

Latent TGF- β binding protein LTBP-2 decreases fibroblast adhesion to fibronectin

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We have analyzed the effects of latent TGF- β binding protein 2 (LTBP-2) and its fragments on lung fibroblast adhesion. Quantitative cell adhesion assays indicated that fibroblasts do not adhere to full-length LTBP-2. Interestingly, LTBP-2 had dominant disrupting effects on the morphology of fibroblasts adhering to fibronectin (FN). Fibroblasts plated on LTBP-2 and FN substratum exhibited less adherent morphology and displayed clearly decreased actin stress fibers than cells plated on FN. These cells formed, instead, extensive membrane ruffles.

LTBP-2 had no effects on cells adhering to collagen type I. Fibroblasts adhered weakly to the NH₂-terminal fragment of LTBP-2. Unlike FN, this fragment did not augment actin stress fiber formation. Interestingly, the adhesion-mediating and cytoskeleton-disrupting effects were localized to the same NH₂-terminal proline-rich region of LTBP-2. LTBP-2 and its antiadhesive fragment bound to FN *in vitro*, and the antiadhesive fragment associated with the extracellular matrix FN fibrils. These observations reveal a potentially important role for LTBP-2 as an antiadhesive matrix component.

Introduction

Latent TGF- β binding protein 2 (LTBP-2) belongs to the LTBP-fibrillin gene family, which contains LTBPs 1–4 and fibrillins 1, 2 (Saharinen et al., 1999), and 3 (Genbank/EMBL/DDBJ accession no. AY165864). These proteins are ECM glycoproteins sharing similar overall domain structure. They consist mainly of cysteine-rich EGF-like and 8-cysteine (8-Cys) repeats. EGF-like repeats exist in a number of ECM proteins, whereas 8-Cys repeats have so far only been found in fibrillins and LTBPs. Cysteine-rich regions are frequently interrupted by variable proline-rich regions in LTBPs, while fibrillins contain only one such region.

Fibrillins were first identified as components of elastic tissue microfibrils. Microfibrils consist, in addition to fibrillins, of several other proteins like microfibril-associated glycoproteins (MAGPs) 1 and 2 (Rosenbloom et al., 1993). Fibrillins are important for the structure of microfibrils, as shown by mice expressing mutated fibrillin-1 forms (Pereira et al., 1997; Kielty et al., 1998; Gayraud et al., 2000). TGF- β s are secreted from cells as latent complexes composed of the mature dimeric growth factor together with its NH₂-terminal latency-associated peptide (Saharinen

et al., 1999; Annes et al., 2003). The secretion of small latent (SL) TGF- β from the cells is augmented by LTBP-1 (Miyazono et al., 1991), and LTBP-1 directs this complex to the ECM (Taipale et al., 1994). At least LTBP-1 has a role in the activation of latent TGF- β (Flaumenhaft et al., 1993). LTBP-1 localizes to the fibrillin-containing ECM microfibrils as well as to the pericellular cell surface-associated fibronectin (FN) fibrils (Taipale et al., 1996; Isogai et al., 2003). The majority of LTBP-1 protein is secreted from the cells without SL-TGF- β (Taipale et al., 1994), suggesting that LTBP-1 has novel functions, like structural roles in the ECM.

After the identification of LTBP-1 (Kanzaki et al., 1990), three other LTBPs have been cloned, namely LTBP-2, -3, and -4 (Moren et al., 1994; Yin et al., 1995; Giltay et al., 1997; Saharinen et al., 1998). LTBP-2 has been localized by immunoelectron microscopy to the elastic microfibrils in bovine tissues (Gibson et al., 1995). While LTBP-1, -3, and -4 are true SL-TGF- β binding proteins, LTBP-2 is unable to bind SL-TGF- β (Saharinen and Keski-Oja, 2000). In addition, gene-targeted mice for LTBP-2 show early embryonic lethality (Shiple et al., 2000) differing from the phenotypes of all TGF- β (TGF- β 1, - β 2, and - β 3) gene knockout mice (Shull et al., 1992; Proetzel et al., 1995;

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Abbreviations used in this paper: FN, fibronectin; LTBP-2, latent TGF- β binding protein 2; MAGP, microfibril-associated glycoprotein; SL, small latent.

Sanford et al., 1997). This indicates that at least LTBP-2 has important functions not related to TGF- β .

Cell interactions with ECM components are a central, regulated cascade of events both during embryo- and organogenesis and during later stages of life (Gumbiner, 1996). Cells adhere to the ECM via cell surface receptors, often via integrin receptors and RGD recognition sequences of ECM proteins (Ruoslahti, 1996). FN (Vaheri and Ruoslahti, 1975) is the most widely studied example of an adhesive ECM component. Earlier studies indicated that LTBP-2-associated ECM components, such as fibrillin-1 and MAGP-2, mediate cell adhesion, at least in part via their RGD sequences (Sakamoto et al., 1996; Gibson et al., 1999). LTBP-2 contains an RGD cell adhesion sequence, which led us to explore the possible adhesive functions of LTBP-2 for human lung fibroblasts. We chose lung fibroblasts for our studies because lung is the major site of LTBP-2 expression (Moren et al., 1994). These cells attached to a specific proline-rich NH₂-terminal fragment of LTBP-2, which did not, however, contain the RGD sequence. Fibroblasts adhering to this fragment did not spread appreciably or form actin stress fibers. In contrast, this fragment exhibited a dominant cytoskeleton formation-disrupting effect on fibroblasts. LTBP-2 and especially its proline-rich NH₂-terminal region exerted modulatory effects on the actin cytoskeleton of fibroblasts and induced a less adherent phenotype. LTBP-2 is thus a new member to the family of extracellular antiadhesive glycoproteins.

Results

Production of LTBP-2 fragments

To explore the effects of LTBP-2 and its domains on cell adhesion, we produced partially overlapping recombinant protein fragments covering the whole LTBP-2 protein (Fig. 1 A). The fragments were expressed either as histidine-tagged fragments or as dimeric fusion proteins with the constant region of immunoglobulin G to aid purification. The reason for using the Ig tag was that the expression levels were generally higher, and some fragments were more soluble when expressed as fusion proteins with a relatively large IgG constant region than those with a small histidine tag (not depicted). However, all the results obtained with Ig-tagged proteins were confirmed with histidine-tagged fragments.

All protein fragments were secreted from the cells, but the expression levels varied. The larger fragments were, in general, expressed at lower levels than the smaller ones. Some proteins were prone to proteolysis by endogenous proteases. This was noted with fragments containing the proline-rich regions before the hybrid domain (fragments L2-IV, L2-VII), and especially with COOH-terminal fragments containing the proline-rich region (fragments L2-XIII and L2-XIV) (Fig. 1 B), suggesting that the COOH terminus of LTBP-2 is sensitive to proteases in intact, full-length protein. A related cleavage has been described earlier with corresponding LTBP-1 fragments (Unsöld et al., 2001). Fragment L2-X containing the previously identified protease-sensitive area (Hyytiäinen et al., 1998) was also slightly degraded. The migration of fragment L2-X was

