Potential Role for Heparan Sulfate Proteoglycans in Regulation of Transforming Growth Factor-β (TGF-β) by Modulating Assembly of Latent TGF-β-binding Protein-1

Qian Chen, Pitchumani Sivakumar, Craig Barley, Donna M. Peters, Ronald R. Gomes, Mary C. Farach-Carson, and Sarah L. Dallas

From the Department of Oral Biology, School of Dentistry, University of Missouri at Kansas City, Kansas City, Missouri 64108, the Wellcome Trust Centre for Cell-Matrix Research, School of Biological Sciences, University of Manchester, Manchester M13 9PT, United Kingdom, the Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, Wisconsin 53706, the Department of Biological Sciences, University of Delaware, Newark, Delaware 19716, and the Department of Orthopedics & Rehabilitation, Pennsylvania State College of Medicine, Hershey, Pennsylvania 17033-0850

Latent transforming growth factor-β-binding proteins (LTBPs) are extracellular matrix (ECM) glycoproteins that play a major role in storage of latent TGF-β in the ECM and regulate its availability. We have previously identified fibronectin as a key molecule for incorporation of LTBPs and TGF-β into the ECM of osteoblasts and fibroblasts. Here we provide evidence that heparan sulfate proteoglycans may mediate binding between LTBPs and fibronectin. We have localized critical domains in the N terminus of LTBPs that are required for co-localization with fibronectin in osteoblast cultures and have identified heparin binding sites in the N terminus of LTBPs between residues 345 and 487. Solid-phase binding assays suggest that LTBPs do not bind directly to fibronectin but that the binding is indirect. Heparin coupled to bovine serum albumin (heparin-BSA) was able to mediate binding between fibronectin and LTBPs. Treatment of primary osteoblast cultures with heparin or heparin-BSA but not with chondroitin sulfate impaired LTBPs deposition onto fibronectin without inhibiting expression of LTBPs. Inhibition of LTBPs incorporation was accompanied by reduced incorporation of latent TGF-β into the ECM, with increased amounts of soluble latent TGF-β. Inhibition of attachment of glycosaminoglycans to the core proteins of proteoglycans by β-D-xylosides also reduced incorporation of LTBPs into the ECM. These studies suggest that heparan sulfate proteoglycans may play a critical role in regulating TGF-β availability by controlling the deposition of LTBPs into the ECM in association with fibronectin.

Interaction between growth factors and extracellular matrix (ECM) molecules may be a major mechanism for regulation of growth factor activity (1–3). The latent transforming growth factor-β-binding proteins (LTBs) are a family of ECM glycoproteins that are key regulators of transforming growth factor-β (TGF-βs) (4–6). These multifunctional growth factors have potent effects in multiple cell types and have been implicated in several human diseases, including cancer (reviewed in Ref. 7).

TGF-βs are produced by virtually all cells as one or more latent complexes, which must be activated in order for TGF-β to exert its biological activities (reviewed in Refs. 5 and 8). The major secreted forms of latent TGF-β are known as the small and large latent TGF-β complexes (6, 9, 10). The small latent complex consists of mature TGF-β, noncovalently bound to its latency-associated peptide. In the large latent TGF-β complex, TGF-β and its latency-associated peptide are associated with a third protein from the family of LTBPs, of which four members (LTBs 1–4) have been identified (reviewed in Refs. 4 and 6).

Several studies have shown that LTBPs are major regulators of TGF-β. LTBPs have been shown to facilitate secretion of latent TGF-β (10), to target latent TGF-β to ECM for storage (11, 12), and LTBPs cleavage may provide a mechanism for release of the latent TGF-β from ECM (13, 14). LTBPs may also be critical players in the activation of TGF-β by specific cell types (15, 16). Interestingly, LTBP3-null mice showed craniofacial malformations and developed osteosclerosis and osteoarthritis (17). The LTBP4-null mice showed pulmonary emphysema, cardiomyopathy, and colorectal cancer (18). In both cases, the phenotypes appear to be due to misregulation of TGF-β activity (17, 18).

LTBP1 shares homology with fibrillins, and it is now clear that the LTBPs 1–4 and fibrillins 1–3 constitute a superfamily of ECM proteins with overlapping and interacting functions (reviewed in Ref. 4). Similar to the fibrillins, 60–70% of the structure of LTBPs consists mainly of two types of cysteine-rich repeats. These include epidermal growth factor (EGF)-like six-cysteine repeats, similar to motifs found in the EGF precursor.

TGF-β, transforming growth factor-β; EGF, epidermal growth factor; HSPG, heparan sulfate proteoglycan; aa, amino acid(s); ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CS, chondroitin 6-sulfate.

© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
and eight-cysteine repeats that are unique to the LTBP1s and fibrillins. The LTBP1s contain 16–18 of the EGF-like repeats and 3–4 copies of the 8-cysteine repeats. The third 8-cysteine repeat of LTBP1 is critical for interaction with latent TGF-β (19, 20). A heparin binding consensus sequence (HRRRPITHHVGGK) is found in the “hinge” region of human LTBP1 at amino acids 414–425 (21).

At present, little is known concerning the mechanisms by which LTBP1 assembles into the ECM. Several studies suggest that the N terminus of LTBP1 contains a critical region for matrix binding (19, 22–24). A binding region in the C terminus was also identified (23). LTBP1 and fibrillin-1 co-localize in the matrix of early and late osteoblast cell cultures (22), and LTBP1 has been immunolocalized to fibrillin-containing microfibrils in the skin (25) and bone (11, 22) as well as to microfibrillar structures in the heart (26). LTBP1 also shows a time-dependent co-localization with fibronectin in osteoblast culture models, where co-localization is seen in early postconfluent cultures (1–3 days) but not in late cultures (14–21 days) (27).

Binding studies have demonstrated direct interactions between LTBP1 and fibrillins (28). However, by overexpressing LTBP1 in UMR-106 cells that lack endogenous LTBP1 or fibrillin-1, we previously showed that fibrillin-1 was not required for LTBP1 deposition into the ECM in association with fibronectin (27).

Fibronectin has been shown to be required for assembly of several matrix proteins, including type I collagen, thrombospondin, fibulin-1, and fibrinogen (29–31). Our recent studies have shown that fibronectin also plays a critical role for LTBP1 assembly into the ECM (27). However, the mechanism by which fibronectin regulates LTBP1 assembly is still unclear.

