Fibronectin Regulates Latent Transforming Growth Factor-β (TGFβ) by Controlling Matrix Assembly of Latent TGFβ-binding Protein-1*

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Latent transforming growth factor-β-binding proteins (LTBPs) are extracellular matrix (ECM) glycoproteins that play a major role in the storage of latent TGFβ in the ECM and regulate its availability. Here we show that fibronectin is critical for the incorporation of LTBP1 and transforming growth factor-β (TGFβ) into the ECM of osteoblasts and fibroblasts. Immunolocalization studies suggested that fibronectin provides an initial scaffold that precedes and patterns LTBP1 deposition but that LTBP1 and fibronectin are later localized in separate fibrillar networks, suggesting that the initial template is lost. Treatment of fetal rat calvarial osteoblasts with a 70-kDa N-terminal fibronectin fragment that inhibits fibronectin assembly impaired incorporation of LTBP1 and TGFβ into the ECM. Consistent with this, LTBP1 failed to assemble in embryonic fibroblasts that lack the gene for fibronectin. LTBP1 assembly was rescued by full-length fibronectin and superfibronectin, which are capable of assembly into fibronectin fibrils, but not by other fibronectin fragments, including a 160-kDa RGD-containing fragment that activates α5β1 integrins. This suggests that the critical event for LTBP1 assembly is the formation of a fibronectin fibrillar network and that integrin ligation by fibronectin molecules alone is not sufficient. Not only was fibronectin essential for the initial incorporation of LTBP1 into the ECM, but the continued presence of fibronectin was required for the continued assembly of LTBP1. These studies highlight a nonredundant role for fibronectin in LTBP1 assembly into the ECM and suggest a novel role for fibronectin in regulation of TGFβ via LTBP1 interactions.

Recent evidence suggests that the binding of growth factors to the extracellular matrix (ECM) is a major mechanism for regulation of growth factor activity and plays a fundamental role in tissue morphogenesis and repair (1). The latent transforming growth factor-β-binding proteins (LTBPs) are members of a family of ECM proteins that are key regulators of transforming growth factor-β (TGFβ) (2–4). LTBP1, the prototype member of this family, regulates TGFβ at multiple levels. First, LTBP1 associates with latent TGFβ inside the cell and facilitates secretion of the latent complex (5). LTBP1 then targets latent TGFβ to the ECM for storage (6, 7). LTBP1 may also provide a vehicle for release of the latent growth factor, following proteolytic cleavage of LTBP1 and release of C-terminal LTBP1 fragments, still bound to the latent TGFβ (7–9). Finally, there is also evidence that LTBP1 plays a role in activation of the latent TGFβ complex (10, 11).

LTBP1s are members of a larger superfAMILY of matrix proteins that include fibrillins 1 and 2, the recently reported fibrillin 3 (12), and the latent TGFβ-binding proteins 1–4 (reviewed in Refs. 2, 3, 13, and 14). At least three of the LTBP1s (LTBP1s 1, 3, and 4) can form complexes with latent TGFβ1, whereas LTBP2 appears not to bind to latent TGFβ (15). LTBP1 is also secreted by many cell types in a free form that is not bound to TGFβ. The percentage of free LTBP1 varies from 10 to 90%, depending on the cell type and the differentiation stage examined (6, 9, 16). These observations raise the possibility that LTBP1 may also have important functions that are independent of TGFβ and may be related to its properties as an ECM protein.

At present, little is known about how LTBP1s function as matrix proteins. Like the fibrillins, their primary structure consists predominantly of 6 cysteine (epidermal growth factor-like) repeats similar to the motifs found in the epidermal growth factor precursor and 8 cysteine repeats (termed “TB repeats”) unique to the LTBP1s and fibrillins. Recent studies have demonstrated that the third TB repeats in LTBP1s 1, 3, and 4 are the sites for covalent binding of latent TGFβ (15, 17).

Immunolocalization studies have shown that LTBP1 co-localizes with fibrillin-1 in 10-nm microfibrils in the ECM of osteoblasts (18). LTBP1 has also been shown to co-localize with fibronectin (18–20), and a binding interaction between LTBP1 and fibronectin has been suggested by ligand blotting studies (19). Fibronectin is required for the assembly of several ECM proteins, including type I collagen (21), fibulin I (22), thrombospondin (23), and fibrinogen (24). We therefore hypothesized that fibronectin may be a critical determinant of latent growth factor activity and that inhibition of LTBP1 assembly by fibronectin may be a potential therapeutic target for the regulation of TGFβ activity.