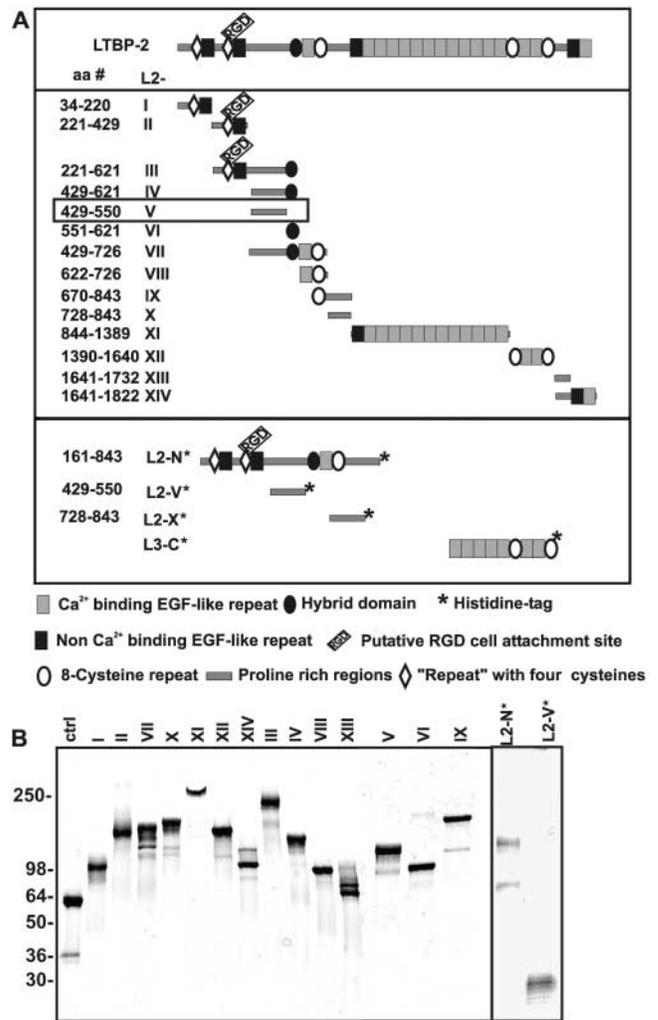


Figure 1. Schematic representation of recombinant LTBP-2 fragments. (A) Domain structure of full-length LTBP-2 (top). LTBP-2 fragments were expressed as fusion proteins with IgG Fc region (middle) or as COOH-terminally histidine-tagged proteins (bottom). The domain map of each fusion protein is illustrated, as well as amino acid numbers of each construct. The characteristics of the domains are described below the figure. The COOH-terminal LTBP-3 fragment L3-C* (Penttinen et al., 2002) used as a control is also illustrated. The antiadhesive fragment L2-V is rectangular. (B) Analysis of expressed and purified LTBP-2 fragments by SDS-PAGE. Aliquots (2 μ g) of each expressed and purified LTBP-2 fragment were analyzed by 4–15% gradient PAGE in the presence of SDS under nonreducing conditions and visualized by Coomassie blue staining. The migration of molecular mass standards (kD) is indicated on the left, and the designations of the fusion proteins on the top. Leftmost, control Fc protein (ctrl).

also anomalous in SDS-PAGE. The calculated molecular mass of this dimeric fragment L2-X together with the Ig tag is \sim 80 kD, but it migrated as an almost 200-kD band. The reason for its aberrant migration in SDS-PAGE is unclear at present.

The histidine-tagged LTBP-2 fragments were purified by metal chelate chromatography as described above. In addition to the fragments, there was occasionally an \sim 65-kD contaminating protein, which copurified from CHO-conditioned medium in metal chelate chromatography (Fig. 1 B, lane L2-N*). It could be removed with gel filtration and was

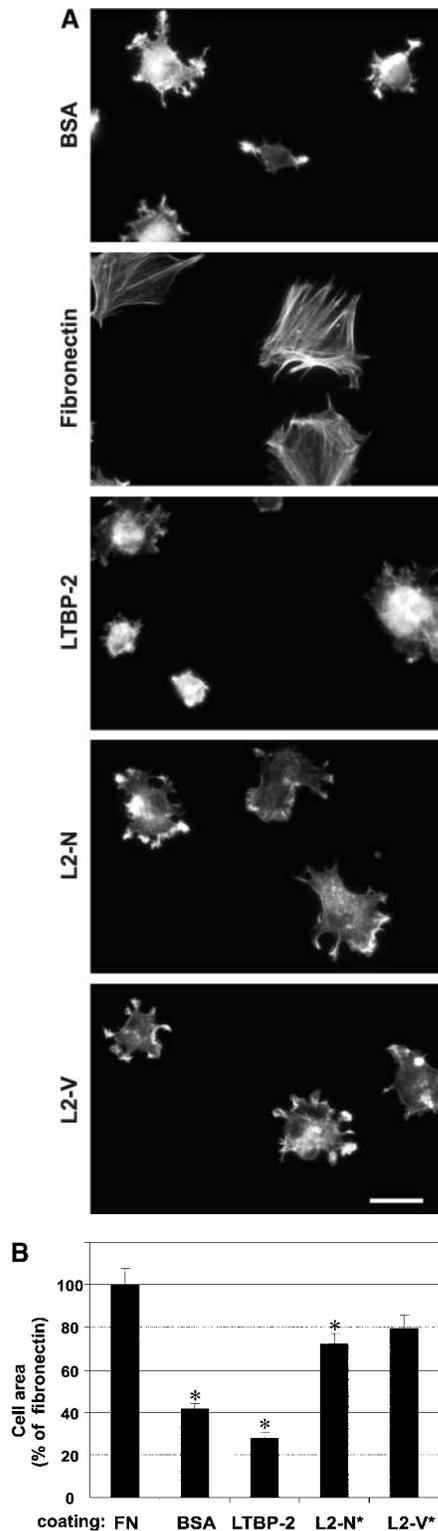


Figure 4. Cells bound to LTBP-2 fragments spread slightly, but do not form actin stress fibers. (A) Morphological analysis. To characterize the actin cytoskeletons of the cells attached to LTBP-2 fragments, glass coverslips were coated with BSA, FN (10 $\mu\text{g/ml}$), full-length LTBP-2 (20 $\mu\text{g/ml}$), or histidine-tagged fragments L2-N* (10 $\mu\text{g/ml}$) or L2-V* (5 $\mu\text{g/ml}$). Fibroblasts were allowed to attach for 90 min, followed by fixation and staining with phalloidin-TRITC. Cells plated on LTBP-2 did not spread or form stress fibers. Cells bound to LTBP-2 fragment-coated plates spread more efficiently than cells on BSA, but did not form stress fibers, as cells on FN. Bar, 20 μm . (B) Quantifica-

response effects: after reaching a critical coating concentration, cell adhesion decreased.

NH₂-terminal fragments of LTBP-2 induce modest cell spreading, but not actin stress fiber formation

To characterize the effects of the fibroblast adhesion–mediating LTBP-2 fragments on cell morphology, we visualized actin cytoskeletons with fluorescently labeled phalloidin after plating the cells on coverslips coated with LTBP-2 or its fragments (Fig. 4 A) and quantified cell spreading on these substrates (Fig. 4 B). The coating concentrations of fragments were chosen for optimal cell binding as analyzed in Fig. 3. Cells seeded on BSA-coated coverslips spread very poorly and did not form stress fibers. Cells seeded on full-length LTBP-2 spread even less (Fig. 4 B). Filamentous actin localized to patches in the cytoplasm, but no distinct structures could be observed. Even lower concentrations of LTBP-2 did not induce cell spreading (not depicted). Cells plated on coverslips coated with NH₂-terminal LTBP-2 fragments spread slightly more, but significantly less than on FN (Fig. 4 B). Phalloidin staining was partially localized to the cytoplasm, but also to the membrane ruffles in the cell periphery (Fig. 4 A). Cells attached to LTBP-2 fragments did not form actin stress fibers.

