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LTBP-2 specifically interacts with the amino-terminal region of fibrillin-1 and competes with LTBP-1 for binding to this microfibrillar protein

Rena Hirani, Eric Hanssen [1], Mark A. Gibson *

From the Discipline of Pathology, School of Medical Sciences, University of Adelaide, Adelaide, South Australia 5005, Australia

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Abstract

LTBP-2 is a matrix protein of unknown function since, unlike other LTBPs, it does not form covalent complexes with latent TGF-β. We have previously shown that LTBP-2 has widespread association with fibrillin-containing microfibrils in developing aorta and other tissues. We have now shown that full-length human recombinant LTBP-2 specifically binds to the amino-terminal region of fibrillin-1, but not to fibrillin-2, in solid phase assays and overlay blotting. The binding was enhanced by the inclusion of 2 mM Ca^{2+} ions in the assay buffer and abolished by 5 mM EDTA indicating that the interaction was directly or indirectly Ca^{2+} ion dependent. The K_d for the interaction was calculated from the specific binding curve as 9.4 nM. A recombinant carboxyl-terminal fragment of LTBP-2 was shown to a) bind the amino-terminal fragment of fibrillin-1 and b) block completely the binding of full length LTBP-2 to fibrillin-1. This result indicates that the major fibrillin-1 binding site resides close to the carboxyl-terminus of LTBP-2. Further competitive binding studies showed that an analogous carboxyl terminal fragment of LTBP-1 was able to block the binding of LTBP-2 to fibrillin-1 and that the C-terminal fragment of LTBP-2 could block the interaction of the LTBP-1 fragment with the fibrillin. Thus the binding site for LTBP-2 on fibrillin-1 appears to be the same or in close proximity to that for LTBP-1. Immunohistochemical analysis of developing human aorta showed distinctive but extensively overlapping distributions for LTBPs-1 and -2. Both LTBPs showed extensive co-localization with fibrillin-1 and elastic lamellae but LTBP-2 had extensive signal throughout the medial layer whereas LTBP-1 showed strong localization only in the outer medial layer. The finding indicates that there is a possibility for LTBP-2 to compete with LTBP-1 for binding to fibrillin-containing microfibrils throughout the aortic wall but particularly in the outer medial region where the LTBP-1 is predominantly located. Overall, the results support the concept that that LTBP-2 may be an indirect negative modulator for storage of the large latent TGF-β complex on microfibrils in aorta and other fibrillin-rich tissues.

Keywords: LTBP fibrillin tgf-beta microfibril

1. Introduction

Transforming growth factors-β are a family of potent multifunctional cytokines which modulate a variety of cellular and physiological processes during tissue development, morphogenesis and homeostasis (Massague, 1990, 2000; Lawrence, 2001). As general characteristics, activated TGF-βs tend to inhibit cell proliferation, stimulate matrix production and exert immunosuppressive effects (Lawrence, 2001). Excessive expression of TGF-βs has been implicated in a range of diseases including pulmonary fibrosis, glomerulonephritis, liver cirrhosis, keloid formation and cancers (Blobe et al., 2000; Elliott and Blobe, 2005). The immunosuppressive properties of many tumor types may be due in part to excess production of these cytokines (Taipale et al., 1998; Massague et al., 2000). TGF-βs are secreted from cells as small latent complexes consisting of dimers of the mature cytokine linked to its pro-region or latency associated peptide, or as large latent complexes in which the small complex is covalently linked via the latency associated peptide to a latent TGF-β binding protein (LTBP) (Rifkin, 2005; Carta et al., 2006). The LTBP appears to target the latent complex to specific sites for storage within the extracellular

Abbreviations: BMP, bone morphogenetic protein; BSA, bovine serum albumin; LTBP, Latent transforming growth factor-β binding protein; RT-PCR, reverse transcription-polymerase chain reaction; TGF-β, transforming growth factor-β; TBS, Tris-buffered saline.

* Corresponding author. Tel.: +61 8 8303 5337; fax: +61 8 8303 4408.
E-mail address: mark.gibson@adelaide.edu.au (M.A. Gibson).
[1] Current address: Department of Biochemistry, La Trobe University, Melbourne, Victoria 3086, Australia.

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matrix where it awaits activation by agents such as thrombospondin, integrin αVβ6, reactive oxygen species or low pH during processes such as tissue remodeling, inflammation and wound repair (Annes et al., 2003, 2004).