In these studies, we sought to determine whether LTBP1 binds directly to fibronectin and to localize the critical domains in LTBP1 that are required for LTBP1 deposition in association with fibronectin. Solid-phase binding assays suggested that fibronectin interacted with LTBP1 through an indirect mechanism. Because fibronectin contains heparin binding sites (32) and LTBP1 contains a consensus heparin binding site, and because fibrillins have been shown to be dependent on heparan sulfate proteoglycans (HSPGs) for their incorporation (33, 34), we examined the potential role of HSPGs in mediating interactions between LTBP1 and fibronectin.

**EXPERIMENTAL PROCEDURES**

Antibodies and Purified Proteins—Antibodies for LTBP1 included an affinity-purified rabbit polyclonal against rat LTBP1 generated in our laboratory (22) (for immunostaining this antibody recognizes rat and mouse LTBP1 but not human) and a rabbit polyclonal against human LTBP1 (Ab39) that cross-reacts with multiple species (a kind gift of Dr. C. H. Hedin, Ludwig Institute for Cancer Research, Uppsala, Sweden) (10). A monoclonal antibody against human LTBP1 was also used (R&D Systems, Minneapolis, MN). Our laboratory has also recently developed a rabbit polyclonal antibody (Ab-K) against a recombinant human LTBP1 fragment (aa 526–1014) (Fig. 1A). This antibody recognizes LTBP1 in human, mouse, and rat cells. Antibodies for fibronectin included a commercially available mouse monoclonal against the ED-A domain (Sigma) that cross-reacts with human, mouse, and rat fibronectin and a rabbit polyclonal antibody that also recognizes human, mouse, and rat fibronectin (Sigma). For detection of His-tagged recombinant LTBP1 fragments, an anti-His monoclonal antibody was used (EMD Biosciences, Madison, WI).

Human plasma fibronectin and N-terminal 70-kDa proteolytic fragment were obtained from commercial sources (Invitrogen or Sigma). The 160-kDa fibronectin fragment was prepared as described previously (35). The H120 fibronectin fragment, consisting of type III repeats 12–15 (36), was provided by Dr. Martin Humphries (University of Manchester, United Kingdom) (Fig. 4C shows a schematic diagram of these fibronectin fragments). HSPG (Perlecan) was purchased from Sigma, and purified perlecan domain I was produced as described elsewhere (37).

Cell Culture—Tissue culture reagents were purchased from Invitrogen or Mediatech Inc. (Herndon, VA). 293-EBNA cells were purchased from the American Type Tissue Culture Collection (Manassas, VA) and were routinely maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 2 mm l-glutamine, 100 units/ml penicillin/streptomycin, 250 μg/ml G418. 2T3 cells were a kind gift of Dr. S. E. Harris (University of Texas Health Science Center at San Antonio, TX). These cells were maintained as described previously (38). Primary cultures of fetal rat calvarial osteoblasts (FRCs) were isolated and maintained as described previously (11). TMLC-C32 cells were a kind gift of Dr. D. B. Rifkin (New York University, New York, NY) and were cultured as described previously (11, 39).

Expression and Purification of Recombinant LTBP1 Peptides—For expression and purification of recombinant LTBP1 peptides, cDNA sequences corresponding to the desired recombinant LTBP1 fragments were generated by PCR amplification using Vent DNA polymerase, a high fidelity DNA polymerase, according to the manufacturer’s instructions (New England Biolabs, Beverly, MA). The template was a human LTBP1 cDNA in the vector pSV7d (10) (kindly donated by Dr. K. Miyazono, University of Tokyo, Tokyo, Japan). A 6- or 10-histidine epitope tag was engineered into the primers at the C terminus of the recombinant LTBP1 fragments, followed by a stop codon and XhoI restriction site. An NheI restriction site was engineered at the N terminus.

Schematic diagrams of the LTBP1 constructs generated are shown in Figs. 1A and 3A. The primer sets used for amplification of these specific fragments are presented in supplemental Table S1. The PCR products were digested with NheI and XhoI and ligated into the pCEP-Pu expression vector (a kind gift of E. Kohfeldt, Max Planck Institut of Biochemistry, Martinsried, Germany) in-frame with the BM40 signal sequence (40, 41). The sequences of the inserts were confirmed by automated sequencing (MWG Co., High Point, NC). The constructs were then transfected into 293-EBNA cells using the Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen).

Transfected cells were selected in puromycin (1 μg/ml), and resistant cells were expanded into triple layer flasks. Recombinant fragments were purified from 2–5 liters of serum-free conditioned media using a nickel- nitritoltriacid agarose col-
Role for HSPG in LTBP1 Assembly

utm according to the manufacturer’s instructions (Qiagen). Bound proteins were eluted either with low pH or with 100–300 mM imidazole. If further purification was required, a mono-Q ion exchange column was used in conjunction with a Bio Cad 700E protein purification system (Applied Biosystems, Foster City, CA). Bound proteins were eluted with a linear 0–1 M NaCl gradient. Coomassie Blue staining was used to visualize the purity of the fragments, and mass spectrometry/peptide mass mapping was used to validate the recombinant LTBP1 fragments. Using this approach, we have obtained 1–2 mg of most of the recombinant LTBP1 fragments (see Figs. 1C and 3B, Coomassie Blue staining).

**2T3/293-EBNA Co-culture System and Transfection of LTBP1 Constructs**—A 2T3/293-EBNA co-culture system was used for transient in vitro expression of human LTBP1 constructs to determine their ability to deposit into the ECM in association with fibronectin. This co-culture system combines the advantages of the 293-EBNA cells, which express high amounts of recombinant proteins but do not produce an extensive extracellular matrix, with the 2T3 calvarial osteoblast cell line, which has a low transfection efficiency but produces an extensive ECM. 2T3 cells at 4 × 10^4 cells/ml and 293-EBNA cells at 4 × 10^5 cells/ml were plated together into eight-chamber Lab-Tek chamber slides in Dulbecco’s modified Eagle’s medium supplemented as described above (0.5 ml per well). 24 h later, the LTBP1 constructs were transfected into the coculture system using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were cultured for 3 more days, and then fixed in 95% ethanol. Double immunofluorescent staining was performed as described previously (22). Recombinant LTBP1 expression was detected with anti-His monoclonal antibody. Fibronectin expression was detected using the rabbit anti-fibronectin antibody (Sigma). Appropriate combinations of Cy3-conjugated detection antibodies or biotinylated antibodies followed by streptavidin-fluorescein isothiocyanate (Jackson ImmunoResearch, West Grove, PA) were used for detection as described previously (22). The slides were viewed and photographed digitally using a Nikon E800 fluorescence microscope with cooled charge-coupled device camera driven by the AnalySIS software (Soft Imaging Systems Corp., Lakewood, CO).