LTBP-2 and its fragments have dominant actin cytoskeleton and focal adhesion–disrupting effects in fibroblasts adhering to FN

To analyze whether LTBP-2 or its fragments can affect cell adhesion and actin cytoskeleton organization in cells adhering to FN, we performed quantitative adhesion assays and phalloidin stainings as above. In preliminary experiments, we noted that simultaneous coating of the plates with both proteins at the concentrations used decreased the coating efficiency of FN. This was determined by semiquantitative immunoblotting analysis of coverslip/cell culture plate-bound FN (unpublished data). To maintain constant levels of coverslip-bound FN, the coverslips were first coated with FN and the binding sites were saturated with BSA. Subsequently, the indicated LTBP-2 fragments were allowed to bind onto coverslips in PBS. Due to this sequence in coating, only the effects of the proteins bound either to FN or nonspecifically to BSA were detected in the attachment assays (Fig. 5). We found that full-length LTBP-2 bound nonspecifically to all coverslips and plates under these coating conditions, even to BSA (not depicted).

Full-length LTBP-2 had a relatively minor effect ($\sim 10\%$ decrease) on the number of cells adhering to FN substratum (Fig. 5 A), and statistical values were $P > 0.05$, when cell adhesion was compared with cells adhering to FN. As LTBP-2 proved later to have antiadhesive effects (see next paragraph), we compared its effects to tenascin-C, a known anti-

tion of cell spreading. Cells were plated and processed as in A, and cell spreading was quantified from three randomly selected fields of each coverslip as described in the Materials and methods. Cells spread to some extent on NH₂-terminal fragments of LTBP-2, whereas no cell spreading was observed in cells plated on full-length LTBP-2 (*, $P < 0.05$, compared with cell spreading on FN).

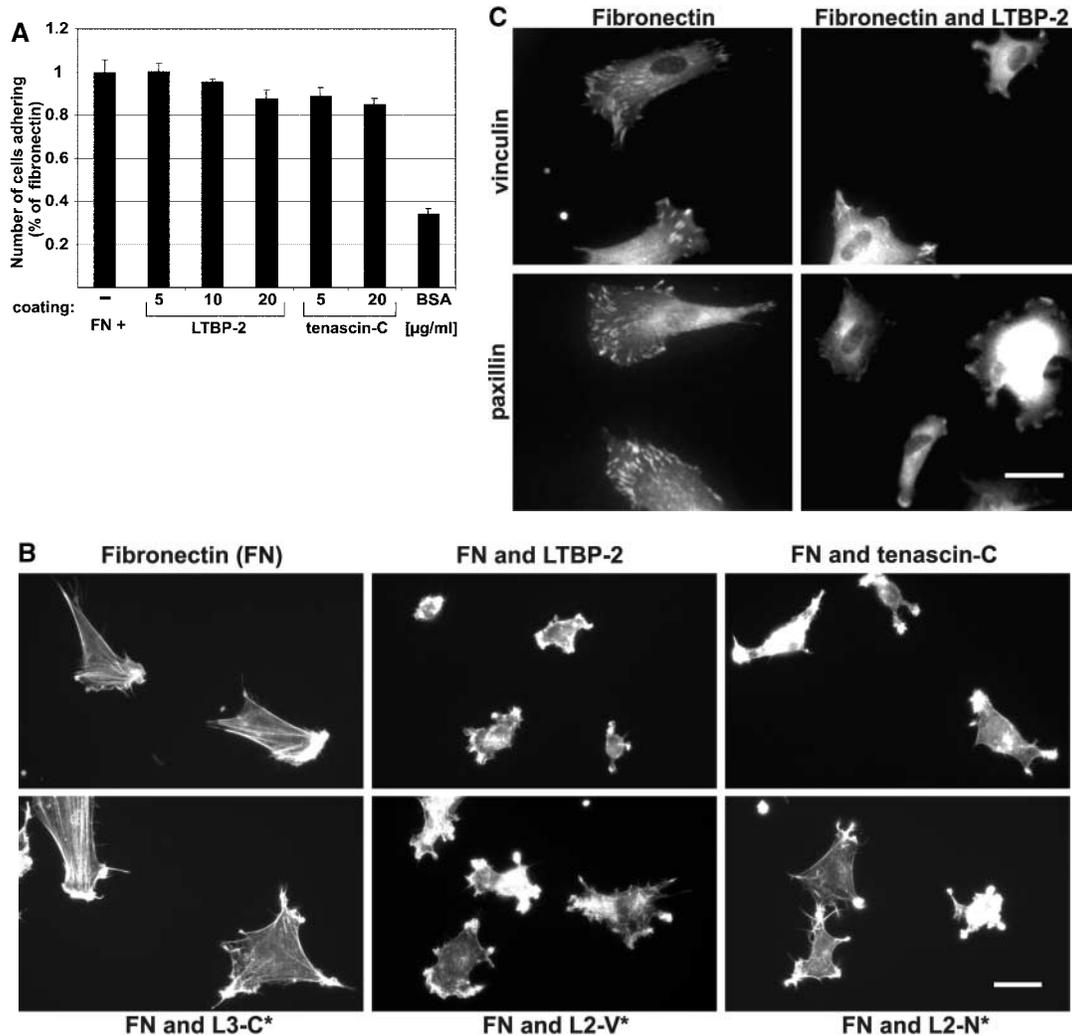


Figure 5. LTBP-2 and its fragments have a dominant actin cytoskeleton formation-disrupting effect on fibroblast spreading on FN. (A) Comparison with tenascin-C. Plastic cell culture plates were coated first with FN (10 μg/ml), and the free protein binding sites were saturated with BSA. The FN-coated plates were then incubated with LTBP-2 or tenascin-C at the indicated concentrations (μg/ml). The plates were washed, and quantitative cell adhesion assays were performed. (B) Morphological analysis. Glass coverslips were coated first with FN (10 μg/ml), and the free protein binding sites were saturated with BSA. FN-coated coverslips were then incubated with LTBP-2, control LTBP-3 fragment L3-C*, tenascin-C (20 μg/ml), or fragments L2-N* (10 μg/ml) or L2-V* (5 μg/ml). Fibroblasts were then allowed to attach and spread for 90 min, followed by fixation and staining with phalloidin-TRITC. Note that actin stress fiber formation was decreased in cells grown on coverslips treated with LTBP-2, its fragments, and tenascin-C. Fragment L3-C* had no effect. Bar, 20 μm. (C) Effects on focal adhesions. Glass coverslips were coated with FN and incubated with LTBP-2 or PBS (concentrations as in B). The cells were plated and fixed as in B, after which vinculin and paxillin were detected by indirect immunofluorescence. Both proteins localized to focal adhesions in control cells, whereas focal adhesions were missing from cells attaching on FN/LTBP-2 substratum. Bar, 20 μm.

adhesive protein (Chiquet-Ehrismann et al., 1988; Huang et al., 2001). The effects of tenascin-C were of the same magnitude as those of LTBP-2.

Next, we investigated the effects of LTBP-2 on actin cytoskeletons of cells attaching to FN. Full-length LTBP-2 and its NH₂-terminal fragments had a dominant actin stress fiber formation-disrupting effect on fibroblasts when compared with cells plated on FN alone (Fig. 5 B). Actin staining was mainly localized to the cell periphery, including membrane ruffles and patch-like structures at the extremities of the cells, resembling very much the cells plated on LTBP-2 fragments only (compare with Fig. 4), whereas cells plated on FN formed extensive stress fibers. The effect resembled that of tenascin-C. In addition, cells cultured on

LTBP-2/FN substratum were less spread than control cells (Fig. 6 A). The COOH-terminal control fragment L3-C* derived from LTBP-3 (Penttinen et al., 2002) did not exhibit any detectable effects on cell morphology or actin cytoskeleton formation (Fig. 5 B).

