Efficient Association of an Amino-terminally Extended Form of Human Latent Transforming Growth Factor-β Binding Protein with the Extracellular Matrix*

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The abbreviations used are: TGF-β, transforming growth factor-β; EGF, epidermal growth factor; HEL, human erythroleukemia; LAP, latency-associated peptide; LTBP, latent TGF-β binding protein; Ref. 11) as a sense primer (nucleotides 487–507) and a sequence of human LTBP-1S (10) as an antisense primer (nucleotides 162–182). PCR was performed using a Perkin-Elmer DNA thermal cycler with Pyrococcus furiosus DNA polymerase (Stratagene) and with linearized U-1240 MG glioblastoma DNA as a template. Thus a 570-base pair fragment specific for TGF-β precursor, which confers the latency of TGF-β (9) forming the small latent complex.

LTBP-1 is disulfide-linked to LAP in the large latent TGF-β complex (10, 11). About two-thirds of the sequence of LTBP-1 is composed of two different types of cysteine-rich repeat sequences; there are 16–18 copies of epidermal growth factor (EGF)-like repeats and 3–4 copies of a repeat containing eight cysteine residues (eight-cysteine repeat). EGF-like repeats are found in many other proteins and are suggested to be important in homophilic or heterophilic protein-protein interactions (12). The functions of the eight-cysteine repeats are unknown. Multiple EGF-like repeats and eight-cysteine repeats have thus far been identified in a family of proteins, consisting of LTBP-1, LTBP-2 (13), LTBP-3 (14), and components of microfibrils of elastic tissues, fibrillin-1 and -2 (15–17).

LTBP-1 is not directly needed for the latency of TGF-β, but it appears to have several important functions. It plays important roles in the assembly and secretion of latent TGF-β complex from certain cell types (6). In human fibroblasts and fibrosarcoma cells, LTBP-1 binds to extracellular matrix after secretion and is released from the matrix by proteolytic digestion (18, 19). In addition, LTBP-1 is important for the activation of latent TGF-β, e.g. in co-culture of endothelial cells and smooth muscle cells (20).

In human platelets, LTBP-1 is observed as multiple components of 125–160 kDa. In contrast, it is observed as 190–210-kDa components in the media from human foreskin fibroblasts (10) and human erythroleukemia (HEL) cells (6). The difference in the sizes may be in part due to proteolytic processing of the protein (6). In addition, two different transcript sizes (approximately 5.2 and 7.0 kb) of LTBP-1 mRNA have been observed, which suggests use of different polyadenylation sites or different promoters or the presence of alternative splice variants (10, 11). Comparison of LTBP-1 cDNAs from human foreskin fibroblast and rat kidney cDNA libraries (10, 11), revealed that the rat clone was longer than the human clone in the 5′-part of the open reading frame. Here we report the cDNA cloning of a longer version of human LTBP-1S (LTBP-1L) and show that the extracellular matrix binding properties of the long and short variants of LTBP-1 differ.