**Incubation of 2T3 Cultures with Recombinant LTBP1 Fragments**—In parallel to the co-culture transfection experiments above, 2T3 cells were also incubated with purified recombinant LTBP1 fragments (10 μg/ml) for 3 days. Cells were then fixed in 95% ethanol. Incorporation of LTBP1 fragments and co-localization of LTBP1 and fibronectin were determined by double immunofluorescence staining as described above.

**Heparin Sepharose Affinity Chromatography**—Affinity chromatography was performed using heparin-Sepharose columns (HiTrap Heparin HP, 1 ml, Amersham Biosciences). The columns were equilibrated in 20 mM Tris-HCl, pH 7.4. Purified recombinant LTBP1 fragments (20–40 μg) were applied manually to the columns in equilibration buffer at an approximate flow rate of 0.1 ml/min, according to the manufacturer’s instructions. After washing the columns with equilibration buffer, bound material was eluted with 1 M NaCl. The eluted volumes were fractionated into 0.5-ml aliquots. The amounts of recombinant LTBP1 fragments in each fraction were determined by ELISA, and the protein was verified by SDS-PAGE and/or Coomassie Blue staining.

**Solid-phase Binding Assays**—Solid-phase binding assays to measure binding of LTBP1 fragments to fibronectin or fibronectin fragments were performed on 96-well ELISA high binding plates (Corning Costar, Acton, MA). Plasma fibronectin, superfibronectin, or fibronectin fragments (100 nM) (see Fig. 4C), diluted in phosphate-buffered saline (PBS) containing 2 mM calcium, were immobilized on high binding ELISA plates overnight at 4 °C. All subsequent steps were performed at room temperature. The wells were washed three times with PBS-T washing buffer (PBS containing 0.05% Tween 20). Blocking of nonspecific binding sites was performed by incubating for 1–2 h with 200 μl of binding buffer (PBS, pH 7.4, plus 5% nonfat dry milk and either 2 mM CaCl₂ or 10 mM EDTA). The wells were incubated for 2 h with recombinant LTBP1 fragments at a starting concentration of 100 or 150 nM, then diluted serially 1:2 in binding buffer (100 μl per well). The plates were then washed three times with washing buffer, and the wells were incubated for 1 h with 100 μl of mouse anti-His monoclonal antibody (1:1000, diluted in binding buffer). After washing three times, the wells were incubated with 100 μl of donkey anti-mouse horseradish peroxidase conjugate (1:1000 diluted in binding buffer, Jackson ImmunoResearch). After washing, the plates were reacted with O-phenylenediamine using 4-μg substrate tablets, according to the manufacturer’s instructions (Sigma). The plates were read on an ELX800 plate reader (Biotek Instruments Inc., Winoski, VT) at 490 nm after treatment with 25 μl of 3 M HCl. Background subtraction was performed using a blank control that was coated with 100 nM bovine serum albumin (BSA) in place of the fibronectin or fibronectin fragments. For solid-phase binding assays to examine binding of LTBP1 fragments to heparin, heparin coupled to BSA was used (Sigma). 100 nM heparin-BSA in PBS, pH 7.4, was coated onto plates overnight at 4 °C. All subsequent steps were performed at room temperature as described above.

**Effects of Heparin and β-D-Xylosides on LTBP1 Deposition in the ECM**—FRC cells were seeded at 2 × 10⁴ cells/cm² of growth area in 8-chamber Lab-Tek slides or 6-well plates (Nalge Nunc International, Rochester, NY) in the presence of heparin (0.1–0.5 mg/ml), chondroitin 6-sulfate (CS, 0.1–0.5 mg/ml), heparin-BSA (0.01–0.1 mg/ml), 4-methylumbelliferol-β-D-xylopyranoside (0.1 mM), or p-nitrophenyl-β-D-xylopyranoside (0.2 mM). After overnight adherence, the cells were washed twice with PBS and then incubated with culture medium containing treatments for 3–6 days. Media was changed every 3 days. Cells in Lab-Tek slides were immunostained for LTBP1 and fibronectin as described above.

To determine the relative amounts of secreted LTBP1 in the conditioned media, the cultures were changed to serum-free culture media (plus treatments) on day 5. After a further 24 h, the conditioned medium was harvested and concentrated 10- to 20-fold using a Centricon membrane (30-kDa cut-off). To test the LTBP1 levels in the ECM, plasmin digestions of detergent-insoluble matrix pellets were performed as described previously (11, 22). Both the conditioned media and plasmin-di-
Role for HSPG in LTBP1 Assembly

**Fig. 1.** Expression and purification of recombinant LTBP1 fragments. A, a schematic diagram showing the domain structure of full-length LTBP1 and the various recombinant LTBP1 fragments that were generated. Black rectangles, 4-cysteine repeat; black oval, 8-cysteine repeats (also known as "TB-motifs"); latent transforming growth factor-β-binding protein-like); gray rectangles, EGF-like repeats; open rectangles, EGF-like repeats with consensus for calcium binding; shaded rectangles, proline-rich "hinge" region. The antibodies used for detection of the various constructs are shown at the top. B, Western blotting using anti-His antibodies showing expression of the constructs in the conditioned media of stably transfected 293-EBNA cells. C, Coomassie Blue-stained gels showing the purified recombinant LTBP1 fragments. Molecular mass markers (kilodaltons) are indicated on the left of the gel images.

Full-length recombinant LTBP1 was produced, as well as a panel of four overlapping recombinant LTBP1 fragments that spanned the complete molecule (Fig. 1A). These included an N-terminal fragment (aa 21–487), a fragment spanning the proline-rich hinge domain, termed the LTBP1-hinge construct (aa 413–545), a fragment consisting of 11 consecutive EGF-like domains, termed the "EGF" construct (aa 526–1014), and a C-terminal fragment (aa 1008–1394). Fig. 1A also indicates the effect of heparin or CS treatment on TGF-β activity of osteoblasts, a co-culture approach was used in which the TMLC-C32 reporter cells were co-cultured with the FRC cells (modified from previous studies (16, 43)). FRC cells were plated into 96-well plates at 8 × 10^3 cells/well. The cells were then treated with or without 0.5 mg/ml heparin or CS 16 h later. TMLC-C32 cells (1.5 × 10^5 cells/well) were then added to the 96-well plates 2 days later in media containing heparin or CS treatments as before. After a further 24 h, TGF-β activity was assessed by measuring luciferase activity in the cell lysates from co-cultures (14, 16). A duplicate 96-well plate was used for cell counting to normalize data to cell number. To exclude the possibility that heparin or CS treatment might interfere with the reporter cells, controls were performed in which TMLC-C32 cells were treated with different doses of heparin or CS to confirm that there was no effect on luciferase.

