Latent TGF-β binding protein LTBP-1 contains three potential extracellular matrix interacting domains

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SUMMARY

Latent TGF-β binding proteins (LTBPs) are components of the extracellular matrix (ECM). They belong to the fibrillin/LTBP-superfamily, and are high molecular weight glycoproteins characterized by EGF-like repeats and 8-Cys repeats. Most LTBPs associate with the small latent forms of TGF-β. Their roles include to facilitate the secretion of latent TGF-β and to target it to the ECM. In order to identify new matrix-binding domains of LTBP-1 and to characterize their association with the extracellular matrix, we have produced (in a mammalian expression system) partly overlapping recombinant fragments of its shorter form, LTBP-1S, and analyzed the binding of the purified fusion proteins to extracellular matrices of cultured human dermal and lung fibroblasts. Recombinant fragments from three different regions of the N- and C-termini showed affinity to the matrix. These interacting regions contain either the first (hybrid), second or fourth 8-Cys domains of the LTBP-1S molecule. They bound independently to the matrix. Each of them had an ability to inhibit the association of native exogenous LTBP-1 with fibroblast extracellular matrix. The interactions of the LTBP-1 fragments with the extracellular matrix resisted treatment with sodium deoxycholate, suggesting strong, possibly covalent binding. The binding occurred in a time- and dose-dependent fashion. The N-terminal fragments bound more readily to the matrices. With all fragments the binding took place both with intact fibroblast matrices and with matrices isolated by sodium deoxycholate. When using CHO cell layers, which form sparse matrices, only the N-terminal fragment of LTBP-1 was efficiently incorporated. The association of the binding fragments with isolated matrices was enhanced by soluble, cell-derived factors. The current data suggest that LTBP-1 contains three different domains with an ability to associate with the extracellular matrix.

Key words: LTBP domain, Matrix binding, Microfibril, Fibronectin

INTRODUCTION

Extracellular matrix (ECM) is a complex network of numerous macromolecules, which fulfill a large number of mechanical, chemical and biological functions. For example, collagens and elastic fibers confer to tissues strength and elasticity, respectively, whereas structural glycoproteins create tissue cohesiveness. ECM molecules can interact with cells, and also with themselves. Numerous components of the matrix have an ability to bind growth factors (Taipale and Keski-Oja, 1997). ECM plays therefore an important role in the regulation of numerous processes, where these growth factors are involved. An example is TGF-β, a multi-functional growth factor that, interestingly, is directly involved in the biosynthesis, degradation and remodeling of ECM itself. Cells secrete TGF-β as a small latent complex composed of the mature dimeric TGF-β molecule and the two respective N-terminal pro-domains (LAP, or latency-associated protein). In most cell types, this complex is secreted in association with a large binding protein, LTBP (latent TGF-β-binding protein). LAP, but not LTBP, is responsible and sufficient for the latency of TGF-β. Dissociation from the latent complex enables the mature TGF-β to bind to its signaling receptors (Gentry and Nash, 1990; Wakefield et al., 1989; reviewed by Saharinen et al., 1999).

LTBPs are components of the extracellular matrix and most of them are responsible for the storage of latent TGF-β in the ECM (Saharinen and Keski-Oja, 2000). LTBPs form a group of high molecular weight proteins that are structurally related to fibrillins. Thus far, two isoforms of fibrillin (fibrillins 1 and 2) (Sakai et al., 1986; Zhang et al., 1994) and four isoforms of LTBP (LTBPs 1-4) (Kanzaki et al., 1990; Moren et al., 1994; Gibson et al., 1995; Giltay et al., 1997; Saharinen et al., 1998; Yin et al., 1995), as well as numerous splice variants, have been molecularly cloned, and together they form the fibrillin/LTBP superfamily. While fibrillins are considered to be integral components of 10 nm diameter microfibrils (Reinhardt et al., 1996), which provide a scaffold for elastin deposition to elastic fibers, little is known about the supramolecular organization of LTBPs. Fibrillins and LTBPs are cysteine-rich proteins, mainly composed of EGF-like repeats, 8-Cys repeats and hybrid domains containing sequences that are similar to these two repeats. Fibrillins and LTBPs, except LTBP-3, display also 1-2 RGD sequences for putative cell adhesion. All LTBP isoforms possess numerous glycosylation sites.

Two forms of LTBP-1 exist, namely LTBP-1S and LTBP-1L, encoded by alternatively spliced RNA of the *LTBP-1* gene (Koski et al., 1999). LTBP-1S possesses 18 EGF-like repeats, 15 of which are of Ca^{2+}-binding type, and four 8-Cys repeats,

where the first one is a hybrid domain. LTBP-1L contains, in addition, a 4-Cys repeat and a Ca^{2+}-binding EGF-like repeat at its N-terminal end. EGF-like repeats are common to various proteins and are known for their roles in protein-protein interactions (Davis, 1990). In contrast, the 8-Cys repeats are a structural feature unique to the fibrillin/LTBP superfamily, the role of which is poorly understood so far. The third 8-Cys domain of LTBP-1 has been identified as the binding site of LAP (Saharinen et al., 1996). The fourth 8-Cys domains in fibrillins-1 and -2 mediate cell adhesion. However, this may be essentially due to the presence of an RGD sequence in this domain, and not specific to the 8-Cys repeat itself (Pfaff et al., 1996; Sakamoto et al., 1996). Nevertheless, the fact that several mutations in the 8-Cys repeats in fibrillin-1 induce a phenotype of the heritable connective tissue disorder, Marfan syndrome (Collod-Beroud et al., 1997), indicates that this domain has also an important role for the integrity of the microfibrils.

After secretion LTBPs associate covalently with ECM via their N-termini, but can be released as truncated forms by treatment with proteases such as plasmin (Hyytiäinen et al., 1998; Saharinen et al., 1998; Taipale et al., 1994). Transfection of cDNA for LTBP-1L and -1S in COS cells reveals that LTBP-1L binds more efficiently to the extracellular matrix than does LTBP-1S. The physiological role of two LTBP-1 splice forms is unknown. They may mediate different localization patterns of latent TGF-β complexes in vivo (Oloffson et al., 1995). By immunohistological analysis of various tissues and cell types, LTBP-1 has been found to co-localize with fibronectin (Taipale et al., 1996), elastin (Karonen et al., 1997) and fibrillin-1 (Raghunath et al., 1998). In a sandwich dot-blot assay, a physical interaction was observed between LTBP-1 and fibronectin, which was abolished by pretreatment of the LTBP-1-containing conditioned medium with plasmin (Taipale et al., 1996). Dallas et al. analyzed the LTBP-1/fibrillin-1 colocalization with fibronectin in osteoblast cultures (Dallas et al., 2000). They found that both molecules initially associate with fibronectin, but display a clearly distinct fibrillar pattern in long-term cell cultures. These results suggest that the LTBP-1/fibrillin-1-positive fibrils, which occur later in culture, represent true microfibrils. Furthermore, LTBP-1 is a substrate for transglutaminase (Nunes et al., 1997). Swiss 3T3 fibroblasts overexpressing tissue transglutaminase develop a more abundant LTBP-1-containing fibrillar network than the control cells. In addition, the analysis of these cells by immunogold electron microscopy revealed co-localization of tissue transglutaminase, LTBP-1 and fibronectin at the cell surface (Taipale et al., 1996; Verderio et al., 1999), indicating a role for transglutaminase in the deposition of LTBP-1 into the matrix.

No diseases associated with LTBPs have been reported so far. However, the importance of LTBP is underlined in LTBP-2 null mice, which have an embryonic lethal phenotype and die between after 3.5 and 6.5 days postcoitus (Shipley et al., 2000). Mutations in fibrillins-1 and -2, which are structurally related to LTBPs, affect the global function of the microfibrils and cause the pathological phenotypes of Marfan syndrome and congenital contractural arachnodactyly, respectively. The possible vital importance of LTBPs in general may be the reason why they have not been associated with diseases so far (Saharinen et al., 1999).

