Latent Transforming Growth Factor-β-binding Protein 2 Is an Adhesion Protein for Melanoma Cells

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Of the four latent transforming growth factor (TGF)-β-binding proteins (LTBPs), LTBP-2 is different in the respect that it does not bind small latent forms of TGF-β. LTBP-2 is therefore likely to have other roles in the extracellular matrix. LTBP-2 contains an RGD putative integrin recognition site, suggesting a role in cell adhesion. We carried out a study on cell attachment to LTBP-2. Purified recombinant LTBP-2 was used as substrate in cell adhesion and migration studies. We found that, unlike most adherent cell lines, all of the melanoma cell lines tested adhered to LTBP-2 very efficiently and in a concentration-dependent manner. Bowes melanoma cells bound most efficiently to LTBP-2 and were used for further characterization. Cell adhesion assays with recombinant LTBP-2 fragments indicated that the adhesive site is located in an N-terminal region of LTBP-2. The attachment of melanoma cells to LTBP-2 was prevented with monoclonal antibody against β1 integrin in a concentration-dependent manner, suggesting an important role for β1 integrin in the process. Antibodies against integrin subunits α3 and α6 decreased melanoma cell adhesion as well. The β1 and α6 integrins were localized on the cell surface, especially in lamellipodia, as observed by immunofluorescence. In addition to integrin antagonists, heparin also markedly decreased melanoma cell adhesion. LTBP-2 also supported Bowes cell migration in modified Boyden chamber assays in a manner similar to the migration on fibronectin. Current data indicate that LTBP-2 can play a role in melanoma cell adhesion.

Latent TGF-β-binding proteins (LTBPs) are known as binding proteins for small latent forms of transforming growth factor βs (1). Three isoforms of TGF-βs (TGF-β1, -2, and -3) have been cloned from humans, and they belong to a large superfamily of growth-modulating polypeptides (2). Members of this family regulate the growth and differentiation of many cell types and have an important role in numerous developmental processes (3). TGF-βs regulate also the formation and proteolytic degradation of the extracellular matrix (4). Most cells secrete TGF-β in a latent form, noncovalently bound to its propeptide latency-associated peptide (LAP). LAP associates with LTBP, which is needed for secretion, correct folding, and matrix deposition of TGF-β (5). Activation of the growth factor requires the dissociation of mature TGF-β from LAP-LTBP complex (1, 6).

LTBPs belong to the LTBp/fibrillin family of extracellular matrix proteins, which includes LTBPs 1–4 and fibrillins 1 and 2. LTBP-1 and -2 co-localize with extracellular fibrillin microfibrils as well as with fibronectin in cultured cells at certain points of matrix formation (7, 8). LTBP-3 is especially important for bone development (9). LTBP-1 and -3 possess the strongest ability to bind small latent TGF-β as assessed by complex formation assays (10). LTBP-4 is also important for regulating TGF-β1 function, since the LTBP-4 gene-targeted mouse suffers from defects in TGF-β targeting (11). Unlike the other LTBPs, LTBP-2 is unable to associate with the small latent TGF-β (10). Human LTBP-2 is expressed mostly in the lung and to a lesser extent in the liver, skeletal muscle, placenta, and heart (12). LTBP-2 deficiency in the mouse causes early embryonic lethality, indicating the importance of the molecule in development, possibly during implantation (13). LTBP-2 contains the common integrin recognition sequence RGD in its N terminus, suggesting a possible role in cell adhesion. Interactions between fibrillin-1 and integrin αβ3 (14) and also between LAP-TGF-β1 and integrins αβ1, αβ6, and αβ9 have been reported (see Refs. 15–18, respectively).