Matrix structures important for latent TGF-β storage appear to be the fibrillin-containing microfibrils (Vehvilainen et al., 2003). These microfibrils are found in the matrix of most tissues often as a component of elastic fibers in association with elastin (Mecham and Davis, 1994; Cleary and Gibson, 1996; Kielty et al., 2002). The microfibrils are composed primarily of one or more members of the fibrillin family of large glycoproteins (fibrillins 1–3) (Sakai et al., 1986; Handford et al., 2000; Kielty et al., 2005). Defects in the fibrillin-1 gene have been linked to the common genetic disorder, Marfan syndrome, characterized by skeletal, cardiovascular, ocular and lung defects (Online Mendelian Inheritance in Man number 154700). Mutations in the fibrillin-2 gene result in a related disorder, congenital contractural arachnodactyly (Online Mendelian Inheritance in Man number 121050). Recent evidence indicates that some of the phenotypic characteristics of Marfan syndrome may be due to inappropriate activation of TGF-β during tissue development and growth (Neptune et al., 2003; Ng et al., 2004). In addition, a Marfan-like phenotype has been recently linked to mutations in the TGF-β receptor 2 (TGFBR2) gene (Mizuguchi et al., 2004). These observations suggest that the fibrillin-containing microfibrils are critical regulators of latent TGF-β complex storage and that reduction in the number of microfibrils in Marfan syndrome can lead to increased activation of the latent TGF-β in tissues, resulting in some aspects of the phenotype (Judge and Dietz, 2005).

Interestingly, fibrillins share similar structural features with the LTBP family which has four members (LTBP-1–4) and the two groups of proteins are considered to compose a superfamily. LTBPs, although smaller in size, share a structure with fibrillins characterized by tandem arrays of epidermal growth factor-like 6-cysteine motifs, interspersed with characteristic 8-cysteine motifs found only in these proteins (Sinha et al., 2002; Hyytiainen et al., 2004; Rifkin, 2005). No evidence has been forthcoming for direct binding of latent TGF-β to fibrillin. However, molecular interactions of LTBPs-1 and -4 with fibrillins-1 and -2 have recently been reported (Isogai et al., 2003), supporting the concept that LTBPs anchor latent TGF-β to the microfibrils.

In contrast to other LTBPs, LTBP-2 does not appear to bind covalently to latent TGF-β (Gibson et al., 1995; Saharinen and Keski-Oja, 2000) However, LTBP-2 has been shown to be abundantly associated with fibrillin-containing microfibrils during development in tissues such as aorta and elastic ligaments (Gibson et al., 1995). Currently, the function of LTBP-2 is unknown although its critical importance in early embryogenesis has been recognized since *Ltbp-2* null mouse embryos were found to die between days 3.5 and 6.5 of embryonic development (Shipley et al., 2000).

In this study we show that LTBP-2 binds specifically to fibrillin-1, but not to fibrillin-2 and that the interaction involves the carboxyl-terminal region of LTBP-2 and the amino-terminal region of fibrillin-1. Moreover, LTBP-2 competes with LTBP-1 for binding to fibrillin-1 *in vitro* suggesting that LTBP-2 may be a negative regulator of attachment of LTBP-1 to the microfibrils in some tissues. This idea is supported by our immunohistochemical studies which show distinctive but extensively overlapping distributions for LTBPs-1 and -2 with fibrillin-1 in developing human aorta.

2. Results

2.1. LTBP-2 specifically binds to the amino-terminal region of fibrillin-1

An expression construct for full length human LTBP-2 with a carboxyl-terminal His_6-tag, LTBP-2(H) (Fig. 1A), was made using vector pCEP-4 and expressed in 293-EBNA cells. The LTBP-2(H) protein was purified from the culture medium and analyzed by SDS-PAGE and immunoblotting under reducing and non-reducing conditions (Fig. 1B). LTBP-2(H) migrated as a doublet of protein bands with apparent molecular weights of 210 kDa and 219 kDa under reducing conditions and 170 kDa and 180 kDa under non-reducing conditions. Both bands