EXPERIMENTAL PROCEDURES

Cloning of a cDNA for LTBP-1L—A partial clone for LTBP-1L was obtained by polymerase chain reaction (PCR) using a sequence of rat LTBP-1S (large subunit of TGF-β masking protein; Ref. 11) as a sense primer (nucleotides 487–507) and a sequence of human LTBP-1S (10) as an antisense primer (nucleotides 162–182). PCR was performed using a Perkin-Elmer DNA thermal cycler with Pyrococcus furiosus DNA polymerase (Stratagene) and with linearized U-1240 MG glioblastoma DNA as a template. Thus a 570-base pair fragment specific for LTBP-1L was generated. A 570-base pair fragment specific for LTBP-1L was obtained by polymerase chain reaction (PCR) using a sequence of rat LTBP-1S (large subunit of TGF-β masking protein; Ref. 11) as a sense primer (nucleotides 487–507) and a sequence of human LTBP-1S (10) as an antisense primer (nucleotides 162–182). PCR was performed using a Perkin-Elmer DNA thermal cycler with Pyrococcus furiosus DNA polymerase (Stratagene) and with linearized U-1240 MG glioblastoma DNA as a template. Thus a 570-base pair fragment specific for LTBP-1L was generated. A 570-base pair fragment specific for LTBP-1L was obtained by polymerase chain reaction (PCR) using a sequence of rat LTBP-1S (large subunit of TGF-β masking protein; Ref. 11) as a sense primer (nucleotides 487–507) and a sequence of human LTBP-1S (10) as an antisense primer (nucleotides 162–182). PCR was performed using a Perkin-Elmer DNA thermal cycler with Pyrococcus furiosus DNA polymerase (Stratagene) and with linearized U-1240 MG glioblastoma DNA as a template. Thus a 570-base pair fragment specific for LTBP-1L was generated. A 570-base pair fragment specific for LTBP-1L was obtained by polymerase chain reaction (PCR) using a sequence of rat LTBP-1S (large subunit of TGF-β masking protein; Ref. 11) as a sense primer (nucleotides 487–507) and a sequence of human LTBP-1S (10) as an antisense primer (nucleotides 162–182). PCR was performed using a Perkin-Elmer DNA thermal cycler with Pyrococcus furiosus DNA polymerase (Stratagene) and with linearized U-1240 MG glioblastoma DNA as a template. Thus a 570-base pair fragment specific for LTBP-1L was generated. A 570-base pair fragment specific for LTBP-1L was obtained by polymerase chain reaction (PCR) using a sequence of rat LTBP-1S (large subunit of TGF-β masking protein; Ref. 11) as a sense primer (nucleotides 487–507) and a sequence of human LTBP-1S (10) as an antisense primer (nucleotides 162–182). PCR was performed using a Perkin-Elmer DNA thermal cycler with Pyrococcus furiosus DNA polymerase (Stratagene) and with linearized U-1240 MG glioblastoma DNA as a template.
nitrocellulose replica filter papers and washing were performed essentially as described (10, 13). Purification of positive bacteriophage plaques was performed as described (22). A clone termed H4 with a 4.1-kb insert was isolated from the cDNA library. The 5'-part of clone H4 was cut by EcoRI, subcloned into pBluescript SK vector, and sequenced by the dideoxy chain termination method (23) on both strands using T7 DNA polymerase (Pharmacia Biotech Inc.). DNA sequences were analyzed by the DNA STAR computer program (DNA STAR, Ltd.). Amino acid sequences were compared by the Clustal method (24).

Northern Hybridization Analysis—A 635-base pair Pst restriction fragment of LTBP-1S (BPA-13, nucleotides 1516–2151) and the PCR product specific for LTBP-1L were radiolabeled by the Megaprime DNA labeling system and used as probes. A Northern hybridization filter with mRNAs from different human tissues (Clontech) was hybridized and washed, essentially as described (13), followed by autoradiography.

Transient Transfection—For the construction of a plasmid for LTBP-1L, the 3'-part of BPA 13 (10) and the H4.4 cDNA clones were ligated at the overlapping EcoRI site (see Fig. 1A) to generate a complete LTBP-1L cDNA. A transient expression plasmid for LTBP-1L was then constructed by subcloning LTBP-1L cDNA into the pSV7d expression vector (25) as described. A transient expression plasmid for LTBP-1S cDNA, subcloned into pSV7d (10), was also used. COS-1 cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 50 μg/ml streptomycin. Transfection of the following day at subconfluency with 4 μg of plasmid and 6 μl of Transfectam (Promega) according to the manufacturer’s protocol.

In brief, cells were seeded into six-well tissue culture dishes (Falcon) and transfected the following day at subconfluency with 4 μg of plasmid and 6 μl of Transfectam. After 12 h of incubation, cells were washed twice with phosphate-buffered saline (137 mM NaCl, 8 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄), and incubated with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 50 μg/ml streptomycin. One and a half days after transfection, the cells were used for metabolic labeling and immunoprecipitation. In the experiment using coated culture dishes, the culture dishes were coated with 0.5 ml of fibronectin (Sigma) and centrifuged, and the supernatants were subjected to immunoprecipitation.