Focal adhesions are another important cellular structure for cell adhesion. We explored the effects of LTBP-2 on focal adhesions of cells adhering to FN by localizing two well-characterized focal adhesion proteins, vinculin and paxillin (Petit and Thiery, 2000). Focal adhesions formed efficiently in cells adhering to FN substratum (Fig. 5 C, left). In contrast, focal adhesion formation was disrupted by LTBP-2 (Fig. 5 C, right). Vinculin and paxillin were mostly cytoplasmic, and no distinct structures could be observed. A small portion of the

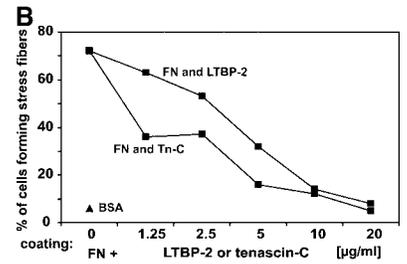
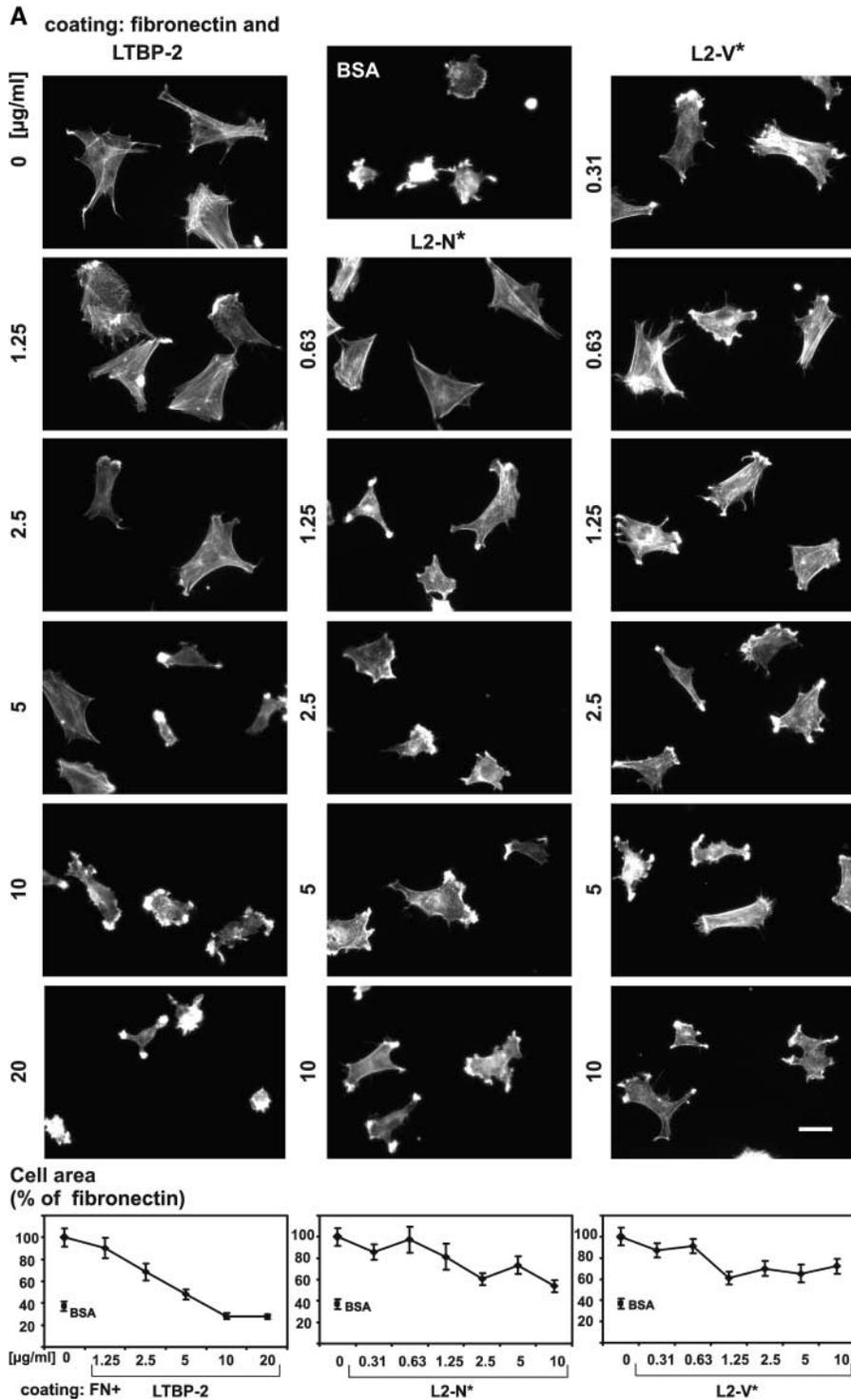


Figure 6. Antiadhesive effects of LTBP-2 and its fragments are concentration dependent. (A) Concentration dependency analysis. Glass coverslips were coated with constant concentration of FN (10 µg/ml). Nonspecific binding sites were saturated with BSA, and the coverslips were incubated with increasing concentrations of LTBP-2 or its fragments L2-N* and L2-V*. Fibroblasts plated on BSA only served as a control. Fibroblasts were fixed 90 min after plating and stained for actin. Cells lost their adherent morphology when the concentrations of LTBP-2 or its fragments were increased. Bottom, quantification of cell spreading. Cell spreading was quantified from the coverslips (three independent fields). Note the differences in maximal effects of LTBP-2 and its fragments. (B) Comparison of LTBP-2 and tenascin-C effects on actin stress fiber formation. Actin stress fiber formation of cells was quantified from coverslips treated with increasing concentrations of LTBP-2 or tenascin-C.

staining was in the tips of the cell extensions and membrane ruffles in a manner similar to actin staining. These findings further support the hypothesis that LTBP-2 acts as an antiadhesive protein that decreases fibroblast adhesion.

Antiadhesive effect of LTBP-2 is concentration dependent

We explored next the concentration dependency of the antiadhesive effect of LTBP-2. Glass coverslips were coated with a constant concentration of FN, and subsequently with in-

creasing concentrations of LTBP-2 or fragment L2-N* or L2-V*. The cells were stained for actin 90 min after plating. Cells were then observed in fluorescence microscopy and the cell spreading was quantified. Low concentrations of LTBP-2 (1.25 µg/ml) had only minor effects (Fig. 6 A). With increasing concentrations of LTBP-2, cell spreading decreased steadily (Fig. 6 A, the lowermost panel), as well as the number of cells forming stress fibers (Fig. 6 B). With the highest LTBP-2 concentration used (20 µg/ml), the cells did not form any stress fibers and spread even less than cells on BSA-

coated substratum. These effects were similar to those obtained by using tenascin-C (Fig. 6 B and not depicted). The fragments L2-N* and L2-V* had quite similar concentration-dependent effects on cell morphology and actin cytoskeleton organization. However, the actin cytoskeleton- and cell spreading-disrupting effects were less efficient than with full-length LTBP-2 (Fig. 6 A). Unlike LTBP-2 itself, the fragments reached a saturating concentration. The increase of the coating concentration over a certain limit (2.5 $\mu\text{g}/\text{ml}$ with L2-N* and 1.25 $\mu\text{g}/\text{ml}$ with L2-V*) did not further inhibit cell spreading or prevent further actin cytoskeleton organization (compare with LTBP-2 panel).

