Expression of alternatively spliced human latent transforming growth factor β binding protein-1

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Abstract Latent transforming growth factor β binding protein-1 (LTBP1) is important in regulating the localisation and activation of transforming growth factor β (TGFβ). Three forms of LTBP1 mRNA have previously been described, LTBP1L, LTBP1S and LTBPΔ53. Here, we have analysed the LTBP1 coding sequence and identified two other spliced forms, LTBP1Δ55 and LTBP1Δ41. LTBP1Δ55 is a short form of LTBP1L which lacks 55 amino acids including two consensus *N*-glycosylation sites and LTBP1Δ41 is a form of LTBP1 which lacks the 12th EGF-like repeat. Furthermore, sequencing of genomic clones showed that splicing to generate LTBP1L occurs using an intra-exonic 3′ splice acceptor site in the first coding exon of LTBP1S and that LTBP1Δ55 arises from the alternative use of an exonic 3′ splice acceptor site at the end of the following intron. LTBP1Δ41 arises from skipping the exon which encodes the 12th EGF-like repeat. LTBP1Δ55 and LTBP1Δ41 mRNA are expressed in a wide variety of human tissues but the proportions of each splice form vary in the tissues.

Key words: Latent transforming growth factor β binding protein; EGF-like repeat; Alternative splicing

1. Introduction

Transforming growth factor β (TGFβ) is a widely expressed cytokine that influences the growth and differentiation of many cell types [1–4]. In addition to important roles in normal physiology, TGFβ has been implicated in several pathological conditions including atherosclerosis [5,6], fibrosis [7] and some cancers [8–10]. TGFβ is secreted from cells as part of small (∼100 kDa) or large (∼270 kDa) latent complexes from which the active protein can be released. The small latent complex consists of the mature TGFβ homodimer (∼25 kDa) non-covalently associated with the latency-associated peptide (LAP, the amino-terminal remnant of the TGFβ precursor) and association of LAP with mature TGFβ is sufficient to confer latency. The large latent complex consists of the small latent complex disulfide-bonded to a latent TGFβ binding protein (LTBP).

To date four LTBP genes have been cloned (*LTBP1*, *LTBP2*, *LTBP3* and *LTBP4*) that encode proteins sharing approximately 40% sequence homology. This family of proteins appears to be closely related to the fibrillins in that its members contain long, tandem arrays of EGF-like repeats and a novel eight-cysteine repeat of unknown function [11]. Some of the EGF-like domains contain a consensus calcium binding sequence and calcium binding has been shown to confer resistance to proteolysis on fibrillin [12] and LTBP1 [13]. The third eight-cysteine repeat in LTBP1 contains a putative *N*-glycosylation site, and is necessary and sufficient for the covalent interaction with the small latent complex of TGFβ [14,15].

In some cell types LTBP1 may be necessary for the normal assembly and secretion of TGFβ [16] although other cells have been shown to secrete the small latent complex of TGFβ [17]. In addition, several studies have suggested that LTBP plays a role in regulating the activity of TGFβ. For example, addition of excess free LTBP, a peptide fragment of LTBP, or an antibody directed against native platelet LTBP inhibits TGFβ activation in a dose-dependent manner [18–20]. LTBP1 has also been shown to associate with the extracellular matrix (ECM) in vivo and in vitro [21–24]. These observations have led to the suggestion that LTBP1 is a component of the ECM. However, it is also possible that the role of the interaction between LTBP1 and the ECM is to localise TGFβ. Immunohistochemistry and in situ hybridisation experiments have shown LTBP1 levels to be altered in certain pathological conditions, including some cancers, although the role of LTBP1 in these diseases remains to be determined [25–32].