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that fibronectin may play a critical role in LTBP1 assembly into the ECM and may thereby play a role in regulation of TGFβ. Multiple approaches were used to disrupt fibronectin assembly, and the effect on LTBP1 and TGFβ incorporation was determined. These studies highlight a novel role for fibronectin in the regulation of TGFβ via LTBP1 interactions.

MATERIALS AND METHODS

Reagents—Human plasma fibronectin was purified as described previously (25) or purchased from Invitrogen. The 70-kDa N-terminal and 160-kDa fibronectin fragments were prepared as described previously (25). 30- and 40-kDa fibronectin fragments as well as superfibronectin were purchased from Sigma. The H120 recombinant fibronectin fragment was prepared as described previously (27). Fibronectin-striped serum was prepared by passing the serum over a gelatin-Sepharose column as described elsewhere (28).

Antibodies—Antibodies against LTBP1 included a rabbit polyclonal antibody (Ab39; kindly supplied by K. Miyazono, Japanese Foundation for Cancer Research, Tokyo) (18, 29). A rabbit polyclonal antibody against a peptide in rat LTBP1 was also used, which recognizes mouse and rat but not human LTBP1 (18). Fibronectin antibodies included a mouse monoclonal antibody (IgM) against cellular fibronectin (Sigma), a mouse monoclonal antibody (IgG) against the human fibronectin ED-A domain (Harlan), a mouse monoclonal antibody IgG (39B6) directed against type II repeats 12–14 of human fibronectin (30), and a rabbit polyclonal antibody against LTBP1 purified from human platelets (Neomarkers, Freemont, CA).

A peroxidase-conjugated donkey anti-rabbit antibody (Amersham Biosciences) was used for Western blotting and ELISA. All secondary antibodies were used in place of primary antibody, testing each primary antibody individually using both secondary antibodies to exclude cross-species reactivity in the gels.

Immunocytochemistry—For immunofluorescent staining were purchased from Jackson ImmunoResearch—West Grove, PA). Various combinations of fluorochrome-conjugated secondary antibodies were used as appropriate for each combination of primary antibodies, as stated in the figure legends. In some experiments, biotinylated secondary antibodies were used in conjunction with fluorescein isothiocyanate-conjugated streptavidin (Vector Laboratories, Burlingame, CA).

Cell culture—The culture reagents were purchased from Invitrogen. Primary fetal rat calvarial (FRC) osteoblasts were described as prepared previously (7, 18). UMR-106 cells were a gift from T. J. Martin (St. Vincent Institute of Medical Research, Fitzroy, Victoria, Australia) and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 100 units/ml penicillin/streptomycin, and 10% fetal bovine serum.

FN-null (FN−/−) and heterozygous (FN+/−) embryonic fibroblasts were derived as described elsewhere (31). For experiments examining LTBP1 assembly in FN-null fibroblasts, the cells were cultured in the presence of FN-striped serum with or without the addition of 10 μg/ml plasma fibronectin. For growth curve experiments, cells were plated in 96-well plates at 20,000 cells/cm². Cells were trypsinized at the specified times, and cell number was determined by using a Coulter counter (Beckman Coulter Inc.).

Immunocytochemistry—For immunocytochemistry, cells were plated in Lab-Tek chamber slides at 20,000 cells/cm² in Dulbecco’s modified Eagle’s medium supplemented with 10% FN-striped fetal bovine serum, 2 mM l-glutamine, and 100 units/ml penicillin/streptomycin with or without 10 μg/ml plasma fibronectin. At confluence, the media were changed to Dulbecco’s modified Eagle’s medium supplemented with 5% FN-striped fetal bovine serum, 50 μg/ml ascorbic acid, and other additives as above, with or without the addition of fibronectin or fibronectin fragments. The cells were cultured for the specified periods, with media changed every 3 days. Co-localization of LTBP1, fibronectin, and type I collagen was performed using double staining indirect immunofluorescence techniques as described previously (18). The specimens were viewed and photographed digitally using a Leica DMRXA microscope with epifluorescence illumination and a Photometric Coolsnap FX CCD camera. For double labeling experiments, multiple controls were performed, including using nonimmune serum or control IgG in place of primary antibody, testing each primary antibody individually using both secondary antibodies to exclude cross-species reactivity of the secondary antibodies, and preincubation with the primary antibody with the immunizing peptide (where available).

TGFβ ELISA and Modified ELISA for Detection of Relative Amounts of LTBP1—A commercial ELISA (TGFβ1 Emax; Promega Corp., Madison, WI) was used for measurement of TGFβ1 in conditioned media samples collected over 48 h or in guandine HCl extracts of the ECM, prepared as described elsewhere (32). Values were normalized to cell number. For measurement of total (active + latent) TGFβ, the samples were acidified using HCl and then reneutralized prior to measurement using NaOH according to the ELISA manufacturer’s instructions.