**RESULTS**

Localization of Domains in LTBP1 Important for ECM Deposition in Association with Fibronectin

Full-length recombinant LTBP1 was produced, as well as a panel of four overlapping recombinant LTBP1 fragments that spanned the complete molecule (Fig. 1A). These included an N-terminal fragment (aa 21–487), a fragment spanning the proline-rich hinge domain, termed the LTBP1-hinge construct (aa 413–545), a fragment consisting of 11 consecutive EGF-like domains, termed the "EGF" construct (aa 526–1014), and a C-terminal fragment (aa 1008–1394). Fig. 1A also indicates the effect of heparin or CS treatment on TGF-β activity of osteoblasts, a co-culture approach was used in which the TMLC-C32 reporter cells were co-cultured with the FRC cells (modified from previous studies (16, 43)). FRC cells were plated into 96-well plates at 8 × 10^3 cells/well. The cells were then treated with or without 0.5 mg/ml heparin or CS 16 h later. TMLC-C32 cells (1.5 × 10^5 cells/well) were then added to the 96-well plates 2 days later in media containing heparin or CS treatments as before. After a further 24 h, TGF-β activity was assessed by measuring luciferase activity in the cell lysates from co-cultures (14, 16). A duplicate 96-well plate was used for cell counting to normalize data to cell number. To exclude the possibility that heparin or CS treatment might interfere with the reporter cells, controls were performed in which TMLC-C32 cells were treated with different doses of heparin or CS to confirm that there was no effect on luciferase.
Role for HSPG in LTBP1 Assembly

A)

| EV  | Full Length | N-term | Hinge | EGF  | C-term |
|-----|-------------|--------|-------|------|--------|
| anti-His |             |        |       |      |        |
| anti-FN  |             |        |       |      |        |
| merge    |             |        |       |      |        |

B)

Western: Anti-His

C)

| Control | Full Length | N-term | Hinge | EGF  | C-term |
|---------|-------------|--------|-------|------|--------|
| anti-His |             |        |       |      |        |
| anti-FN  |             |        |       |      |        |
| merge    |             |        |       |      |        |
and was co-localized with fibronectin fibrils (Fig. 2A). These data suggest that amino acids 21–487 are sufficient for co-distribution of LTBP1 and fibronectin. Some association of the C-terminal LTBP1 construct to the ECM was also observed. However, unlike the N-terminal construct, the C-terminal fragment did not appear to co-localize well with fibronectin. The EGF and hinge constructs did not incorporate into the ECM.

Western blotting of conditioned media confirmed that the hinge-LTBP1, EGF-LTBP1, and C-terminal LTBP1 constructs were efficiently secreted into the media of the co-cultures (Fig. 2B), suggesting that the lack of incorporation into the ECM was not due to differences in expression of the various constructs. Only small or undetectable amounts of full-length LTBP1 and N-terminal-LTBP1 were found in the conditioned media, suggesting that these fragments were mainly assembled into the matrix after secretion.

Results from transient transfection experiments were confirmed by incubating 2T3 cultures with the purified recombinant LTBP1 fragments (Fig. 2C). In agreement with the transient transfection studies, full-length and N-terminal fragments were efficiently incorporated into the matrix in association with fibronectin. Hinge-LTBP1-, EGF-LTBP1-, and C-terminal-LTBP1-purified fragments failed to assemble into the ECM. In both the transient transfection assays and the recombination peptide incubation assays, antibodies internal to the LTBP1 sequence were also used to confirm results using anti-His (data not shown).

Based on the results of the above ECM deposition assays, a series of shorter N-terminal constructs was generated to further define the critical regions in the N terminus of LTBP1 that were important for co-localization with fibronectin (Fig. 3A). Constructs N4 (aa 205–487), N5 (aa 299–487), and N9 (aa 345–487) were expressed efficiently and successfully purified (Fig. 3B). Constructs N6 (aa 67–343) and N7 (aa 21–299) only expressed at low levels that were detectable by Western blotting (Fig. 3B) but did not provide sufficient protein for purification.

Transient transfection experiments were performed using these smaller N-terminal constructs in 2T3/293-EBNA co-cultures as described above. Double immunostaining using anti-His and anti-fibronectin antibodies showed that N4-LTBP1, N5-LTBP1, and N7-LTBP1 were able to incorporate into the ECM in association with fibronectin (Fig. 3C). Although construct N6 was expressed at very low levels (see Fig. 3B), this construct did show a few sparse ECM fibrils, suggesting ECM incorporation in association with fibronectin (Fig. 3C, arrowheads). In contrast, N9-LTBP1 failed to assemble into ECM fibrils, even though cells expressing the construct could be identified (Fig. 3C) and the construct was efficiently expressed and secreted (Fig. 3B). Together, these data suggest that there are two separate sites that can specify co-localization with fibronectin. One is between aa 67–299 and the second is between aa 299 and 487.

**Solid-phase Binding Assays Suggest an Indirect Binding Mechanism between LTBP1 and Fibronectin**—Solid-phase binding assays were performed to determine whether there is a direct binding interaction between LTBP1 and fibronectin and to localize the important domains for binding. These assays were performed using plasma fibronectin (30 nM) immobilized on 96-well plates and then incubating with full-length LTBP1 and recombinant LTBP1 fragments. Binding of the recombinant LTBP1 fragments to fibronectin was monitored using anti-His antibodies and confirmed using appropriate internal LTBP1 antibodies. Surprisingly, neither full-length LTBP1 nor any of the purified recombinant LTBP1 fragments showed direct binding with immobilized fibronectin (see Fig. 4A). To confirm the lack of direct binding between LTBP1 and fibronectin, solid-phase assays were also performed in reverse by immobilizing full-length and N-terminal LTBP1 onto the ELISA plate and using soluble fibronectin as the ligand. Again, no direct binding was observed (see supplemental Fig. S1). Two potential explanations for this are (i) that interactions between LTBP1 and fibronectin are indirect and one or more additional ECM components are required or (ii) that the binding sites for LTBP1 in fibronectin are not exposed in the globular dimeric plasma form of fibronectin. To exclude the second possibility, solid-phase binding assays were performed using monomeric 70-kDa, H120, and 160-kDa proteolytic fragments of fibronectin or using superfibronectin in which the addition of a recombinant III-C fibronectin fragment to soluble fibronectin exposes more of the fibronectin self-interacting domains, resulting in formation of high molecular weight “superfibronectin” aggregates (44) (see Fig. 4C for a diagram of the fibronectin fragments). In these experiments, no direct binding was observed between LTBP1 or any of the LTBP1 fragments and fibronectin fragments or superfibronectin (data not shown). As a positive control, solid-phase binding assays were performed using perlecan as the ligand, because perlecan is known to bind to fibronectin (Fig. 4B). Perlecan was found to bind with immobilized fibronectin but not with N-terminal LTBP1 or with BSA.