LTBP1 purified from different cell types varies in molecular weight from 125 kDa in platelets to 190 kDa in fibroblasts [11,33]. Northern analysis has shown that most tissues express two isoforms of *LTBP1* mRNA, a shorter 5.2 kbp mRNA (*LTBP1S*) and a longer 7.0 kbp mRNA (*LTBP1L*) [34]. *LTBP1L* mRNA encodes an additional 346 amino acids and the LTBP1L protein has a greater affinity for the ECM than LTBP1S [34]. In addition to these two forms of LTBP1, we and others have recently described a form of *LTBP1* mRNA (LTBP1Δ53) which encodes a protein that lacks 53 amino acids including the eighth cysteine of the first cysteine repeat and a consensus heparin binding sequence [35,36]. Alternative splicing or post-translational modifications may therefore account for some of the variations in the molecular weight of LTBP1 protein. In this study we have analysed *LTBP1* mRNA to determine other regions of the *LTBP1* message which can be alternatively spliced.

2. Materials and methods

2.1. Human tissue samples

Human heart, aorta and lung specimens were obtained during heart and lung transplantation at Papworth Hospital (Cambridge, UK), immediately frozen in liquid nitrogen and stored at −80°C until analysed. Human spleen, liver, gall bladder, cervix, ovary, tonsil, foreskin and veins were obtained from Addenbrooke's Hospital (Cam-

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Abbreviations: TGFβ, transforming growth factor β; LTBP, latent transforming growth factor β binding protein; LAP, latency-associated peptide; LR-PCR, long-range polymerase chain reaction; RT, reverse transcriptase; EGF, epidermal growth factor

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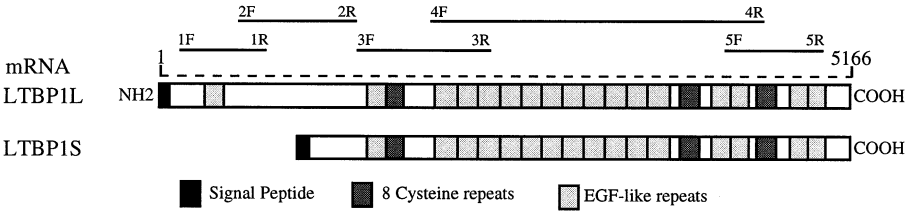

Fig. 1. Regions of LTBP1 amplified to identify alternatively spliced forms. Schematic representation of the LTBP1 proteins LTBP1L and LTBP1S. The dashed line represents the mRNA and the solid lines above show the regions of mRNA amplified by primer pairs 1–5. Primers are labeled F for forward and R for reverse. The LTBP1 mRNA is numbered as described in Section 2.

bridge, UK) surgical tissue bank. Normal human breast epithelial cells were provided by Dr. Mike O'Hare, Department of Surgery, University College/Middlesex Medical School, London.

2.2. RNA and DNA manipulations

Total RNA was extracted from normal human tissues using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Three micrograms of total RNA was reverse transcribed as previously described [35]. The PCR products were separated by electrophoresis using a 1.5% agarose gel and DNA was visualised by staining with ethidium bromide. PCR products from the gel were purified using a gel extraction kit (Qiagen) and cloned into pGem-T-Easy vector (Promega). DNA sequencing was performed using the ABI Prism automated DNA sequencer using the dye terminator procedure. Southern blot analysis, library screening, short-range PCR and long-range (LR) PCR with plasmid DNA, cDNA and cosmid DNA were carried out as previously described [35]. A human chromosome 2 cosmid library (LL02NC02) on stamped filters was obtained from the UK HGMP Resource Center and was screened by hybridisation to a ^{32}P-labeled 924 bp PCR product from primer pair B (Fig. 1). For Southern analysis, ^{32}P-labeled probes were made using PCR products from primers 10F and 2R, and 6F and 18R, as described above, for detecting LTBP1Δ55 and LTBP1Δ41, respectively.