To quantitate the relative amounts of LTBP1 in the ECM, a modified ELISA was used. Cells were grown in 96-well plates in media with or without fibronectin. The plates were fixed in 5% ethanol, then blocked with 1% milk for 2 h, followed by incubation in primary antibodies against LTBP1. After washing, the amount of bound LTBP1 antibody was determined using the Vectorstain-Elite ABC immunodetection kit (Vector Laboratories, Burlingame, CA) in conjunction with O-phenylenediamine as a reaction substrate. Alternatively, the vector VIP substrate kit was used, followed by solubilization of the reaction product by incubating for 10 min at room temperature in 50 μl of 2 M KCl, then adding 50 μl of Me3SO and incubating for a further 10 min. The plates were read on an ELx 800 plate reader (Bio-Tek Instruments, Winooski, VT) at 450 nm, and background subtraction was performed using a blank control that had been incubated with nonspecific IgG in place of primary antibody. Values were normalized to total protein content of the cell lysate or to cell number as determined using a Coulter counter.

Immunoelectron Microscopy—For immunoelectron microscopy, FN-null and heterozygous fibroblasts were cultured on Thermofax coverslips (Nalge Nunc International) in media with or without added fibronectin as described above. Media were changed every 3 days, and coverslips were harvested on days 6, 14, and 21. Immunogold staining was performed on unfixed specimens as described previously (18) using anti-LTBP1 and anti-fibronectin antibodies each diluted 1:10. For detection, a donkey anti-rabbit secondary antibody-6-nm gold conjugate and a donkey anti-mouse secondary antibody-18-nm gold conjugate were used (Jackson ImmunoResearch). The samples were fixed after immunogold staining and processed for transmission electron microscopy as described previously (18). They were then embedded in Taab epoxy resin (Taab Laboratories Equipment Ltd., Aldermaston, UK). After polymerization, the coverslips were removed to leave the cell layer exposed on the outer surface of the block. Ultrathin sections were cut at a slight angle to the growth substrate by using a diamond knife. Sections were examined with or without contrast in a Philips EM 301 transmission electron microscope at an accelerating voltage of 60 kV.

LTBP1 Stably Expressing Cell Lines—To generate UMR-106 cell lines stably overexpressing LTBP1, a full-length human LTBP1 construct in the pSV7d vector (kindly provided by K. Miyazono, Japanese Foundation for Cancer Research, Tokyo) was co-transfected at a 10:1 ratio with an RVSVneo selection vector, using calcium phosphate precipitation as described elsewhere (33). The transfected cells were selected with 440 μg/ml G418, and resistant single cell clones were screened for LTBP1 expression using an ELISA, as described previously (7). Three high expressing clones (0.2–1.5 μg/ml) were selected for further study. Control transfectants were selected with RVSVneo alone were used for comparison.

FPLC Analysis—FPLC fractionation was performed as described previously (16) using 48-h serum-free, phenol red-free conditioned media harvested from 90–95% confluent cultures. 150 ml of conditioned medium was concentrated 10-fold over a 50-kDa cut-off membrane using a minisette concentrator (Millipore, Bedford, MA). The samples were acidified using HCl and then reneutralized prior to measurement using NaOH according to the ELISA manufacturer’s instructions. Samples were acidified using HCl and then reneutralized prior to measurement using NaOH according to the ELISA manufacturer’s instructions.

Metabolic Labeling and Immunoprecipitation—For metabolic labeling and immunoprecipitation, cells were plated into 12-well multwell plates at 10,000 cells/cm² growth area. At 90% confluence, the cells were metabolically labeled as described previously (8) by using 100 μCi/well [35S]cysteine for 6 h. LTBP1 was measured in the culture supernatants as well as the ECM by immunoprecipitation followed by SDS-PAGE and autoradiography as described previously (8, 16). A plasmid digestion was used to release the ECM-bound LTBP1 (7, 19).