**Identification of Heparin Binding Sites in LTBP1**—A recent study by Tiedemann and co-workers (33) showed that HSPGs play an important role in the assembly of fibrillin-1 into the ECM. Interestingly, the proline-rich hinge region of LTBP1 contains a consensus heparin binding site, and fibronectin is also known to contain at least two heparin-binding sites. We therefore hypothesized that HSPGs may mediate binding.

![Figure 2](image_url)
Role for HSPG in LTBP1 Assembly

A) N-term LTBP1 (21-487)
N4-LTBP1 (205-487)
N5-LTBP1 (299-487)
N6-LTBP1 (67-343)
N7-LTBP1 (21-299)
N9-LTBP1 (345-487)

B) Western: anti-His

C) EV  N-term  N4  N5  N6  N7  N9
  anti-His  anti-His  anti-His  anti-His  anti-His  anti-His  anti-His
  anti-FN  anti-FN  anti-FN  anti-FN  anti-FN  anti-FN  anti-FN
  anti-FN  anti-FN  anti-FN  anti-FN  anti-FN  anti-FN  anti-FN
Role for HSPG in LTBP1 Assembly

between LTBP1 and fibronectin. To address this, solid-phase binding assays were performed to determine whether recombinant LTBP1 fragments bound to heparin. Because plastic surfaces cannot readily be coated with soluble heparin due to its high negative charge, heparin coupled to BSA was used as described by Tiedemann and co-workers (33). Heparin-BSA is thought to mimic the clustered glycosaminoglycan chains of heparan sulfate containing proteoglycans.

Solid-phase binding assays showed that full-length LTBP1 as well as the larger N-terminal LTBP1 construct and constructs N4, N5, and N9 bound to heparin-BSA (Fig. 5A). No binding was seen in controls in which the ELISA plates were coated with BSA alone. In contrast to the N-terminal construct, the hinge-LTBP1, EGF-LTBP1, and C-terminal-LTBP1 constructs showed no binding to heparin-BSA. The binding of N-terminal LTBP1 to heparin-BSA was unaffected by the presence of 10 mM EDTA, suggesting that the interaction is calcium-independent (data not shown). Together, these data suggest the presence of a heparin binding site between aa 345 and 487. Constructs N6-LTBP1 and N7-LTBP1 were expressed at levels too low for purification. Therefore, it was not possible to confirm whether additional heparin binding sites may be present between aa 21 and 345.

Heparin binding of constructs N-LTBP1, N4-LTBP1, N5-LTBP1, and N9-LTBP1 was confirmed by heparin-Sepharose chromatography (Fig. 5B). Consistent with findings from the solid-phase binding assays, the other constructs tested, including hinge-LTBP1, EGF-LTBP1, and C-LTBP1, showed no binding to heparin-Sepharose (data not shown).

Heparin-BSA Can Mediate Binding between N-terminal LTBP1 and Fibronectin—To further test the hypothesis that heparan sulfate-containing proteoglycans may mediate binding interactions between fibronectin and LTBP1, a modified solid-phase binding assay was performed. High binding ELISA plates were first coated with plasma fibronectin, superfibronectin, or a 70-kDa fibronectin N-terminal fragment. Nonspecific binding was blocked using 5% nonfat dry milk. The plates were then incubated with heparin-BSA or BSA alone and blocked again. Next, the purified N-terminal LTBP1 fragment (aa 21–487) was incubated and the amount of bound N-terminal fragment was detected by anti-His monoclonal antibody.

As observed previously, no direct binding between the N-terminal LTBP1 fragment and any of the fibronectin fragments was observed in the presence of BSA alone (Fig. 6). However, when the fibronectin-coated plates were preincubated with heparin-BSA, the N-terminal LTBP1 fragment was found to bind to both plasma fibronectin and superfibronectin. Heparin-BSA did not appear to mediate binding between N-terminal LTBP1 and an N-terminal 70-kDa fragment of fibronectin, suggesting that the Hep I domain in the N terminus of fibronectin may not be the critical heparin binding site for interactions between LTBP1 and fibronectin.

FIGURE 3. The N terminus of LTBP1 contains two binding sites that specify co-localization with fibronectin. A, schematic diagram showing the N-terminal LTBP1 construct and the shorter N-terminal constructs that were generated within this N-terminal region (see Fig. 1 legend for key to symbols). B, Western blot using anti-His antibodies and Coomassie Blue staining showing expression and purification of the recombinant LTBP1 fragments. Molecular mass markers (kilodaltons) are indicated on the left of the gel images. C, immunostaining from co-cultures of 2T3 cells and 293-EBNA cells transiently transfected with N-terminal LTBP1 constructs or empty vector (EV) as a control. The top panel (red) shows staining for the recombinant LTBP1 constructs with anti-His antibodies, the middle panel (green) shows staining with anti-fibronectin, and the lower panel shows the merged images. Arrowheads indicate sparse fibrils observed with the N6 construct. Bar = 20 μm.

FIGURE 4. Solid-phase binding assays suggest no direct binding of full-length LTBP1 or LTBP1 fragments with fibronectin. A, solid-phase binding assay to determine the binding of full-length LTBP1 or LTBP1 fragments to immobilized fibronectin. Detection of the bound LTBP1 fragments was performed using anti-His monoclonal antibody followed by a peroxidase-conjugated detection antibody reacted with O-phenylenediamine substrate. B, solid-phase binding assay to show the binding of perlecan to immobilized fibronectin but not N-terminal LTBP1 or a BSA control. Data in A and B are expressed as the absorbance at 490 nm (mean from duplicate determinations). C, schematic diagram showing the modular structure of a monomer of plasma fibronectin. Open rectangles, type I repeats; black ovals, type II repeats; gray rectangles, type III repeats; V, variable region; A, ED-A domain; and B, ED-B domain. Various fibronectin fragments that were used in the present study are indicated below, and the location of known heparin binding sites is indicated.
Heparin and Heparin-BSA but Not Chondroitin 6-Sulfate Impairs LTBP1 Incorporation into the ECM—Because the N-terminal LTBP1 fragment appears to contain a heparin binding site and because heparin-BSA was able to mediate binding interactions between LTBP1 and fibronectin, we next determined the effect of soluble heparin or heparin-BSA on LTBP1 assembly in FRC cultures. We hypothesized that exogenous heparin would prevent LTBP1 incorporation into the ECM by competing for binding to heparin binding sites, thereby preventing association of LTBP1 with HSPG in the ECM.