The goals of the present work were to identify and map the domains of LTBP-1 with potential ability to interact with the extracellular matrices of human dermal and lung fibroblasts. LTBP-1L is a splice variant, which contains an N-terminal extension of 346 amino acids not present in LTBP-1S. Both LTBP-1S and the longer form, LTBP-1L, have the ECM-binding function, and it has already been reported that the longer form binds more efficiently to the ECM (Olofsson et al., 1995). Both forms are regulated by independent promoters in a cell type-specific manner (Koski et al, 1999), probably to target them to different tissues. For the current analyses we produced recombinant Fc tail-tagged protein fragments covering the entire LTBP-1S molecule, and characterized their binding to human fibroblast matrices. We find here that the N terminus contains two putative matrix-binding domains, and also that the region containing the fourth 8-Cys domain in the C-terminus of LTBP-1 has an affinity for and can bind to the matrix.

MATERIALS AND METHODS

Reagents and antibodies

All restriction enzymes (*Xba*I, *Bgl*II, *Bam*HI, *Hin*dIII) were from New England Biolabs (Beverly, MA). Biotinylated protein A and monoclonal anti-fibronectin (clone FN-15) antibody were from Sigma (St. Louis, MO), rabbit anti-human IgG antibodies and FITC-conjugated anti-mouse antibodies from DAKO (Glostrup, Denmark), and Cy3-coupled streptavidin from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit Ab39, raised against human platelet LTBP-1, was a gift from Dr C.-H. Heldin (Ludwig Cancer Institute, Uppsala, Sweden).

Cell culture

Human foreskin fibroblasts were isolated from skin biopsies as described elsewhere (König, et al., 1992). Human lung fibroblasts (WI-38 and CCL-137) and CHO K7 cells were obtained from American Type Culture Collection (Rockville, MD). All cells were cultured in Eagle's modification of minimal essential medium (MEM), supplemented with 10% fetal calf serum (Life Technologies, Täby, Sweden), 4 mM L-glutamine, 100 IU/ml penicillin and 50 μg/ml streptomycin. For incubation of the cells with the fusion proteins, 0.2% BSA was added to the serum-free cell culture medium.

Cloning of LTBP-1S fragments

Diverse fragments spanning the entire sequence of human LTBP-1S (see Fig. 1 for designations) containing appropriate restriction endonuclease recognition sites at their 5′ and 3′ ends were generated by PCR. The design of the primers was based on the LTBP-1S sequence published under the GenBank Accession Number M34057 (Kanzaki et al., 1990). Using the Rapid DNA Ligation Kit (Boehringer Mannheim, Mannheim, Germany) the PCR products were ligated into a Signal pIg plus vector (R&D Systems, Minneapolis, MN) as *Xba*I-*Bam*HI fragments (I: 20-105, II: 20-299), *Xba*I-*Bgl*II fragments (III: 20-545, IV: 300-545, V: 340-545) and *Hin*dIII-*Bam*HI fragments (VI: 543-1008, VII: 1005-1198, VIII: 1005-1394, IX: 1139-1394, X: 1242-1394). All inserts were verified by sequencing with an ABI 373A automatic DNA sequencer (Applied Biosystems, Foster City, CA).

Expression of LTBP-1 fragments in CHO cells

Cells were seeded in 100 mm diameter cell culture dishes. At semi-confluency, the cells were transfected with 5 μg of plasmid-DNA using a Lipofectamine Transfection Kit (Gibco, BRL) according to the manufacturer's instructions. Selection was carried out for two weeks in the presence of G418 (1.5 mg/ml) with 2-3 medium changes per week. After the selection period, the cells were dilution cloned in

96-well plates. Cells were cultivated at a concentration of G418 of 0.4 mg/ml. Clones were screened by a dot blot assay for the secretion of the fusion proteins, and positive clones were characterized further by immunoblotting after separation of the proteins by SDS-PAGE (4-15% polyacrylamide gradient) in order to verify the correct sizes of the secreted fusion proteins.

Purification of LTBP-1 fusion proteins

Stably transfected CHO cells overexpressing either LTBP-1S fragments or the control fusion protein lacking the LTBP-1S sequence were seeded in 135 mm diameter tissue culture dishes and grown to confluency. Subsequently, the cell culture medium was changed to serum-free medium, and collected after an incubation time of two days, followed by another medium change and incubation of two days. After collection, aminoethylbenzene sulfonylfluoride (1 mM final concentration, Calbiochem) was added to the medium to prevent degradation of the fusion proteins. Purification was carried out by affinity chromatography, using HiTrap protein A columns (Amersham Pharmacia Biotech, Uppsala, Sweden). The fusion proteins were eluted with 0.58% acetic acid, 140 mM NaCl. Affinity chromatography was followed by gel filtration with a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) with phosphate buffered saline (PBS; 0.14 M NaCl in 10 mM sodium phosphate buffer, pH 7.4) as the buffer system. The purity of all fractions and the presence of degradation products after affinity chromatography and gel filtration, were visualized by Coomassie Blue staining of SDS-PAGE (4-15% polyacrylamide gradient) and immunoblotting. The fractions containing the highest amounts of fusion protein were pooled. The protein concentrations were determined from the pooled samples by using the 'Micro BCA Protein Assay' (Pierce, Rockford, IL).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting

SDS-PAGE was carried out using commercial pre-cast 4-15% polyacrylamide gradient gels (BioRad., Richmond, VA) in the Laemmli buffer system (Laemmli, 1970). Electrophoretically separated proteins were transferred onto nitrocellulose membranes. The transfer of proteins was carried out in 30 mM Tris/200 mM glycine buffer (pH 8.3) containing 0.01% SDS and 10% methanol. To prevent nonspecific binding, the membranes were treated with 5% nonfat milk in blot wash buffer (0.05 M Tris-HCl, 0.5 M NaCl, 0.1% BSA, 0.1% Tween-20, pH 9.0) for 30 minutes. The membranes were then reacted with anti-human IgG antibodies (DAKO A/S, Glostrup, Denmark) or biotinylated protein A (Sigma), washed, and the bound antibodies were detected using biotinylated streptavidin and enhanced chemiluminescence (Amersham). Antibody incubations and washes were carried out in the blot wash buffer.

Indirect immunofluorescence

Cells were seeded in Lab-Tek chamber slides (Nunc, Naperville, IL), and treated as indicated in the respective experiments. The samples were washed twice with PBS, fixed with methanol at –20ºC for 15 minutes and air-dried. All subsequent incubation steps were carried out at room temperature. The specimens were first incubated in the blocking solution (2% BSA in PBS) for 30 minutes, and then with the primary antibodies for 1.5 hours. Unbound antibodies were removed with PBS in three washing steps, 10 minutes each. The binding of the primary antibodies was revealed with fluorescence-conjugated antibodies or streptavidin. The samples were then washed three times for 10 minutes each with PBS, embedded with Mowiol (Calbiochem, La Jolla, CA), and stored at –20°C until microscopy. For microscopy, a fluorescence microscope (Axiovert 135, Zeiss) was used. Images were taken with a digital camera (C4742-95, Hamamatsu Photonics) and processed with the software Image-Pro® Plus (Media Cybernetics®, Silver Spring, MD). In the case of double staining (fusion proteins/fibronectin), the incubation with the primary antibodies was carried out simultaneously. The bound fusion proteins were detected by staining with Cy3-coupled streptavidin, whereas fibronectin was detected by FITC-labeled secondary antibody. As for the primary antibodies, the secondary antibodies were incubated simultaneously.