Integrins are heterodimeric transmembrane receptors consisting of α and β subunits (reviewed in Refs. 19 and 20). They mediate cell-matrix and cell-cell interactions and play important roles in many biological processes such as wound healing, maintenance of tissue integrity, cell growth, and survival. Integrins participate also in various pathological conditions like inflammation and invasion of cancer cells. Integrins are widely distributed on various cell types, and 24 αβ heterodimers are currently known. Their ligands are usually immobilized extracellular matrix proteins, and their intracellular tail is anchored to the actin cytoskeleton through proteins like talin, paxillin, and vinculin (21–23). Integrin receptors lack intrinsic enzymatic activity, but the cytoplasmic domains are the site of accumulation of many signaling molecules such as Src-type kinases and Src substrates (21). In addition, there are proteins that interact with the transmembrane part of an integrin-like adaptor protein, caveolin (24, 25). Through these multiple interactions, integrins mediate signals from the cell surroundings into the cell and vice versa.

The β1 subfamily of integrin receptors includes 12 members, which have partially overlapping ligand specificities (26, 27). Fibronectin, collagens, and laminins are common ligands, but...
Melanoma Cells Adhesion to LTBP-2

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The mouse monoclonal anti-human integrin antibodies against β1 (clone 6G6 and clone P5D2), α5 (clone FB12), α6 (clone 1HE6), α5 (clone ASC-6), α6 (clone P1H4), αv (clone P1D6), αv (MAB1980), and αvβ3 (clone LM090) were purchased from Chemicon International Inc. (Temecula, CA). The goat monoclonal antibody against the human integrin α6 subunit (clone Go63) and anti-paxillin antibody were from BD Transduction Laboratories (Lexington, KY). Mouse monoclonal anti-vinculin antibody was from Sigma. Biotinylated goat anti-mouse immunoglobulins and streptavidin-fluorescein isothiocyanate conjugate were from DAKO D/S (Denmark). Fluorescein isothiocyanate-conjugated anti-mouse was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Laminin (from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma), fibronectin (from human plasma), GRGDTP peptide, and heparin sulfate (from porcine intestinal mucosa) were purchased from Sigma, and heparin was from Leiras (Turku, Finland).

Cells and Cell Culture Conditions—Cell lines used were human embryonic lung fibroblasts (HEL-299; CCL-39, American Type Culture Collection, Manassas, VA) and human melanoma cell lines Bowes (CRL-9607; ATCC); G361 (CRL-1424; ATCC); WM793, WM852, WM163, WM164, and WM239 (WM, Wistar melanoma; isolated from melanoma metastasis, established at Wistar Institute); and Chinese hamster ovary cell clones expressing recombinant LTBP-2 (31) or its fragments. Cells were cultured in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen). Mouse monoclonal anti-vinculin antibody was from Sigma. Biotinylated goat anti-mouse immunoglobulins and streptavidin-fluorescein isothiocyanate conjugate were from DAKO D/S (Denmark). Fluorescein isothiocyanate-conjugated anti-mouse was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Laminin (from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma), fibronectin (from human plasma), GRGDTP peptide, and heparin sulfate (from porcine intestinal mucosa) were purchased from Sigma, and heparin was from Leiras (Turku, Finland).

Production and Purification of Recombinant LTBP-2 and LTBP-2 Fragments—The cloning of the expression construct, production, and purification of full-length LTBP-2 have been described elsewhere (31).

Briefly, conditioned medium from CHO-L2 clone was precipitated with 30% (w/v) (NH4)2SO4 at +4 °C. The precipitate was dissolved in 50 mM Tris-HCl, pH 7.0, after which it was filtered and applied to MonoQ HR (MAB1980), and LTBP-2-containing fractions were then washed twice with PBS and placed in 24-well cell culture plates. Bowes melanoma cells were detached with trypsin-EDTA, and cell suspension (500 105 cells/ml) was used as a control. The cells were suspended in MEM containing 10% heat-inactivated fetal calf serum, and the absorbance was measured at 620 nm.