2.3. Numbering of LTBP1 sequence

The first base of LTBP1L cDNA (accession number L48925) is defined as base 1 (Fig. 1). The longer form of LTBP1 therefore contains a coding sequence of 5166 bases. The LTBP1S message (accession number M34057) contains 145 bases not present in the LTBP1L sequence, designated S1–S145 (Fig. 3a).

Table 1
Primer sequences used

Primer	Sequence
1F	5′-TCTTCGCGGACATTCAACGTCGCGCTCAAC-3′
1R	5′-ACGAGGTGTCCTGGGCAGCTATTGTTTC-3′
2F	5′-CCTAGCTGTGTTCCGCCATGTCAGAATG-3′
2R	5′-CTGGACAGGAAGCCCTTGGTACGAGACTTG-3′
3F	5′-CAAGGTGTATGCCCTAATGGTGAGTGTTTG-3′
3R	5′-CTCACTGGCCATAAATCCTGCTGGGCAAATG-3′
4F	5′-CGCAGGCCAATCCATCACCATGTAGGTAAAG-3′
4R	5′-GCTACACACGTATTCATCACTCAGATGTTC-3′
5F	5′-GCGGCATCCTCAATGGATGTGAAAATG-3′
5R	5′-GCCACACCTATCGGGCTCATCCTAAATC-3′
6F	5′-GCAAGGGGAGTGCCTAAACACAGAG-3′
7F	5′-CACTGTTTGTGACAGTCACGGGTTTTGTGAC-3′
8R	5′-CCTGGGGCTTGAAAGCCCTGATAACAGAG-3′
9R	5′-CAGGAAGGACCCTTCCACGTTTTCACAGAAG-3′
10F	5′-CAGGAGAGCAGTCCACTGAAGGTTC-3′
11R	5′-AAGGGAAAGAACCTTCAGTGGACTGCTCTC-3′
12F	5′-CCTTCCAAGGCAAGTTCATGGATACTAAGCTG-3′
13R	5′-GTGGTGTTCCCCTCCTCACAGCTGTTC-3′
14F	5′-GTAAAGTGACCTGCACCAAGGGCAGCTG-3′
15F	5′-CCATGGTGCCAGCGTGCCTAAACTTTATC-3′
16R	5′-GCTAGTCACGGTCAGAGGCAAGGTATGTGTTG-3′
17F	5′-CAACCAGGCCAATCCCAAGTCTCGTACCAAG-3′
18R	5′-CCACCAGCTTCAGAAGTAGATTCTCCAG-3′

3. Results

3.1. Identification of alternatively spliced LTBP1 mRNA

To identify alternatively spliced forms of the *LTBP1* gene, RNA was isolated from human aorta and human breast epithelial cells. This RNA was reverse transcribed using random hexamers and amplified using five primer pairs (1–5 refer to a forward and a reverse primer, Table 1) designed to amplify overlapping fragments covering bases 151–5266 of *LTBP1* (Fig. 1).

Primer pair 2 generated three products of 924 bp, ~890 bp and 756 bp (Fig. 2, lane 1). The sequence of the largest fragment was identical to the corresponding region of the LTBP1L sequence described by Olofsson et al. [34] while that of the shortest lacked bases 1037–1204, suggesting that the mRNA was alternatively spliced. We define the form of *LTBP1* mRNA with this deletion as LTBP1Δ55. When the fragment of intermediate size (~890 bp) was cloned, the sequence obtained corresponded only to either the 924 bp or the 756 bp fragment. Mixing the purified DNAs of the large and small fragments followed by PCR in the absence of *Taq* polymerase produced a gel pattern identical to that of the RT-PCRs, indicating that the middle fragment was a heteroduplex of the other two products (data not shown).