Enzyme-linked immunoassay (EIA) for the assessment of the binding of LTBP-1S fusion proteins to ECM

Cells were seeded in 24-well plates at a cell density of 10^5 cells/cm^2. At confluency, the cells were washed and the fusion proteins were added into fresh serum-free medium, unless otherwise indicated. After incubation at 37°C for the time intervals described in the experiments, the cell layers were washed twice with PBS, fixed with absolute methanol at –20°C for 15 minutes and air dried. In some instances, deoxycholate insoluble ECM was extracted prior to fixation. Nonspecific antibody binding sites were saturated by treatment with 5% non-fat milk in blot wash buffer (0.05 M Tris-HCl, 0.5 M NaCl, 0.1% BSA, 0.1% Tween-20, pH 9.0) for 30 minutes. This buffer was also used for washes and antibody incubations. Biotinylated anti-human IgG (DAKO) was used as the primary antibody. Incubation was carried out for 45 minutes. After three washes the cell layers were incubated for 20 minutes with biotinylated HRP-conjugated streptavidin (Amersham Pharmacia Biotech). The cell layers were then washed again three times with the blot wash buffer, and twice with PBS. Subsequently, a substrate solution containing 0.03% o-phenylenediamine, 0.01% H_2O_2 in 25 mM citric acid/50 mM Na_2HPO_4 was added to each well. The enzymatic reaction was terminated by addition of H_2SO_4 (0.4 M final concentration). The absorbance was measured at 450 nm.

Isolation of sodium deoxycholate insoluble ECM

The cell layers were washed twice with PBS at room temperature, followed by three incubations for 10 minutes each, on a slowly moving shaker, with 0.5% sodium deoxycholate/10 mM Tris-HCl buffer, pH 8.0, at 4°C. For this treatment, a buffer volume of 0.25 ml/cm^2 was applied to the cell culture plates (Hedman et al., 1979). The substratum-attached material was washed twice with the same buffer, twice with PBS, and allowed to dry overnight at room temperature. These preparations were then incubated with LTBP-1S fragments and/or analyzed by indirect immunofluorescence.

Matrix binding inhibition assay

For the production of secreted soluble [^{35}S]methionine/cysteine-labeled fibroblast-derived proteins, confluent cultures of human embryonic lung fibroblasts (CCL-137) were incubated under serum-free conditions with methionine- and cysteine-free medium for 4 hours. The cells were then radiolabeled with [^{35}S]methionine/cysteine (140 µCi/ml; Amersham Pharmacia Biotech, UK) for 20 hours.

To analyze the inhibition of matrix incorporation of radiolabeled LTBP-1 we proceeded as follows. Confluent cultures of skin fibroblasts were incubated with serum-free cell culture medium containing 0.2% BSA for 1 hour, and were subsequently used as recipient cells. The medium from radiolabeled cells was collected, clarified by centrifugation and used as a source of LTBP-1. The recipient cells were then incubated for 24 hours with the pIg fusion proteins II (20-299), V (340-545) or IX (1139-1394), as well as an Ig fragment lacking the LTBP-part, in the conditioned medium containing radiolabeled proteins from human lung fibroblasts. To detect the LTBP-1 associated with ECM, the extracellular matrix was extracted with sodium deoxycholate as described above (Taipale et al., 1994). The matrix preparations were then digested with plasmin (0.3 U/ml) at 37°C for 1 hour. The digests were collected by rubber policeman and clarified by centrifugation with a microfuge for 10 minutes at 13,000 rpm.

The supernatants of the plasmin digests were used for immunoprecipitation analysis as described elsewhere (Taipale et al., 1994). Briefly, the samples were preabsorbed by incubation with normal rabbit serum at 4°C overnight, followed by the addition of

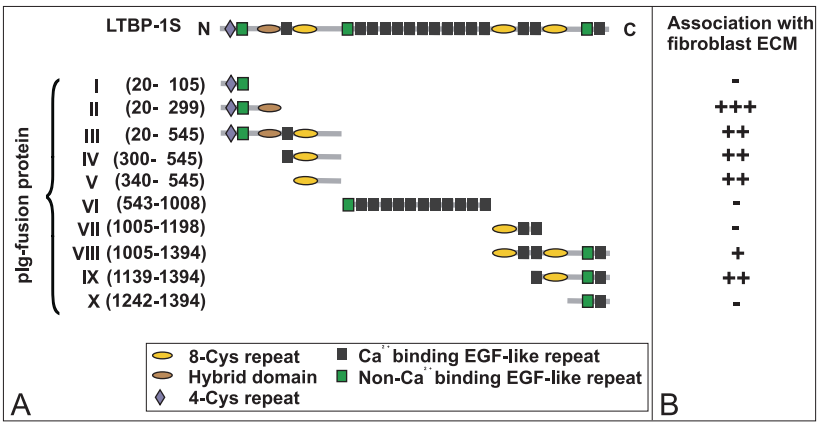

Fig. 1. Design of fusion proteins spanning the full-length LTBP-1S sequence. Fragments of LTBP-1S were stably expressed in CHO cells. Purified LTBP-1S fragments were used for matrix-incorporation assays. (A) Sequences of the domain-specific fusion proteins. The Roman number designations are included for clarity and brevity. (B) Summary of the results of indirect immunofluorescence analysis obtained after incubation of intact human fibroblast cell layers with the respective fusion proteins (2 μg/ml) in serum-free medium; –, no binding; + to +++, weak, intermediate or strong binding.

protein A-Sepharose beads. After 2 hours of incubation at 4°C, the beads were collected by centrifugation at 13,000 rpm for 10 minutes. The supernatants were then transferred to fresh tubes and the preadsorption procedure was repeated. Subsequently, the samples were incubated with the specific anti-LTBP-1 antiserum (IgG fraction, Ab39), or purified IgG for 2 hours, followed by incubation with protein A-Sepharose for 1 hour. The beads were collected by centrifugation at 2,000 rpm for 5 minutes. The beads were then washed twice with PBS, twice with detergent buffer (1% sodium deoxycholate, 1% TX-100, 150 mM NaCl in 50 mM Tris-HCl buffer, pH 7.0), once with high salt buffer (500 mM NaCl, 0.2% TX-100, in 20 mM Tris-HCl buffer, pH 7.0), followed by three washes with PBS. The immunoprecipitated polypeptides were eluted from the beads by treatment at 95°C for 5 minutes in gel sample buffer containing 2% SDS. The samples were analyzed by SDS-PAGE (4-15% polyacrylamide gradient) under non-reducing conditions with subsequent fluorography. The obtained LTBP-1 bands were quantified by scanning the X-ray films, and analyzed by densitometry using the computer program Multi-Analyst (BioRad).

RESULTS

Production of recombinant LTBP-1S fragments

In order to identify the putative matrix binding sites of LTBP-1, we designed ten different partially overlapping recombinant fragments (I-X) spanning the entire LTBP-1S sequence (Fig. 1A), and containing in their C-terminal ends the sequence of the constant region of human immunoglobulins as a tag. The resulting fusion proteins are secreted as dimers, where two protein chains are linked via a disulfide bond. The Arabic numbers refer to the positions of the amino acids in the published sequence (Kanzaki et al., 1990). Stably transfected clones of CHO cells were produced, and the expression levels of the fusion proteins were analyzed by Coomassie Blue staining after SDS-PAGE, and by immunoblotting. All fusion proteins were expressed and secreted into the cell culture medium. Subsequent purification was carried out in two steps, by affinity chromatography and gel filtration. The latter was used to change the buffer to PBS, since the fusion proteins were intended to be used in cell culture, and to eliminate co-purified degradation products. After gel filtration, all protein-containing fractions were analyzed by immunoblotting for the integrity of the fusion proteins. Fractions containing high amounts of degradation products were discarded. Only the fractions containing highly intact protein were pooled and subsequently used in cell culture (Fig. 2).

Immunoblotting revealed minor degradation products in the pools of the N-terminal fragments II (20-299), IV (300-545) and V (340-545), and the C-terminal fragments VIII (1005-1394), IX (1139-1394) and X (1242-1394). In the case of the C-terminal fusion proteins we observed a similar degradation product of about 50 kDa (Fig. 2, three rightmost lanes). This degradation product is probably generated by cleavage in the region between amino acids 1242 and 1297. Proteinase-sensitive regions have been proposed in both the N-terminal (two cleavage sites starting at amino acids 415 and 461) and the C-terminal parts (one cleavage site starting at amino acid 1257) of LTBP-1 (Kanzaki et al., 1990; Tsuji et al., 1990).

