et al., 1999), we selected two amplified areas; the first at the C-terminus and the second around the 3rd 8-Cys repeat (Fig. 1A). No alternatively spliced LTBP isoforms have earlier been identified around these areas. Since the exon-intron structures of human LTBP-2 (Bashir et al., 1996) and LTBP-3 (J.S. et al., unpublished) are known, we used this knowledge to design primers that would probably amplify regions spanning exon-intron boundaries. Data obtained by RT-PCR from these two different regions within each LTBP was very similar. Since the amplification efficiency of the different primer pairs varied to a certain extent, the levels of the different LTBP isoforms in each cell line could be compared only in a semi-quantitative manner (Fig. 1B). The two bands detected with LTBP-4.2 primers are discussed below.

All cell lines assayed expressed at least some of each LTBP (summarized in Fig. 1C). The expression levels of the panel of cell lines were compared to those of CCL-137 cells. LTBP-1 was abundant in embryonic lung fibroblasts and fibrosarcoma cells, while only low levels of expression could be detected in transformed VA-13 cells. LTBP-2 expression was most prominent in skin fibroblasts and endothelial cells, whereas only low levels were expressed in VA-13 cells. LTBP-3 expression was more uniform with highest expression in HaCat keratinocytes, and lowest in VA-13 cells. LTBP-4 was most prominent in embryonic lung fibroblasts and HaCat keratinocytes and lowest levels of expression were detected in skin fibroblasts and endothelial cells. Also, LTBP-4 was downregulated in VA-13 cells compared with the parental WI-38 cells, suggesting that malignant transformation downregulates LTBP production in general (see also Taipale et al., 1996; Koski et al., 1999).



C.

	Skin fibroblast	CCL-137 (lung)	WI-38 (lung)	VA-13	HT-1080	HUVEC	HaCat
LTBP-1	++	+++	+++	+	+++	++	++
LTBP-2	++++	+++	+++	+	+++	++++	++
LTBP-3	+++	+++	+++	+	+++	+++	++++
LTBP-4	+	+++	+++	++	++	+	+++

Fig. 1. RT-PCR analysis of LTBP expression in human cell lines. Total RNA isolated from different cell lines was reverse transcribed followed by PCR amplification using two different primer pairs per each LTBP. (A) LTBP-x.1 primers amplify products spanning from the last EGF-repeats to the noncoding region, whereas LTBP-x.2 primers amplify regions around the 3rd 8-Cys repeat (for primer sequences see Table 1). 'RPA' and '3rd 8-Cys' probes used in northern blotting and RNase protection assays are indicated under LTBP-4. In B, human lung fibroblast (CCL-137) RNA was used in RT-PCR reaction with the eight different primer pairs. Agarose gel electrophoreses of the amplified fragments are shown. An arrowhead indicates the LTBP-4Δ8-Cys3rd form. The molecular weight markers (nt) are shown on the left. (C) A summary of the arbitrary quantitation of the amounts of different LTBPs in the panel of the indicated cell lines. The amounts of specific products were equalized using G3PDH levels in each cell line, and compared with those of CCL-137 cells. These cell lines included human embryonic lung fibroblasts (CCL-137, WI-38), human skin fibroblasts, SV-40 transformed embryonic lung fibroblasts (VA-13; counterpart of WI-38 cells), human fibrosarcoma cells (HT-1080), human umbilical vein endothelial cells (HUVEC) and immortalized human skin keratinocytes (HaCat). The results, obtained from several independent experiments using two different primer pairs of each LTBP, were combined and are expressed arbitrarily from positive (+) to strong positive (++++).

Identification of a new LTBP-4 splice form

Amplification with LTBP-4 primers around the 3rd 8-Cys repeat (primers LTBP-4.2) resulted in two products (Fig. 2), the expected 486 bp product and an additional 147 bp product. The small product was sequenced. Nucleotides 3216-3554 encoding the 3rd 8-Cys repeat as well as flanking regions were missing from this newly identified LTBP-4 splice form, which we named LTBP-4Δ8-Cys3rd. The 147 bp fragment amplified more readily and was always the prominent product seen in RT-PCR. All cell lines studied expressed the LTBP-4Δ8-Cys3rd splice variant. Since only the 3rd 8-Cys repeat is responsible for covalent binding to small latent TGF-β (Saharinen et al., 1996), the splice variant is therefore unable to associate with small latent TGF-β (Saharinen and Keski-Oja, 2000).