Western Blotting—Proteins in concentrated conditioned media samples or plasmid matrix digests were separated by SDS-PAGE using 4–20% gradient polyacrylamide gels, and immunoblotting was performed as described previously (7). The immunobound proteins were visualized using the Renaissance ECL detection system according to manufacturer’s instructions (PerkinElmer Life Sciences). Samples were normalized to total protein content or to cell number prior to loading on the gels.
RESULTS

Time Course of LTBP1 and Fibronectin Assembly in Primary Osteoblast Cultures—To determine the time course of LTBP1 and fibronectin assembly, immunolocalization studies were performed over a time course of 1–21 days (Fig. 1). Fibronectin initially appeared as short fibrillar structures on the surface of fetal rat calvarial cells after 24 h in culture (Fig. 1, d1). At this stage, no immunoactive LTBP1 was present on the cell surface or in the extracellular matrix. By 2 days in culture (Fig. 1, d2), the fibronectin became organized into a fibrillar network in the ECM, and a small amount of LTBP1 was observed that co-localized with fibronectin. By 3 and 5 days of culture (Fig. 1, d3 and d5), LTBP1 was localized in a fibrillar network that co-distributed with fibronectin. The incorporation of LTBP1 therefore lagged 1–2 days behind that of fibronectin, and there was always a proportion of fibronectin-positive fibrils that did not stain for LTBP1. Between days 5 and 21, there was an increase in formation of LTBP1 fibrils, which became organized into long parallel fibrillar arrays. At the same time, there was a progression from distinct fibronectin fibrils to fibrils with a more diffuse appearance. The co-localization of LTBP1 and fibronectin started to diverge until day 21, when LTBP1 became localized in fibrils that were clearly distinct from fibronectin. Identical results were obtained by using the human MG63 osteosarcoma cell line as well as with human skin fibroblasts and using two independent antibodies for LTBP1 and fibronectin (data not shown).

Double-labeled immunogold localization for LTBP1 and fibronectin demonstrated co-localization of these two molecules in fibrillar structures in 6-day fetal rat calvarial cell cultures (Fig. 2). In later (14-day) cultures, some fibrils could be found that were labeled with both antibodies; however, LTBP1 was also frequently found in bundles of 10-nm microfibrils that were not labeled with fibronectin antibodies (Fig. 2).

Inhibition of LTBP1 and TGFβ1 Incorporation by a 70-kDa N-terminal Fibronectin Fragment—To determine whether fibronectin was required for assembly of LTBP1 into bone ECM, fetal rat calvarial cell cultures were treated for 6 days with a 70-kDa N-terminal fibronectin fragment (Fig. 3a). This fragment blocks assembly of both endogenous and exogenous fibronectin in fibroblasts by occupying binding sites that are required for fibronectin self-association (28). Treatment with 70 μg/ml of the fibronectin fragment (Fig. 3a, 70K) resulted in a dramatic reduction in fibronectin incorporation compared with untreated controls (control) or control cultures treated with plasma fibronectin (pFN). This was paralleled by a reduction in LTBP1 incorporation into the ECM. Dose-response experiments showed a significant reduction in LTBP1 incorporation at doses between 10 and 70 μg/ml, as determined by immunofluorescence (data not shown) and also by quantitation of the relative amounts of incorporated LTBP1 using a modified ELISA (Fig. 3b).

Time course experiments indicated that at early time points (days 1–3) there was a virtual absence of both fibronectin and LTBP1 in the ECM of cells treated with the fibronectin 70-kDa fragment. However, by day 5, fibronectin and LTBP1 incorporation began to partially recover. This recovery of LTBP1 incorporation appeared to be due to the fact that even the highest dose of the 70-kDa fibronectin fragment failed to completely block fibronectin incorporation. The above results were replicated by using an LTBP1 antibody that recognizes the proline-rich hinge region and using MG63 human osteosarcoma cells (data not shown).

TGFβ1 is the major TGFβ isoform produced in bone (7, 34). To determine whether inhibition of fibronectin assembly also affected incorporation of TGFβ into the ECM, the amount of TGFβ1 in the ECM was measured using a commercial ELISA (Fig. 3c). Treatment of fetal rat calvarial cells with the 70-kDa fibronectin fragment reduced the amount of latent TGFβ1 stored in the ECM by 80%. This was not because of reduced synthesis and secretion of TGFβ as increased amounts of latent
TGFβ were detected in the conditioned media of the treated cultures (Fig. 3c).

LTBP1 Assembly in Fibronectin-null Embryonic Fibroblasts—To define further the role of fibronectin in the assembly of LTBP1 into the ECM, we examined LTBP1 assembly in fibroblasts differentiated from mouse embryonic stem cells that were null for the fibronectin gene (FN-null) (Fig. 4). Both heterozygous (+/−) and FN-null (−/−) embryonic fibroblasts synthesized and secreted LTBP1 in amounts that were grossly equivalent to the amounts produced by fetal rat calvarial cells. By day 14, many microfibrillar bundles (MF) can be found that contain LTBP1 but not fibronectin. A control in which a nonimmune IgG was used in place of the primary antibody is shown on the right.