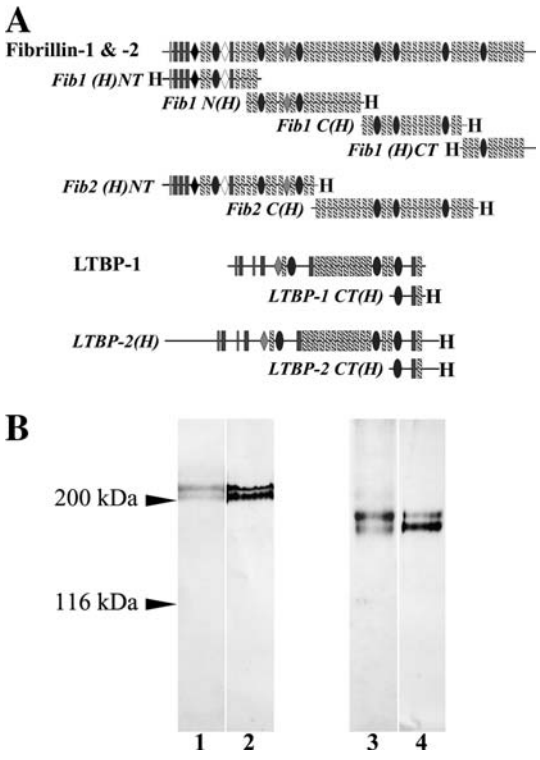

Fig. 1. Recombinant human fibrillin and LTBP fragments. (A). Domain structure of fibrillins and LTBPs (bold) and recombinant protein constructs (italics). Motif structures: Light gray box, 4-Cys motif; black box, EGF-like motif; black diamond, 9-Cys motif; crosshatched box, Ca-binding EGF-like motif; black ovoid, 8-Cys motif; open diamond, 'hinge' region; grey diamond, hybrid 8-Cys motif; H, His6-tag. (B) SDS-PAGE of recombinant human LTBP-2 [LTBP-2 (H)]. Purified LTBP-2(H) was analyzed on a 6.5% gel under reducing (left panel) and non-reducing (right panel) conditions. Lanes: 1 and 3, Coomassie blue stained gel; 2 and 4, immunoblot with anti-[LTBP-2-peptide] polyclonal antibody (1:500 dilution) following transfer onto polyvinylidene difluoride membrane. The arrowheads indicate the relative mobilities of concurrently run protein standards.

stained with the specific anti-LTBP-2 antibody. The lower apparent sizes of the species under non-reducing conditions are consistent with the molecules being stabilized in a folded conformation by extensive disulfide bonding. The measured molecular masses are higher than the theoretical value of 195 kDa calculated from the predicted protein sequence of the mature recombinant protein. However, digestion with N-glycosidase (but not O-glycosidase) caused a reduction in size of the doublet bands by approximately 15 kDa indicating that LTBP-2(H) was significantly N-glycosylated (data not shown). The yield of LTBP-2(H) was consistently around 4 mg/L of conditioned medium.

LTBP-2(H) was tested for binding to a range of contiguous recombinant human fibrillin-1 and -2 fragments (Fig. 1A) which have been described previously (Hanssen et al., 2004). Specific binding of LTBP-2 was identified by solid phase assay (Fig. 2A) and overlay blotting (Fig. 2B) to the amino-terminal fibrillin-1 fragment, Fib1(H)NT. No binding to the analogous fibrillin-2 fragment Fib2NT(H) was detected. In similar experiments no LTBP-2(H) binding was detected to tropoelastin, MAGPs-1 and -2, decorin, byglycan and collagen types I, III, V and VI (data not shown). The binding of LTBP-2(H) to the fibrillin-1 fragment Fib1(H)NT was enhanced by inclusion of 2 mM Ca^{2+} ions in the binding buffer and abolished by the addition of 5 mM EDTA, indicating that the interaction was directly or indirectly calcium ion dependent (Fig. 3). SDS-PAGE analysis of the Fib1(H)NT after the incubation showed that no degradation of the