Besides LTBP-4, both LTBP-1 and -3 bind small latent TGF-β readily (Saharinen et al., 1996; Gleizes et al., 1996; Saharinen and Keski-Oja, 2000). No other splice forms could be detected using the primers to amplify regions around the 3rd 8-Cys repeats in other LTBPs (see Fig. 1). This suggests that LTBP-4Δ8-Cys3rd is a unique splice form, although it cannot be ruled out that for some reason the RT-PCR method would have failed in amplifying a smaller product from the other LTBPs.

Genomic structure of LTBP-4 around the 3rd 8-Cys repeat

To analyze the genomic structure of LTBP-4 around the 3rd 8-Cys repeat, a PAC-clone containing LTBP-4 genomic sequences (Saharinen et al., 1998) was used as a template in PCR amplification. Primers LTBP-4.2 amplified a fragment of ~4.5 kb indicating the presence of ~4 kb of intron sequences (Fig. 3A). Primers ('3rd 8-Cys', see Materials and Methods) that amplify the 339 bp fragment absent from LTBP-4Δ8-Cys3rd cDNA resulted in a ~600 bp amplification product from the PAC clone. This indicated that intron sequences were also present between nucleotides 3216-3554 in LTBP-4 cDNA sequence.

The PCR products were cloned and partially sequenced. The positions of exon-intron boundaries as well as the flanking sequences are presented in Fig. 3B. The junctional sequences fit well with the consensus sequences at exon-intron boundaries in protein-coding genes. The 3rd 8-Cys repeat is encoded by two exons analogous to the exon-intron pattern in LTBP-2 and LTBP-3.

LTBP-4 mRNA expression analyses

RNase protection assays were used to analyze the expression of LTBP-4 mRNAs in HT-1080, WI-38 and VA-13 cells. The

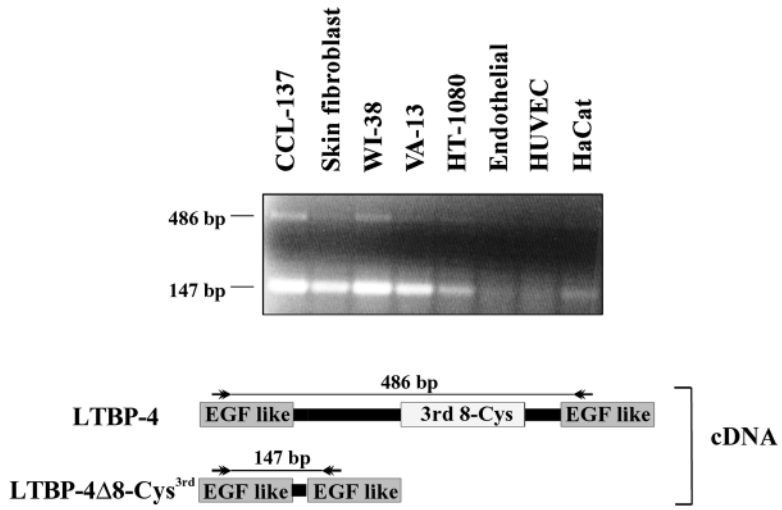


Fig. 2. Identification of a new LTBP-4 splice form. (Top) RT-PCR amplification of RNA from different cell lines using LTBP-4.2 primers. The migration of the expected 486 bp product as well as the novel 147 bp product in an agarose gel are indicated on the left. The cell line designations are indicated on the top. Endothelial denotes human microvascular endothelial cells. Both PCR products were cloned and sequenced. (Bottom) A schematic view of the amplified sequences. The new splice variant, LTBP-4Δ8-Cys^{3rd} is formed by splicing of a proline rich region and the 3rd 8-Cys repeat.