Immunoblotting of the purified LTBP-1S fragments indicated that all fusion proteins migrated as slightly higher molecular mass bands than predicted from their calculated values (Table 1; Fig. 2). This may be explained by the presence of glycosylation sites both in the LTBP-1 molecule, as well as in the Fc tail of human IgG.

The highest yields of protein were obtained with fragments V (340-545), IV (300-545) and IX (1139-1394), even though some protein was lost due to degradation. The lowest amounts

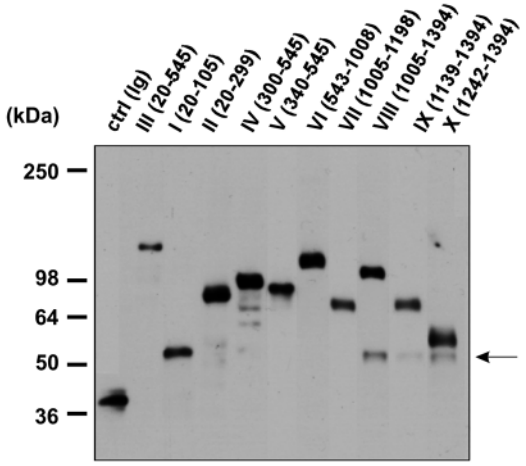

Fig. 2. Analysis of the molecular sizes of affinity-purified LTBP-1S fusion proteins. Reduced samples were separated by SDS-PAGE (4-15% polyacrylamide gradient) and detected by immunoblotting using biotinylated rabbit anti-human IgG. Protein bands were visualized by enhanced chemiluminescence. The migration of the molecular mobility standards is indicated on the left. The common cleavage product of the three C-terminal fragments is indicated by an arrow.

of protein were recovered from fragments VI (543-1008), X (1242-1394), VII (1005-1198) and II (20-299) (Table 1). While fragments VI and VII remained intact during purification, fragment X showed the highest degree of degradation of all the fusion proteins produced, as seen by immunoblotting of the single fractions after gel filtration. In the case of the N-terminal fragment II (20-299), in addition to degradation, significant loss of protein occurred during gel filtration, probably as a result of nonspecific binding to surfaces of that fragment.

Mapping of the domains of LTBP-1S interacting with the ECM of human fibroblasts

Confluent cultures of normal human skin fibroblasts or human embryonic lung fibroblasts (CCL-137 or WI-38 cells) were incubated in serum-free medium containing LTBP-1S fusion proteins (2 μg/ml) at 37°C for 24 hours. The Ig fusion protein lacking the LTBP-1S sequence was used as a control. In order to determine the optimal concentrations, the range of 0.1-10 μg/ml was tested in preliminary experiments for every fusion protein. The cell layers were then washed and processed for indirect immunofluorescence by using rabbit anti-human IgG as the primary antibody. Staining with a fibronectin-specific monoclonal antibody monitored the presence of a well-developed matrix network. The control pIg fusion protein did not associate with the ECM, as shown by the absence of staining (Fig. 3). A clear pattern of fibrillar structures was observed with the fragments II (20-299), III (20-545), IV (300-545), V (340-545), VIII (1005-1394) and IX (1139-1394) (Fig. 3, summarized in Fig. 1B), whereas the other fragments were negative. Similar results were obtained with all three fibroblast strains. This suggests that ECM-interacting domains exist both at the N-terminal and the C-terminal regions of LTBP-1S. No binding was observed with fragment I (20-105) if analyzed via indirect immunofluorescence, but a weak signal was obtained by the EIA method (data not shown). This indicates that the amino acids 105-299 (covered by fragment II), including the hybrid domain, mainly contribute to the ECM interaction of the extreme N terminus.

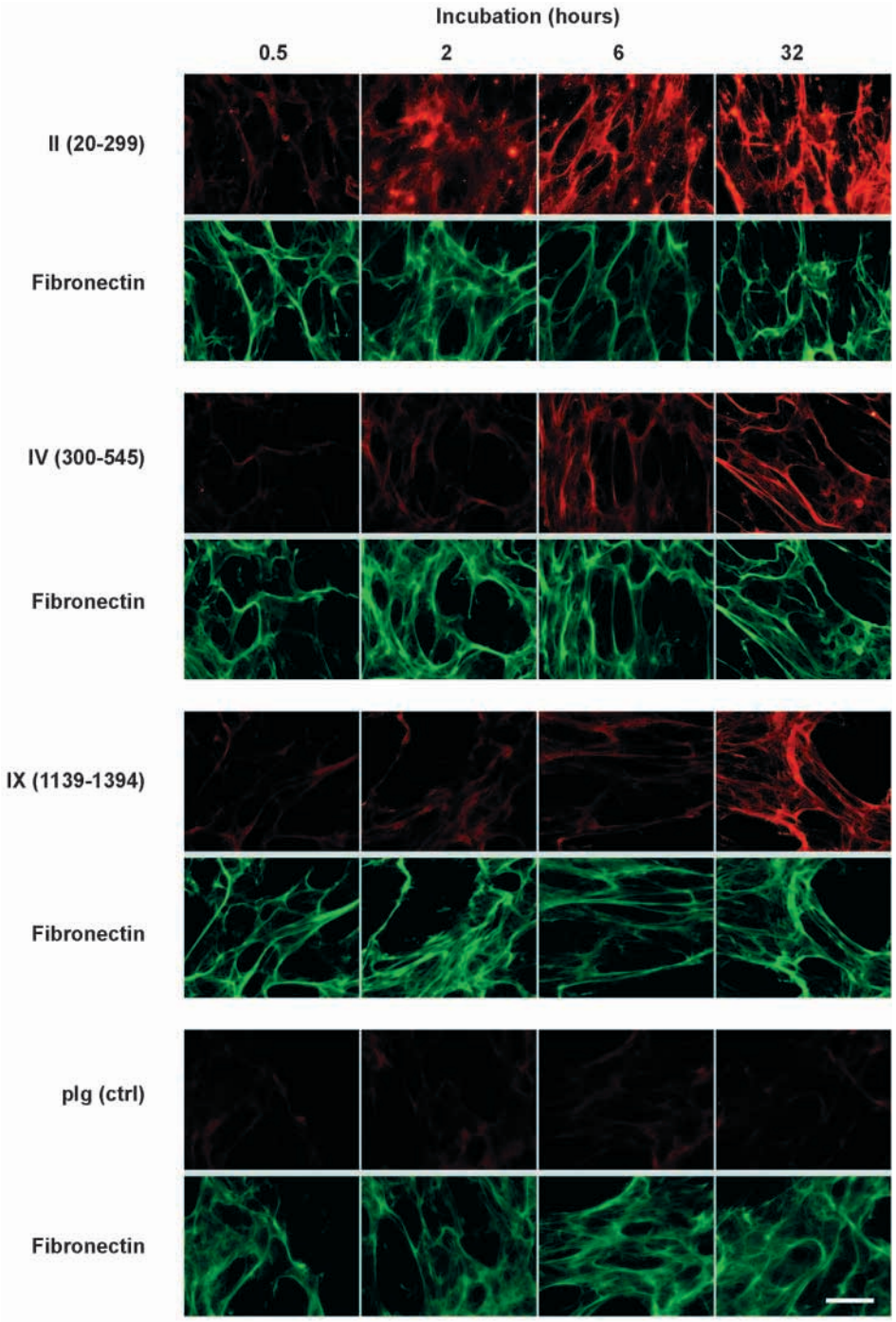

Fig. 3. Timecourse of the association of recombinant LTBP-1S fragments with the ECM of human skin fibroblasts. Cell layers were incubated with the fusion proteins (II, IV and IX) at a concentration of 2 μg/ml in serum-free medium for the indicated time intervals. The cells were then washed, and the bound fusion proteins were detected with rabbit anti-human IgG antibodies combined with Cy3-coupled streptavidin (red color). The fibronectin network was visualized by using a monoclonal anti-fibronectin antibody, and subsequently a FITC-conjugated antibody (green color). All photographs were taken with the same exposure time and processed with the same computer settings. Scale bar: 10 μm.