Immunofluorescence Stainings—Glass coverslips were coated with either LTBP-2 or laminin (10 or 20 µg/ml) at +37 °C for 2 h and then treated with 1% heat-denatured BSA at room temperature for 1 h to prevent nonspecific binding of cells. BSA-coated coverslips were used as negative controls. Bowes melanoma cells were detached with trypsin-EDTA and suspended at 1 × 105 cells/ml in serum-free MEM. The coated coverslips were placed in six-well plates, and 1 × 105 cells were transferred to each well. The cells were then allowed to attach at +37 °C for 2 h, and nonattached cells were removed by washing with PBS, after which the cells were fixed with 3% paraformaldehyde for 15 min. The cells were then treated with 5% BSA for 30 min, washed with PBS, and incubated with monoclonal antibodies against the integrin β3 subunit (P5D2), paxillin, or vinculin at pretested concentrations for 1 h. Unbound proteins were removed by washing with PBS with subsequent incubation either with biotinylated secondary antibodies or with fluorescein isothiocyanate-conjugated anti-mouse antibody. The coverslips were washed again and Streptavidin-fluorescein isothiocyanate was used as fluorescence conjugate when needed. The coverslips were washed and mounted on glass slides using Vectashield (Vector Laboratories, Burlingame, CA). The fluorescent images were obtained using epifluorescent microscope.

Cell Migration Assays—Migration assays were performed using modified Boyden chambers (Falcon cell culture inserts with 8-µm pores). The upper and/or lower sides of the membranes of inserts were coated on either with the indicated concentrations of LTBP-2 or fibronectin at +37 °C for 1 h, washed with PBS, and then treated with 1% heat-inactivated BSA for 30 min to prevent nonspecific binding. The inserts were then washed twice with PBS and placed in 24-well cell culture plates. Bowes melanoma cells were detached with trypsin-EDTA, and cell suspension (500 105 cells/ml) was used as a control. The cells were suspended in MEM containing 10% FCS at a density of 1 × 105 cells/ml. Cell suspension (500 µl) was applied to upper chamber, and 750 µl of MEM with 0.1% FCS was added to lower chamber. The cells were allowed to migrate in 5% CO2 at +37 °C for 6 h. The cells were then fixed and stained with 4% methanol, 10% acetic acid, and 0.1% Coomassie Blue for 5 min and washed with the same fixative without the dye. The cells that had not migrated through the pores were removed from the upper surface of the membrane with cotton swabs. The number of migrated cells was counted from three randomly chosen microscopic fields of each membrane.

RESULTS

Melanoma Cells Attach to LTBP-2—To explore the effects of LTBP-2 on cell adhesion, we produced recombinant LTBP-2 and partially overlapping fragments that covered the whole protein. We tested several cell lines of different origin, such as human lung fibroblasts, endothelial cells, and fibrosarcoma and osteosarcoma cells, to analyze their abilities to bind to purified LTBP-2 immobilized to plastic (data not shown). We found that among these cell lines all of the melanoma cell lines tested were able to adhere to LTBP-2 (Fig. 1A). Human embryonic lung fibroblasts (CCL-137) and fibrosarcoma cells (HT-1080) are shown as examples of cells that cannot adhere to LTBP-2.

Most efficient adhesion to LTBP-2 was detected using MCF-7 cells. The cells were adherent to LTBP-2 at 1 h, and the cell number increased with time. The number of migrated cells was counted from three randomly chosen microscopic fields of each membrane.
Cell adhesion assays were carried out in 96-well tissue culture plates. The wells were coated with 10 μg/ml LTBP-2, and nonspecific binding was prevented by BSA treatment. 3 × 10^4 cells were seeded per well, the cells were allowed to attach for 1 h, and nonbound cells were removed by washing. The adhered cells were stained with 0.1% Coomassie Blue and lysed in 1% SDS. The amount of stain associated with adherent cells correlates with cell number and was measured by a spectrophotometer. The A<sub>620</sub> values represent the mean value of three wells. The binding to BSA has been subtracted. A, all the melanoma cell lines attached to LTBP-2, whereas the binding of control CCL-137 fibroblasts and HT-1080 fibrosarcoma cells was negligible. Bowes cells adhered to LTBP-2 most efficiently. B, binding of Bowes cells is dependent on the amount of substratum bound LTBP-2. Wells were coated with increasing concentrations of LTBP-2. Bowes melanoma cells adhered to LTBP-2 in a concentration-dependent manner. C, Bowes cell adhesion to LTBP-2 is associated with cell spreading. Glass coverslips were coated with LTBP-2 and treated with 1% heat-inactivated BSA. Control coverslips were treated with BSA only. Bowes cells were seeded on coverslips and permitted to adhere for 90 min, after which the cells were fixed with 3% paraformaldehyde and subsequently permeabilized and treated with phalloidin-TRITC, which stains the actin cytoskeleton. Cells adhered to LTBP-2 spread and expressed organized actin cytoskeletons, especially extended filopodia, in contrast to cells attached to BSA, which were rounded. The number of bound cells was lower on BSA-coated coverslips.
mostly seen at the cell cortex, which is typical for these cells. Cells seeded on LTBP-2 formed also extensive filopodia, in contrast to cells adhered to BSA, which appeared more round and less spread.