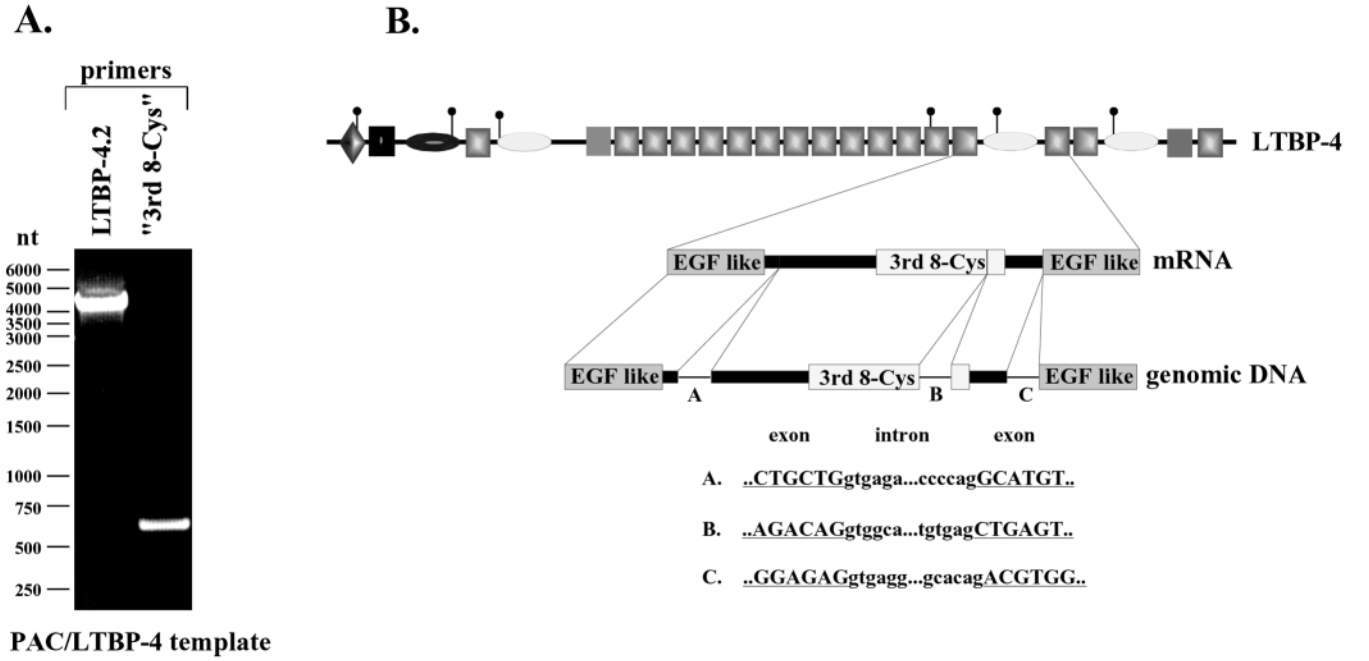


Fig. 3. Genomic structure of LTBP-4 around the 3rd 8-Cys repeat. (A) PAC/LTBP-4 clone containing genomic sequences from LTBP-4 was used as a template in PCR amplification using LTBP-4.2 and '3rd 8-Cys' primers. The '3rd 8-Cys' primers amplify a region that is absent from LTBP-4Δ8-Cys^{3rd} (for primer sequences see Table 1). The migration of ~4.5 kb and ~650 bp PCR products in an agarose gel is shown. The molecular weight markers (nt) are shown on the left. (B) Schematic representation of LTBP-4 as well as the exon-intron structure around the 3rd 8-Cys repeat. The sequences obtained from splice-site junctions of introns A, B and C are shown.

probe was designed to result in different sizes of protected fragments for mRNAs, whether or not they contained the 3rd 8-Cys repeat (see Materials and Methods). Fragment sizes of 331 bp and 225 bp corresponded to mRNAs containing or not containing the 3rd 8-Cys repeat, respectively. Both mRNAs were expressed in all three cell lines (Fig. 4A). In the transformed cell lines VA-13 and HT-1080, the expression of both LTBP-4 forms was lower than in WI-38 cells. These results are well in accordance with data obtained by RT-PCR.

Northern blotting of CCL-137 total RNA was performed with two different probes to analyze the mRNA species detected by these probes for subsequent use in Multiple Tissue Expression arrays (see below). 'RPA' probe contains sequences from the 3rd 8-Cys repeat as well as from the following EGF-repeat and detects both LTBP-4 splice forms. The '3rd 8-Cys' probe detects only mRNA species containing the 3rd 8-Cys repeat. The sizes of the two mRNAs differ only by 339 bp and they migrate at the same level in northern blots. Both probes detected the expected mRNA species of ~5 kb (Fig. 4B). Unexpectedly, a minor mRNA species of approximately 2 kb was also detected. When the northern filters were washed under high stringency conditions it was still detected, suggesting that it is specific and contains the 3rd 8-Cys repeat since both probes detected it. The identity of this minor mRNA was not further