![Image](image-url)

**Fig. 2. Double-labeled immunogold EM localization of LTBP1 and fibronectin in 6- and 14-day cultures of fetal rat calvarial cells.** Fibronectin was localized using 18-nm gold-labeled secondary antibodies and LTBP1 was localized using 6-nm gold particles. Note that at 6 days, fibrils are seen that are positive for both LTBP1 and fibronectin. By day 14, many microfibrillar bundles (MF) can be found that contain LTBP1 but not fibronectin. A control in which a nonimmune IgG was used in place of the primary antibody is shown on the right.

6 day 14 day control

of the primary antibody is shown on the right.
Hocking (23) has shown that the continual assembly of fibronectin is required to maintain the stability of several ECM components. To determine whether the continual assembly of fibronectin is required to maintain the presence of LTBP1 in the ECM, we performed rescue experiments in FN-null cells followed by withdrawal of fibronectin (Fig. 9). FN-null cells were rescued with fibronectin treatment (10 μg/ml) for 3 days, after which the cultures were either fixed immediately or continued for a further 3 days in the presence of fibronectin. Immunostaining confirmed that FN-null cells rescued with fibronectin treatment for 3 days showed a well-organized fibronectin fibrillar network and incorporated LTBP1 into the ECM. If the cultures were continued for a further 3 days in the presence of fibronectin, additional fibronectin and LTBP1 assembly occurred. However, withdrawal of fibronectin for days 4–6 resulted in loss of fibronectin immunoreactivity from the ECM, suggesting that the continual presence of fibronectin is required to maintain stability of the fibronectin fibrillar network. In contrast, withdrawal of fibronectin did not result in significant loss of the LTBP1 that had already been incorporated, but it did prevent further LTBP1 assembly (i.e. LTBP1 staining in cultures following withdrawal of fibronectin for days 4–6 resembled cultures that had been fixed on day 3).

Type I Collagen and Fibrillin-1 Are Not Required for Association of LTBP1 with Fibronectin—Similar to the report of Velling et al. (21), we also found that type I collagen incorporation was compromised in FN-null fibroblasts compared with heterozygous controls (data not shown). To exclude the possibility that the lack of incorporation of LTBP1 into the ECM in FN-null fibroblasts was secondary to an effect on collagen assembly, experiments were performed in the absence of ascorbic acid. Under these conditions, no fibrillar collagen was detected, and all the type I collagen was localized intracellularly. However, the relationship between LTBP1 and fibronectin was unaffected by the absence of ascorbic acid, i.e. LTBP1 incorporation in FN-null cells could be rescued by addition of fibronectin in the absence of fibrillar collagen (data not shown).

In addition to reduced incorporation of LTBP1 and type I collagen, we also found that fibrillin-1 assembly was impaired in FN-null fibroblasts. Because LTBP1 and fibrillin-1 both appear to be dependent on fibronectin for assembly into the ECM and because we have previously reported that LTBP1 and fibrillin-1 co-localize in 10-nm microfibrils, we next examined whether fibrillin-1 was required for incorporation of LTBP1 into the ECM in association with fibronectin. LTBP1 was stably transfected into UMR-106 rat osteosarcoma cells, which do not produce endogenous LTBP1 or fibrillin-1. We have shown previously (16) that these cells produce exclusively the 100-kDa small latent TGFβ complex that lacks LTBP1. However, these cells do produce an organized fibronectin matrix, making them a useful host cell for LTBP1 overexpression to examine its association with fibronectin in the absence of fibrillin-1.

