THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 27, Issue of July 4, pp. 24705–24713, 2003
Printed in U.S.A.

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

Received for publication, December 19, 2002, and in revised form, April 2, 2003
Published, JBC Papers in Press, April 25, 2003, DOI 10.1074/jbc.M212953200

Piia Vehviläinen, Marko Hyytiäinen, and Jorma Keski-Oja‡

From the Departments of Pathology and Virology, Haartman Institute, University of Helsinki, and Helsinki University Hospital, FIN-00014 Helsinki, Finland

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 substratum 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 β_1 integrin in a concentration-dependent manner, suggesting an important role for β_1 integrin in the process. Antibodies against integrin subunits α_3 and α_6 decreased melanoma cell adhesion as well. The β_1 and α_3 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)[1] 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 $\alpha_V\beta_3$ (14) and also between LAP-TGF-β1 and integrins $\alpha_V\beta_1$, $\alpha_V\beta_6$, and $\alpha_8\beta_1$ 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 β_1 subfamily of integrin receptors includes 12 members, which have partially overlapping ligand specificities (26, 27). Fibronectin, collagens, and laminins are common ligands, but

* This work was supported by the Academy of Finland, the Sigrid Juselius Foundation, Biocentrum Helsinki, the Helsinki University Hospital Fund, the Novo Nordisk Foundation, the Finnish Cancer Foundation, TEKES, and the University of Helsinki. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Department of Pathology, P.O. Box 63, Biomedicum Helsinki, University of Helsinki, FIN-00014 Helsinki, Finland. Tel.: 358-9-191-25566; Fax: 358-9-191-25573; E-mail: Jorma.Keski-Oja@Helsinki.fi.

[1] The abbreviations used are: LTBP, latent TGF-β-binding protein; TGF, transforming growth factor; ECM, extracellular matrix; LAP, latency-associated peptide; BSA, bovine serum albumin; MEM, minimal essential medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; mAb, monoclonal antibody; TRITC, tetramethylrhodamine isothiocyanate.

there are also receptors for tenascin and VCAM-1 in this family. Melanoma cells are known to express a wide variety of integrin receptors, of which the function of the $\alpha_V\beta_3$ is best characterized in the context of malignant melanoma (28–30).

In the current work, we have analyzed the ability of LTBP-2 to act as an adhesion protein for different cultured cells. In general, LTBP-2 did not mediate cell adhesion but acted as an adhesion protein for all melanoma cell lines tested. This feature of LTBP-2 is new and provides new views to the regulation of melanoma cell adhesion and spreading.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The mouse monoclonal anti-human integrin antibodies against β_1 (clone 6S6 and clone P5D2), α_1 (clone FB12), α_2 (clone 1IE6), α_3 (clone ASC-6), α_4 (clone P1H4), α_5 (clone P1D6), α_V (MAB1980), and $\alpha_V\beta_3$ (clone LM609) were purchased from Chemicon International Inc. (Temecula, CA). The goat monoclonal antibody against the human integrin α_6 subunit (clone GoH3) 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 heparan 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-137, American Type Culture Collection, Manassas, VA) and human melanoma cell lines Bowes (CRL-9607; ATCC); G361 (CRL-1424; ATTC); 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), 100 IU/ml penicillin, and 50 μg/ml streptomycin. Stably transfected LTBP-2 expressing CHO-L2 clone was grown in MEM containing 0.2% bovine serum albumin, supplemented with FCS and antibiotics as above and 0.4 mg/ml neomycin, G418 (Invitrogen).

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) $(NH_4)_2SO_4$ 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 5/5 column (Amersham Biosciences). The bound proteins were eluted with a 210–700 mM NaCl gradient. LTBP-2-containing fractions were combined, and 4 M urea was added to achieve the final concentration of 2 M. The sample was applied to a MonoQ 5/5 column equilibrated in 50 mM Tris-HCl, pH 7.0, containing 2 M urea. Proteins were eluted with a 0–1 M NaCl gradient. The fractions containing LTBP-2 were fractionated on a Superdex 200 gel filtration column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 2 M urea. The buffer of LTBP-2 fractions was then changed to phosphate-buffered saline (PBS) with Fast Desalting column HR 10/10 (Amersham Biosciences). Expression and purification of LTBP-2 fragments as IgG Fc-tail fusion proteins was carried out essentially as described for LTBP-1 (32).

Cell Adhesion Assays—Plates of 96 wells were coated with the indicated proteins diluted in PBS at +37 °C for 2 h. The residual protein binding sites were saturated with 1% heat-denaturated bovine serum albumin (BSA) in PBS (0.14 M NaCl, 10 mM sodium phosphate buffer, pH 7.4) at room temperature for at least 30 min. The wells were washed with PBS before the addition of cells. Semiconfluent cultures of cells were detached with trypsin-EDTA and suspended at a density of 6.0×10^5 cells/ml in serum-free minimal essential medium. Cell suspension (50 μl) was then transferred to each well. In inhibition studies, the cells were incubated with antibodies or other agents of interest for 15 min before transfer to the wells. The antibodies were used at concentrations up to 25 μg/ml, the RGD peptide was used at 100–500 μM, and heparin was used at up to 800 IU/ml. Each experimental condition was tested in triplicate. The cells were permitted to attach at +37 °C for 1 h, after which the wells were washed with PBS to remove nonattached cells. Cells were then fixed and stained simultaneously with 0.1% Coomassie Blue in 10% acetic acid and 40% methanol and washed with the same fixative without the dye. Subsequently, the cells were lysed in 100 μl of 1% SDS in PBS, 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-denaturated 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 1×10^5 cells/ml in serum-free MEM. The coated coverslips were placed in six-well plates, and 1×10^5 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. Permeabilization of cells was performed with 0.5% Nonidet P-40 for 5 min. The cells were then treated with 5% BSA for 30 min, washed with PBS, and incubated with monoclonal antibodies against the integrin β_1 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.