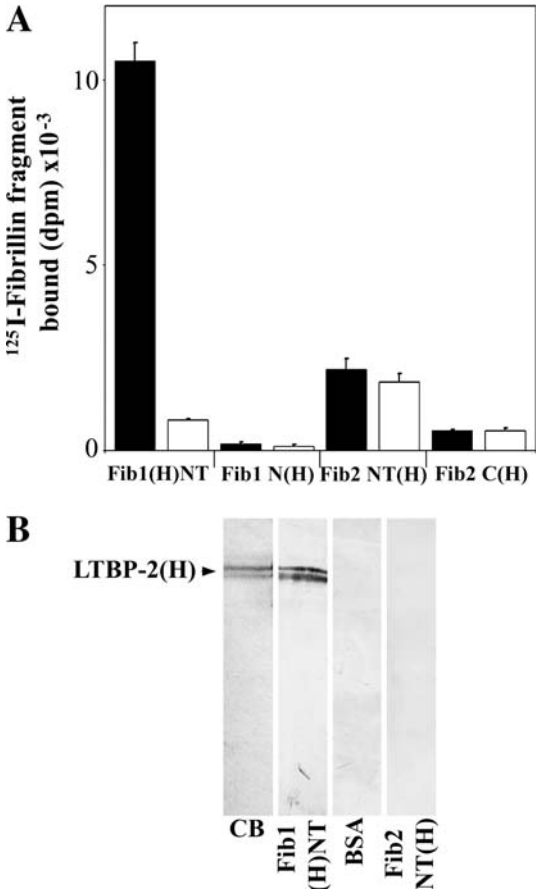

Fig. 2. LTBP-2 specifically binds to the amino-terminal region of fibrillin-1. (A) Solid phase binding. Wells were coated with LTBP-2(H) (200 ng/well) (black columns) or BSA (62 ng/well) as a control (white columns). After blocking, the wells were incubated for 3 h at 37 °C with 1.28×105 dpm of a ^{125}I-labeled fibrillin fragment, Fib1(H)NT, Fib1N(H), Fib2NT(H) or Fib2C(H) (each of specific activity 1.28×107 dpm/μg). After washing, binding was measured by direct γ counting. The means±S.D. of triplicate determinations are shown. (B) Affinity blotting. LTBP-2(H) (1 μg/lane) was resolved by SDS-PAGE on an 8% gel under reducing conditions, stained with Coomassie blue (CB) or transferred onto polyvinylidene difluoride membrane. After equilibration in binding buffer (TBS containing 2 mM $CaCl_2$) and blocking with 3% milk, duplicate blots were incubated with 5 μg/mL Fib1(H)NT or BSA for 8 h. Specific binding to LTBP-2(H) (200 kDa) was detected with anti-[fibrillin-1] antibody MAB2502. A further control blot, incubated with Fib2NT(H) (5 μg/mL) then anti-[fibrillin-2] antibody 16E12 showed no binding to LTBP-2(H).

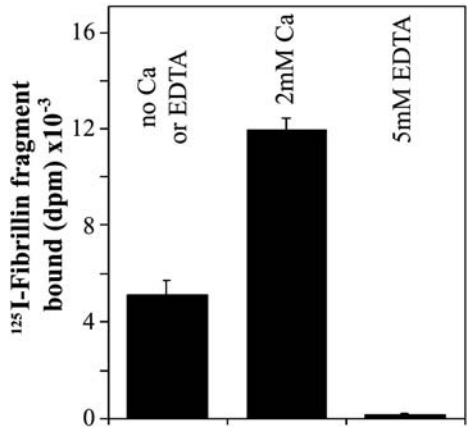

Fig. 3. The interaction of LTBP-2 with fibrillin-1 is calcium dependant. The wells of a microtiter plate were coated with LTBP-2(H) (200 ng/well) or BSA (62 ng/well) as a negative control. After blocking, 2.56×104 dpm of ^{125}I-labeled Fib1(H)NT (specific activity 1.28×10 dpm/μg) was added and the wells were incubated at 37 °C for 3 h in the absence of added $CaCl_2$ (No Ca or EDTA), with 2 mM $CaCl_2$, or 5 mM EDTA. After washing, binding was measured by direct γ counting. The means±S.D. of quadruplicate determinations are shown.

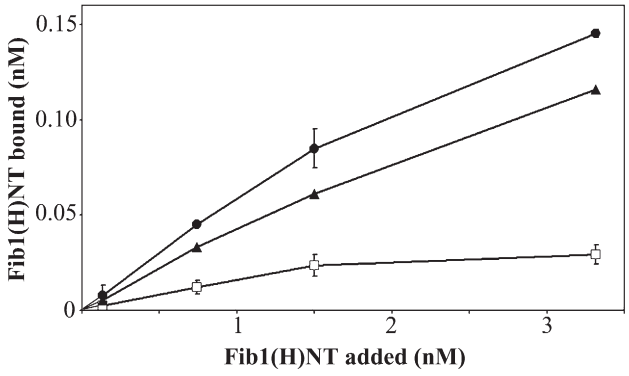

Fig. 4. Calculation of K_d for the interaction of LTBP-2 with fibrillin-1. Microtiter plates were coated with LTBP-2(H) (75 ng/well) or BSA control (20 ng/well) and incubated for 3 h at 37 °C with ^{125}I-labeled Fib1(H)NT at concentrations of 0 to 25 ng/100 μl (specific activity 1.28 ×107 dpm/μg). The liquid phase was then removed, the wells were washed and the amount of bound and unbound radioactivity in each well was determined by direct γ counting. Specific binding (triangles) was calculated as the amount of Fib1(H)NT bound to LTBP-2(H)-coated wells (circles) minus the average amount bound to the corresponding BSA-coated wells (squares). The K_d was calculated as 9.4 nM using non-linear regression analysis of the specific binding curve (see Experimental procedures). The means±S.D. of triplicate determinations are shown.

fibrillin had occurred even in the presence of EDTA (data not shown). LTBP-2 binding was measured at a range of Fib1(H) NT concentrations and a specific binding curve was plotted (Fig. 4). A K_d of 9.4×10^{-9} M was calculated from non-linear regression analysis of the curve using the Prism program.