Several stable single cell clones were obtained, which expressed LTBP1 in the range 0.2–1.5 μg/ml, as determined by ELISA. FPLC analysis was used to examine the forms of latent TGFβ produced by the transfected UMR-106 cell lines (Fig. 10a). In empty vector-transfected cells, a single peak of TGFβ activity was observed, eluting at 0.2 M NaCl (Fig. 10a, gray bars), consistent with the known elution position of the 100-kDa small latent TGFβ complex (16, 35). LTBP1 was undetectable by ELISA in empty vector-transfected cells (Fig. 10a, solid lines). Transfection of UMR-106 cells with LTBP1 altered the elution profile so that ~30% of the latent TGFβ activity eluted at a later position (0.3 M NaCl), corresponding to the known elution position of the 290-kDa large latent TGFβ complex, containing LTBP1 (16, 35). Consistent with this, the LTBP1 peak, as measured by ELISA, overlapped with the large latent TGFβ complex peak. The LTBP1 peak also extended beyond the TGFβ peak, which presumably represents free, uncomplexed LTBP1. Fig. 10b shows immunoprecipitation analysis on two LTBP1-overexpressing UMR-106 clones. As expected, LTBP1 was undetectable in empty vector-transfected cells in either the media or matrix. In contrast, when LTBP1 was overexpressed in UMR-106 cells, bands were observed in the conditioned medium and in plasmid digests of the ECM at the expected size for free LTBP1 (Fig. 10b, black arrowhead) and LTBP1 complexed to TGFβ (Fig. 10b, white arrowhead), suggesting that the overexpressed protein was incorporated into the ECM. Double-stained immunofluorescence confirmed that of fibronectin for days 4–6 resulted in loss of fibronectin immunoreactivity from the ECM, suggesting that the continual presence of fibronectin is required to maintain stability of the fibronectin fibrillar network. In contrast, withdrawal of fibronectin did not result in significant loss of the LTBP1 that had already been incorporated, but it did prevent further LTBP1 assembly (i.e. LTBP1 staining in cultures following withdrawal of fibronectin for days 4–6 resembled cultures that had been fixed on day 3).
LTBP1 was undetectable in the ECM of empty vector-transfected cells (Fig. 10c). In contrast, in LTBP1-overexpressing cells, abundant LTBP1 staining was observed in the matrix, which co-localized with fibronectin. No fibrillin-1 immunostaining was detected in either control or LTBP1-transfected cells. Identical results were obtained with three independent LTBP1-overexpressing clones. Thus, in the absence of fibrillin-1, LTBP1 still co-localized with fibronectin.
Role for Fibronectin in LTBP1 Assembly

FIG. 6. Immunofluorescent staining showing rescue of LTBP1 incorporation in FN-null embryonic fibroblasts treated with 10 μg/ml human plasma fibronectin for 6 days. a, FN-null (+/−) and heterozygous (+/+ ) cultures double-stained using an anti-LTBP1 polyclonal antibody (against proline-rich domain) and anti-fibronectin (monoclonal against the ED-A domain). Note that addition of fibronectin (+/FN) completely rescues LTBP1 incorporation in FN-null cells. However, no fibronectin is detected in these rescued cultures using the ED-A antibody that only recognizes the endogenously produced cellular form of fibronectin and not the added plasma fibronectin. b, FN-null embryonic fibroblasts rescued by addition of 10 μg/ml plasma fibronectin and double-stained using anti-LTBP1 and anti-fibronectin monoclonal antibody against type III repeats 12–14. In this case the fibronectin antibody recognizes the added plasma fibronectin. Note that the added fibronectin has been assembled into the ECM and that the rescued LTBP1 is co-localized with it. Bar, 50 μm.

FIG. 7. Western blot (a) and modified ELISA (b) showing LTBP1 incorporation in FN-null (−/−) and heterozygous (+/−) embryonic fibroblasts cultured for 14 days with and without addition of 10 μg/ml fibronectin (+/FN). Note that by Western analysis (a), there is a virtual absence of LTBP1 (indicated by open arrow) and large latent TGFβ complex (indicated by black arrow) in the ECM of FN-null cells, which is rescued by addition of fibronectin. Samples were normalized to cell number prior to loading on the gel. The ELISA (b) confirms a significant reduction in relative amounts of LTBP1 in the ECM of FN-null fibroblasts compared with heterozygous controls. c, growth curves of FN-null and heterozygous fibroblasts. Note that the FN-null cells (closed circles) actually grow faster than the heterozygous controls (closed triangles). Addition of fibronectin has little effect on the growth of either cell type (open circles and triangles). *, significantly different from heterozygous control, p < 0.05 by analysis of variance/Student’s Newman Keuls test.

FIG. 8. Rescue of LTBP1 incorporation in FN-null fibroblasts treated with full-length plasma fibronectin and superfibronectin but not with various fibronectin fragments. a, schematic diagram showing full-length fibronectin and the various fibronectin fragments added to FN-null fibroblasts at 10 μg/ml (gray rectangles, type I repeats; black ovals, type II repeats; white rectangles, type III repeats; V, variable region; A, ED-A domain; B, ED-B domain). b, immunostaining of FN-null cell cultures treated with the various fibronectin fragments. The cultures were stained with anti-LTBP1 antibodies. Note that only plasma fibronectin and superfibronectin were able to rescue LTBP1 incorporation. Bar, 50 μm.