The α_3 integrin staining was performed as described above with one exception; the primary antibody, mAb ASC-6, was permitted to bind to living cells on ice for 20 min before fixation. The following steps were carried out as for other stainings.

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 the cell number was determined by an optical electronic counter. The cells were suspended in MEM containing 0.1% FCS at a density of 1×10^5 cells/ml. Cell suspension (500 μl) was applied to upper chambers, and 750 μl of MEM with 0.1% FCS was added to lower chambers. The cells were allowed to migrate in 5% CO_2 at +37 °C for 6 h. The cells were then fixed and stained with 40% 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 microscope 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. 1*A*). 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 Bowes cells; therefore, we selected this melanoma cell line for further characterization. The adhesion of Bowes cells to LTBP-2 was dependent on the protein concentration used in coating (Fig. 1*B*).

To test the effects of LTBP-2 on cell spreading, we coated glass coverslips with 10 μg/ml LTBP-2. Coverslips coated with 1% denatured BSA were used as controls. Cells were seeded on coated coverslips and allowed to adhere for 90 min, after which they were fixed. The cell morphology was visualized by staining the actin cytoskeleton with phalloidin-TRITC. By microscopic analysis, we found that Bowes cell adhesion to LTBP-2 was associated with cell spreading (Fig. 1*C*). Actin staining was

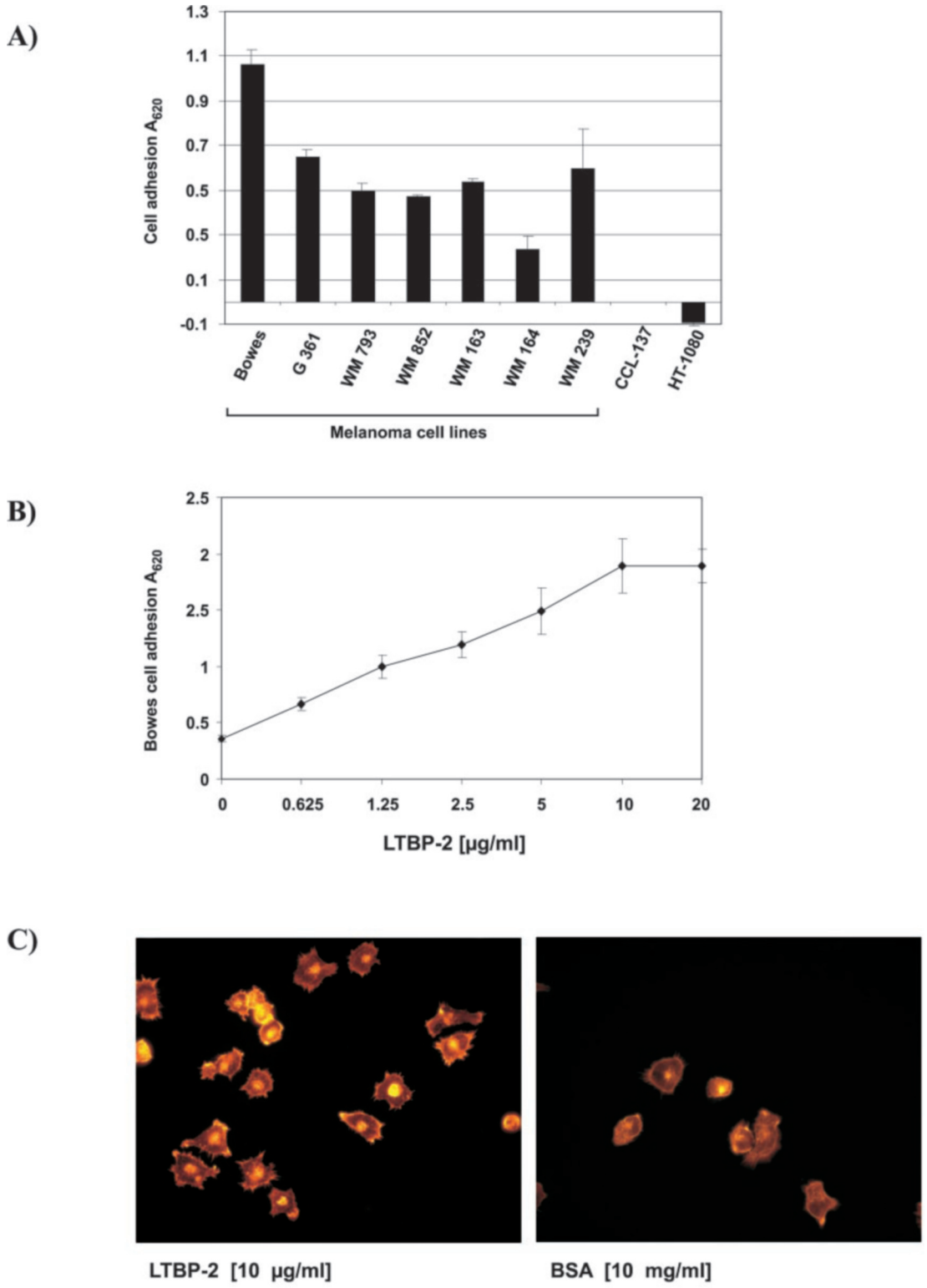

FIG. 1. **Adhesion of melanoma cells to LTBP-2.** Cell adhesion assays were carried out in 96-well tissue culture plates. The wells were coated with 10 μg/ml LTBP-2, and nonspecific binding was prevented by BSA treatment. 3×10^4 cells were seeded per well, the cells were allowed to attach for 1 h, and 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_{620} 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.