Primer pair 3 also produced three bands consistent with splicing to generate LTBP1 and LTBP1Δ53, as previously described, in addition to the formation of another heteroduplex which was confirmed by sequencing. Primer pair 4 produced two products, one of the expected size (~2400 bp) and one about 150 bp shorter. These fragments were cloned into pGem-T-Easy and sequenced. The 3′ and 5′ se-

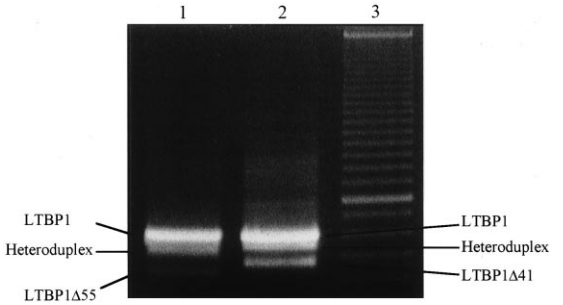

Fig. 2. PCR products of LTBP1 cDNA from human lung tissue. RNA was extracted from human lung tissue, reverse transcribed and amplified as described in Section 2. Lane 1: LTBP1 and LTBP1Δ55 amplified using primers 10F and 2R (Table 1). Lane 2: LTBP1 and LTBP1Δ41 amplified using primers 6F and 18R. Lane 3: 100 bp ladder. The middle band in lanes 1 and 2 is a heteroduplex of the outer two bands.