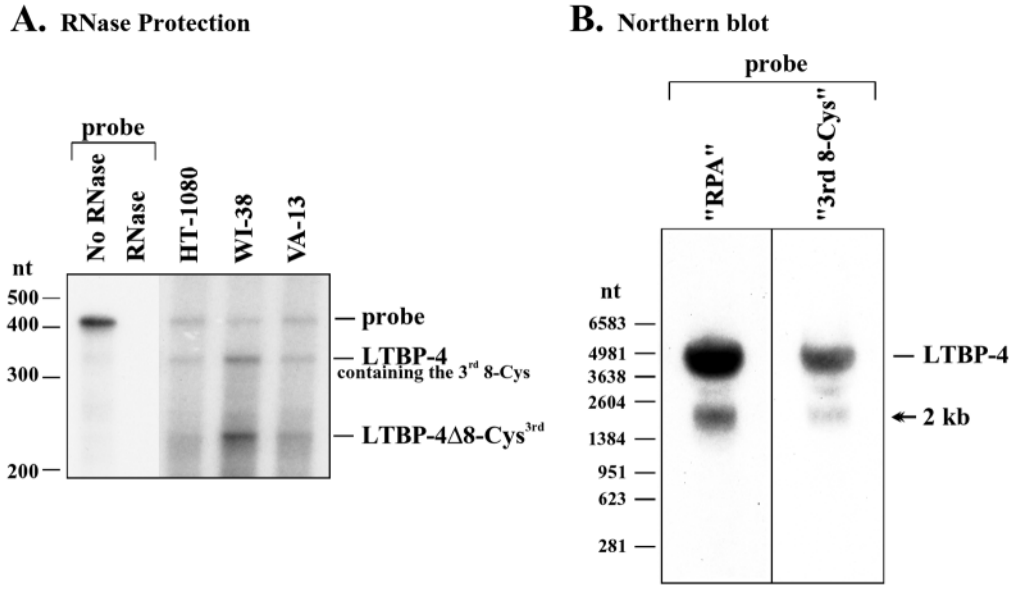


Fig. 4. Expression of LTBP-4 in cell lines. (A) RNase protection assay. Total cellular RNA was extracted from HT-1080, WI-38 and VA-13 cells. Total RNA (5 μg) was used in the assay. The probe was radioactively labeled and incubated with or without cellular RNA. After incubation RNA not protected with the probe was digested with RNase. After precipitation the protected fragments were separated in 6% polyacrylamide gels containing 8 M urea. Intact probe was used as a control. The two protected fragments corresponding to LTBP-4 containing the 3rd 8-Cys repeat as well as LTBP-4Δ8-Cys3rd are indicated on the right. The mobilities of molecular weight markers (nt) are shown on the left. (B) Northern blot analysis of human lung fibroblast (CCL-137) total RNA (10 μg). The RNA-filter was probed with 'RPA' probe detecting both LTBP-4 forms or with '3rd 8-Cys' probe detecting specifically LTBP-4 containing the 3rd 8-Cys repeat. An expected band of ~5 kb was detected. In addition, a minor mRNA species of ~2 kb was detected by both probes (arrowhead). The migration of RNA markers (nt) is shown on the left.

investigated. Northern blotting analysis of WI-38 lung fibroblasts gave similar results (data not shown).

Expression of LTBP-4 in different tissues

A multiple tissue expression array filter containing mRNAs from different tissues, was probed with both 'RPA' and '3rd 8-Cys' probes that detect total LTBP-4 (including LTBP-4Δ8-Cys3rd), or specifically LTBP-4 containing the 3rd 8-Cys repeat, respectively. Radioactivity was quantified using a phosphoimager. Each phosphoimager value obtained with one probe, after subtracting the background, was divided by the average value from all tissues. The expression pattern of LTBP-4 obtained using these two probes correlated well with our previous results (Saharinen et al., 1998) revealing high expression in aorta, heart ileum, jejunum, uterus and thyroid gland (data not shown). The relative values obtained with the two different probes were very similar in most tissues, indicating that the proportional expression of LTBP-4 forms were similar. However, in lymph node, bone marrow, peripheral blood leukocyte, thymus, lung, kidney and liver the relative values differed considerably, suggesting a different ratio of the two LTBP-4 isoforms compared with most other tissues (data not shown). Those tissues were selected for further analyses by RNase protection assay.

RNase protection assay from tissue mRNA

To analyze the expression of LTBP-4Δ8-Cys3rd and LTBP-4 containing the 8-Cys repeat in selected tissues, RNase protection assay was first performed using the 'RPA' probe as in Fig. 4. From the commercial tissue mRNAs, isolated from pooled tissue specimen, two additional protected fragments of ~240 bp and ~90 bp were detected. This was probably due to allelic variation at position 3685 leading to a mismatch with the probe and degradation by RNase at this site (see below and Fig. 6). LTBP-4Δ8-Cys3rd was found to be expressed in all tissues analyzed, but accurate quantification was not feasible (data not shown).