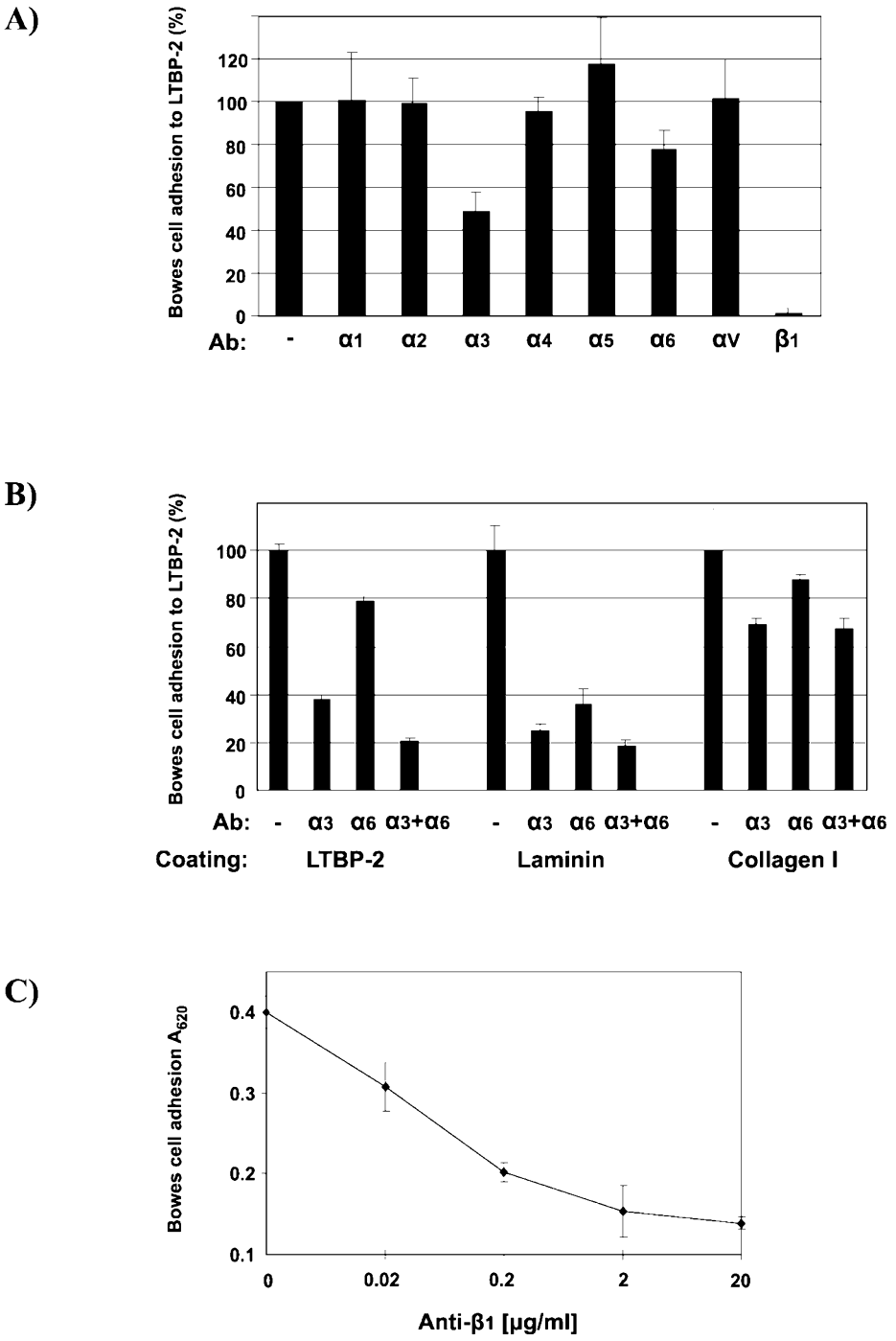

FIG. 2. **Effects of anti-integrin antibodies on Bowes cell adhesion to LTBP-2.** In inhibition assays, the cells were incubated with antibodies (20 μg/ml unless indicated otherwise) for 15 min before seeding onto LTBP-2-coated wells. *A*, monoclonal anti-integrin β_1 antibody 6S6 totally prevented the adhesion of Bowes melanoma cells to LTBP-2. Antibody against integrin α_3 subunit decreased the adhesion, whereas the other integrin antibodies did not have significant effects. The adhesion of Bowes melanoma cells to LTBP-2 is marked as 100%. Adhesion to BSA has been subtracted. *B*, when anti-α_6 mAb was used together with anti-α_3 mAb, it enhanced the inhibitory effect of the α_3 mAb on Bowes cells plated on LTBP-2. Both the anti-α_6 and the anti-α_3 antibodies markedly decreased Bowes cell binding to laminin, but they did not markedly prevent adhesion to collagen I. *C*, the inhibitory antibody against β_1 integrin prevented Bowes cell adhesion to LTBP-2 in a concentration-dependent manner.