Fibrillar structures of equal intensity and density were observed with fragments IV (300-545) or V (340-545), suggesting that the EGF-like repeat comprised in fragment IV does not contain structures for matrix association. The central fragment VI (543-1008) containing the tandem of EGF-

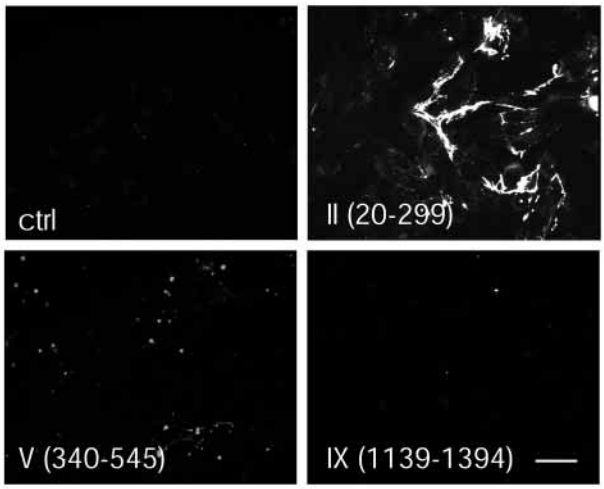

Fig. 4. Association of LTBP-1 fusion proteins with CHO cell matrices. Subconfluent CHO cell layers were incubated with the fusion proteins (II, V or IX) at a concentration of 5 μg/ml in serum-free medium for 16 hours. The bound fusion proteins were detected with rabbit anti-human IgG antibodies combined with Cy3-coupled streptavidin. Scale bar: 10 μm.

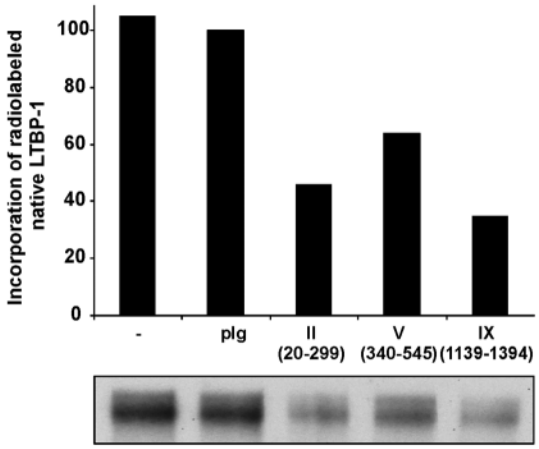

Fig. 5. Inhibition of the matrix association of exogenous native LTBP-1 by recombinant LTBP-1 fragments. Fibroblast-conditioned medium was produced by incubating confluent human lung fibroblast cultures (CCL-137) in serum-free medium (MEM) for 20 hours in the presence of [^{35}S]methionine/cysteine. After radiolabeling, cell-conditioned medium was collected and clarified by centrifugation. To prepare recipient cells, confluent cultures of skin fibroblasts were incubated under serum-free conditions in medium containing 0.2% BSA. Subsequently, skin fibroblasts were incubated with recombinant LTBP-1 fragments II (20-299), V (340-545) or IX (1139-1394) at a final concentration of 30 μg/ml for 24 hours. Control samples received no fusion protein (–) or the pIg fusion protein lacking the LTBP part (pIg). Protein synthesis was prevented with cycloheximide (40 μg/ml). Extracellular matrix was then extracted and solubilized by digestion with plasmin (0.3 U/ml) for 1 hour at 37°C. The digests were subjected to immunoprecipitation with Ab39. The immunoprecipitates were analyzed by SDS-PAGE (4-15% polyacrylamide gradient) with subsequent fluorography. The intensity of the LTBP-1 bands was quantified by densitometry. The intensity of the band obtained from the sample incubated with the control fusion protein (pIg) was set to 100% of incorporation of the native LTBP-1. Similar results were obtained from three independent experiments.

like repeats did not associate with the matrix (shown in Fig. 8).

In the C-terminal part, neither the fragment VII (1005-1198), containing the third 8-Cys repeat with two neighboring EGF-like repeats, nor the fragment X (1242-1394), containing the two last C-terminal EGF-like repeats, showed any interaction with the ECM. However, the two C-terminal fragments containing the fourth 8-Cys repeat and the preceding EGF-like repeat (fragments VIII and IX) were clearly interactive in the assay.

In order to rule out the possibilities of nonspecific interactions we isolated the sodium deoxycholate insoluble ECM from confluent fibroblast cultures subsequent to the incubation of the cell layers with LTBP-1S fusion proteins (5 μg/ml) in serum free medium at 37°C for 48 hours. The isolation of the ECM by sodium deoxycholate did not abolish the fibrillar staining of the LTBP-1 fragments (data not shown). However, some reduction of the background staining was observed mainly with the N-terminal fragments II (20-299), III (20-545), IV (300-545) and V (340-545). The positive staining of the fibrils after removal of soluble, extractable components indicates that the recombinant LTBP-1S fragments get tightly assembled to the ECM.

Table 1. Molecular masses of the LTBP-1S fusion proteins and the yields after purification

LTBP-1S fragment	Numbers of amino acids	Calculated molecular mass of monomeric protein (kDa)	Apparent molecular mass of reduced protein (kDa)	Yield of intact protein (μg/ml)
Control	Ig	25	40	1.4
I	(20-105)	34	52	1.0
II	(20-299)	54	80	0.5
III	(20-545)	79	136	1.2
IV	(300-545)	50	98	2.1
V	(340-545)	46	88	4.0
VI	(543-1008)	76	118	0.2
VII	(1005-1198)	47	68	0.5
VIII	(1005-1394)	69	100	0.6
IX	(1139-1394)	54	68	2.0
X	(1242-1394)	43	54	0.4

IgG-Fc tagged LTBP-1S fusion proteins were expressed in CHO cells and purified from serum-free conditioned medium by affinity chromatography and subsequent gel filtration. The apparent molecular masses were obtained by comparison of the mobility with molecular weight standards after SDS-PAGE (4-15% polyacrylamide) and immunoblotting. The yields of the fusion proteins are expressed as the amount of intact protein obtained from 1 ml of conditioned medium. The Roman number designations on the left are included for clarity and brevity.

CHO cells can assemble N-terminal LTBP-1 fragment into their extracellular matrix

Chinese hamster ovary cells are known to produce sparse extracellular matrices. We analyzed, therefore, whether incubation of CHO cells with the dimeric LTBP-1S fusion proteins would result in the formation of fibrillar structures. Subconfluent cultures of CHO cells were cultivated with fragments II, V or IX under serum-free conditions overnight. Indirect immunofluorescence analysis revealed fibrillar structures when the N-terminal fragment II (20-299) was used, whereas no fibrils were observed after incubation of the cells with fragments V (340-545) or IX (1139-1394) (Fig. 4). Fibrillar structures were also observed after shorter incubation times (30 minutes-2 hours) (data not shown).

Recombinant LTBP-1S fragments inhibit the incorporation of native LTBP-1 into fibroblast matrix

We analyzed next, whether the ECM interactive LTBP-1 fragments II (20-299), V (340-545), and IX (1139-1394) could inhibit the incorporation of radiolabeled, exogenous fibroblast-derived native LTBP-1. For this purpose, human foreskin fibroblasts were incubated with radiolabeled proteins from fibroblast-conditioned medium in the presence of recombinant LTBP-1 fragments (30 μg/ml) under serum-free conditions.