A new probe for RNase protection assay was designed ('RPA-2', see Materials and Methods), and polyA+ RNAs from heart, lung, lymph node, bone marrow, fetal liver, kidney and thymus were used in the assay. Three protected fragments of different sizes were detected and found to correlate with full length LTBP-4 (439 bp), LTBP-4 lacking the 13th EGF like repeat (LTBP-4ΔEGF-13, 313 bp) described previously (Saharinen et al., 1998), as well as LTBP-4Δ8-Cys3rd (274 bp). A faint band corresponding to the size of a splice form lacking both the EGF-like repeat and the 3rd 8-Cys repeat was consistently seen in lung mRNA samples, but quantification of this minor fragment was not possible. Radioactivity of the protected fragments was quantified using a phosphoimager (Fig. 5). LTBP-4Δ8-Cys3rd was found to be expressed in all tissues analyzed, but the relative ratios of the full length LTBP-4 and LTBP-4Δ8-Cys3rd were similar in tissues analyzed, not supporting data obtained with multiple tissue expression arrays. RNase protection assays from at least two different tissue mRNA preparations indicated that LTBP-4 is very prominently expressed in the adult lung, which was not clearly observed with the multiple tissue expression arrays. This indicates that only a limited amount of information can be obtained from those filters. More extended analyses of the expression of different splice variants in various tissues will probably provide clues to the functions of the different LTBP-4 forms.

LTBP-4 splice variant from HL-60 cells

HL-60 promyelocytic leukemia cells were included in the multiple tissue expression arrays and originally showed a different relative ratio for the LTBP-4 splice variants. It was therefore subjected to further analysis. RT-PCR assay from HL-60 mRNA revealed another LTBP-4 splice variant, which

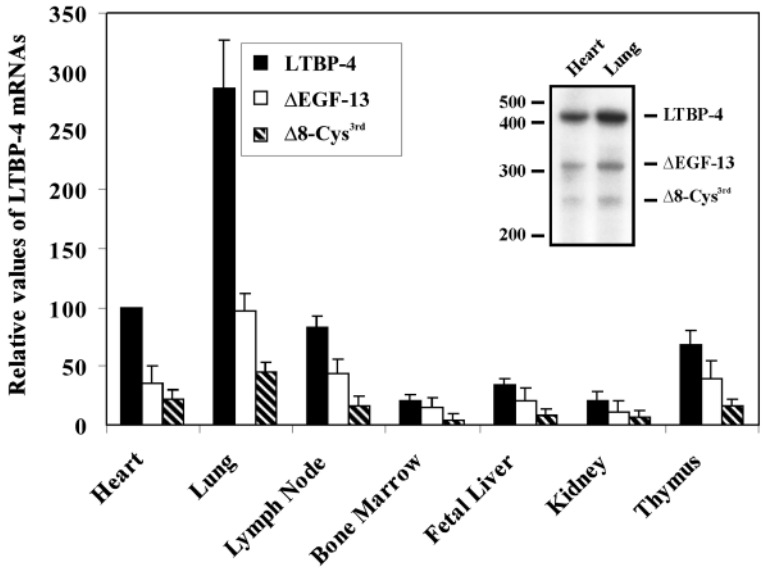


Fig. 5. RNase protection assay from tissue-derived mRNA. RNase protection assays were performed with 0.5 μg of polyA$^+$ RNA derived from different tissues as illustrated. The probe, 'RPA-2', was radioactively labeled and incubated with or without tissue mRNA as in Fig. 4A. Relative values of LTBP-4 mRNAs (see text for details) compared with LTBP-4 levels in the heart (set as 100%). Phosphoimager quantification of two separate experiments is presented with error bars. (Inset) RNase protection assay of heart and lung mRNAs. The protected fragments corresponding to LTBP-4 containing the 3rd 8-Cys repeat, LTBP-4Δ8-Cys^{3rd} as well as LTBP-4ΔEGF-13 are indicated on the right. The mobilities of the molecular weight markers (nt) are shown on the left of the inset.

lacks only the second exon (nt 3483-3553) of the 3rd 8-Cys repeat (Fig. 6). This exon codes for 24 amino acids, including the last cysteine residue of the 8-Cys repeat, and its absence probably has an effect on the structure of the rest of the domain. An LTBP-3 variant that lacks the first exon, coding the first seven cysteines, of the fourth 8-Cys repeat has been described (Yin et al., 1998). The variant identified from HL-60 cells, named LTBP-4Δ24 8-Cys, was not observed from the tissue mRNAs (data not shown), suggesting that it may be specific for cells of haematopoietic origin or related to the malignant phenotype of HL-60 cells. Sequencing of several RT-PCR products from separate experiments indicated that in the heart or in the HL-60 cells there was cytosine or thymidine at position 3685, respectively. This, in addition to the sequence data from LTBP-4 cloning papers (Saharinen et al., 1998; Giltay et al., 1997), suggests that there is allelic variation at position 3685. The amino acid sequence does not differ whether there is cytosine or thymidine at this position.