**Antibodies to β1 Integrin Totally Prevent Bowes Cell Adhesion to LTBP-2**—Since LTBP-2 contains an RGD sequence in its N terminus, we assumed that the interaction of Bowes melanoma cells with LTBP-2 could be mediated by one or more integrin receptors. We took the approach of inhibiting cell adhesion by a number of monoclonal anti-integrin antibodies. Cells were incubated with function-blocking antibodies for 20 min at room temperature before seeding onto LTBP-2-coated wells. Among the antibodies, the anti-β1 antibody 6S6 completely prevented melanoma cell adhesion to LTBP-2 (Fig. 2A). The inhibitory effect was clearly concentration-dependent (Fig. 2C). The antibody against the integrin α3 subunit decreased cell adhesion by 50%, whereas mAbs recognizing the α subunits 1, 2, 4, 5, and V did not have any effect on Bowes cell adhesion to LTBP-2 (Fig. 2A). mAb against the integrin α6 subunit had a small decreasing effect on the adhesion and enhanced the inhibitory effect of the anti-α3 antibody when used in combination (Fig. 2B). Together, the antibodies against α3 and α6 integrins decreased Bowes cell adhesion to LTBP-2 almost by 80%, which was comparable with their effect on Bowes cell adhesion to laminin. However, when used individually, both anti-α3 and anti-α6 antibodies were more effective in preventing Bowes cell adhesion to laminin, as could be expected, since α3β1 and α6β1 are known as laminin receptors (summarized in Ref. 33). The effect of these antibodies on Bowes cell adhesion to collagen I was negligible. Since antibod-
ies against integrin α3 and α6 subunits did not totally prevent Bowes cell adhesion to LTBP-2, some other interactions are evidently involved in the adhesion.

αvβ3 integrin has been shown to be an important integrin receptor in melanoma cell adhesion and invasion in various studies (28, 29, 34). Although the antibody against αv subunit did not have any effect on the cell membrane, in control cells plated on laminin, the β3 integrin was seen in lamellipodia and also inside the cell. The staining in cells attached to BSA was more diffuse. The α3 integrin staining resembled that of β1. Cells bound to LTBP-2 were able to form focal contacts, which is demonstrated by paxillin and vinculin stainings (two bottom panels). They are localized just beneath the cell membrane both in cells adhered to LTBP-2 and laminin. Marginal staining was observed also in cells attached to BSA.

**Fig. 3.** Bowes cells on LTBP-2 substratum express β1 and α3 integrins on the cell membrane and form focal contacts. The involvement of the β1 integrin was verified with immunofluorescence staining. Bowes cells were seeded on LTBP-2-or laminin-coated (10 µg/ml) coverslips and fixed after the cells were attached. The cells were then stained with anti-integrin β1 antibody and subsequently with green fluorescent conjugate. In cells attached to LTBP-2, the staining was located mostly on cell edges, especially in lamellipodia. In control cells plated on laminin, the β3 integrin was seen in lamellipodia and also inside the cell. The staining in cells attached to BSA was more diffuse. The α3 integrin staining resembles that of β1. Cells bound to LTBP-2 were able to form focal contacts, which is demonstrated by paxillin and vinculin stainings (two bottom panels). They are localized just beneath the cell membrane both in cells adhered to LTBP-2 and laminin, which indicates that cells are firmly attached and form focal contacts.