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 β_1 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 96-well plates. Among the antibodies, the anti-β_1 antibody 6S6 completely prevented melanoma cell adhesion to LTBP-2 (Fig. 2*A*). The inhibitory effect was clearly concentration-dependent (Fig. 2*C*). The antibody against the integrin α_3 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. 2*A*). mAb against the integrin α_6 subunit had a small decreasing effect on the adhesion and enhanced the inhibitory effect of the anti-α_3 antibody when used in combination (Fig. 2*B*). Together, the antibodies against α_3 and α_6 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-α_3 and anti-α_6 antibodies were more effective in preventing Bowes cell adhesion to laminin, as could be expected, since $\alpha_3\beta_1$ and $\alpha_6\beta_1$ 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-

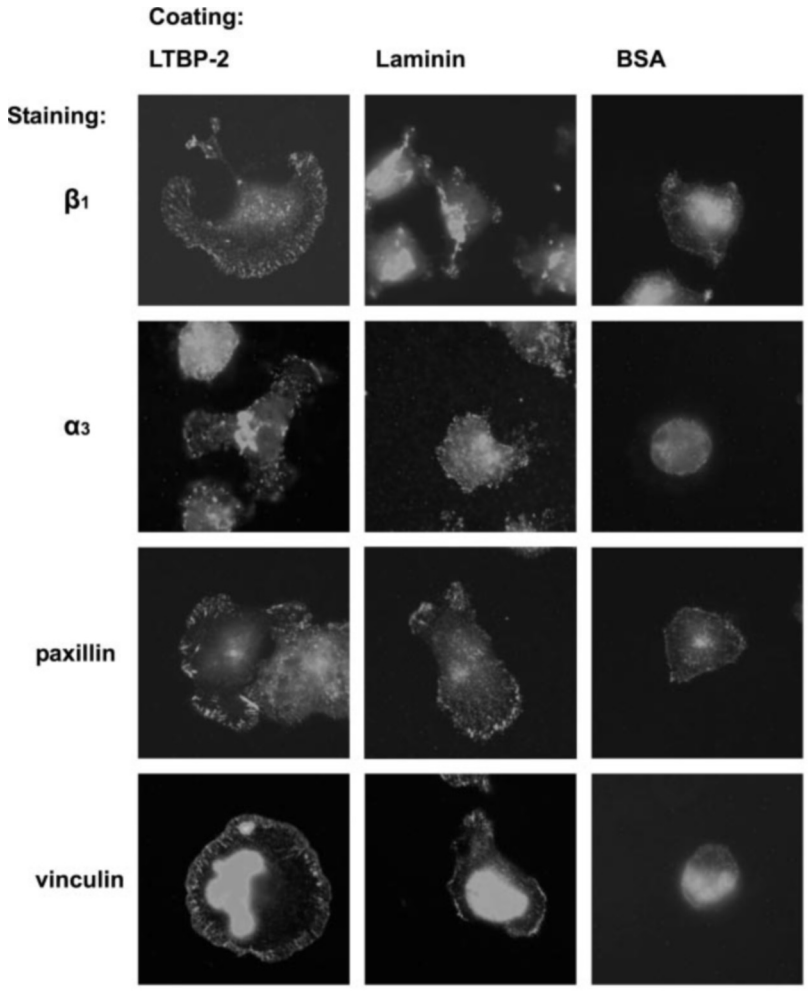

FIG. 3. **Bowes cells on LTBP-2 substratum express β_1 and α_3 integrins on the cell membrane and form focal contacts.** The involvement of the β_1 integrin was verified with immunofluorescence staining. Bowes cells were seeded on LTBP-2- or laminin-coated (10 μg/ml) coverslips and fixed after the cells were attached. The cells were then stained with anti-integrin β_1 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 β_1 integrin was seen in lamellipodia and also inside the cell. The staining in cells attached to BSA was more diffuse. The α_3 integrin staining resembled that of β_1. 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.

ies against integrin α_3 and α_6 subunits did not totally prevent Bowes cell adhesion to LTBP-2, some other interactions are evidently involved in the adhesion.

$\alpha_V\beta_3$ 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 α_V subunit did not have any effect on melanoma cell adhesion to LTBP-2, we tested monoclonal antibodies against the β_3 subunit and an antibody against the entire $\alpha_V\beta_3$ in inhibition assays. We could not observe any effect on cell adhesion (data not shown), confirming that $\alpha_V\beta_3$ integrin is not involved in adhesion to LTBP-2.

Cells Seeded on LTBP-2 Express the β_1 and α_3 Integrins on Their Surface and Form Focal Adhesions—The involvement of the β_1 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, β_1 integrin was seen in lamellipodia and also inside the cell (Fig. 3). Marginal, more diffuse staining was seen in cells attached to BSA. The α_3 integrin staining was similar to β_1 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 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.

Melanoma Cell Attachment to LTBP-2 Is Not Dependent on RGD Sequence—Since the $\alpha_3\beta_1$ 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. Cells were allowed to attach to LTBP-2-coated wells in the presence of RGD peptide. In our experiments, the RGD peptide decreased Bowes cell adhesion to LTBP-2 by 40% at a concentration of 500 μM. Lower concentrations did not have any effect (data not shown). To control the function of the RGD peptide, we tested its ability to inhibit Bowes cell adhesion to fibronectin and vitronectin. The peptide totally prevented both at a concentration of 500 μM (Fig. 4*A*).