LTBP-2 and its antiadhesive fragments bind to FN

To gain an insight into the mechanisms of the antiadhesive property, we analyzed whether LTBP-2 or its antiadhesive fragments could bind directly to FN or collagen type I. For this purpose, we established an enzyme-linked immunosorbent assay. Plates of 96 wells were first coated with increasing concentrations of FN or collagen type I. Both proteins coated the plates effectively under these conditions. Soluble LTBP-2 or its fragments were then incubated in the wells in a buffer containing BSA to prevent binding of LTBP-2 to BSA. Bound LTBP-2 was detected with polyclonal affinity-purified antibodies, and histidine-tagged fragments were detected with monoclonal antibody recognizing the histidine tag (Fig. 7 A). We observed that LTBP-2 and its fragments L2-V* and L2-N* bound to FN in a concentration-dependent and saturable manner, showing the specificity of binding (Fig. 7). In contrast, no binding of LTBP-2 to collagen type I was observed.

LTBP-2 fragment L2-V* associates with the ECM

To analyze the extracellular localization of the NH₂-terminal antiadhesive fragments in overexpressing cells, we transfected COS-7 cells and detected the expressed proteins by indirect immunofluorescence using antibodies recognizing the histidine tag. Secreted and either cell surface- or ECM-bound histidine-tagged fragments (Fig. 8) were detected in nonpermeated cells. To detect possible colocalization with LTBP-2 fragments, we performed FN immunofluorescence analysis of the same cells.

An antibody recognizing the histidine tag gave only faint background staining in nontransfected control COS-7 cells (Fig. 8, no transfection). The long fragment L2-N* localized clearly to ECM fibers, colocalizing with FN. Part of the staining localized to the surface of cells (Fig. 8, L2-N*). Several ECM binding regions have been localized to the corresponding NH₂-terminal region of LTBP-1 (Nunes et al., 1997; Unsold et al., 2001), and this pattern was therefore expected.

The antiadhesive fragment L2-V* localized to the extracellular/pericellular matrix as well (Fig. 8, L2-V*). The staining was more discontinuous than that of fragment L2-N*. The staining clearly colocalized with FN in many, but not all, locations.

Histidine-tagged fragment L2-X* was used as a control. This fragment was detected at the surfaces of the cells (Fig. 8, L2-X*), and no obvious colocalization with FN was detected.

COS-7 cells form relatively sparse extracellular matrices. We have therefore repeated the assays using human lung fi-

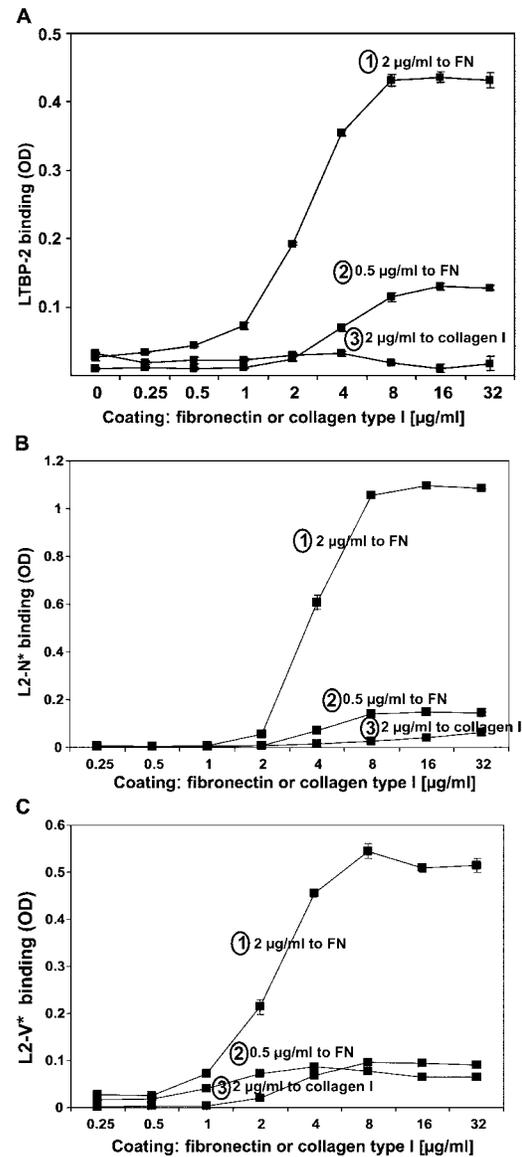


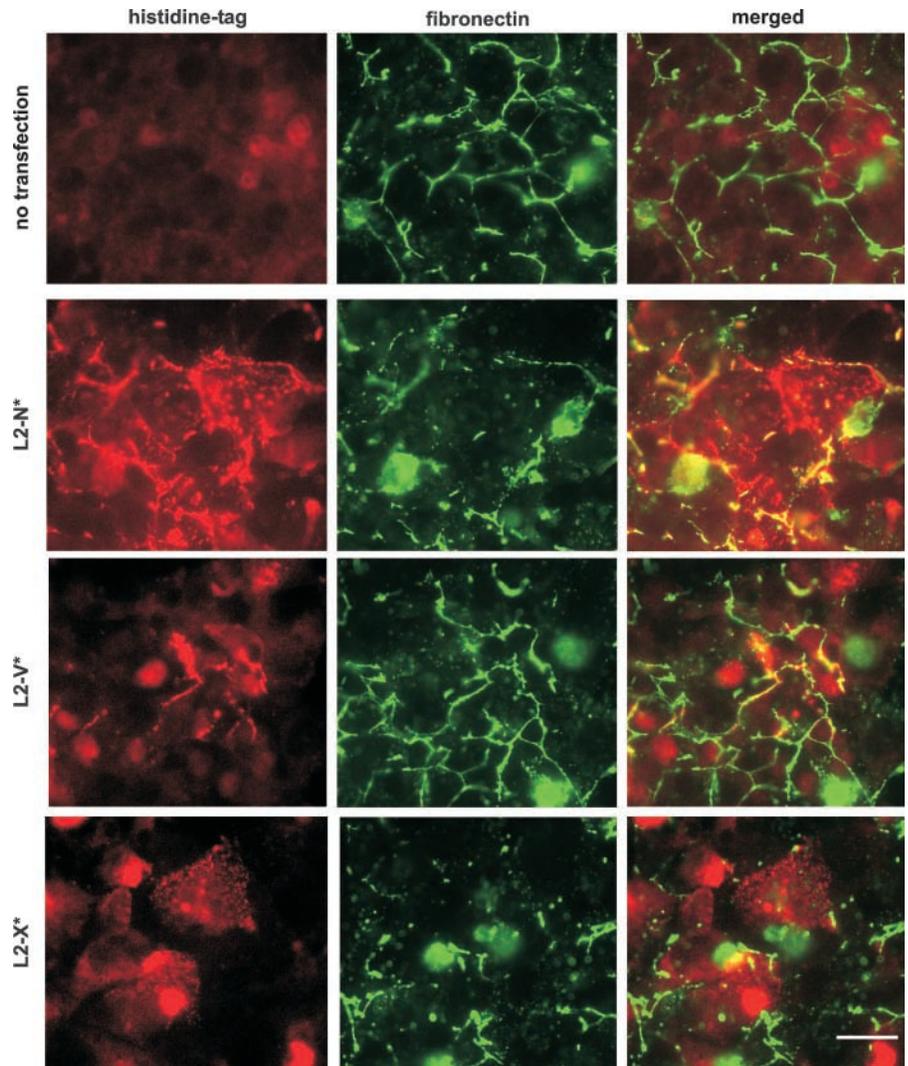
Figure 7. Soluble LTBP-2 and its antiadhesive fragments bind to immobilized FN but not collagen type I. 96-well plates were coated with the indicated concentrations of FN (1 and 2) or collagen type I (3), after which nonspecific binding sites were saturated with BSA. LTBP-2 (A), fragment L2-N* (B), or fragment L2-V* (C) (1 and 3, 2 $\mu\text{g}/\text{ml}$; 2, 0.5 $\mu\text{g}/\text{ml}$) was then incubated in the wells for 1 h in a buffer containing BSA, to lower the background binding, after which the wells were washed. Bound LTBP-2 was detected with anti-LTBP-2 antibodies and horseradish peroxidase-conjugated secondary antibody. LTBP-2 binds to FN in a concentration-dependent and saturable manner, whereas binding to collagen type I was not observed.

broblasts. The results were similar (unpublished data), but the transfection efficiencies of fibroblasts were very poor, which prevented their use in the assays.