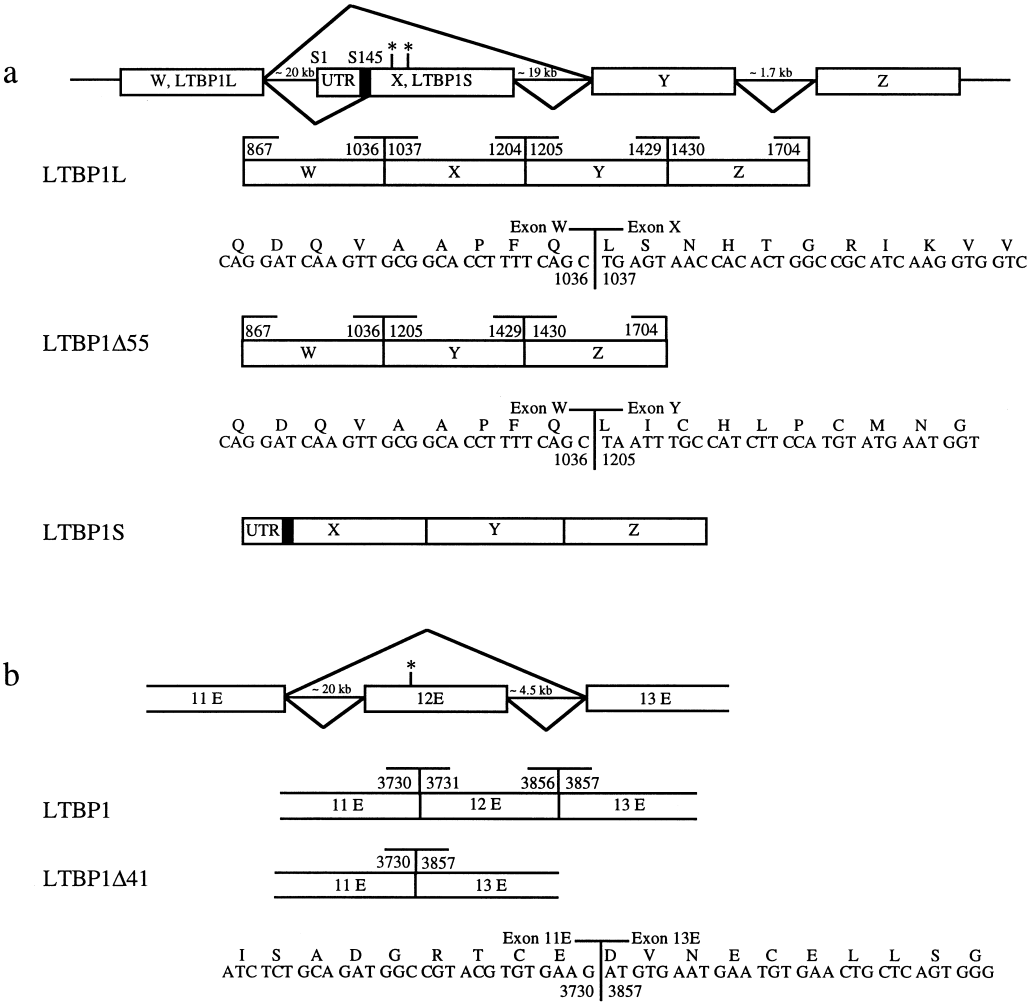

Fig. 3. Intron/exon structure of the LTBP1 gene in the regions spliced to generate LTBP1Δ55 and LTBP1Δ41. a: Nucleotide positions of the four exons of LTBP1 are shown as defined in Section 2. LTBP1L: exon W is joined to exon X through the use of an intra-exonic 3′ splice acceptor site. LTBP1Δ55: exon X is omitted. LTBP1S: bases S1–S145 (the 5′ untranslated region and signal peptide) are included in exon X. b: LTBP1: exon 12E included. LTBP1Δ41: exon 12E omitted. The asterisks represent putative *N*-glycosylation sites.

quences of both clones were identical to those of LTBP1 cDNA (M34057) indicating that the shorter fragment had an internal deletion. Comparison of this region of LTBP1 cDNA with the Unigene EST database (Unigene code HS.119574) identified cDNA clones AA522895 and AA056948 which have bases 3731–3856 deleted. Therefore four new primers (6F, 7F, 8R and 9R, Table 1) were synthesised and used for PCR and sequencing of the long and short clones. In addition to the expected products, PCR using primers 6F and 18R produced an intermediate-sized fragment which was also confirmed to be a heteroduplex by sequencing (Fig. 2, lane 2). Sequencing from primers 6F, 7F, 8R and 9R showed that the shorter clone we had identified was identical to the above EST sequences. The amino acid sequence encoded by bases 3731–3856 is the 12th EGF-like repeat in LTBP1L and we define the form of LTBP1 with this deletion as LTBP1Δ41. Primer pairs 1 and 5 both produced single products which were of the expected size and sequence, indicating that alternative splicing does not occur in these regions of LTBP1 mRNA in breast epithelial cells and human aorta examined here.

3.2. Genomic confirmation of the corresponding LTBP1Δ55 and LTBP1Δ41 cDNA

To confirm the cDNA data which suggested LTBP1Δ55 and LTBP1Δ41 as new forms of *LTBP1* mRNA, genomic structures of the relevant regions were defined. A chromosome 2 cosmid library was screened with the radioactively labeled 924 bp PCR product from primer pair 2. Ten cosmids were isolated (AE70c1, AE39g13, AE58m17, AE76g21, AE76g19, AE31a24, AE26c8, AE28n16, AE32o19, and AE23d3) and restriction endonuclease digests indicated genomic inserts to range from 40 to 50 kbp.

Southern analysis and PCR (both short- and long-range) were used to map the cosmid inserts and to select four cosmids covering bases 867–1704 of the cDNA sequence. Sequencing cosmid AE31a24 with primers 10F and 11R showed that bases 867–1036 form a discrete exon (Figs. 3a and 4). Sequencing of this cosmid from primer 12F did not produce a product suggesting that this cosmid does not contain the bases S1–S145. LR-PCR of human genomic DNA using primers 10F and 13R produced a product of ~20 kbp.