Computer search for human LTBP-4 sequences lacking the 3rd 8-Cys repeat

Full length LTBP-4 was used in a BLAST search (Altschul et al., 1990) to obtain LTBP-4 sequences that lacked one or both of the exons coding for the 3rd 8-Cys repeat. Two sequences lacking the second exon were retrieved from GenBank Human EST database (NCBI, NLM, NIH). They were cDNA sequences from libraries made from bulk tissue of serous papillary carcinoma (GenBank accession number: Aw118247) and normal germinal center B-cells (Aa832367). This suggests that the splice form found in HL-60 cells is also present in normal B-cells. cDNA sequences lacking the 3rd 8-Cys repeat were more abundantly found and included clones from libraries made from total fetus and adult brain (Ai039262, R87406, H46427, A1453016, H40747). Similar searches with full length LTBP-1, -2 or -3 did not result in sequences lacking the respective 8-Cys repeats, suggesting that this kind of splicing may be unique to LTBP-4.

Expression of LTBP-4 fragments and immunoblotting from cell conditioned medium

Since the expression of the full length recombinant LTBP-4 in cultured cells is very inefficient (Saharinen et al., 1998), we constructed expression plasmids spanning the region from the 3rd 8-Cys repeat to the end of the 4th 8-Cys repeat. pFull sequence is derived from the published LTBP-4S sequence (Saharinen et al., 1998), whereas pΔ8-Cys^{3rd} and pΔ24 8-Cys plasmids lack sequences that are missing from the newly identified splice variants, LTBP-4Δ8-Cys^{3rd} and LTBP-4Δ24 8-Cys, respectively. The expression constructs were transfected into 293T human kidney epithelial cells and aliquots of the conditioned media were subjected to immunoblotting analyses as described in Materials and Methods. All constructs were expressed and secreted to the conditioned media as detected by anti-HA antibodies (Fig. 7). As expected, the LTBP-4 antibody

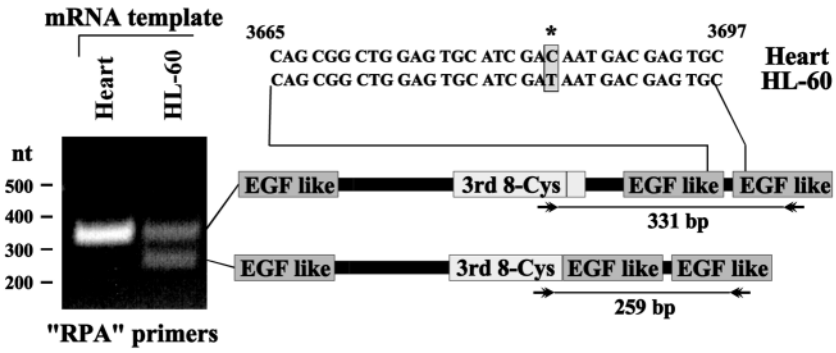


Fig. 6. RT-PCR from tissue mRNA with 'RPA' primers. RT-PCR amplification of polyA$^+$ RNA from heart tissue and HL-60 promyelocytic leukemia cells was performed using LTBP-4 primers ('RPA', see Materials and Methods). The migration of the expected 331 bp product as well as a novel 259 bp product is indicated and presented schematically. The molecular weight markers (nt) are shown on the left. The sequence between nucleotides 3665 and 3697 is also presented. An asterisk indicates a single nucleotide difference in the LTBP-4 sequence obtained from the heart and HL-60 cells.

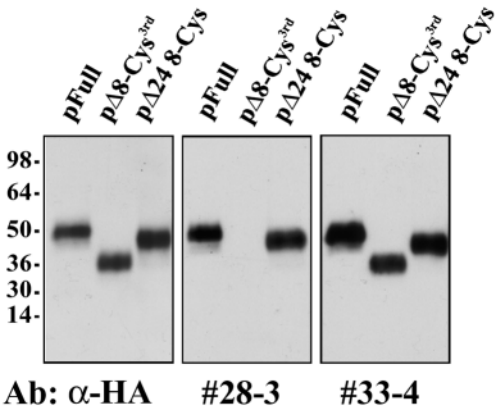


Fig. 7. Expression of LTBP-4 fragments and immunoblotting from cell conditioned medium. Expression constructs pFull, pΔ8-Cys^{3rd} and pΔ24 8-Cys were transfected into 293T cells and serum-free cell conditioned media were collected for 48 hours. Aliquots of the media were separated by 4-15% SDS-PAGE under reducing conditions, transferred to nitrocellulose filters and immunoblotted with the indicated antibodies. α-HA antibody recognizes the C-terminal HA-tag of the constructs, whereas #28-3 and #33-4 recognize the 3rd and 4th 8-Cys repeats of LTBP-4, respectively (Saharinen et al., 1998). The mobilities of the molecular weight markers (kDa) are shown on the left.