Metabolic Labeling and Immunoprecipitation—Metabolic labeling of transfected COS cells was performed for 36 h with 100 μCi/ml of [35S]methionine and [35S]cysteine (Amersham) in methionine- and cysteine-free MCDB 104 medium (13). After labeling, the conditioned media were collected. The cells were washed with phosphate-buffered saline and then solubilized in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate, 1.5% Trasylol (Bayer), and 1 mM phenylmethylsulfonyl fluoride (Sigma). After 15 min on ice, the cell lysates were recovered by scraping and centrifuged, and the supernatants were subjected to immunoprecipitation.

For preparation of extracellular matrix proteins, the media were collected and the cells were washed three times with 0.5% sodium deoxycholate, 10 mM Tris-HCl, pH 8.0, for 20 min each time on ice, followed by a single short wash with phosphate-buffered saline. The plates with the remaining matrix were then dried at room temperature overnight and subjected to digestion with 0.3 units of plasmin (Sigma)/ml for 1 h at 37 °C in phosphate-buffered saline containing 1 mM CaCl₂, 1 mM MgSO₄, and 0.1% N-octyl-β-D-glucopyranoside (Sigma). The digested extracellular matrix proteins were then extracted by adding the same volume of lysis buffer followed by incubation for 10 min on ice. The supernatant was recovered and subjected to immunoprecipitation using a protein G-antiserum to LTBP, Ab 39 (10) as described (13). The immune complexes were heated at 95 °C for 5 min in SDS-sample buffer (26) in the presence of 20 μl diethiothreitol, and analyzed by SDS-gel electrophoresis using 5–15% polyacrylamide gels (26). Gels were fixed, incubated with Amplify (Amersham) for 20 min, and subjected to fluorography.

RESULTS

Cloning of a cDNA for Human LTBP-1L—A part of the NH₂-terminal region of LTBP-1L was obtained by PCR using sequences of rat LTBP-1L (11) and human LTBP-1S (10) as primers. Screening the HEL cell cDNA library with the PCR product as a probe, a clone (H4) with a 4.1-kb insert was obtained (Fig. 1A). Nucleotide sequencing of the cDNA clone revealed that it has an NH₂-terminal region of 346 amino acid residues (L-region) that is not found in LTBP-1S (Fig. 1B). In the L-region, an EGF-like repeat (amino acid residues 188–220) is present, which is less conserved compared with the other EGF-like repeats found in the molecule. Alternative splicing appears to have occurred between codons 145 and 146 of LTBP-1S. The L-region of LTBP-1L is highly similar (77%) to that of rat LTBP-1 (Fig. 1C). Moreover, it is also similar (23%) to the corresponding region of LTBP-2; in addition to the presence of an EGF-like repeat, a sequence containing four cysteine residues with a high amino acid sequence similarity is found in both LTBP-1L and LTBP-2 (Fig. 1C).

Distribution of Two LTBP-1 Transcripts in Different Human Tissues—The expression of the two different transcripts of LTBP-1 in human tissues was investigated using human multiple-tissue Northern blots. The 7.0-kb transcript was seen in heart, placenta, kidney, and prostate, whereas the 5.2-kb transcript was more widely expressed and was seen also in lung, skeletal muscle, testis, and ovary (Fig. 2A).