Cell surface proteoglycans are important modulators of cell adhesion, and heparan sulfate proteoglycans cooperate with integrins in many cell-matrix interactions (38). This led us to examine whether Bowes cell adhesion to LTBP-2 would be affected by treating cells with heparin, a wide range inhibitor of cell adhesion (38), before seeding cells onto LTBP-2-coated wells. Heparin inhibited Bowes cell adhesion to LTBP-2 in a concentration-dependent manner (Fig. 4*B*). The effect of hepa-

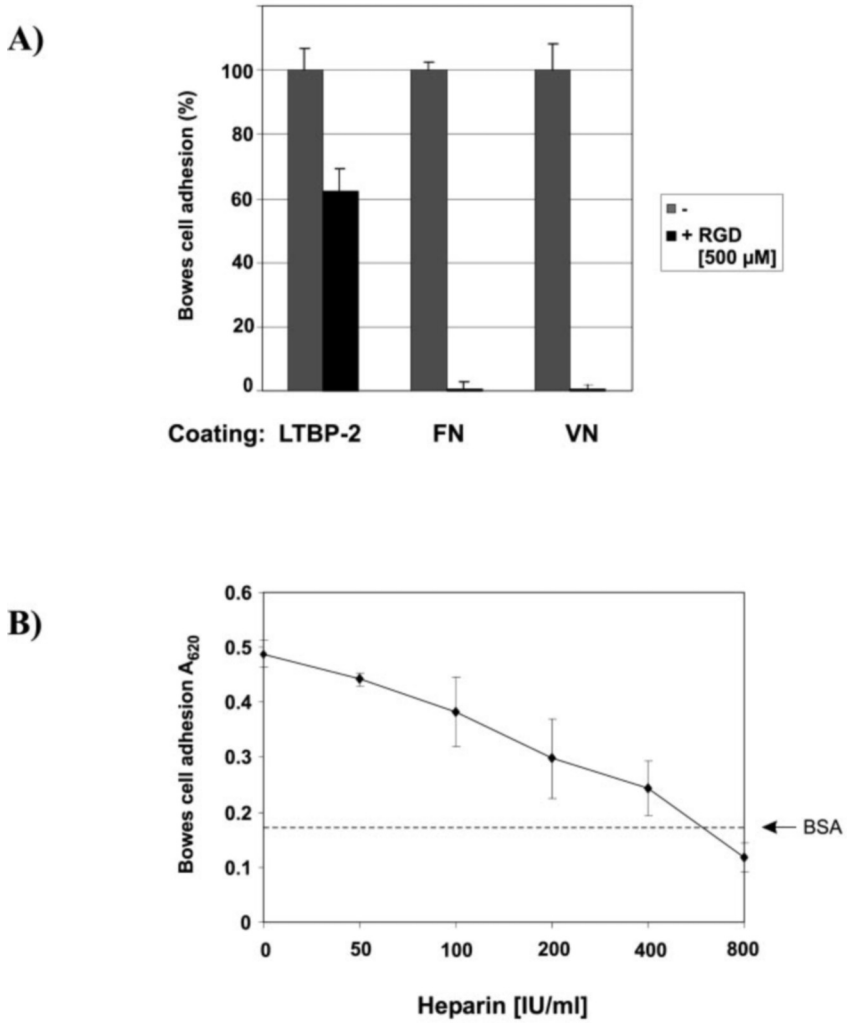

FIG. 4. **Inhibition of Bowes cell adhesion to LTBP-2 by RGD peptide and heparin.** Bowes cells were incubated with the indicated concentrations of RGD peptide or heparin for 10 min before seeding into protein coated wells. *A*, RGD peptide inhibited the adhesion of Bowes cells to LTBP-2 by ~ 40% at concentration of 500 μM. The same RGD concentration totally prevented Bowes cell adhesion to both fibronectin and vitronectin. The adhesion of Bowes melanoma cells to LTBP-2, fibronectin and vitronectin in the absence of any inhibitors is marked as 100%. *B*, heparin inhibited Bowes cell adhesion to LTBP-2 in a concentration-dependent manner and totally prevented it at a concentration of 800 IU/ml. Cell adhesion to BSA is marked with *dashed line*.

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.

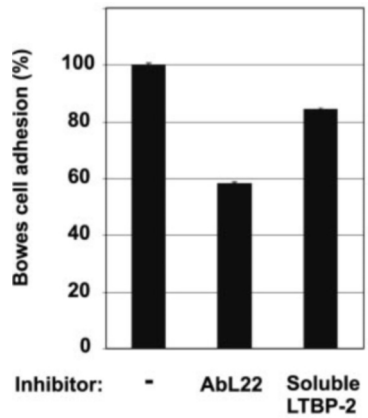

FIG. 5. **Bowes cell adhesion to LTBP-2 is decreased by polyclonal antibody against LTBP-2 but not with the soluble form of LTBP-2.** 96-Well tissue culture plates were coated with LTBP-2 and treated with 1% BSA. Before seeding the cells, the wells were incubated with 100 μg/ml of polyclonal IgG antibodies against recombinant LTBP-2 (AbL22) for 10 min. Soluble LTBP-2 (5 μg/ml) was incubated with cells for 10 min before plating. The treatment of wells with AbL22 decreased Bowes cell binding by >40%, whereas the effect of soluble LTBP-2 was only an ~15% decrease.

We observed that soluble LTBP-2 decreased melanoma cell adhesion by ~15%, suggesting that cells cannot efficiently bind soluble LTBP-2 and that the adhesive properties of LTBP-2 are dependent on its deposition to substratum, namely tissue culture plate in this case.

[2] M. Hyytiäinen, P. Vehviläinen, and J. Keski-Oja, unpublished data.