Treatment of FRC cells with heparin at 0.1–1 mg/ml dose-dependently inhibited LTBP1 incorporation with only a small apparent effect on fibronectin assembly. In contrast, 0.1–1.0 mg/ml CS, used as a control, had no effect. Heparin-BSA was ∼10-fold more potent than heparin, with inhibitory effects between 0.05 and 0.1 mg/ml. Fig. 7A shows immunostaining results from heparin and CS treatment at 0.5 mg/ml and heparin-BSA at 0.1 mg/ml. Western blotting confirmed the reduction of LTBP1 incorporation into the ECM in heparin-treated cultures compared with untreated or CS-treated controls (for representative gel, see Fig. 7B). In plasmin-treated matrix extracts, bands corresponding to the expected size for the plasmin-cleaved fragment of free LTBP1 and LTBP1 complexed to TGF-β (i.e. large latent complex) were observed, whereas only a band corresponding to free LTBP1 was observed in the conditioned media. Specificity of these bands was confirmed by preincubating the antibody with recombinant EGF-LTBP1 (data not shown). Densitometric quantitation of 4 samples from pooled experiments showed that there was a reduction of ∼65% in the amount of LTBP1 in the ECM in heparin-treated cultures compared with either untreated or CS-treated controls (Fig. 7C, left panel). The effect of heparin was not due to impaired synthesis or secretion of LTBP1, because there was no reduction in the amount of LTBP1 secreted into the culture media (Fig. 7, B and C). In fact, there was actually a small increase in the amount of LTBP1 in both heparin-treated and CS-treated cultures compared with untreated controls (Fig. 7C, middle panel), but this only reached significance for the CS-treated group. Although the bands for the large latent TGF-β complex were much less intense than those for free LTBP1, densitometric quantitation also suggested a similar reduction in the matrix-bound large latent complex (Fig. 7C, right panel).
β-D-Xylosides Inhibit LTBP1 Incorporation into the ECM

To further support the hypothesis that HSPGs may mediate or nucleate LTBP1 assembly into the ECM, primary fetal rat calvarial osteoblasts were cultured in the presence of β-D-xylosides. These reagents are chemical analogues of xylose, the first monosaccharide in glycosaminoglycan biosynthesis that is covalently attached to a serine residue in the core protein of proteoglycans. Thus, β-D-xylosides reduce or abolish the attachment of glycosaminoglycans to proteoglycans by competing with the authentic substrates (33, 45). Treatment of FRC cells with 0.1 mM 4-methylumbelliferyl-β-D-xylopyranoside or 0.2 mM p-nitrophenyl-β-D-xylopyranoside for 6 days was found to inhibit assembly of LTBP1 into the ECM, as shown by immunofluorescent staining and quantitation of the fluorescence intensity (see Fig. 8, A and B).

Heparin but Not Chondroitin 6-Sulfate Inhibits TGF-β Storage in the ECM—LTBP1 is a major regulator of TGF-β storage and availability (11–16). Because HSPGs appear to play an important role in LTBP1 assembly into matrix, we next determined whether they may also play a role in regulation of TGF-β storage in the ECM and/or TGF-β availability.

Treatment of fetal rat calvarial cells with 0.5 mg/ml heparin, a dose that efficiently disrupts LTBP1 incorporation into the ECM, resulted in a 70% reduction in the total amount of TGF-β stored in the ECM compared with untreated or CS-treated controls (see Fig. 9A). There was no significant effect of heparin or CS treatment on secretion of latent TGF-β into the media (Fig. 9A). Total TGF-β (combined matrix and media values) were also not significantly different (data not shown).

Active TGF-β was undetectable using this commercial ELISA (detection limit = 30 pg/ml). Therefore, TGF-β activity in the culture supernatant was also measured as described previously (14, 16) using the mink lung epithelial cell luciferase bioassay. Initial experiments again suggested that active TGF-β was undetectable in the conditioned media of FRC cells treated with heparin or CS (data not shown). However, because active TGF-β can rapidly be lost from solution due to binding to culture plastics and/or to serum or other proteins, we also performed experiments in which the mink lung epithelial indicator cells (TMLC-C32) were co-cultured with FRC cells. As shown in Fig. 9B, using this more sensitive co-culture assay system, heparin treatment at 0.5 mg/ml was associated with a 50% increase in the amount of active TGF-β in the culture supernatant. In contrast, CS had no effect (Fig. 9B).

DISCUSSION

We have previously shown that fibronectin is a key regulator of LTBP1 deposition into the ECM of osteoblasts and fibroblasts and thereby regulates TGF-β (27). However, how fibronectin regulates LTBP1 and TGF-β incorporation into the ECM remains unclear. This study has investigated the nature of the association between LTBP1 and fibronectin in osteoblast culture systems. The main findings are: (i) specific domains in the N terminus of LTBP1 appear to be responsible for co-localization of LTBP1 with fibronectin, (ii) the interaction of LTBP1 with fibronectin appears to be indirect and may be mediated by heparan sulfate-containing proteoglycans, and (iii) treatment of primary osteoblasts with heparin or with inhibitors of GAG chain addition impairs ECM incorporation of both LTBP1 and TGF-β. Together, these data suggest that HSPGs may play an important role in regulation of TGF-β through regulation of ECM deposition of LTBP1.

Data on the C-terminal ECM binding domains in LTBP1 fragments, showed that overlapping constructs N6 (aa 67–343) and N7 (aa 21–299) were deposited into the ECM in association with fibronectin, suggesting that a binding domain exists between residues 67 and 299. Construct N5 (aa 299–487), which does not overlap with N7, also bound to the ECM in association with fibronectin, suggesting a second binding site between residues 299 and 487. These observations are consistent with the results of Unsold et al. (23) and further refine the binding sites they reported in the N terminus between residues 20 and 299 and between 300 and 545. Both studies are also consistent with the findings of Nunes et al. (24), who reported that residues 294–441 in the N terminus are critical for transglutaminase-dependent matrix incorporation of LTBP1S.