DISCUSSION

In this study we provide evidence that fibronectin is a key regulator of LTBP1 and TGFβ incorporation into the ECM of osteoblasts and fibroblasts. Immunolocalization studies showed that fibronectin is assembled into the ECM prior to LTBP1 and that LTBP1 is then deposited onto and co-localizes with fibronectin. However, in long term cultures of both primary osteoblasts and fibroblasts, fibronectin and LTBP1 are localized in separate fibrillar networks. These data suggest that fibronectin provides an initial template for deposition of LTBP1 (i.e., formation of a fibronectin matrix precedes and patterns LTBP1 deposition) but that this template only acts as a temporary scaffold (i.e., the initial fibronectin template is lost).

Consistent with a critical role for fibronectin in LTBP1 assembly, disruption of fibronectin fibrillogenesis using a 70-kDa N-terminal fibronectin fragment impaired incorporation of LTBP1 and latent TGFβ1 into the ECM. Furthermore, embryonic fibroblasts that lacked the fibronectin gene failed to incorporate LTBP1 into the ECM even though they secreted a large amount of LTBP1 into the culture medium. Addition of exogenous fibronectin was able to completely rescue fibrillar assembly of fibronectin and to rescue LTBP1 incorporation into the ECM as well as its co-localization with fibronectin. Together, these data suggest a crucial non-redundant role for fibronectin in the assembly of LTBP1 into the ECM. The lack of LTBP1 assembly in the absence of fibronectin suggests that LTBP1 does not use the same integrin receptor system as fibronectin (i.e., α5β1 or α4β1) directly for its assembly, since these integrins are expressed in the FN-null cells (31) and their presence is not sufficient for LTBP1 assembly in the absence of fibronectin. The assembly of LTBP1 therefore appears to be fundamentally different from that of fibronectin.

Although LTBP1 assembly was virtually absent in 1–6-day cultures of FN-null fibroblasts, with extended culture times (14–21 days) a small amount of LTBP1 incorporation was observed, which was localized in areas where the cells were multilayered and had the appearance of short, disorganized fibrils. One possibility is that this small amount of LTBP1 incorporation may be due to low levels of residual fibronectin remaining in the stripped fetal calf serum used for culturing. However, by using two independent antibodies that recognize plasma fibronectin, we have not detected any incorporation of
plasma fibronectin into the ECM of these cells by immunofluorescence. This suggests that a very limited amount of LTBP1 assembly can occur via an alternative pathway that does not require fibronectin.

In rescue experiments using a panel of fibronectin fragments, only fragments that were capable of assembly into fibronectin fibrils were able to rescue LTBP1 incorporation in FN-null cells. This suggests either that the critical event for LTBP1 assembly is the formation of a fibrillar fibronectin network and/or that multiple interacting sites on the fibronectin molecule are required for LTBP1 incorporation and that the individual fragments tested were lacking one or more of these sites. Future studies will therefore require the use of fibronectin constructs that contain all the necessary domains for assembly into fibrils but contain deletions in other internal domains (36). The ability of the 70-kDa fibronectin fragment to inhibit incorporation of LTBP1 in primary osteoblasts favors the hypothesis that fibronectin fibril assembly is the critical event, since this fragment works by blocking fibronectin self-interacting domains but does not inhibit binding of fibronectin to cell surface integrins. Furthermore, the 160-kDa fibronectin fragment that contains the RGD sequence and is known to stimulate integrin-mediated signaling was unable to rescue LTBP1 incorporation in FN-null cells, suggesting that integrin ligation alone is not the critical event for LTBP1 assembly.

Similar to LTBP1, we have observed a time-dependent co-localization between fibrillin-1 and fibronectin, with initial deposition in association with fibronectin, followed by localization in separate fibrillar networks. We have also found that fibrillin-1 assembly was disrupted by inhibition of fibronectin assembly. However, fibrillin-1 showed a somewhat greater capacity for assembly in the absence of fibronectin compared with LTBP1. Together, these data show that fibronectin is a critical regulator of assembly for at least two members of the fibrillin superfamily and that these microfibrillar proteins may have a limited capacity for self-assembly in living cell systems in the absence of fibronectin.

A number of studies have shown that fibronectin acts as an orchestrator for the assembly of other ECM proteins. For example, assembly of type I and III collagen is impaired in FN-null fibroblasts (21, 23). Although collagens are clearly capable of self-assembly in various cell-free systems, these studies suggest that additional levels of control may be exerted in living cell systems. In addition to reduced LTBP1 and fibrillin-1 incorporation, we also observed that assembly of type I collagen was compromised in FN-null cells. However, the fact that LTBP1 assembly could be rescued in FN-null cells in the absence of ascorbic acid (i.e. in the absence of fibrillar collagen) suggests that the impairment of LTBP1 incorporation was not a secondary consequence of reduced collagen assembly.