#28-3 made against the 3rd 8-Cys repeat did not detect pΔ8-Cys^{3rd}, but was able to detect pΔ24 8-Cys. #33-4 antibody, which recognizes the 4th 8-Cys repeat was able to react with all three constructs. These results suggest that cultured cells can produce LTBP-4 fragments lacking parts of the 8-Cys repeat and secrete them into the culture medium.

To demonstrate binding of full length LTBP-4 with TGFβ1•LAP, Saharinen et al. encountered problems with very low expression levels of the LTBP-4 protein (Saharinen et al., 1998). Also, smaller protein fragments containing the TGF-β binding domain showed lower binding capacity to TGFβ1•LAP than LTBP-1 fragments. We carried out co-transfection experiments to demonstrate complex formation between the mutated proteins and TGFβ1•LAP as described previously (Saharinen et al., 1998). Our numerous attempts proved to be constantly negative (data not shown; see also Saharinen and Keski-Oja, 2000).

DISCUSSION

Although LTBPs are known to bind small latent TGF-β and associate with the ECM, the specific functions of the four different isoforms are still relatively unclear. The expression of LTBPs is partially overlapping and there is little information on the expression levels in different cell types. Our current findings clarify the following aspects: (1) in a panel of fibroblasts of different origin, and in a few other cell types, LTBPs 1-4 were found to be expressed in an overlapping manner, but the levels of expression varied; (2) a general downregulation of LTBP expression was observed in an SV-40 (simian virus-40) transformed fibroblast cell line; (3) a novel LTBP-4 splice form lacking the TGF-β binding 8-Cys repeat (LTBP-4Δ8-Cys^{3rd}) was identified; (4) LTBP-4Δ8-Cys^{3rd} was found to be produced by alternative splicing over two exons; (5) the tissue expression profiles of LTBP-4 and LTBP-4Δ8-Cys^{3rd} indicated that they were expressed in the same tissues; and (6) a previously described LTBP-4 variant lacking an EGF-like repeat was expressed in a similar manner.

Latent TGF-β complexes are integral parts of the extracellular structures and, when activated, can regulate extracellular matrix composition by increasing the synthesis of matrix components and decreasing protease activity (Piek et al., 1999; Taipale et al., 1998). Matrix association and activation of TGF-β complexes form a finely tuned control network for the maintenance of the organization of extracellular structures. LTBPs function both as TGF-β-binding proteins and as structural components of the extracellular matrix (Taipale et al., 1996). The folding and secretion of TGF-β is dependent on simultaneously produced LTBP (Miyazono et al., 1991). LTBPs target TGF-β to extracellular structures for storage as well as for activation, where LTBPs play a role as well (Flaumenhaft et al., 1993). A role for LTBPs as components of the extracellular matrix 10 nm microfibrils as well as thicker fibronectin rich fibers has been observed (M. Hyytiäinen and J.K.-O., unpublished; Gibson et al., 1995; Taipale et al., 1996; Dallas et al., 2000). The existence of four different LTBPs with only partially overlapping expression patterns suggests important functions for LTBPs. LTBP-1 is mainly expressed in the heart, placenta, lung, spleen, kidney and stomach, and LTBP-2 in lung, skeletal muscle, liver and placenta (Kanzaki et al., 1990; Morén et al., 1994; Tsuji et al., 1990). LTBP-3 (J.S. et al., unpublished) and -4 have similar expression patterns, predominantly found in aorta, heart, small intestine and ovaries (Saharinen et al., 1998; Giltay et al., 1997). In fetal tissues, LTBP-1 and -2 are expressed more abundantly than LTBP-3 and -4 (reviewed by Saharinen et al., 1999).

We analyzed here the relative patterns of expression of LTBPs in cultured fibroblasts of different origin as well as in umbilical vein endothelial cells and HaCat keratinocytes by a semi-quantitative RT-PCR. We observed different expression levels in cells from different tissues. This suggests differential regulation of the LTBP/TGF-β system in tissues, as well as possible tissue-specific composition of the microfibrils. Fibrillins-1 and -2 are the major components of the 10 nm microfibrils and show tissue specificity in their expression levels (Sakai et al., 1986; Zhang et al., 1995). Together with different LTBPs and other components such as the microfibril associated glycoprotein (MAGP, Gibson et al., 1998), the composition and function of microfibrils may be regulated in a tissue specific manner.