LTBP-2 is not antiadhesive for cells adhering to type I collagen

We determined next whether the actin cytoskeleton-disrupting effect of LTBP-2 would be specific for FN. For this purpose, we coated coverslips with FN or collagen type I. Fibroblasts adhered more avidly to collagen type I than FN

Figure 8. Fragment L2-V associates with the ECM. COS-7 cells were transfected with the expression constructs (indicated on the left). 48 h after transfection, the cells were fixed. The expressed histidine-tagged proteins were visualized by a mAb recognizing histidine tag and Texas red–conjugated anti–mouse secondary antibody. FN was visualized from the same cells with polyclonal rabbit anti-FN antibodies and FITC-conjugated anti–rabbit antibodies. Both L2-N* and L2-V* fragments labeled fibrillar structures in the ECM. The staining colocalized partially with FN. Fragment L2-X*, which was used as a control, localized to cell surfaces, not to ECM fibers. Bar, 20 μ m.



(unpublished data), permitting the use of lower concentrations of it (Fig. 9). After saturation of nonspecific binding sites with BSA, the coverslips were incubated with LTBP-2. Under these conditions, LTBP-2 bound to both types of coverslips, coated either with FN or collagen type I (not depicted). Cells adhered and formed stress fibers efficiently on coverslips coated either with FN or collagen type I (Fig. 9). However, in the presence of LTBP-2, the cells adhering to FN were clearly less adherent and more rounded, whereas LTBP-2 had no appreciable effects on cells adhering to collagen type I (Fig. 9). We analyzed a wide range of FN and collagen type I coating concentrations in the assays. The effects of LTBP-2 on cells adhering to FN were always prominent, whereas effects on cells adhering to collagen type I were negligible (unpublished data). These results indicated that even though LTBP-2 is present on coverslips coated with both FN and collagen type I, the cytoskeleton-disrupting effect of LTBP-2 is effective only for cells adhering to FN.

Discussion

LTBPs have generally been considered proteins that target the effects of TGF- β by augmenting its secretion from the

cells and targeting the latent complexes to appropriate sites in the ECM (Saharinen et al., 1999). So far relatively few other functions have been identified for LTBPs.

Fibrillins and other microfibril components, MAGP-2 (Gibson et al., 1999; Bax et al., 2003), mediate cell adhesion via RGD sequences. Fibrillin-1 seems to mediate cell adhesion also via non-RGD-dependent ways (Sakamoto et al., 1996). LTBP-2 possesses a putative cell adhesion–mediating RGD sequence, which led us to examine the effects of LTBP-2, and especially its NH₂ terminus, on cell adhesion.

Although full-length LTBP-2 did not mediate cell adhesion, we found with Ig-tagged fragments an area that supported fibroblast binding in cell adhesion assays. This activity was localized to the NH₂ terminus of LTBP-2 at the variable region toward the COOH terminus from the second EGF-like repeat and toward the NH₂ terminus from the hybrid domain. This region is rich in proline and positively charged amino acids and has no defined domain properties. The RGD sequence is not located at this area. When reanalyzing the results with histidine-tagged fragments, we observed that cell adhesion to plates coated with recombinant fragments of this region did not induce actin stress fiber formation, even though the cells spread slightly more efficiently

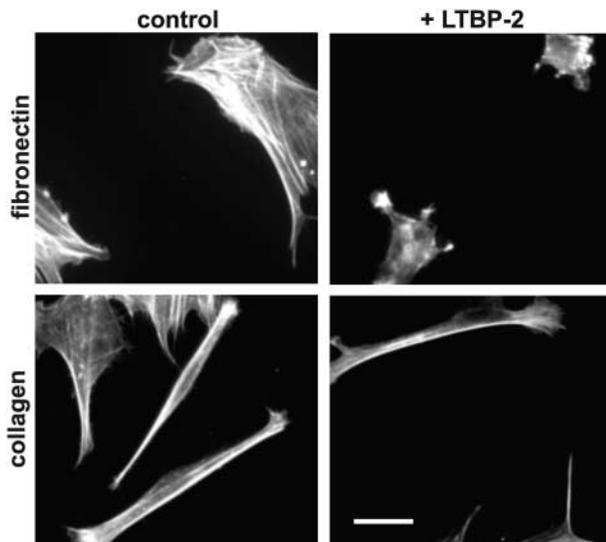


Figure 9. LTBP-2 affects actin stress fibers only in cells adhering to FN, not to collagen type I. Glass coverslips were coated either with FN (10 $\mu\text{g/ml}$) or with collagen type I (0.33 $\mu\text{g/ml}$) as indicated, treated with BSA, and incubated with LTBP-2 (20 $\mu\text{g/ml}$) as indicated. Fibroblasts were then plated, allowed to attach for 90 min, fixed, and stained for filamentous actin. Substratum-bound LTBP-2 completely prevented actin stress fiber formation in cells adhering to FN, but had no effect on cells adhering to collagen type I. Bar, 20 μm .

than cells attaching onto BSA-coated coverslips. Actin was localized in these cells to membrane ruffles. In addition, we found that this region of LTBP-2 exhibited a concentration-dependent, bell-shaped effect on cell adhesion. This suggested that LTBP-2 might have antiadhesive properties and guided us to explore possible combinatory effects of LTBP-2 and the adhesive ECM components FN and type I collagen on cell adhesion and morphology.

We found that full-length LTBP-2, its NH_2 -terminal half, and the proline-rich region (fragment L2-V) had dramatic and dominant effects on the morphology of cells adhering to FN. Both LTBP-2 and its NH_2 -terminal fragments prevented stress fiber formation and caused actin redistribution to the cell periphery. Cell spreading was also slightly impaired, and the cells were more rounded, contributing to a less adherent morphology. Accordingly, LTBP-2 disrupted focal adhesion formation. Paxillin and vinculin, proteins localizing normally to focal adhesions in adherent cells, localized mostly to patchy structures in the cytoplasm and partially to membrane ruffles in the presence of LTBP-2. Although the effect of LTBP-2 on the number of attaching cells was minor, the cytoskeletal changes were comparable to the other antiadhesive proteins such as SPARC (Sage et al., 1989) and thrombospondin (Murphy-Ullrich and Höök, 1989). This justifies the usage of the term antiadhesive with LTBP-2.

The effect was characteristic of FN; cells adhering to type I collagen exhibited normal cell morphology and actin cytoskeletons in the presence of LTBP-2. The tested proteins comprise only a small number of adhesive ECM components, and it will be of interest to analyze whether the antiadhesive effect of LTBP-2 is specific for example for certain integrins.

To obtain mechanistic insights for the antiadhesive effect of LTBP-2, we established a binding assay for LTBP-2. We noted that LTBP-2 binds to FN in a specific, saturable manner but not to type I collagen. In addition, in binding experiments with histidine-tagged fragments, we found that the fragments with antiadhesive properties bound to FN as well. Interestingly, the same fragments associated with the ECM and colocalized with FN in cell cultures.