At present, the mechanism by which fibronectin and LTBP1 interact is unclear. Solid phase binding assays performed in our laboratory by using purified LTBP1 and fibronectin fragments suggest that there is no direct binding interaction between LTBP1 and fibronectin and that the binding is indirect. Although Taipale et al. (6) reported a binding interaction between LTBP1 and fibronectin by ligand blotting, these studies were performed with conditioned media rather than purified LTBP1 and are therefore consistent with an indirect binding mechanism. Because it has been reported that LTBP1 can interact directly with fibrillin-1 (37) and because fibrillin-1 co-localizes with fibronectin in a manner similar to LTBP1, we examined whether fibrillin-1 may mediate LTBP1 binding to fibronectin. By overexpressing LTBP1 in UMR-106 cells that lack endogenous LTBP1 or fibrillin-1, we showed that fibrillin-1 was not required for LTBP1 incorporation in association with fibronectin. Our current data suggest that heparan sulfate proteoglycans may be responsible.

In primary osteoblasts, human fibroblasts, and osteoblastic cell lines, we have consistently observed that LTBP1 initially co-localizes with fibronectin but later is localized in a separate fibrillar network. In accordance with this, we have also reported that LTBP1 and fibronectin show a partial overlap in distribution in periosteal osteoblasts in vivo (18). At present, the mechanism for the change in co-distribution patterns is unknown. One possibility is that LTBP1 is laid down in association with fibronectin as part of an initial phase of ECM assembly designed to rapidly assemble a supportive "tempo-rary ECM." However, this ECM is later remodeled to produce a more highly organized ECM containing specific fibrillar structures. This could be achieved either by breakdown and reassembly of the original ECM or by an active cell-mediated reorganization of the original ECM. Recent studies (23) have shown that the continual assembly of fibronectin into the ECM is required to maintain stability of ECM components, such as type I collagen and thrombospondin, suggesting that the ECM is a highly dynamic structure, which may be constantly undergoing remodeling.

Consistent with the results of Sottile and Hocking (23), we have observed that in FN-null cells that were first rescued with fibronectin supplementation followed by withdrawal of fibronectin, there is a loss of fibronectin from the ECM. This suggests that the continual presence of fibronectin is required to maintain stability of the fibronectin fibrillar network. Although the LTBP1 fibrillar network appeared to be more stable and was not degraded upon withdrawal of fibronectin, withdrawal of fibronectin halted further LTBP1 assembly so that cultures treated with fibronectin on days 1–3 followed by fibronectin withdrawal for days 4–6 resembled cultures that had been fixed on day 3. In contrast, cultures in which fibronectin treatment was continued for days 4–6 continued to assemble more LTBP1 into the ECM. These data suggest that not only is fibronectin required for the initial assembly of LTBP1 into the ECM but that the continual presence of fibronectin is essential for LTBP1 assembly to continue.

3 S. L. Dallas, manuscript in preparation.
LTBP1 is a multifunctional regulator of TGFβ activity by facilitating secretion of latent TGFβ from the cell (5), targeting latent TGFβ to the ECM for storage (6, 7), providing a vehicle for release of the growth factor upon proteolytic cleavage (8, 9), and facilitating activation of latent TGFβ (10, 11). Our data showing a critical role for fibronectin in regulating LTBP1 incorporation therefore suggest a previously unknown function for fibronectin as a regulator of TGFβ via regulation of LTBP1 assembly. In support of this, we have shown reduced latent TGFβ1 incorporation into the ECM of FN-null fibroblasts together with a corresponding increase in latent TGFβ1 in the conditioned medium. Interestingly, Globus et al. (38) showed that antibodies to fibronectin induced apoptosis of osteoblasts and that this could be reversed by addition of TGFβ, suggesting...
an interaction between fibronectin and TGFβ in regulation of osteoblast function. The present findings showing that fibronectin is critical for LTBP1 and TGFβ assembly into the ECM of osteoblasts may provide a molecular explanation for such interactions between fibronectin and TGFβ in bone cells.

In summary, we have demonstrated a novel function for fibronectin in regulating the incorporation of LTBP1 and latent TGFβ into the ECM. Because LTBP's are major regulators of transforming growth factor-βs, these studies may have important implications for diseases in which TGFβ plays a pathogenic role, such as fibrotic diseases, cancers, osteoporosis, and arthritis. These studies will also have implications for current models of assembly of microfibrillar proteins.

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