Data on the C-terminal ECM binding domains in LTBP1 appear to be more variable. Unsold et al. (23) identified a matrix binding site in the C terminus of LTBP1 (aa 1139–1394) that appeared to co-localize with fibronectin in fibroblasts. Here, we report a matrix binding site in the C terminus (aa 1008–1394). However, this fragment did not show robust co-localization with fibronectin in osteoblasts. One possible explanation for these apparently contradictory findings comes from the recent report that a C-terminal fragment (aa 1181–1394) of LTBP1 interacts with fibrillin-1 (28). Thus, it seems likely that the C-terminal fragment may deposit into the ECM in association with fibrillin. This would mean that the ability of recombinant C-terminal LTBP1 fragments to associate with the ECM in different culture systems may be variable, depending on whether fibrillin is present. Furthermore, the ability of the C-terminal construct to co-localize with fibronecin may depend on the stage of culture, because fibrillin shows a time-dependent co-
localization with fibronectin in fibroblast and osteoblast cultures. Two other studies have shown that longer C-terminal fragments of LTBP1 that only lack the N-terminal 527 amino acids fail to assemble into the ECM (19, 22). Thus, in longer forms of the LTBP1 molecule the C-terminal binding sites may not be accessible. A likely interpretation of all these observations may be that intact LTBP1 first binds to the ECM via its N terminus and that this then induces a conformational change in the molecule that exposes binding sites for fibrillin in the C terminus.

Surprisingly, solid-phase binding assays using full-length LTBP1 as well as purified LTBP1 fragments spanning the entire molecule showed no direct binding activity between LTBP1 and fibronectin. In contrast, Fontana and co-workers (46) reported that in ELISA or blot overlay assays, a purified LTBP1 peptide (rL1N, aa 21–629) bound directly to fibronectin. The reason for the apparent discrepancy between these two studies is unclear. Because their fragment (aa 21–629) is longer than the N-LTBP1 fragment (aa 21–487) used in the present study, one possibility may be that additional sequences between residues 487 and 629 may be required for interaction. Although Fontana et al. did not examine binding of full-length LTBP1, our data suggest no binding of the full-length molecule to fibronectin. It may therefore be that the folded structure of the rL1N, LTBP1 21–629 fragment may be different from full-length LTBP1, such that the binding properties of the fragment are also different. If so, an implication of this may be that selective proteolytic cleavage of LTBP1 could alter the binding properties of the molecule, which might add an additional level of complexity to the regulation of TGF-β.

Our findings suggest that HSPGs may mediate binding interactions between fibronectin and LTBP1. An N-terminal LTBP1 fragment (aa 21–487) was found to bind to heparin. The heparin binding domain was further localized to between aa 345 and 487. However, we cannot exclude that one or more additional heparin binding sites may be present between aa 21 and 345, because all the constructs that were generated spanning this region either failed to express or expressed at very low levels that precluded purification. This failure in protein expression seemed to be a common feature of all the N-terminal constructs that lacked sequences between aa 412 and 487 suggesting that

3 S. L. Dallas, unpublished observations.
these sequences may be important for protein expression/folding and/or secretion. A surprising finding was that the LTBP1 hinge construct (aa 413–454), by itself, was unable to bind to heparin, even though this construct does include a consensus heparin binding sequence at aa 414–425. This construct also failed to assemble into the ECM, suggesting that additional sequences/domains in the LTBP1 molecule may be required for binding to heparin and/or for ECM assembly.

Interestingly, the short N9-LTBP1 fragment (345–487) bound to heparin but did not assemble into the ECM in transient transfection assays, whereas the longer N5 fragment (299–487) did incorporate into fibronectin-containing fibrils. These observations suggest that, in living cell systems, in addition to heparin binding, sequences between 299 and 345 may also be important for LTBP1 assembly, perhaps by stabilizing molecular interactions or facilitating interactions with cell surface receptors involved in the assembly process.

Consistent with a potential role for HSPGs in mediating binding between fibronectin and LTBP1, heparin-BSA was able to induce binding interactions between the N terminus of LTBP1 (aa 21–487) and fibronectin in solid-phase binding assays. Our finding, that inhibition of GAG chain assembly or treatment with heparin and heparin-BSA inhibits LTBP1 assembly, further supports a role for HSPGs in assembly of LTBP1 into the ECM. These observations complement those of Tiedemann et al. (33) and Ritty et al. (34) who showed a similar role for HSPGs in assembly of fibrillins into the ECM. It therefore remains to be determined whether the involvement of HSPGs in the assembly pathway may be a common mechanism utilized by other microfibrillar proteins in the fibrillin superfamily.

At present, the specific HSPGs that mediate LTBP1-fibronectin interactions or that mediate fibrillin assembly into the ECM are unknown. Double immunostaining indicated colocalization of perlecans with both fibronectin and LTBP1 in early fetal rat calvarial cell osteoblast cultures (data not shown). However, solid-phase binding assays with a commercial HSPG preparation consisting mainly of perlecans (data not shown) or with recombinant perlecan domain I showed binding to fibronectin but not to LTBP1. Other potential HSPGs that may be important in LTBP1 assembly include members of the syndecan family of cell surface heparan sulfate proteoglycans.

LTBP1s are thought to be major regulators of TGF-β activity and availability (4–6). Therefore, via regulation of LTBP matrix deposition, HSPGs could also play a role in regulation of TGF-β. In support of this, a dramatic reduction in ECM-bound TGF-β was observed in heparin-treated fetal rat calvarial cell cultures, as measured by ELISA on ECM extracts. Furthermore, heparin treatment actually resulted in a small increase in the amount of active TGF-β detected in the conditioned media of fetal rat calvarial osteoblasts, suggesting that some of the TGF-β that fails to be assembled into the ECM may be converted into the active form. Although these data are consistent with a role for HSPGs in regulating TGF-β storage in the ECM via LTBP1, there are other ECM molecules besides LTBPls/fibrillins (e.g., decorin and biglycan) that can also sequester TGF-βs (mainly the active form) in the extracellular space (48, 49). Furthermore, because heparin itself can bind directly to the active growth factor, presumably other heparan sulfate-containing ECM macromolecules may also bind. This adds additional complexity to interpretation of the data. However, we were able to confirm by Western blotting that heparin treatment resulted in a reduction in both free LTBP1 and large latent TGF-β complex in the ECM. This supports the hypothesis that at least a proportion of the heparin effect is explained by inhibition of LTBP1 incorporation.

In summary, our data suggest that HSPGs mediate binding interactions between LTBP1 and fibronectin, thereby controlling incorporation of LTBP1 and latent TGF-β into the ECM.
Role for HSPG in LTBP1 Assembly

These findings may have important implications for diseases in which TGF-β has been implicated, such as fibrotic diseases, osteoarthritis and rheumatoid arthritis, and cancer.

Acknowledgments—We thank Dr. Lynda F. Bonewald for critical review of the manuscript and Mark Dallas for expert technical assistance with the microscopy and image analysis.