Sequencing cosmid AE23d3 with primers 12F, 13R and 14F

Exon		Intron/Exon sequence	Encoded feature
		Intron 5' Exon 3' Intron	
W:	170 bp	ttttgtttcttcccagAGTGACTCCT.....CCTTTTCAGCgtgagtatagtctta	?
X:	168 bp	**tgcctccgctcctag**TGAGTAACCA.....TTCCGAGTGGgtgagttcctccacg	2 N-Glycosylation sites
Y:	225 bp	ctcttttccctttagTAATTTGCCA.....GGAGTCAAAGgtaagctttttcatt	N-Glycosylation site
Z:	275 bp	tcccaatctgttgtagTGAAATTTCC.....TGGGTCACAGgtaaacatcatcaccg	?
12E:	126 bp, 3'end	.....GGGTGTGTGGgtgagttttagattt	EGF-like repeat
13E:	5' end	gtgctccctttccagATGTGAATGA.....	EGF-like repeat
Splice consensus sequence		$(y)_n$n(c/t)agG.....AGgtaagt	

Fig. 4. Intron/exon boundaries of exons W, X, Y, Z, 12E-3′ end and 13E-5′ end. Intron sequences are in lower case and exon sequences are in capitals. The sequence in bold for exon X encodes part of the LTBP1S signal peptide and is the intra-exonic 3′ splice acceptor used to generate LTBP1L. Exon sizes are in bp.

showed that bases S1–S145 and 1037–1204 are in the same exon and comparison of the genomic DNA sequence with the cDNA sequence data shows that the long LTBP1 message is formed by the use of an intra-exonic splice junction (Figs. 3a and 4). This intra-exonic splice acceptor site is 3′ to the putative signal peptide sequence cleavage site of LTBP1S. Sequencing cosmids AE39g13 and AE32o19 with primers 2R, 15F, 16R, and 17F showed that bases 1205–1429 and 1430–1704 form discrete exons. Comparison of these data with the cDNA sequences shows that the shorter form of LTBP1L is formed by use of the 3′ splice acceptor site at the end of the intron separating nucleotides 1204 and 1205. LR-PCR using primers 2R, 14F, 15F and 16R showed that a ~19 kbp intron separates exons X and Y and that a ~1.7 kbp intron separates exons Y and Z (Fig. 3a).

To define the genomic structure of the cDNA forms obtained from PCR with primer pair 4, human genomic DNA was amplified by LR-PCR from primers 6F and 8R, and 7F and 9R. These reactions produced products of ~20 kbp and ~4.5 kbp. Direct sequencing of the genomic amplification product from primers 7F and 9R showed that nucleotides 3856 and 3857 were in adjacent exons separated by a 4.5 kbp intron (Figs. 3b and 4). Alignment of the EST sequences and the sequences presented here, in conjunction with the LR-PCR data, indicates that nucleotides 3730 and 3731 are in adjacent exons separated by a 20 kbp intron.

3.3. Tissue distribution of the corresponding RNA for LTBP1Δ55 and LTBP1Δ41

To determine the tissue-specific expression of the spliced variants, RNA was isolated from a series of human tissues and reverse transcribed using random hexamers. The cDNA generated was amplified with primers 10F and 2R, and primers 6F and 18R to show the expression pattern for LTBP1Δ55 and LTBP1Δ41 DNA, respectively, and Southern blotted. Southern blot analysis show both splice variants to be widely expressed in human tissues in varying proportions. LTBP1Δ55 is most strongly expressed in lungs, breast epithelial cells, cervix, ovaries and tonsils (Fig. 5). LTBP1Δ41 is the minor form in most tissues but is the predominant form in heart muscle (Fig. 6).

4. Discussion

The data presented define two further alternative splices in the *LTBP1* gene and show the genomic structure in the vicinity of the splice sites. One region which can be alternatively spliced generates two forms of LTBP1L. In the longer form, splicing joins nucleotides 1036 and 1037 of LTBP1L cDNA using a 3′ splice acceptor site within the first coding exon of LTBP1S (Fig. 3a). The shorter form of LTBP1L (LTBP1Δ55) joins nucleotides 1036 and 1205 of the cDNA and uses the splice acceptor site at the 3′ end of the intron following the first coding exon of LTBP1S. Intra-exonic splicing normally occurs when there are two possible (C/T)AG sequences close