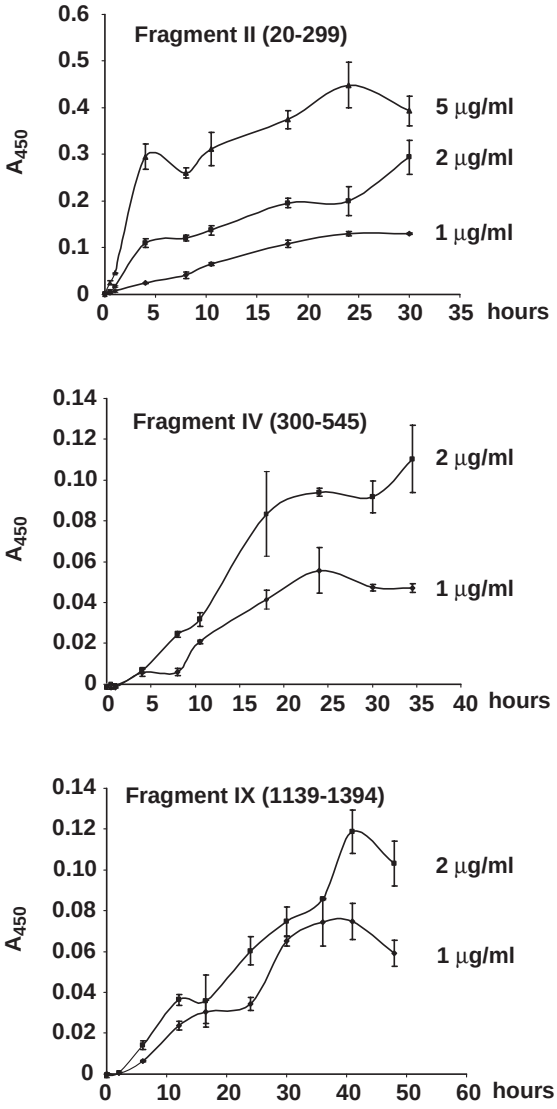

Fig. 6. Assessment of matrix association of LTBP-1S fragments by an enzyme-linked immunoassay. Human skin fibroblasts were incubated with ECM-binding fusion proteins (II, IV and IX) at concentrations of 1, 2 and 5 μg/ml in serum-free medium for increasing periods of time at 37°C. Cell layers were washed, the ECM was extracted with sodium deoxycholate, and the bound fusion proteins were revealed by EIA (see Materials and Methods for details). After incubation with the substrate solution, the absorbance of the supernatant was measured at 450 nm. Error bars represent the standard error determined from triplicate measurements.

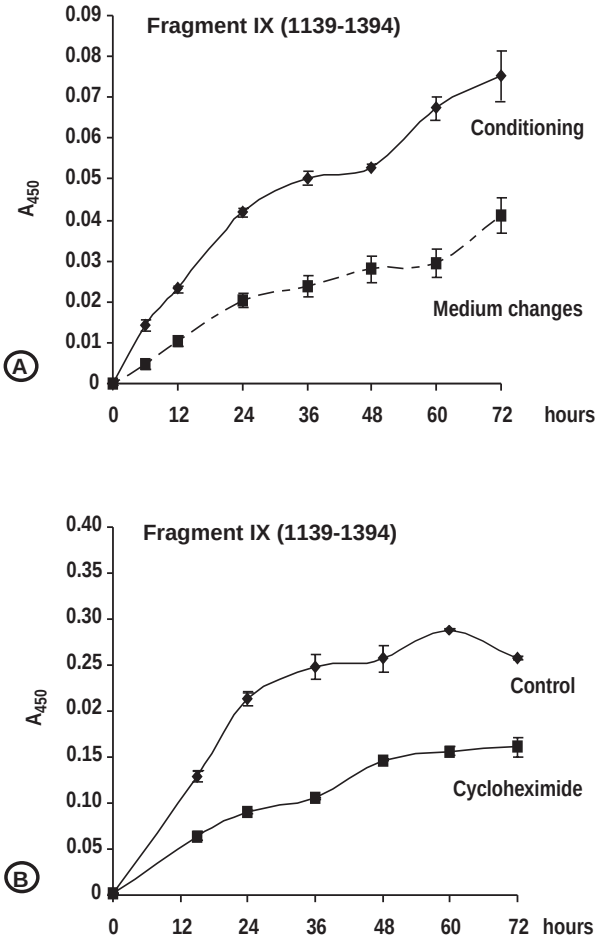

Fig. 7. Association of the C-terminal LTBP-1S fragment IX into the ECM of human skin fibroblasts. Fragment IX (1139-1394) was added to cultures of human skin fibroblasts (5 μg/ml) in serum free medium, and incubation was carried out for the indicated time intervals. (A) Every 4-6 hours, the cultures were washed once and received thereafter a new dose of the fusion protein in serum-free medium (Medium changes). The control cells were incubated in the same medium containing fragment IX (Conditioning). (B) The cells were treated with cycloheximide during incubation with the fusion protein. Treatment with cycloheximide (40 μg/ml) was started 1 hour before addition of the LTBP-1S fragments. Unbound fusion proteins were removed by two washes with PBS. Incorporation of the fusion protein into the matrix was assessed with an enzyme-linked immunoassay, using biotinylated rabbit anti-human IgG, and HRP-coupled streptavidin. Samples were then incubated with the substrate solution. The data represent the averages of triplicate determinations.

After 24 hours of incubation, plasmin digests from the respective extracellular matrix extracts were prepared and subjected to immunoprecipitation with antibody Ab39, which is known to specifically recognize LTBP-1. Separation of the immunoprecipitated polypeptides by SDS-PAGE was followed by autoradiography. Densitometric analysis of the autoradiograms of the samples revealed that the incorporation of native radiolabeled LTBP-1 into fibroblast matrix was inhibited by all three fragments (Fig. 5). The control fusion protein did not have any inhibitory effect. These results, obtained by LTBP-1 binding inhibition by fusion protein fragments suggest that, in addition to the two N-terminal binding domains, also the C-terminal binding site may be involved in the association of native LTBP-1 with fibroblast ECM.

LTBP-1 fragments associate with fibroblast ECM in a time-dependent manner

In order to study the timecourse of the interaction of the recombinant LTBP-1S fusion proteins, confluent fibroblast cultures were incubated with fragments II (20-299), IV (300-545) and IX (1139-1394) at a concentration of 2 μg/ml for increasing periods of time (30 minutes to 32 hours; Fig. 3). The pIg fusion protein, lacking the LTBP-1S fragment was used as a control. In order to estimate the areas of equally dense ECM, a double staining of fibronectin and the fusion proteins was performed. The incorporation of fragment II (20-299) occurred relatively rapidly, reaching positive levels within 2 hours of the onset of incubation, as seen by faint staining of the fibrils. The intensity of the staining and the number of stained fibrils increased with prolonged incubation times. For the fragment IV (300-545) positively stained fibrils were detectable within 6 hours. Also in this case, the intensity of specific fluorescence increased with time. Intense staining of fibrils with fragment IX (1139-1394) was not seen until 32 hours. However, some fibrils were stained around 21 hours after the onset of the incubation (data not shown). These data indicate that the binding of the recombinant LTBP-1S fragments to the ECM increased with time and occurred in a time-dependent manner. The binding domains corresponding to the N-terminal part (fragments II and IV) of LTBP-1S associated faster with the ECM than of the C-terminal part (fragment IX).

Recombinant LTBP-1S fragments associate with fibroblast matrices in a dose-dependent manner

In order to determine whether the association of the recombinant LTBP-1S fragments is dependent on their amounts in the incubation medium, we developed an EIA for LTBP-1 fragment binding to the ECM. Human fibroblasts seeded onto 24-well plates. Upon confluency the cultures were changed to serum free medium, and the LTBP-1S fusion proteins II (20-299), IV (300-545) or IX (1139-1394) were added at final concentrations of 1-5 μg/ml, as illustrated in Fig. 6. Incubation was carried out for the indicated time periods (Fig. 6). Subsequently, deoxycholate insoluble ECM was extracted, and the binding of the fusion proteins was assessed by EIA. It was found that all three LTBP-1 fragments accumulated to the fibroblast matrix in a relatively dose-dependent manner. Increasing concentrations also accelerated the matrix interaction to some extent (Fig. 6), indicating that the saturation of this process was not reached with the concentrations used.