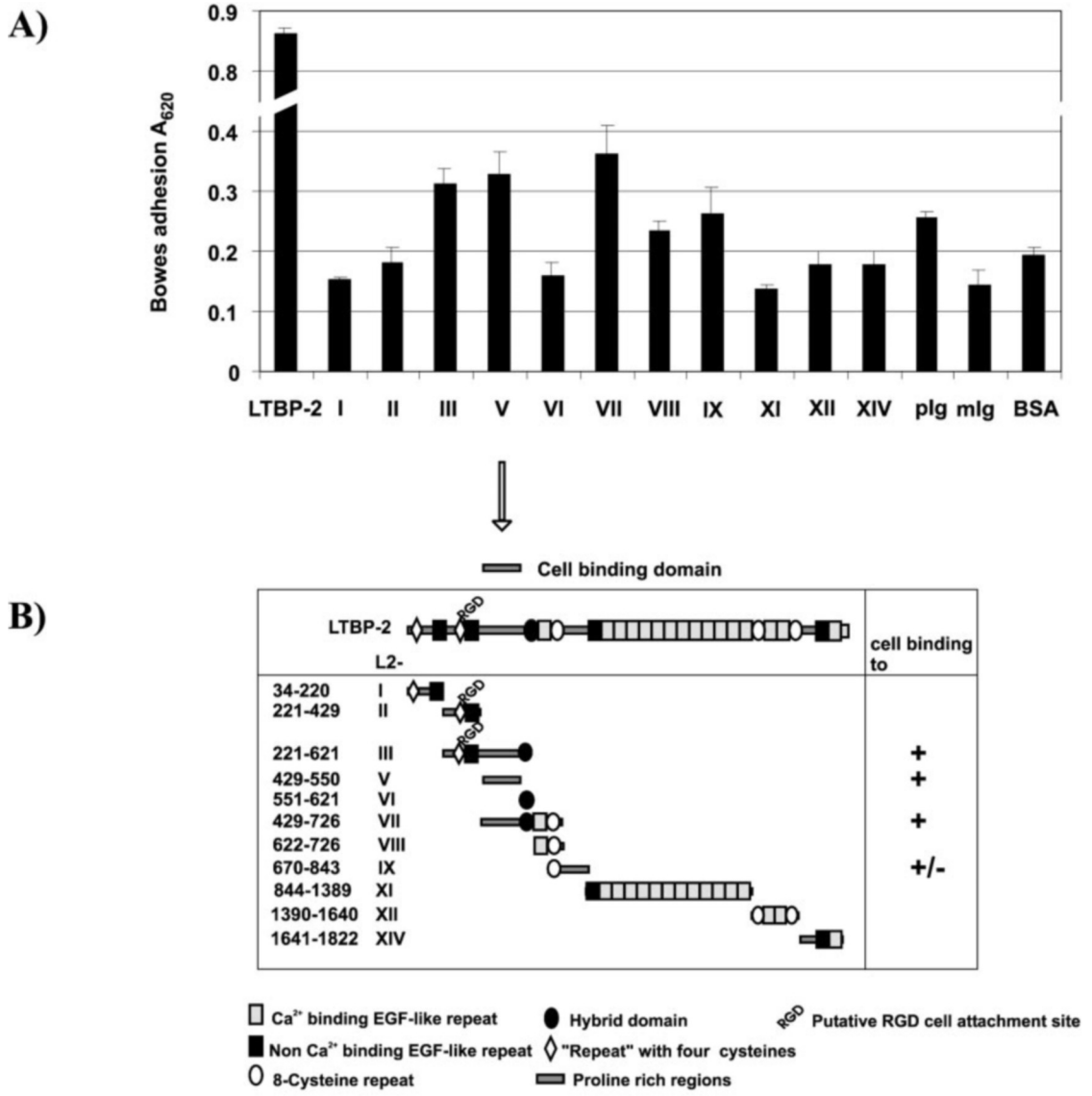

FIG. 6. **N-terminal proline-rich fragment of LTBP-2 is responsible for Bowes cell adhesion.** *A*, Bowes cell binding to LTBP-2 fragments. The fragments were produced as Ig fusion proteins and were used for coating 96-well tissue culture plates at a concentration of 20 μmol/ml. A cell adhesion assay was carried out for 3 h instead of 1 h, which was sufficient with the full-length protein. The cells attached to the N-terminal fragments L2-III, L2-V, and L2-VII, fragment V being the shortest one to mediate adhesion. This fragment does not contain the RGD sequence. *B*, schematic illustration of LTBP-2 and Ig fusion fragments.

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. 6*B*). 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 (Fig. 6*A*). 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 pIg 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 (39) 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

LTBPs were originally identified as binding proteins for small latent TGF-β complexes, and they augment the secretion

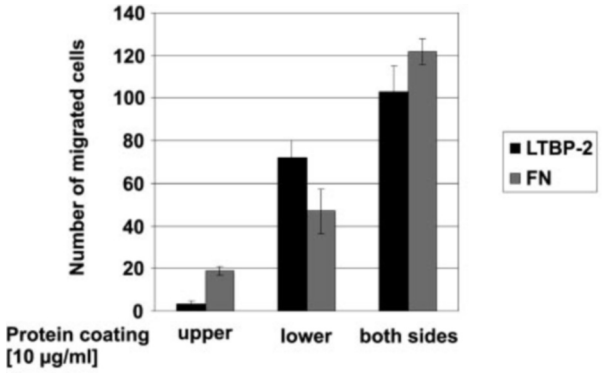

FIG. 7. **LTBP-2 supports the migration of Bowes melanoma cells.** Cell migration assays were carried out in modified Boyden chambers. The membranes of the cell culture inserts were coated with LTBP-2 or fibronectin on either upper or lower surface or on both as indicated. Melanoma cells (5×10^4 cells in MEM containing 0.1% FCS) were seeded in each insert and allowed to migrate at +37 °C for 6 h. The cells were then fixed and stained as described under "Experimental Procedures." The results indicate the number of cells that migrated through the pores to the lower surface. Bowes melanoma cells migrated toward LTBP-2 when it was coated on the lower side of the cell culture insert membrane, but the strongest migration was achieved when both sides of the membrane were coated with LTBP-2. The pattern is similar to Bowes cell migration on fibronectin. The number of migrated cells represents the mean value of three calculated microscopic fields.