REFERENCES

1. Taipale, J., Saharinen, J., and Keski-Oja, J. (1998) Adv. Cancer Res. 75, 87–134
2. Charbonneau, N. L., Ono, R. N., Corson, G. M., Keene, D. R., and Sakai, L. Y. (2004) Birth Defects Res. C Embryo Today 72, 37–50
3. Ramirez, F., Sakai, L. Y., Dietz, H. C., and Rifkin, D. B. (2004) Physiol. Genomics 19, 151–154
4. Hyytiainen, M., Penttinen, C., and Keski-Oja, J. (1998) Ciba Found. Symp. 157, 81–89; discussion 89–92
5. Annes, J. P., Munger, J. S., and Rifkin, D. B. (2003) Adv. Cancer Res. 87–134
6. Saharinen, J., Hyytiainen, M., Taipale, J., and Keski-Oja, J. (1999) Cytokine Growth Factor Rev. 10, 99–117
7. Blobe, G. C., Schildmamm, W. P., and Lodish, H. F. (2000) Nature 406, 293–297
8. Miyazono, K., Olofsson, A., Wernstedt, C., Hellman, U., Miya-zono, K., Claesson-Welsh, L., and Heldin, C. H. (1990) EMBO J. 9, 1091–1101
9. Abe, M., Oda, N., and Sato, Y. (1998) J. Cell Biol. 140, 171–181
10. Miyazono, K., Skerry, T. M., Mundy, G. R., and Bonewald, L. F. (1995) J. Cell Biol. 131, 539–549
11. Taipale, J., Miyazono, K., Heldin, C. H., and Keski-Oja, J. (1994) J. Cell Biol. 124, 171–181
12. Taipale, J., Lohi, J., Saharinen, J., Kovanen, P. T., and Keski-Oja, J. (1995) J. Biol. Chem. 270, 4689–4696
13. Dallas, S. L., Saharinen, J., Kovanen, P. T., and Keski-Oja, J. (1999) J. Biol. Chem. 274, 12132–12136
14. Fläumenhaft, R., Abe, M., Sato, Y., Miyazono, K., Harpel, J., Heldin, C. H., and Rifkin, D. B. (1993) J. Cell Biol. 120, 995–1002
15. Dallas, S. L., Miyazono, K., Chen, Y., Munger, J. S., and Rifkin, D. B. (2004) J. Cell Biol. 165, 723–734
16. Dabovic, B., Chen, Y., Colarossi, C., Obata, H., Zambuto, L., Perle, M. A., and Rifkin, D. B. (2002) J. Cell Biol. 156, 227–232
17. 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
18. Saharinen, J., Taipale, J., and Keski-Oja, J. (1996) EMBO J. 15, 245–253
19. Gleizes, P. E., Beavis, R. C., Mazzerie, R., Chen, Y., and Rifkin, D. B. (1996) J. Biol. Chem. 271, 29891–29896
20. Oklu, R., Metcalfe, J. C., Hesketh, T. R., and Kemp, P. R. (1998) FEBS Lett. 425, 281–285
21. Dallas, S. L., Keene, D. R., Bruder, S. P., Saharinen, J., Sakai, L. Y., Mundy, G. R., and Bonevald, L. F. (2000) J. Bone Miner. Res. 15, 68–81
22. Unsold, C., Hyytiainen, M., Bruckner-Tuderman, L., and Keski-Oja, J. (2001) J. Cell Sci. 114, 187–197
23. Nunes, I., Gleizes, P. E., Metz, C. N., and Rifkin, D. B. (1997) J. Cell Biol. 136, 1151–1163
24. Raghunath, M., Unsold, C., Kubitscheck, U., Bruckner-Tuderman, L., Peters, R., and Meuli, M. (1998) J. Invest. Dermatol. 111, 559–564
25. Nakajima, Y., Miyazono, K., Muto, K., Takase, M., Yamagishi, T., and Nakamura, H. (1997) J. Cell Biol. 136, 193–204
26. Dallas, S. L., Sivakumar, P., Jones, C. J., Chen, Q., Peters, D. M., Mosher, D. F., Humphries, M. J., and Kielty, C. M. (2005) J. Biol. Chem. 280, 18871–18880
27. Isogai, Z., Ono, R. N., Ushiro, S., Keene, D. R., Chen, Y., Mazzieri, R., Charbonneau, N. L., Reinhardt, D. P., Rifkin, D. B., and Sakai, L. Y. (2003) J. Biol. Chem. 278, 2750–2757
28. Sottile, J., and Hocking, D. C. (2002) Mol. Biol. Cell 13, 3546–3559
29. Godyna, S., Mann, D. M., and Argraves, W. S. (1995) Matrix Biol. 14, 467–477
30. Pereira, M., Rybarczyk, B. J., Orlid, T. M., Hocking, D. C., Sottile, J., and Simpson-Haidaris, P. J. (2002) J. Cell Sci. 115, 609–617
31. Dalton, B. A., McFarland, C. D., Underwood, P. A., and Steele, J. G. (1995) J. Cell Sci. 108, 2083–2092
32. Tiedemann, K., Batge, B., Muller, P. K., and Reinhardt, D. P. (2001) J. Biol. Chem. 276, 36035–36042
33. Ritby, T. M., Broekelmann, T. J., Werneck, C. C., and Mechem, R. P. (2003) Biochem. J. 375, 425–432
34. Peters, D. M., and Mosher, D. F. (1987) J. Cell Biol. 104, 121–130
35. Mould, A. P., Askari, J. A., Craig, S. E., Garratt, A. N., Clements, J., and Humphries, M. J. (1994) J. Biol. Chem. 269, 27224–27230
36. Yang, W. D., Gomes, R. R., Jr., Allieknavitch, M., Farach-Carson, M. C., and Carson, D. D. (2005) J. Biol. Chem. 280, 414–516
37. Pfeilschifter, J., Laukhuf, F., Muller-Beckmann, B., Blum, W. F., Pfister, T., and Ziegler, R. (1995) J. Clin. Invest. 96, 767–774
38. Abe, M., Oda, N., and Sato, Y. (1998) J. Cell Biol. 140, 186–193
39. Mayer, U., Nischt, R., Posch, E., Mann, K., Fukuda, K., Gerl, M., Yamada, Y., and Timpl, R. (1993) EMBO J. 12, 1879–1885
40. Kohfeldt, E., Maurer, P., Vannahme, C., and Timpl, R. (1997) FEBS Lett. 414, 551–564
41. Pfeilschifter, J., Laukhuf, F., Muller-Beckmann, B., Blum, W. F., Pfister, T., and Ziegler, R. (1995) J. Biol. Chem. 270, 527–534