**Melanoma Cell Adhesion to LTBP-2 Is Not Dependent on RGD Sequence**—Since the αvβ3 integrin is known to recognize the RGD sequence in some ligands (36, 37), and the LTBP-2 molecule also contains one at its N terminus, we tested whether an RGD peptide could interfere with Bowes melanoma cell adhesion to LTBP-2. We carried out immunofluorescence stainings with antibodies against paxillin and vinculin after Bowes cells were allowed to attach to LTBP-2-coated coverslips. Both paxillin and vinculin were localized to the cell periphery in dot- or plaque-like structures in cells adhered to LTBP-2 or laminin, which indicates that cells are firmly attached and form focal contacts.

**Cells Seeded on LTBP-2 Express the β3 and α3 Integrins on Their Surface and Form Focal Adhesions**—The involvement of the β3 integrin in melanoma cell attachment to LTBP-2 was further verified by immunofluorescence. Cells were allowed to adhere to LTBP-2- or laminin-coated (20 µg/ml) coverslips for 2 h before fixing and staining. The fluorescence signal was localized mostly at the cell membrane, especially in the lamellipodia (Fig. 3, uppermost panel, on the left). Some perinuclear staining was observed as well. In control cells seeded on laminin, β3 integrin was seen in lamellipodia and also inside the cell (Fig. 3). Marginal, more diffuse staining was seen in cells attached to BSA. The α3 integrin staining was similar to β3 localizing mainly to the cell cortex in Bowes cells seeded on either LTBP-2 or laminin (Fig. 3). Both integrins were seen in plaque-like structures on the cell membrane or just beneath it, indicating receptor clustering after ligand recognition.

Connection to actin cytoskeleton is important for integrin functions. The connection is formed through many adaptor proteins (e.g. vinculin and talin at the ECM contact sites called focal contacts or focal adhesions) (35). We explored next whether Bowes cells form this kind of structure when attached to LTBP-2. We carried out immunofluorescence stainings with antibodies against integrin subunits and the RGD peptide. Cells were allowed to attach to LTBP-2-coated coverslips. Both paxillin and vinculin were localized to the cell periphery in dot- or plaque-like structures in cells adhered to LTBP-2 or laminin, which indicates that cells are firmly attached and form focal contacts.
rin was similar to the inhibition of cell adhesion to fibronectin (data not shown). Total inhibition, comparable with adhesion to BSA, was achieved at a concentration of 800 IU/ml. We explored also the effect of heparan sulfate in the same assay but did not observe any effect on Bowes cell adhesion to LTBP-2 (data not shown). This may implicate the involvement of very specialized form of cell surface heparan sulfate proteoglycan.

Bowes Cell Adhesion to LTBP-2 Is Decreased by Polyclonal Antibody against Recombinant LTBP-2 but Only Slightly by the Soluble Form of the Protein—To confirm that the adherence of Bowes cells to LTBP-2 is not caused by some impurity in the protein preparation, we tested the effect of polyclonal antibody, abL22 (affinity-purified IgG), generated against recombinant LTBP-2, in the adhesion assay. Before seeding the cells into LTBP-2-coated wells, the wells were incubated with the antibody at room temperature for 20 min. After that, the adhesion assay was carried out as described under “Experimental Procedures.” Treatment of the LTBP-2-coated wells with abL22 decreased the melanoma cell adhesion by 40% (Fig. 5). We have observed that abL22 does not recognize the N-terminal epitopes of LTBP-2 as well as the C-terminal ones, which may explain the inhibitory effect being only partial.2

We analyzed next whether soluble LTBP-2 could compete with melanoma cell adhesion to an immobilized form of the protein. To test this, Bowes cells were incubated before plating with soluble LTBP-2 (5 μg/ml) at room temperature for 20 min.