2.2. LTBP-2 competes with LTBP-1 for binding to fibrillin-1

LTBP-1 has been reported to bind to fibrillin-1 via its carboxyl terminal region (Isogai et al., 2003). To determine if LTBP-2 had similar fibrillin binding characteristics to LTBP-1, recombinant carboxyl-terminal fragments of both proteins, LTBP-2CT(H) and LTBP-1CT(H) were made in the pCEP-4/293-EBNA system (Fig. 5). Purified LTBP-2CT(H) migrated as a single 45 kDa band which stained with the anti-LTBP-2 antibody, 3504. Purified LTBP-1CT(H) migrated as a doublet of 35 kDa and 40 kDa bands, both of which stained with anti-LTBP-1 antibody 388 (Fig. 5A). Using the solid phase assay, fragment LTBP-2CT(H) was found specifically to bind fibrillin-1 fragment Fib1(H)NT although not as extensively as an

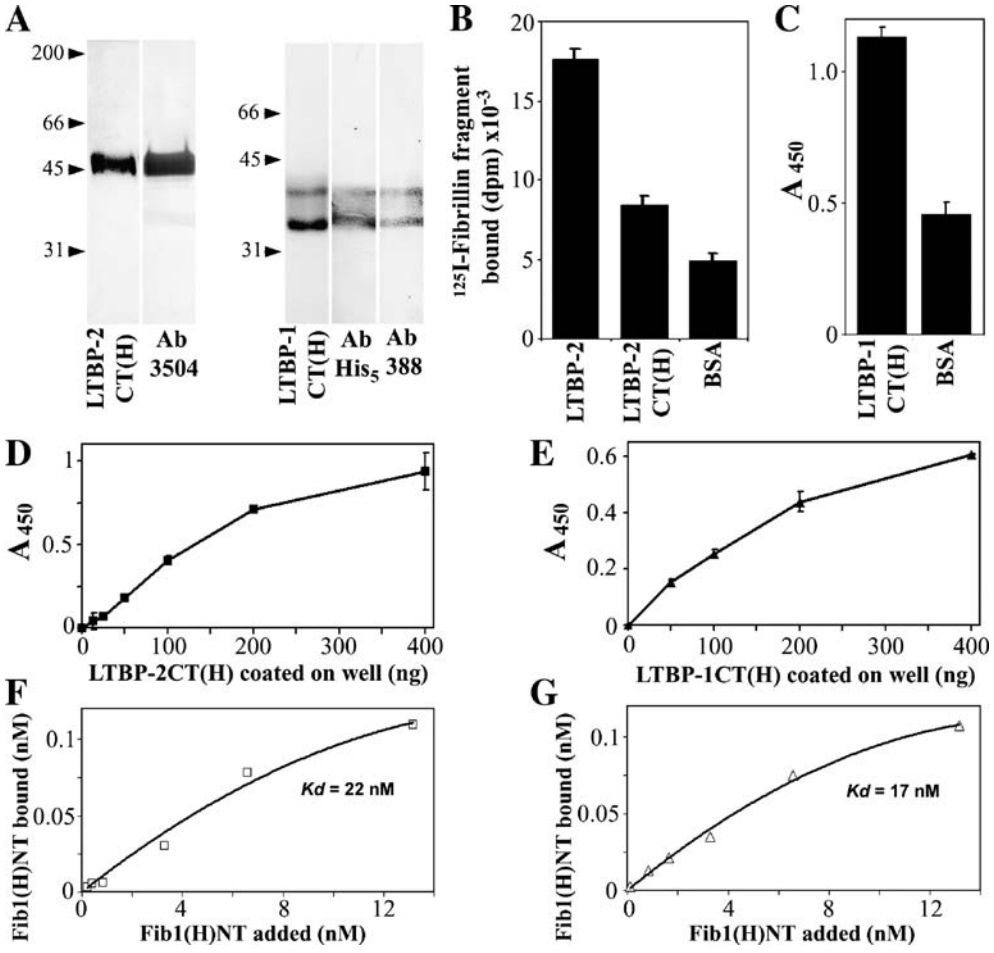

Fig. 5. Recombinant carboxyl-terminal fragments of LTBPs-1 and -2 bind to fibrillin-1. (A) SDS-PAGE analysis of recombinant carboxyl-terminal fragments of LTBP-2 [LTBP-32 2CT(H)] (left panel) and LTBP-1 [LTBP-1CT(H)] (right panel) analyzed on 12% gels under reducing conditions followed by immunoblotting. Left lanes, Coomassie Blue stained gels. Immunoblots: Ab 3504, anti-LTBP-2 antibody 3504; AbHis5, anti-pentahis antibody; Ab 388, anti-LTBP-1 antibody 388. The relative mobilities of protein standards are indicated by arrowheads. (B) Solid phase binding assay of LTBP-2CT(H) with fibrillin-1 fragment Fib-1(H)NT. Microtiter wells were coated with LTBP-2(H) (200 ng/well), fragment LTBP-2CT(H) (43 ng/well) or BSA (62 ng/well) as a negative control. After blocking, 6.4×104 dpm of ^{125}I-labeled Fib1(H)NT (specific activity 1.28×107 dpm/μg) was added and the wells were incubated at 37 °C for 3 h in the presence of 2 mM $CaCl_2$. After washing, binding was measured by direct γ counting. (C) Solid phase binding assay of LTBP-1CT(H) with Fib-1(H)NT. Microtiter wells were coated with LTBP-1CT(H) (400 ng/well) or BSA (125 ng/well). After blocking, each well was incubated as above with Fib1(H)NT (280 ng/well). After washing, specific binding was detected using 1:2000 dilution of anti-fibrillin-1 antibody 2502, followed by the peroxidase system of color development detected at 450 nm. (see Experimental procedures). Color was developed for 20 min. (D) Specific binding curve for LTBP-2CT(H). Increasing amounts of LTBP-2CT(H) (12.5–400 ng) were coated onto wells and incubated as above with Fib1(H)NT (250 ng/well). After washing, specific binding was detected using antibody 2502 as in (C) except color development was for 40 min. (E) Specific binding curve for LTBP-1CT(H). Increasing amounts of LTBP-1CT(H) (12.5–400 ng) were coated onto wells and incubated as above with Fib1 (H)NT (200 ng/well). After washing, specific binding was detected using antibody 2502 as in C) except color development was for 10 min. In all