and correct folding of TGF-βs as well as their deposition into the extracellular matrix (5, 40, 41). However, LTBPs are often secreted in large excess to TGF-β, suggesting additional, possibly structural roles for them in ECM. LTBPs share a similar domain structure with fibrillins, 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 10-nm microfibrillar structures and also to thicker fibronectin fibrils in the vicinity of the cell surface (7) (see also Ref. 43). The role of LTBPs in these structures has been unclear so far. The early lethality of LTBP-2 null mice suggests a crucial role for LTBPs 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 N-terminal fragment, L2-V, which mediates melanoma cell adhesion, acts as an antiadhesive 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 $\alpha_V\beta_3$ 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 α_3, α_V, and β_1 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 LTPB-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 bound to an adjacent short fragment, L2-V. β_1 integrin family contains both kind of receptors, namely RGD-binding and RGD-independent (45). Integrin $\alpha_3\beta_1$ 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 $\alpha_V\beta_3$ 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 $\alpha_V\beta_3$ integrin. Inhibition assays indicated that adhesion to LTBP-2 was mediated by β_1 integrin, the α_3 subunit being the most obvious partner. The mAb against the α_6 subunit had an additive inhibitory effect on melanoma cell adhesion. However, cell adhesion to LTBP-2 does not depend only on the expression of $\alpha_3\beta_1$ or $\alpha_6\beta_1$ 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. 1*A*). 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 $\alpha_3\beta_1$ 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 $\alpha_3\beta_1$ 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 heterogenic. Melanoma cells express cell surface chondroitin sulfate proteoglycans that modulate tumor cell adhesion and motility (reviewed in Ref. 52). chondroitin sulfate proteoglycans bind the $\alpha_4\beta_1$ integrin and are needed for $\alpha_4\beta_1$-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

[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, the ability to adhere to different ECM proteins is beneficial for tumor cells during migration and invasion and when adapting to a new environment after invasion. LTBP-2 is an abundant protein in human lung, and it is also expressed in the liver, where it may serve as an adhesion protein for melanoma cells during cancer spreading.

Acknowledgment—We thank Sami Starast for fine technical assistance.