2 M. Hyytiäinen, P. Vehviläinen, and J. Keski-Oja, unpublished data.
N-terminal Fragment of LTBP-2 Is the Domain Responsible for Bowes Cell Adhesion—We took the recombinant fragment approach to identify the adhesive site in the LTBP-2 protein. To investigate this, we produced several partially overlapping LTBP-2 fragments as Ig fusion proteins covering the whole protein and used the purified fragments in adhesion assays (Fig. 6A). As expected, Bowes cells did not adhere to any of the fragments as efficiently as to full-length LTBP-2. The N-terminal fragments L2-III, L2-V, and L2-VII were the most potent in mediating cell attachment. These fragments contain a common proline-rich region. Of these fragments, L2-III contains the RGD sequence, but the shorter fragment L2-V, lacking this RGD, was slightly more potent in mediating Bowes cell adhesion. Fragment L2-II containing the RGD sequence was ineffective, confirming that Bowes cell adhesion to LTBP-2 is independent of the RGD sequence. Some binding was observed to fragment L2-IX, which contains another proline-rich region, and to the plg control protein. On the other hand, cells did not adhere to purified mouse IgG.

LTBP-2 Supports Migration of Bowes Melanoma Cells—We explored next whether LTBP-2 supports melanoma cell migration in addition to adhesion. The migration assay measures the ability of cells to migrate through a porous membrane, which can be coated with the test protein either on one side or both. We used purified LTBP-2 for coating and made control experiments with fibronectin, since fibronectin is haptotactic for a variety of cell lines. In the experiment where the LTBP-2 was coated only on the upper surface of the membrane of the cell culture insert, the cells migrated very poorly (Fig. 7). When LTBP-2 was coated on the lower surface of the membrane, the cells migrated toward it. Coating of both sides with LTBP-2 further increased cell migration, the pattern being very similar to Bowes cell migration on fibronectin. These data suggest that LTBP-2 can support melanoma cell migration and that LTBP-2 is sufficient for induction of cell migration by itself in the absence of other attractants.

DISCUSSION

LTBP-2s were originally identified as binding proteins for small latent TGF-β complexes, and they augment the secretion
and correct folding of TGF-β as well as their deposition into the extracellular matrix (5, 40, 41). However, LTBP-2s are often secreted in large excess to TGF-β, suggesting additional, possibly structural roles for them in ECM. LTBP-2s share a similar domain structure with fibrillin, which are integral components of ECM microfibrils (1). Mutations in fibrillin-1 and -2 can lead to severe connective tissue disorders, like the Marfan syndrome and congenital contractual arachnodactyly, respectively (42). LTBP-1 from fibroblast cultures is deposited to fibrils in the vicinity of the cell surface (7) (see also Ref. 43). The role of LTBP-2s in these structures has been unclear so far. The early lethality of LTBP-2 null mice suggests a crucial role for LTBP-2s as components of ECM (13). None of the three TGF-βs is co-expressed with LTBP-2 in mouse testis, which further indicates a role for LTBP-2 independent of TGF-β.

Our earlier observation was that LTBP-2 is unable to bind small latent TGF-β (10). Since the LTBP-2 null mice die during the time of implantation, we hypothesized that LTBP-2 could have a cell adhesion-mediating or -modulating function in the ECM. The current study was carried out to explore the effects of LTBP-2 on cell adhesion. We tested several adherent cell lines, both normal diploid and transformed cells, and found that only melanoma cells were able to adhere to immobilized recombinant LTBP-2. We have, in fact, observed that LTBP-2, and especially its C-terminal fragment, L2-V, which mediates melanoma cell adhesion, acts as an adhesion molecule for at least fibroblastic cells, leading to decreased actin stress fiber formation.3 Melanoma cell adhesion to LTBP-2 was accompanied by actin stress fiber formation as well as focal contact formation in a manner similar to cells adhering to laminin. This indicates that LTBP-2 acts as a real adhesion-mediating protein, not just as a passive binding partner.

Integrins are the most common cell surface receptors for extracellular matrix proteins. They are found in a number of species from sponges to vertebrates. In fibrillin-1, fibroblasts adhere to its RGD site via αvβ3 integrin, but in addition, some cell types recognize another, non-RGD binding site within the fibrillin molecule (14). The nature of the second adhesion site is not known. Integrins αv, αv, and β3 are involved in human skin fibroblast and murine L-cell adhesion to fibrillin-2 (44). The motif mediating the adhesion was localized to the 12th module of the fibrillin-D segment, which contains an RGD sequence, and the adhesion could be prevented by RGD peptides. Although RGD sequence is present in LTBP-2, RGD peptides could not compete melanoma cell adhesion to LTBP-2. In addition, melanoma cells did not bind to the smallest RGD-containing fragment but bond to an adjacent short fragment, L2-V. β3 integrin family contains both kind of receptors, namely RGD-binding and RGD-independent (45). Integrin αvβ3 recognizes RGD sequence in some ligands, but it also recognizes several ligands lacking a functional RGD sequence (36). For example, the RGD sites in laminins are often cryptic and inaccessible to integrin receptor. Our findings suggest that melanoma cells adhere to LTBP-2 in an RGD-independent manner.