Efficient association of the C-terminal fragment IX (1139-1394) with ECM needs soluble, cell-derived factors

The recombinant C-terminal fragment IX (1139-1394) interacted with the fibroblast matrix much more slowly than the N-terminal LTBP-1 fragments. We therefore examined the possibility that this fragment would need soluble, cell-derived factors for its efficient association to the ECM. We seeded fibroblasts into 24-well plates, and analyzed the effects of repeated medium changes. Upon confluency, the cell layers were washed with serum-free medium and the fusion protein was added at a concentration of 5 μg/ml in fresh serum-free medium. In parallel we analyzed cells, onto which fragment IX was added with continuous medium changes (every 4-6 hours), i.e. their medium was changed with fresh medium containing the same concentration of this fragment. The association of the fusion protein with ECM was assessed by EIA at the indicated time points. We found that this process was decreased about 50% when the medium was repeatedly changed (Fig. 7A). It thus appeared that the cells secreted factors that enhanced the association of the C-terminal fragment of LTBP-1S with the ECM. To confirm this observation we added cycloheximide (40 μg/ml), an inhibitor of protein synthesis, to the serum-free medium of the cultures during incubation with fragment IX. When protein synthesis was prevented, the binding of this fragment was also reduced by about 50%, confirming that its association with the ECM needs proteinaceous factors (Fig. 7B). To exclude the possibility that the decreased amount of fragment IX detected would be a consequence of the decrease of the cell number caused by the toxicity of cycloheximide, we observed the cultures by light microscopy. In addition, the cell layers were stained with Coomassie Blue to determine the relative cell densities of the treated and nontreated samples. The absorbances obtained after elution of the stain did not reveal any differences between the cycloheximide-treated and the untreated cells (data not shown).

LTBP-1S fusion proteins can interact with isolated extracellular matrices

In order to analyze whether the matrix-deposition of the LTBP-1S fragments requires the participation of cells or cell-derived factors, sodium deoxycholate insoluble ECM was isolated from human fibroblasts. The isolated ECM was incubated with the fragments at a concentration of 5 μg/ml, either in serum-free fresh or conditioned medium collected from fibroblast cultures (24 hour collection), at 37°C for 36-72 hours. For comparison, intact living cell layers were incubated with the fusion proteins diluted in non-conditioned medium, under the same conditions. The matrices were then washed, fixed and processed for indirect immunofluorescence analysis. Microscopic analysis indicated that when using fragments II (20-299), III (20-545), IV (300-545), V (340-545) or IX (1139-1394) thin fibrils were stained positively (Fig. 8). As a negative control, we tested fragment VI (543-1008), which initially had not shown any interactivity with complete cell layers. Incubation of this fragment with isolated matrices did not yield stained fibrillar structures either (Fig. 8). The staining patterns of isolated matrices were much less intense than the ones of complete cell layers, and with fragment IX (1139-1394), positively stained fibers were very rare. These results indicate that the recombinant fragments

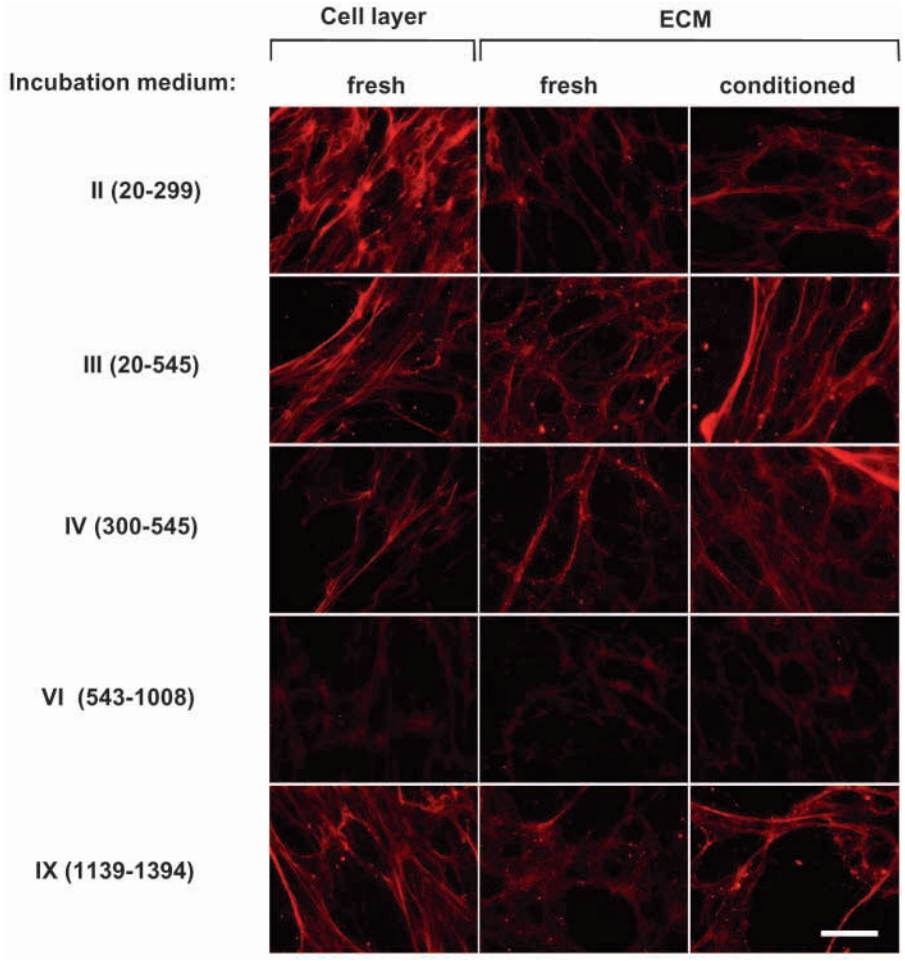

Fig. 8. Association of LTBP-1S fragments with either intact cell layer or isolated skin fibroblast matrices. Extracellular matrices were prepared from confluent human skin fibroblasts by extraction with sodium deoxycholate. ECM preparations were incubated with the indicated fusion proteins (5 μg/ml) either in serum-free, fresh, nonconditioned or in fibroblast-conditioned serum-free medium. Cell layers were incubated with the fusion proteins only in serum free, fresh, nonconditioned medium. Incubation was carried out at 37°C for 72 hours. The samples were then washed and processed for indirect immunofluorescence. Scale bar: 10 μm.

corresponding to both N-terminal and C-terminal regions of LTBP-1S can, to some extent, associate with the matrix without interaction with the cell surface or any enzymatic system. The incorporation into the ECM was increased and the staining pattern of the fibrils appeared more organized if the fusion proteins were incubated with the ECM preparations in 24-hour cell-conditioned medium (Fig. 8). This suggests that cells secrete into their medium proteinaceous factors that augment the interaction between the LTBP-1 fragments with isolated ECM. In both cases, we re-treated the isolated ECM with sodium deoxycholate after incubation with fusion proteins. This re-extraction procedure did not markedly affect the staining patterns (data not shown).