REFERENCES

1. Saharinen, J., Hyytiäinen, M., Taipale, J., and Keski-Oja, J. (1999) *Cytokine Growth Factor Rev.* **10,** 99–117
2. Kingsley, D. (1994) *Genes Dev.* **8,** 133–146
3. Massague, J. (1990) *Annu. Rev. Cell Biol.* **6,** 597–641
4. Laiho, M., and Keski-Oja, J. (1989) *Cancer Res.* **49,** 2533–2553
5. Miyazono, K., Olofsson, A., Colosetti, P., and Heldin, C. H. (1991) *EMBO J.* **10,** 1091–1101
6. Annes, J. P., Munger, J. S., and Rifkin, D. B. (2002) *J. Cell Sci.* **116,** 1–8
7. Taipale, J., Saharinen, J., Hedman, K., and Keski-Oja, J. (1996) *J. Histochem. Cytochem.* **44,** 875–889
8. Gibson, M. A., Hatzinikolas, G., Davis, E. C., Baker, E., Sutherland, G. R., and Mecham, R. P. (1995) *Mol. Cell. Biol.* **15,** 6932–6942
9. Dabovic, B., Chen, Y., Colarossi, C., Obata, H., Zambuto, L., Perle, M.-A., and Rifkin, D. B. (2002) *J. Cell Biol.* **156,** 227–232
10. Saharinen, J., and Keski-Oja, J. (2000) *Mol. Biol. Cell* **11,** 2691–2704
11. Sterner-Kock, A., Thorey, I. S., Koli, K., Wempe, F., Otte, J., Bangsow, T., Kuhlmeier, K., Kirchner, T., Jin, S., Keski-Oja, J., and von Melchner, H. (2002) *Genes Dev.* **16,** 2264–2273
12. Moren, A., Olofsson, A., Stenman, G., Sahlin, P., Kanzaki, T., Claesson-Welsh, L., ten Dijke, P., Miyazono, K., and Heldin, C. H. (1994) *J. Biol. Chem.* **269,** 32469–32478
13. Shipley, J. M., Mecham, R. P., Maus, E., Bonadio, J., Rosenbloom, J., McCarthy, R. T., Baumann, M. L., Frankfater, C., Segade, F., and Shapiro, S. D. (2000) *Mol. Cell. Biol.* **20,** 4879–4887
14. Sakamoto, H., Broekelmann, T., Cheresh, D. A., Ramirez, F., Rosenbloom, J., and Mecham, R. P. (1996) *J. Biol. Chem.* **271,** 4916–4922
15. Munger, J. S., Harpel, J. G., Giancotti, F. G., and Rifkin, D. B. (1998) *Mol. Biol. Cell* **9,** 2627–2638
16. Munger, J., Huang, X., Kawakatsu, H., Griffiths, M., Dalton, S., Wu, J., Pittet, J.-F., Kaminski, N., Garat, C., Matthay, M., Rifkin, D., and Sheppard, D. (1999) *Cell* **96,** 319–328
17. Annes, J. P., Rifkin, D. B., and Munger, J. S. (2002) *FEBS Lett.* **511,** 65–68
18. Lu, M., Munger, J. S., Steadele, M., Busald, C., Tellier, M., and Schnapp, L. M. (2002) *J. Cell Sci.* **115,** 4641–4648
19. Hynes, R. O. (1992) *Cell* **69,** 11–25
20. Hynes, R. (2002) *Cell* **110,** 673–687
21. Yamada, K. M., and Miyamoto, S. (1995) *Curr. Opin. Cell Biol.* **7,** 681–689
22. Liu, S., Calderwood, D. A., and Ginsberg, M. H. (2000) *J. Cell Sci.* **113,** 3563–3571
23. Zamir, E., and Geiger, B. (2001) *J. Cell Sci.* **114,** 3577–3579
24. Wary, K. K., Mainiero, F., Isakoff, S. J., Marcantonio, E. E., and Giancotti, F. G. (1996) *Cell* **87,** 733–743
25. Wei, Y., Yang, X., Liu, Q., Wilkins, J. A., and Chapman, H. A. (1999) *J. Cell Biol.* **144,** 1285–1294
26. Ivaska, J., and Heino, J. (2000) *Cell Mol. Life Sci.* **57,** 16–24
27. Schwartz, M. A., and Ginsberg, M. H. (2002) *Nat. Cell Biol.* **4,** E65–E68
28. Albelda, S. M., Mette, S. A., Elder, D. E., Stewart, R., Damjanovich, L., Herlyn, M., and Buck, C. A. (1990) *Cancer Res.* **50,** 6757–6764
29. Seftor, R. E., Seftor, E. A., Gehlsen, K. R., Stetler-Stevenson, W. G., Brown, P. D., Ruoslahti, E., and Hendrix, M. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 1557–1561
30. Seftor, R. E., Seftor, E. A., and Hendrix, M. J. (1999) *Cancer Metastasis Rev.* **18,** 359–375
31. Hyytiäinen, M., Taipale, J., Heldin, C. H., and Keski-Oja, J. (1998) *J. Biol. Chem.* **273,** 20669–20676
32. Unsöld, C., Hyytiäinen, M., Bruckner-Tuderman, L., and Keski-Oja, J. (2001) *J. Cell Sci.* **114,** 187–197
33. van der Flier, A., and Sonnenberg, A. (2001) *Cell Tissue Res.* **305,** 285–298
34. Li, X., Chen, B., Blystone, S. D., McHugh, K. P., Ross, F. P., and Ramos, D. M. (1998) *Invasion Metastasis* **18,** 1–14
35. Zamir, E., and Geiger, B. (2001) *J. Cell Sci.* **114,** 3583–3590
36. Plow, E. F., Haas, T. A., Zhang, L., Loftus, J., and Smith, J. W. (2000) *J. Biol. Chem.* **275,** 21785–21788
37. Akula, S. M., Pramod, N. P., Wang, F. Z., and Chandran, B. (2002) *Cell* **108,** 407–419
38. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) *Annu. Rev. Biochem.* **68,** 729–777
39. Postlethwaite, A. E., Keski-Oja, J., Moses, H. L., and Kang, A. H. (1987) *J. Exp. Med.* **165,** 251–256
40. Miyazono, K., Hellman, U., Wernstedt, C., and Heldin, C. (1988) *J. Biol. Chem.* **263,** 6407–6415
41. Wakefield, L., Smith, D., Flanders, K., and Sporn, M. (1988) *J. Biol. Chem.* **263,** 7646–7654
42. Ramirez, F. (1996) *Curr. Opin. Genet. Dev.* **6,** 309–315
43. Dallas, S. L., Keene, D. R., Bruder, S. P., Saharinen, J., Sakai, L. Y., Mundy, G. R., and Bonewald, L. F. (2000) *J. Bone Miner. Res.* **15,** 68–81
44. D'Arrigo, C., Burl, S., Withers, A. P., Dobson, H., Black, C., and Boxer, M. (1998) *Connect. Tissue Res.* **37,** 29–51
45. Ruoslahti, E. (1996) *Annu. Rev. Cell Dev. Biol.* **12,** 697–715
46. Xue, W., Mizukami, I., Todd, R. F., III, and Petty, H. R. (1997) *Cancer Res.* **57,** 1682–1689
47. Berditchevski, F., Chang, S., Bodorova, J., and Hemler, M. E. (1997) *J. Biol. Chem.* **272,** 29174–29180
48. Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle, M. V., and Chapman, H. A. (1996) *Science* **273,** 1551–1555
49. Chen, H., Herndon, M. E., and Lawler, J. (2000) *Matrix Biol.* **19,** 597–614
50. Melchiori, A., Mortarini, R., Carlone, S., Marchisio, P. C., Anichini, A., Noonan, D. M., and Albini, A. (1995) *Exp. Cell Res.* **219,** 233–242
51. Yoshinaga, I. G., Vink, J., Dekker, S. K., Mihm, M. C., Jr., and Byers, H. R. (1993) *Melanoma Res.* **3,** 435–441
52. Iida, J., Meijne, A. M., Knutson, J. R., Furcht, L. T., and McCarthy, J. B. (1996) *Semin. Cancer Biol.* **7,** 155–162
53. Iida, J., Meijne, A. M., Oegema, T. R., Jr., Yednock, T. A., Kovach, N. L., Furcht, L. T., and McCarthy, J. B. (1998) *J. Biol. Chem.* **273,** 5955–5962