In contrast to NH_2 -terminal fragments, full-length LTBP-2 had no effects on cell adhesion, when coated separately. This fact suggests that the cell adhesive function is cryptic in full-length LTBP-2. However, FN-bound full-length LTBP-2 had antiadhesive effects on fibroblasts. We therefore propose that the binding of LTBP-2 to FN may result in exposure of cryptic, antiadhesive regions of LTBP-2.

We performed assays where we studied the concentration dependency of the antiadhesive effect of LTBP-2 and its fragments. Full-length LTBP-2 was the most effective in mediating antiadhesion, and the effects became more prominent when increasing the concentration of LTBP-2. In contrast, the fragments of LTBP-2 reached a saturation point. When increasing the coating concentration above a critical point, the effect was saturated. The maximal effects were not as drastic as with full-length LTBP-2. This effect may result from the saturation of LTBP-2 fragment binding sites in FN. LTBP-2 fragments may act by binding to FN (compare Figs. 6 and 7) and blocking some adhesive epitopes in FN. Full-length LTBP-2 may contain some additional binding sites/mechanisms for antiadhesion.

The above-mentioned observations suggest that LTBP-2 has an ability to bind to FN localized to ECM and mask its adhesive epitopes. Tenascin-C, a related antiadhesive ECM component, has been proposed to exert its effects via this mechanism. Tenascin-C binds FN and prevents syndecan-4 binding to it, causing a less adhesive phenotype in cells (Huang et al., 2001). Interestingly, the effects of tenascin-C were very similar to those of LTBP-2 in our studies. Major antiadhesive effects were observed as decreased stress fiber formation as well as cell spreading. Its effects on the number of attaching cells were minor. In studies by others (Huang et al., 2001) tenascin-C decreased the number of cells attaching to mixed tenascin-C/FN substratum. The noted difference possibly results from different experimental settings, such as the number of cells used in assays.

Fibroblasts were observed to bind to the antiadhesive fragment of LTBP-2. The morphology of the cells attaching to the fragments was strikingly similar to cells attaching to FN/LTBP-2 substratum. This suggests that the cell-binding activity is related to its antiadhesive effect. Furthermore, the fact that cells bind to the antiadhesive fragment suggests that there might be a cell surface receptor recognizing this region of LTBP-2. This hypothetical cell surface component could act either in integrin adhesion complexes by sterically hindering integrin binding to its ligand, or alternatively as separate signaling receptors, affecting signal transduction and preventing integrin activation and cell adhesion. This kind of mechanism has been proposed for thrombospondin, another antiadhesive ECM protein. The NH_2 -terminal fragment of thrombospondin binds to calreticulin at the cell surface, which affects the focal adhesion integrity (Goicoechea

et al., 2000). However, so far we have not been able to detect such receptors.

We have observed recently that LTBP-2 acts as an adhesion protein for cultured melanoma cells (Vehviläinen et al., 2003). Melanoma cell adhesion leads to efficient cell spreading, and the actin cytoskeletons appeared comparable to cells adhered to FN. The adhesion was at least in part mediated by $\alpha_3\beta_1$ and, to a lesser extent, $\alpha_6\beta_1$ integrins. Interestingly, the melanoma cell adhesion site in LTBP-2 was localized to the same proline-rich fragment L2-V, which was antiadhesive for lung fibroblasts in the current study.

FN is a component of the so-called provisional matrices during development and wound healing (Mosher, 1995). We noted LTBP-2 to be antiadhesive of the tested ECM components only for cells adhering to FN. Therefore, it is possible that LTBP-2 has an antiadhesive role only for short periods of time during development when FN exists in ECM fibers. However, the antiadhesive role of LTBP-2 needs to be investigated in regard to the other adhesive ECM components.

Antiadhesion or counteradhesion is a relatively rare, but not extinct, phenomenon among ECM proteins (Chiquet-Ehrismann, 1995). Examples of antiadhesive proteins include thrombospondin, tenascin, and SPARC (BM-40). The modulation of cell adhesion is often related to cell migration. Filopodia and lamellipodia formation is crucial for cell movement, and the actin cytoskeleton plays an important role in these processes (Lauffenburger and Horwitz, 1996). As decreased actin stress fiber formation and increased membrane ruffle formation caused by LTBP-2 and its NH₂-terminal fragment L2-V are consistent with the promotion of cell migration, it is tempting to speculate that the antiadhesive property of LTBP-2 is linked to the modulation of fibroblast migration in the lung and other tissues where LTBP-2 is expressed.

Materials and methods

Cell culture

Human embryonic lung fibroblasts (CCL-137; HEL-299, American Type Culture Collection) were maintained in MEM containing 10% fetal calf serum (Life Technologies, Inc.), 100 IU/ml penicillin, and 50 μ g/ml streptomycin. CHO cells were maintained in MEM, 10% fetal calf serum supplemented with 0.2% BSA. Stable neomycin-resistant CHO clones were maintained in MEM containing 0.2% BSA, 10% fetal calf serum, penicillin/streptomycin, and 0.4 mg/ml G418 (Calbiochem). COS-7 cells were maintained in DME supplemented with penicillin/streptomycin and 10% fetal calf serum.

Fibroblast adhesion assays

Tissue culture 96-well plates were coated with LTBP-2 or its fragments at the concentrations indicated (50 μ l/well) either at 4°C for 16 h, or at 20°C for 1 h in PBS (140 mM NaCl, 10 mM sodium phosphate, pH 7.4). Nonspecific cell adhesion was prevented by incubating the wells with 100 μ l of 1% heat-treated (70°C for 1 h) BSA at 22°C for 1 h. The wells were washed with PBS before the adhesion assays.

To detect the effects of LTBP-2 on cells adhering to FN or type I collagen, the tissue culture plates or glass coverslips were first coated with FN (10 μ g/ml) or type I collagen (0.33 μ g/ml). The proteins were prediluted with PBS at room temperature before coating. Nonspecific binding sites were then saturated with heat-treated BSA as before. Coverslips or plates were then incubated with LTBP-2 (Hyytiäinen et al., 1998; Vehviläinen et al., 2003), its fragments, or recombinant tenascin-C (Fischer et al., 1997) (a gift of R. Chiquet-Ehrismann, Friedrich Miescher Institute, Basel, Switzerland) in PBS containing 1 mM CaCl₂, 1 mM MgCl₂ for 1 h. The plates were then washed twice with PBS containing 1 mM CaCl₂, 1 mM MgCl₂.

Cells were detached from plates with trypsin-EDTA and suspended into serum-free MEM. The cells were counted, spun down by centrifugation at 500 g for 5 min, and resuspended in serum-free medium (5×10^5 cells/ml). Aliquots of the cell suspension (100 μ l; 5×10^4 cells) were plated in each well. The plates were incubated in a humidified incubator at 37°C for 1 h. Nonattached cells were removed by washing the wells twice with PBS. The cells were then fixed and stained by filtered 0.1% Coomassie blue dissolved in 30% methanol, 10% acetic acid. Subsequently, the cells were washed extensively with 30% methanol/10% acetic acid and once with PBS. The cells were lysed in 1% SDS, and A₆₂₀ was measured. The results are presented as the mean value obtained from three different wells and compared with the data obtained from cell adhesion to FN. Error bars represent the standard error. Each experiment was performed at least three times.