binding experiments means ± S.D. of quadruplicate determinations are shown. (F) Calculation of K_d for the interaction of LTBP-2CT(H) with fibrillin-1. Microtiter plates were coated with LTBP-2CT(H) (50 ng/well) or BSA control (60 ng/well) and incubated for 3 h at 37 °C with ^{125}I-labeled Fib1(H)NT at concentrations of 0 to 100 ng/100 μl (specific activity 1×107 dpm/μg). The liquid phase was then removed, the wells were washed and the amount of bound and unbound radioactivity in each well was determined by direct γ counting. The liquid phase was then removed, the wells were washed and the amount of bound and unbound radioactivity in each well was determined by direct γ counting. Specific binding was determined as described in Fig. 4. and the K_d was calculated as 22.0 nM. (G) Calculation of K_d for the interaction of LTBP-1CT (H) with fibrillin-1. The procedure outlined in (F) was repeated with LTBP-1CT(H) (32 ng/well) in place of LTBP-2CT(H). The K_d was calculated as 17.0 nM.

equimolar quantity of full length LTBP-2 (Fig. 5B). This lower binding may be due to reduced accessibility of the binding site on the small fragment LTBP-2CT(H) coated on the well, as opposed to the full length LTBP-2 molecule. In a subsequent assay, LTBP-1CT(H) also showed specific binding to Fib1(H)NT (Fig. 5C). The specificity of both interactions was confirmed by the production of authentic binding curves (Fig. 5D and E). The K_ds for the interactions of LTBP-2CT(H) and LTBP-1CT(H) with fibrillin-1 were calculated as 2.2×10^{-8} M and 1.7×10^{-8} M respectively using non-linear regression analysis of specific binding curves with the Prism program (Fig. 5F and G). Thus the binding affinity of LTBP-1 appears to be similar in strength to that of LTBP-2 for interaction with fibrillin-1 (see above). The K_d calculated for LTBP-2CT(H) was higher than that for full length LTBP-2(H) which is consistent with the lower fibrillin-1 binding signal observed for the LTBP-2CT(H) fragment in solid phase assays.

Inhibition experiments were conducted to determine if LTBP-2 contained more than one fibrillin binding region and to determine if the two LTBPs could compete for binding. Firstly, radiolabeled Fib1(H)NT was treated with 7-fold molar excess of LTBP-2CT(H) prior to incubation in wells coated with full length LTBP-2(H) (Fig. 6). In positive controls the LTBP-2CT(H) was replaced with a molar equivalent of BSA. The LTBP-2CT(H) specifically and completely blocked the binding of Fib1(H)NT to LTBP-2(H) indicating that all of the significant fibrillin-1 binding activity of LTBP-2 was confined to the carboxyl-terminal region defined by LTBP-2CT(H) and suggesting that LTBP-2 contained only one major fibrillin-1 binding site. To determine if LTBP-2 and LTBP-1 could compete for binding to fibrillin-1, radiolabeled Fib-1(H)NT was