The filter was then hybridized using a PCR product corresponding to the L-region of LTBP-1L as a probe; only the 7.0-kb transcript hybridized with this probe (Fig. 2B), consistent with the conclusion that 7.0- and 5.2-kb transcripts encode the LTBP-1L and LTBP-1S, respectively.
Expression of LTBP-1L and -1S in COS Cells—The cDNAs for LTBP-1L and -1S were transfected into COS cells, and the expression and localization of the proteins were studied by metabolic labeling followed by immunoprecipitation with Ab 39. LTBP-1S was observed as a 195-kDa protein mainly in the conditioned medium, and only a minor amount of LTBP-1S was found in the cell lysates (Fig. 3A). LTBP-1L was seen as 210- and 180-kDa forms in the medium; the 180-kDa form may be a proteolytically processed form of the 210-kDa form. A small amount of LTBP-1L was found in the cell lysate fraction, mainly as a 210-kDa form.

In order to investigate whether the different forms of LTBP-1 observed in the cell lysate were bound to the extracellular matrix, the transfected COS cells were first treated with 50 mM Tris-HCl, pH 8.0, containing 0.5% sodium deoxycholate and washed. The remaining matrix was subjected to digestion by plasmin. The released matrix-bound proteins were recovered and subjected to immunoprecipitation with Ab 39. As shown in Fig. 3B, LTBP-1L was found as a 170-kDa band in the extracellular matrix digests, whereas only a small amount of LTBP-1S was found in this fraction.

In order to show that LTBP-1 isolated from matrix by digestion by plasmin is due to LTBP-1 bound to matrix and not unspecifically bound to plastic, we coated the cell culture wells with collagen, gelatin, or fibronectin before the cells were seeded and repeated the above experiments. Analysis by plasmin digestion indicated that more LTBP-1L than LTBP-1S was associated with the matrix-coated dishes. The association was more pronounced to culture dishes coated with matrix proteins than to uncoated dishes; the most efficient association was achieved after coating with monomeric collagen (Fig. 3C). In the media there were similar amounts of LTBP-1S and LTBP-1L.

DISCUSSION

We describe here the cloning of human LTBP-1L cDNA. Similar to rat LTBP-1 obtained from a rat kidney cDNA library (11), LTBP-1L has a region of 346 amino acid residues (L-region) in its NH₂-terminal part, which is not found in LTBP-1S obtained from a human foreskin fibroblast cDNA library (10). A region similar to the L-region of LTBP-1L was also found in LTBP-2 (13). The overall structure of LTBP-2 is very similar to that of LTBP-1L; LTBP-2 contains multiple copies of EGF-like repeats and eight-cysteine repeats. Similar

3 A. Olofsson, unpublished data.
matrix (13). It will be interesting to determine whether the sequence in LTBP-2 that is homologous to the L-region of LTBP-1L mediates the association with extracellular matrix. In the L-regions, EGF-like repeats are found both in LTBP-1L and LTBP-2, which are less conserved compared with those in other parts of the molecules. In addition, a conserved sequence containing four cysteine residues is also present in both LTBP-1L and LTBP-2. Notably there is also a proline-rich region (amino acid residues 101–109) in the L-region, similar to that found in fibrillin-1 (17). The L-region is highly basic. It is possible that the positive charges in this region are important for interactions with the extracellular matrix.

The functional roles of the different parts of LTBP-1 are still not fully understood. We have found that both LTBP-1S and LTBP-1L form complexes with the TGF-β1 precursor in transfected COS cells (13). Thus, it is unlikely that the L-region of LTBP-1L is critical for the complex formation with the TGF-β1 precursor. The finding that LTBP-1L associates more efficiently than LTBP-1S with the extracellular matrix suggests a role of the L-region in targeting of latent TGF-β to different tissues. It is not known which matrix components LTBP-1L associates with in vivo, but in experiments using tissue culture plates coated with various extracellular matrix proteins, we could see an efficient association of LTBP-1L with collagen and, to a lesser extent, with gelatin and fibronectin. LTBP-1 was also released from the matrix by collagenase but considerably less efficiently than by plasmin. Latent complexes composed only of TGF-β and LAP, or with LTBP-2 and other components, also exist in certain tissues (7, 8, 13). Thus, the localization and availability in vivo of TGF-β is regulated, at least in part, by the proteins that form complexes with the TGF-β precursors.

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