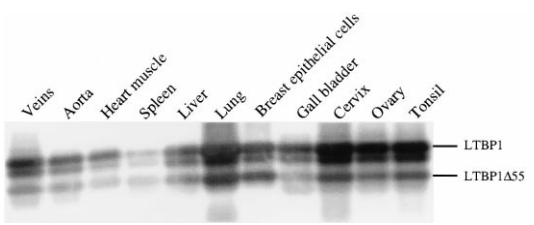

Fig. 5. Expression pattern of LTBP1 and LTBP1Δ55. RNA was isolated from human tissues, reverse transcribed, amplified using primers 10F and 2R and the product detected by Southern analysis using an LTBP1 DNA probe.

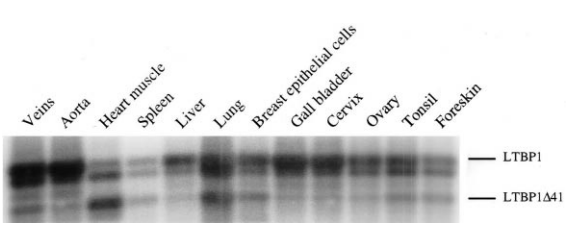

Fig. 6. Expression pattern of LTBP1 and LTBP1Δ41. RNA was isolated from human tissues, reverse transcribed, amplified using primers 6F and 18R and the product detected by Southern analysis using an LTBP1 DNA probe.

together (e.g. as described for rat pro-opiomelanocortin [37]). However, as in LTBP1Δ53, there is another complete splice acceptor site within the coding sequence for LTBP1S [35]. The amino acids deleted in LTBP1Δ55 encode two *N*-glycosylation sites, identified by sequence homology. The functional significance of these alternative forms remains to be elucidated.

The other region in which we observed alternative splicing contains the tandem EGF-like repeats. In the long form of the mRNA, nucleotides 3730 and 3731 are joined whereas in the shorter form nucleotides 3730 and 3857 are joined. The genomic structure defined by PCR and sequencing from human genomic DNA showed that nucleotides 3731–3856 form a discrete exon and this exon is skipped in the short form of the mRNA. These nucleotides code for a single EGF-like repeat (the 12th EGF-like repeat of LTBP1) which contains a consensus calcium binding site and a putative *N*-glycosylation site.

EGF-like repeats have been shown to be important in protein-protein interactions. For example, deletion mutagenesis of Notch, a receptor involved in cell fate determination, identified two EGF-like repeats which are necessary and sufficient for binding the Notch ligands Delta and Serrate [38]. The interaction between protein S and complement C4b-binding protein [39] and the fibronectin binding sites of fibulin-1 [40] have also been mapped to calcium binding EGF-like repeats. In addition, calcium binding to EGF-like repeats in fibrillin and LTBP1 stabilises the proteins and protects them from proteolysis [12,13]. There are several examples where loss of an EGF-like repeat occurs through splicing [41–43]. Although an altered function for these proteins has been suggested, the functional effects of deleting an exon encoding an EGF-like repeat is still unclear. However, mutations that cause skipping of exons containing EGF-like repeats in the fibrillin gene can cause a severe form of the connective tissue disorder Marfan's syndrome [44]. LTBP1, which is structurally similar to fibrillin, has been suggested to be an ECM-associated protein [21–23]. It is therefore possible that loss of the EGF-like repeat in LTBP1Δ41 alters the protein-protein interactions of LTBP1, resulting in a different function or localisation for this form of the protein.

In conclusion, we have described two new alternative splices of the *LTBP1* gene, LTBP1Δ55 and LTBP1Δ41, and showed their expression pattern in human tissues. Whether these truncated forms of the LTBP1 protein have functions in TGFβ activation or LTBP localisation, and whether their expression is correlated with disease remains to be determined.

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