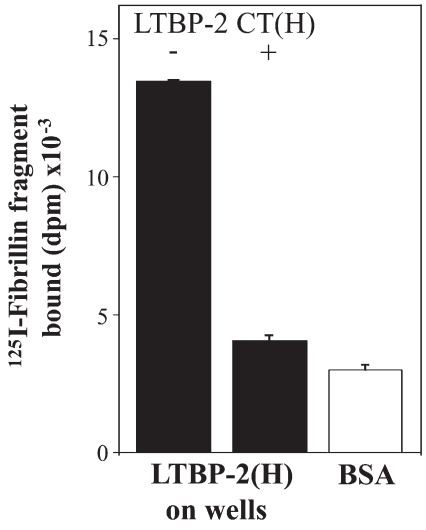

Fig. 6. The carboxyl terminal region of LTBP-2 contains the major fibrillin-1 binding site(s). Purified LTBP-2(H) (black columns) was coated into microtiter plates (200 ng/well). Control wells were coated with a molar equivalent (62 ng/well) of BSA (white columns). After blocking, each well was incubated at 37 °C for 3 h with 6.4×104 dpm of ^{125}I-labeled Fib1(H)NT (specific activity 1.28×107 dpm/μg), which had been pre-treated overnight at 37 °C with a 7-fold molar equivalent of LTBP-2CT(H) (+), or BSA control (−). All reactions were carried out in the presence of 2 mM $CaCl_2$. After washing, binding was measured by direct γ counting. Means±S.D. of quadruplicate determinations are shown.

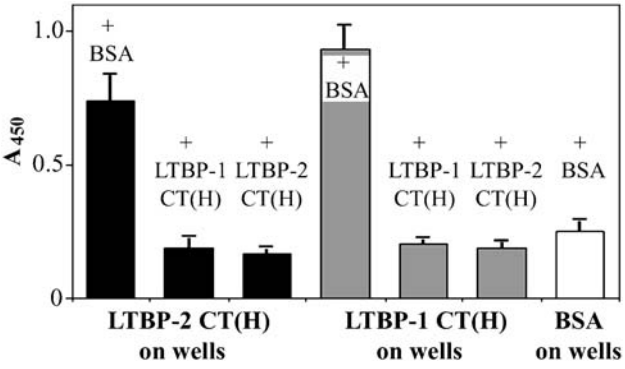

Fig. 7. LTBP-2 competes with LTBP-1 for binding to Fibrillin-1. Purified recombinant C-terminal regions LTBP-2CT(H) (400 ng/well) (black columns) and LTBP-1CT(H) (400 ng/well) (grey columns) were coated onto microtiter plates. Control wells were coated with BSA (125 ng/well) (white column). After blocking, each well was incubated at 37 °C for 3 h with Fib1 34 (H)NT (280 ng/well), which had been pre-treated overnight at 37 °C with a 10-fold molar equivalent of LTBP-2CT(H), LTBP-1CT(H) or BSA (positive control). All reactions were carried out in the presence of 2 mM $CaCl_2$. After washing, specific binding was detected at 450 nm using anti-fibrillin-1 antibody 2502, followed by peroxidase detection method (see Experimental procedures). Means±S.D. of quadruplicate determinations are shown.

treated with 10-fold excess of LTBP-2CT(H) or LTBP-1CT(H) and then incubated in wells coated with one of these two fragments (Fig. 7). Both LTBP-2CT(H) and LTBP-1CT(H) were shown to completely block the fibrillin binding to each other. SDS-PAGE analysis of the proteins in the liquid phase after the incubation showed that no degradation of the proteins had occurred (data not shown). The findings indicate that LTBP-2 and LTBP-1 share the same binding site, or have binding sites in close proximity, on fibrillin-1. Inhibition curves were constructed with serial dilutions of each LTBP fragment incubated with Fib1(H)NT in the liquid phase prior to incubation with LTBP-2CT(H) or LTBP-1CT(H) coated on the wells. There proved to be no statistical difference between the curves (data not shown). The result indicating that LTBP-2CT(H) and LTBP-1CT(H) were equally effective in blocking Fib1(H)NT binding to both LTBP fragments in the solid phase, consistent with the two LTBPs having similar binding affinities for fibrillin-1.

2.3. LTBP-2 and LTBP-1 have overlapping distributions in developing human aorta

The immunohistological localizations of LTBPs-1 and -2 were investigated in developing human aorta (Fig. 8). The two LTBPs showed overlapping but distinctive distributions in the aortic wall. LTBP-1 was localized most intensely to the outermost region of medial layer of the aortic wall and showed a decreasing signal through the medial layer towards the intima. There was much reduced signal in the inner half of the media, except for the internal elastic lamina which stained strongly (Fig. 8A). In contrast, LTBP-2 showed strong relatively uniform staining of most of the media, but had reduced signal in the very outermost regions where the signal for LTBP-1 was strongest (Fig. 8B). LTBP-2 did not stain the internal elastic lamina very