Expression constructs

LTBP-2 fragments were expressed in stably transfected CHO cells as dimeric NH₂-terminal fusion proteins with the Fc portion of immunoglobulin G as follows. Fragments of LTBP-2 cDNA were amplified by PCR using Pfu or Pfu Turbo polymerase (Stratagene) with primers designed to maintain the open reading frame after the signal sequence and to continue to Fc tail in signal plg+ (R&D Systems) vector. Primer sequences contained HindIII (5' primers, all constructs except L2-X) or BamHI (3' primers, all constructs except L2-VII and L2-VIII) restriction enzyme recognition sites in the 5' end of the primers. Construct L2-VII and L2-VIII 3' primers contained NotI recognition sites, and construct L2-X 5' primer contained KpnI recognition site. L2 constructs contained as a fusion partner amino acids of LTBP-2 as described and illustrated in Fig. 1 A. Amino acid numbering is according to the translated cDNA sequence in Genbank/EMBL/DBJ (accession no. Z37976). Control protein containing only the constant region of human IgG was obtained by expression of vector plg+.

Histidine-tagged (six histidines) monomeric fragments were produced using pSecTagA vector (Invitrogen). cDNA fragments were produced by PCR with the primers containing HindIII (5') and NotI (3') restriction enzyme recognition sites. L2-N* contains amino acids 161–843, L2-V* amino acids 429–550, and L2-X* amino acids 728–843 (also illustrated in Fig. 1 A).

PCR fragments were digested with the corresponding restriction enzymes and ligated to vector cut with the same enzymes. In constructs L2-VII and L2-VIII and pSecTag constructs, adenosine nucleotides were first inserted into the 3' ends of the PCR product with Taq polymerase, after which the PCR fragment was cloned into pGEM-T cloning vector (Promega), from which the fragment was cloned to the target vector. All constructs were sequenced to confirm that there were no PCR-derived mutations. Histidine-tagged human LTBP-3 COOH-terminal fragment L3-C* hL3/911–1153 was expressed and purified as described previously (Penttinen et al., 2002).

Expression and purification of LTBP-2 fragments

Stable neomycin-resistant CHO cell clones were obtained by transfecting the CHO cells with lipofectamine (Promega) with each expression construct as described earlier (Hyytiäinen et al., 1998). 1 d after transfection, the cells were changed to medium containing 1.5 mg/ml G418 (plg+) or 0.5 mg/ml zeocin (pSecTag), after which the cells were maintained in the selection medium for 2 wk. Stable drug-resistant cells were diluted and cloned to 96-well plates, and the expression of fusion protein was estimated by dot blotting culture medium 1–2 wk after dilution cloning, using biotinylated protein A (Sigma-Aldrich) or penta-his monoclonal antibody (QIAGEN) as the first conjugate. The IgG fusion proteins were purified from the conditioned medium with protein A affinity chromatography.

Histidine-tagged proteins were first precipitated with 35% (NH₄)₂SO₄. The precipitate was dissolved in the wash buffer (2 M urea, 300 mM NaCl, 50 mM Tris-HCl buffer, pH 7.2). The sample was loaded to Talon metal chelate (CLONTECH Laboratories, Inc.) column. The column was washed with the wash buffer, and the bound proteins were eluted with the same buffer containing 100 mM imidazole. The buffer of the fractions containing the fusion proteins was then exchanged to PBS with Fast Desalting column HR 10/10 (Amersham Biosciences).

Fluorescence staining of the cells

For the immunofluorescence detection of FN and histidine-tagged proteins in COS-7 cells, the cells were plated to six-well plates containing glass coverslips. After reaching semiconfluency, the cultures were transfected with the indicated expression constructs using Fugene 6 (Roche) according to the manufacturer's instructions. Cells were then maintained in normal serum containing medium for 48 h before fixation.

The transfected cultures were washed with PBS. The cells were then fixed with 3% paraformaldehyde for 15 min. The cells were washed three

times with PBS, and nonspecific binding sites were saturated by incubating the cells with 5% BSA in PBS for 30 min. The coverslips were then treated simultaneously with penta-his monoclonal antibody (QIAGEN) and anti-FN rabbit polyclonal antibodies (Sigma-Aldrich), followed by Texas red-conjugated anti-mouse secondary antibody and fluorescein isothiocyanate-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories). After washes, the coverslips were mounted with Vectashield (Vector Laboratories, Inc.).

For actin, vinculin, or paxillin staining of CCL-137 fibroblasts, subconfluent cultures of cells were detached from tissue culture plates with trypsin-EDTA and washed with serum-free medium. The cells were resuspended in serum-free MEM, containing the indicated proteins, and plated onto acid-treated glass coverslips on six-well plate wells (10^5 cells/well). Coverslips were coated before plating as indicated in the Fibroblast adhesion assays section. After 90 min, the medium was removed, and the cells were fixed with 3% paraformaldehyde in PBS for 20 min. The cells were permeated with 0.1% TX-100 in PBS for 5 min, and nonspecific staining was prevented by incubation with 5% BSA in PBS for 30 min. The cells were finally stained for filamentous actin with tetramethyl rhodamine isothiocyanate-conjugated phalloidin (Sigma-Aldrich) in PBS, 0.5% BSA. To detect vinculin, the cells were incubated with anti-vinculin monoclonal antibody (clone hVIN-1; Sigma-Aldrich), and to detect paxillin with anti-paxillin monoclonal antibody (clone 349; BD Biosciences), and subsequently with Texas red-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories). After washes, the coverslips were mounted with Vectashield.

Coverslips were observed with an Axioplan 2 microscope (Carl Zeiss Microimaging, Inc.) using 40× objective. Pictures were taken with Axio-Cam HR (Carl Zeiss Microimaging, Inc.) digital camera and AxioVision 3.1 (Carl Zeiss Microimaging, Inc.) software. Brightness and contrast of the images were adjusted with Corel Photo-Paint (Corel Inc.) and decorated with CorelDRAW (Corel Inc.) software.

Cell spreading and stress fiber quantification

Three independent and randomly chosen pictures were taken at 10× magnification from actin-stained cells. The cell spreading was determined by counting the area of each cell with the aid of Image-Pro software (Media Cybernetics). The average of >100 cells was calculated, and cell spreading on FN was set to one. The cell spreading values of cells on other substrates was then compared with this value. Cells in contact with other cells were excluded from the analysis. Actin stress fibers were quantified from similar pictures by manually counting the percentage of cells forming stress fibers on each substratum.

ELISA

Human plasma FN (Sigma-Aldrich) or native rat tail type I collagen (Upstate Biotechnology) were diluted from stock solutions in acetic acid to PBS to the indicated concentrations. MaxiSorp 96-well plates (Nunc) were then coated with these proteins in PBS at 23°C for 1 h with shaking (as all subsequent steps). Nonspecific protein binding sites were saturated with 3% BSA in PBS (23°C, 1 h). Wells were then washed once with incubation buffer (PBS containing 1 mM CaCl₂, 1 mM MgCl₂, and 0.5% BSA). This buffer was used in all subsequent washes and incubations. Purified LTBP-2 or fragment L2-N* or L2-V* were diluted and incubated in the wells for 1 h. The wells were next washed twice with the incubation buffer (5 min each). Bound LTBP-2 was detected by incubating the wells with polyclonal affinity-purified antibody ab-L22 for 1 h (Hyytiäinen et al., 1998). LTBP-2 fragments were detected with the penta-his monoclonal antibody. The plates were washed twice (5 min each) and incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody for 30 min. The wells were finally washed (4 × 10 min) with the incubation buffer, and the bound immune complexes were detected with 1,2-phenylenediamine dihydrochloride (DakoCytomation) according to the manufacturer's instructions. The reaction was terminated with H₂SO₄, and the color reactions were measured with a spectrophotometer at wavelength 450 nm.

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