DISCUSSION

So far it is known that LTBP-1 associates with the extracellular matrix at least by its N-terminal part (Nunes et al., 1997; Olofsson et al., 1995; Saharinen et al., 1996; Taipale et al., 1994). We find here for the first time that a fusion protein corresponding to the C-terminal region of the LTBP-1S molecule has affinity for fibroblast extracellular matrix. In addition, two other fusion proteins corresponding to two separate regions in the N-terminus of LTBP-1 became associated with the fibroblast extracellular matrix. All formed interactions were resistant to treatment with sodium deoxycholate indicating their strong forces. The three fragments were able to inhibit specifically the incorporation of

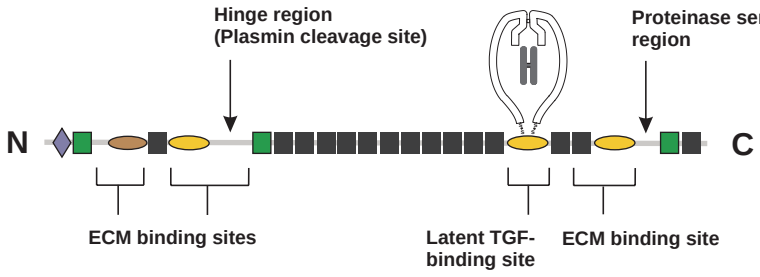

Fig. 9. Organization of the functional regions of LTBP-1S. LTBP-1S associates with the matrix by two N-terminal domains and with one region in the C-terminal part. The third 8-Cys repeat binds covalently to the small latent TGF-β complex via LAP (modified from Saharinen et al., 1999). The truncated form of LTBP-1 can be released from the matrix by plasmin and different elastases. The cleavage site for LTBP-2 has been located at the hinge region. The hinge region of LTBP-1 is thus unlikely responsible for the N-terminal binding to the ECM. Another proteinase-sensitive site exists in the C-terminal part and results in the cleavage of a 50 kDa fragment (see text).

radiolabeled fibroblast-derived LTBP-1 into extracellular matrix. The C-terminal LTBP-1 fragment had an inhibitory effect comparable with that observed with the recombinant N-terminal LTBP-1 parts.

The interaction with fibroblast-ECM could be observed with fragments containing the first 8-Cys repeat (hybrid domain), the second, or the fourth 8-Cys repeat (illustrated in Fig. 9). Our data suggest that the ECM interaction of LTBP-1S involves at least three distinct regions, all containing an 8-Cys repeat. The 8-Cys repeat is a unique feature of the members of the fibrillin/LTBP superfamily, and its functions are poorly understood. In previous studies the 8-Cys repeats were observed to be involved in protein-protein interactions, where the third 8-Cys repeats of LTBP-1, -3 and -4 bind covalently to LAP of the small latent TGF-β complex (Gleizes et al., 1996; Saharinen et al., 1996). According to our data it can be speculated that the 8-Cys domains may mediate the binding of LTBP-1S to fibroblast ECM. The results obtained with fragments IV (300-545) and V (340-545) do not exclude the possible involvement of the hinge region in the ECM interaction. However, the hinge region contains a putative protease cleavage site, which makes it an unlikely candidate for the binding to the ECM.

In a recent study Dallas et al. have used a cell culture system with transiently expressed LTBP-1 fragments (Dallas et al., 2000). They could not detect incorporation to osteoblast matrix of a fragment lacking the N-terminal part of LTBP-1. This might be explained by the possibility that the C-terminal ECM binding site is not accessible when longer LTBP-1 sequences are used. This would be consistent with the current observation that fragment VIII, which contains one more EGF-like repeat and additionally the third 8-Cys repeat, exhibits weaker binding capacity than the shorter fragment IX (1139-1394). However, the C-terminal fragment IX prevented incorporation of full-length LTBP-1 to the matrix, indicating the importance of the binding event in the assembly of LTBP-1. In accordance with the current results we found earlier that incorporation of LTBP-1 into CHO cell matrix did not occur when recombinant LTBP-1 fragment lacking the N-terminal part was used (Saharinen et al., 1996). It appears that the binding of the C-terminus of LTBP-1 requires abundant and well-elaborated matrices. In contrast to the former studies, the fusion proteins used in the present work were expressed as dimeric proteins. There is a possibility that the binding of the C-terminal LTBP-1 domain may require a dimeric conformation, but in view of the lack of understanding of the supramolecular structures of LTBPs this remains a hypothetical view.

Recombinant LTBP-1S fragments bound to fibroblast ECM in a relatively time- and dose-dependent manner. The fragments corresponding to the N-terminal part of LTBP-1 associated more readily with the ECM than the fragment corresponding to the C-terminal part. On the basis of these findings it can be suggested that LTBP-1 is incorporated into the ECM in a multistep process. The first interactions with the ECM may involve the very N-terminal region (amino acids 105-299) of the LTBP-1 molecule followed by additional interactions in the region towards the C terminus (amino acids 340-545), whereas the interaction of the C-terminal LTBP-1 region occurs much later. The association of full-length LTBP-1 with fibroblast matrix would therefore be a dynamic process. Accordingly, we found that the C-terminal fragment IX (1139-1394) inhibited the ECM association of native full-length LTBP-1 after 24 hours, but not after 5 hours (data not shown).

The interactions with the ECM of the two N-terminal and the C-terminal LTBP-1S fragments occurred independently, suggesting that these binding regions are non-cooperative. However, endogenous LTBP-1 and the other four LTBP isoforms are expressed by fibroblasts (K. Koli, J. Saharinen, M. Kärkkäinen and J. K.-O., unpublished observations) and are deposited to the matrix. It cannot be ruled out that these endogenous LTBPs may participate in the ECM-deposition of the exogenously added LTBP-1 fragments (Hyytiäinen et al., 1998). Even though three different ECM-binding sites were detected in the LTBP-1S sequence, a single recombinant LTBP-fragment, used as competitor was sufficient to inhibit the binding of native LTBP-1 into the extracellular matrix. This was an unexpected result, and we cannot exclude the possibility that the Ig part in the fusion protein causes a steric hindrance once the fusion protein is bound to the matrix, and thus masks the binding sites for the two other LTBP-1 binding sites in the matrix. However, simultaneous incubation of fibroblast layers with both the N-terminal fragment V (340-545) and the C-terminal fragment IX (1139-1394) only slightly increased the inhibitory effect on native LTBP-1 binding as compared with fragment IX when it was used alone (data not shown).

The direct association of the matrix-interactive fragments with the ECM seems not to require cell-surface interactions, since the fragments II (20-299), III (20-245), IV (300-545) and IX (1139-1394) became, at least to some extent, associated into cell-free ECM preparations. The mechanism of this basal association is not known at present. Verderio et al. reported co-distribution of LTBP-1 and tissue transglutaminase at the cell surface of tissue transglutaminase overexpressing Swiss 3T3 fibroblasts, which may point at least to indirect actions of the cell surface to the formation of microfibrils (Verderio et al., 1999).

When non-conditioned, fresh medium was used as the reactional environment, the degree of matrix interaction was low, especially in the case of fragment IX (1139-1394). For fragment IX (1139-1394), this finding is in accordance with the results obtained by EIA, where the continuous changes of fusion protein containing medium or the treatment of the cells with cycloheximide reduced the association of the fragment with the matrix significantly. Thus, the presence of proteinaceous factors seems to be indispensable for efficient association. LTBP-1 is a substrate for tissue transglutaminase, and this enzyme is implicated in the process of LTBP-1 incorporation into the ECM. Transglutaminase reactive sites have been proposed to exist both in the N-terminal part and the C-terminal regions (Nunes et al., 1997). Our findings that the interaction of LTBP-1 fragments with isolated fibroblast matrices is enhanced by soluble, cell-derived factors suggest that one of these factors may be transglutaminase.

The current data suggests that the association of LTBP-1 with human fibroblast ECM is a multistep process and involves several regions in the LTBP-1 molecule, both at its N-terminal and C-terminal parts. The observed three binding regions appear to interact in different manners. The most plausible explanation is that the strong N-terminal binding sites are used for anchoring of the protein, and the apparently weaker C-terminal one for the stabilization of the binding. At present, it

is not clear whether LTBP-1 simply binds to different matrix components, such as fibronectin or fibrillin-1, or whether it can form autonomous LTBP microfibrils. Recent studies point to the fact that LTBPs are able to aggregate into microfibrils and microfibrillar bundles in an ordered manner (Dallas et al., 2000). It is also possible that LTBP-1 molecules use other ECM proteins as a scaffold that would help to build up an autonomous network of LTBP-1-containing microfibrils. Further studies will elucidate the potential binding partners of LTBP-1 and their roles in LTBP-1-containing fiber formation.

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