In VA-13 cells (SV-40 virus-transformed WI-38 fibroblasts) the expression of all four LTBPs was downregulated. This notion is in accordance with previous reports indicating that malignant transformation can lead to decreased LTBP levels (Koski et al., 1999; Eklöv et al., 1993; Mizoi et al., 1993). Our earlier results indicate that VA-13 cells produce very little fibronectin matrix, which is paralleled with a marked decrease in LTBP-1 fibers (Taipale et al., 1996). The current results suggest that, in addition to LTBP-1, the other LTBPs are downregulated, and that the regulation is at the level of transcription. Cancer cells have often been found to produce aberrant amounts of TGF-β. They also fail to deposit TGF-β complexes to their extracellular matrices, probably due to decreased fibronectin matrix in cancer cells as well as

decreased LTBP production. This may be beneficial for tumor progression since the production of soluble TGF-β forms instead of tight LTBP-directed control may favor various paracrine effects in tumors including stimulation of angiogenesis and connective tissue formation.

LTBPs and fibrillins show structural variation. Proteins with different N-terminal regions are commonly produced and they are thought to bind extracellular matrix components with divergent affinities and possibly also specificities. For example, the production of the two forms of LTBP-1 (the longer protein having a 346 amino acid extension in the N-terminus) is regulated by independent promoters (Koski et al., 1999). Whether this is also the case for the LTBP-4 isoforms with differing N-termini (Giltay et al., 1997; Saharinen et al., 1998) is still unclear. Deletions or insertions of EGF-like domains are commonly found, and these probably alter the structure and function of the produced proteins (see Saharinen et al., 1999; Sakai et al., 1986; Koli et al., 2001). EGF-like domains participate in protein-protein interactions and via calcium binding also provide stability to protein structures. Changes in the number of EGF-like repeats alter the length of the molecule and possibly modulate its properties in the association with microfibrils. Of the 34 known 8-Cys repeats found in LTBPs and fibrillins only three are known to bind small latent TGF-β (Saharinen and Keski-Oja, 2000). An LTBP-1 splice variant that lacks a consensus heparin-binding domain has been suggested to be less protease sensitive, changing the ability of this particular variant to be released from the extracellular matrix (Michel et al., 1998; Öklü et al., 1998).

We discovered here an LTBP-4 splice variant that lacks the TGF-β binding domain, namely the 3rd 8-Cys repeat (LTBP-4Δ8-Cys3rd). This variant was produced by alternative splicing over two exons. The exon-intron structure of LTBP-4 around this area was similar to those of human LTBP-2 (Bashir et al., 1996) and LTBP-3 (J.S. et al., unpublished). In the mouse LTBP-3, a variant lacking most of the last 8-Cys repeat has been described (Yin et al., 1998). Although no specific function for the non-TGF-β binding 8-Cys repeats have been suggested, they are crucial to the structure of fibrillin-microfibrils (Lee et al., 1991). LTBP-1 and LTBP-3, which also bind small latent TGF-β via their respective 3rd 8-Cys repeats, were not found as similar splice variants in RT-PCR analyses. The expression of LTBP-4 and LTBP-4Δ8-Cys3rd in cell lines was analyzed by RNase protection assays and both forms were expressed, but major differences in expression levels were not noted. Also, LTBP-4Δ8-Cys3rd was expressed in a variety of normal human tissues.

Regulation of the relative amounts of LTBP-4Δ8-Cys3rd and LTBP-4 expressed by cells provides a novel level for the regulation of the TGF-β system. The existence of LTBP-4Δ8-Cys3rd, a variant unable to bind small latent TGF-β, is compatible with the idea of LTBPs having functions not restricted to the TGF-β system only. These include the regulation of cell adhesion to fibronectin or chemotactic properties, as described for LTBP-2 (M. Hyytiäinen and J.K.-O., unpublished) and -1, respectively (Kanzaki et al., 1998). Interestingly, the coding sequence between the 3rd 8-Cys repeat and the previous EGF-repeat in LTBP-4 is considerably longer than that in the other LTBPs and contains proline-rich sequences. These regions are thought to provide flexibility and protease sensitive sites to LTBP molecules. A proline-rich region is also spliced out in the LTBP-4Δ8-Cys3rd, which may lead to a more rigid structure and new functional properties.

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