Cell adhesion to ECM via integrins regulates cell behavior and fate in many ways being crucial for tissue morphogenesis and in guiding cell migration during embryonic development. Melanoma cell adhesion via αvβ3 integrin is widely characterized and appears to be important in melanoma metastasis (30). In our studies, melanoma cells did not adhere to LTBP-2 by αvβ3 integrin. Inhibition assays indicated that adhesion to LTBP-2 was mediated by β3 integrin, the αv subunit being the most obvious partner. The mAb against the αv subunit had an additive inhibitory effect on melanoma cell adhesion. However, cell adhesion to LTBP-2 does not depend only on the expression of αvβ3 or αvβ3 integrins, since many of the cell lines we have tested (e.g., HT-1080 fibrosarcoma cells) express either one or both of these receptors (46, 47) and still could not bind to LTBP-2 (Fig. 1A). The ligand binding specificities of integrins are known to vary from cell type to another, and various other cell surface proteins, like urokinase-type plasminogen activator receptor, thrombospondin, or tetraspanins, modulate the integrin functions (48, 49). Thus, this kind of regulation of integrin activity may explain why melanoma cells but none of the other tested cells adhered to LTBP-2. The αvβ3 integrin has a role in melanoma cell migration toward laminin, fibronectin, and collagen IV and in invasion through Matrigel (50), and its expression is increased in some metastatic melanomas (51). Our results indicate that melanoma cell adhesion to LTBP-2 could be mediated at least partially by the αvβ3 integrin receptor.

In our studies, heparin markedly decreased Bowes melanoma cell adhesion to LTBP-2. This could implicate that cell surface proteoglycans play a role in the adhesion. We were not able to inhibit the adhesion by competing with heparan sulfate, which could imply the involvement of very specialized form of heparan sulfate proteoglycan, since they are very heterogeneous. Melanoma cells express cell surface chondroitin sulfate proteoglycans that modulate tumor cell adhesion and motility (reviewed in Ref. 52). Chondroitin sulfate proteoglycans bind the αvβ3 integrin and are needed for αvβ3-mediated melanoma cell adhesion (53). Involvement of chondroitin sulfate proteoglycans in melanoma cell adhesion to LTBP-2 has not been ruled out here.

Soluble LTBP-2 did not efficiently prevent Bowes cell adhesion to the substratum-bound form of the protein. This suggests that in solution LTBP-2 adopts a conformation in which cell adhesion sites are masked and not accessible to cell surface receptors. When coated on a solid surface, the conformation is obviously changed, exposing a plausible cryptic site in LTBP-2, and may act more like in extracellular matrix in vivo.

The polyclonal antibody generated against the full-length LTBP-2, AbL22, inhibited only partially Bowes cell adhesion to

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3 M. Hyytiäinen and J. Keski-Oja, unpublished observations.
LTBP-2. This may be due to possible differences between the protein conformations in solution and in immobilized form, since these antibodies have been generated against the soluble form. Melanoma cells adhered to some extent to an N-terminal fragment of LTBP-2, and the AbL22 does not recognize the N-terminal fragments as well as the C-terminal ones. This may explain the relatively inefficient inhibitory effect of AbL22 in the adhesion assays.

LTBP-2 deficiency in the mouse leads to embryonic lethality during the time of implantation (13), which raises a question of whether cell adhesion to LTBP-2 would play a role during development. Our findings indicate that LTBP-2 can mediate melanoma cell adhesion in an integrin-dependent manner. Bowes cells were also able to migrate on LTBP-2. In general, melanoma cell adhesion in an integrin-dependent manner.

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