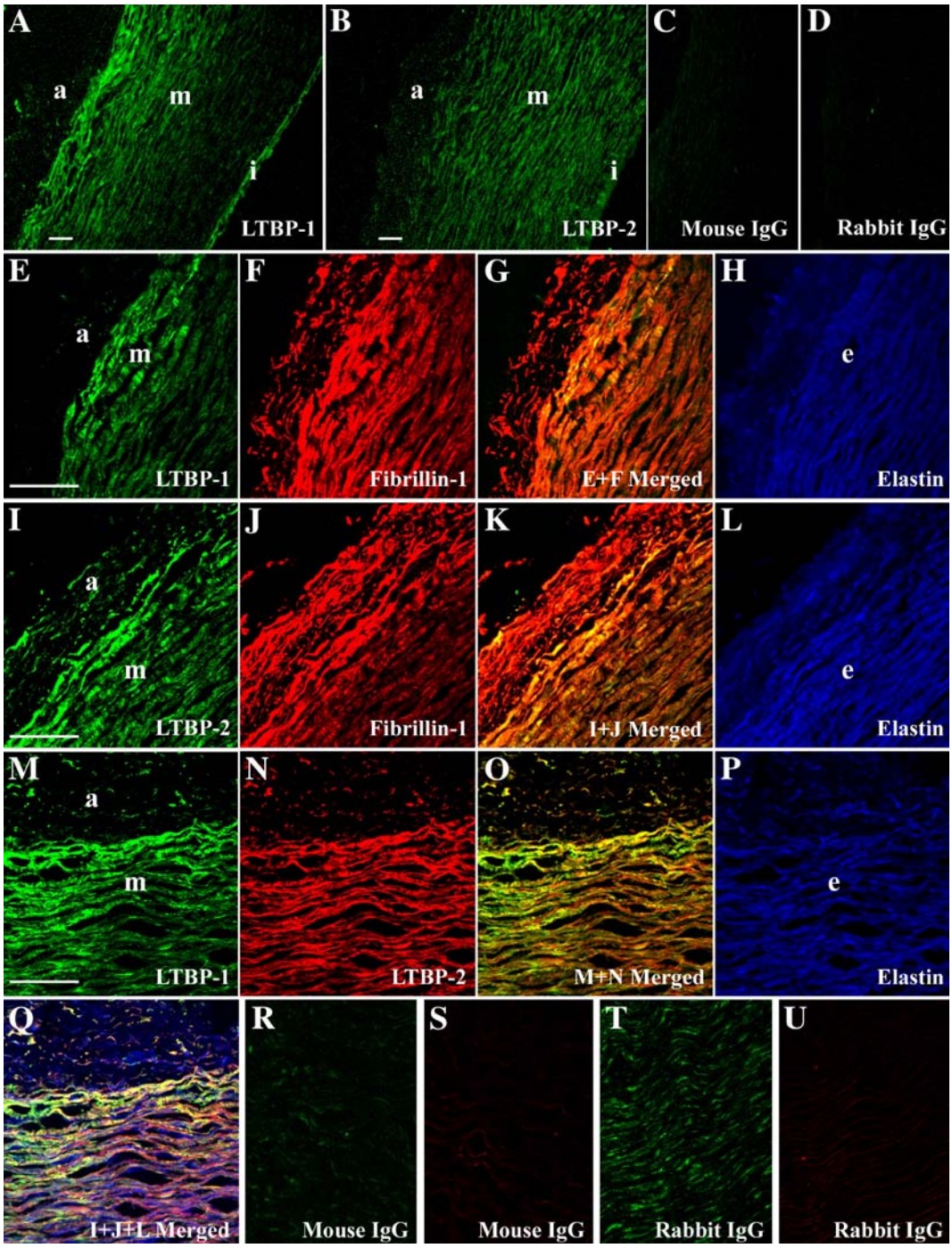

Fig. 8. LTBP-2 and LTBP-1 have distinct distributions in the matrix of developing human aorta. (A–D) Immunofluorescence analysis of late-trimester human fetal aorta. Cryostat sections (5 μm) were incubated with antibodies to LTBP-1, and LTBP-2 diluted in phosphate buffered saline. Primary antibody binding was detected with goat anti-mouse IgG antibody /fluorescein conjugate. (A) mouse anti-LTBP-1 monoclonal antibody 388 (10 μg/mL); (B) rabbit anti-[LTBP-2 peptide] polyclonal antibody 3504 (10 μg/mL); (C) mouse IgG control; (D) rabbit IgG control. Magnification bars = 300 μm. (E–U) Confocal analysis of outer medial layer of the aortic wall. Further cryostat sections were incubated with antibodies to fibrillin-1, LTBP-1, and/or LTBP-2 as outlined above. Primary antibody binding was detected using an appropriate secondary antibody conjugated to fluorophore alexa 488 (green) or Cy5 (red). (E and M), mouse anti-LTBP-1 monoclonal antibody 388 (10 μg/mL); (F) rabbit anti-[fibrillin-1 peptide] polyclonal antibody Fib1A (500 μg/mL); (G) E and F merged; (H,L and P), elastin autofluorescence visualized in the Cy3 channel and digitally displayed in blue; (I and N) rabbit anti-[LTBP-2 peptide] polyclonal antibody 3504 (10 μg/mL); (J) mouse anti-fibrillin-1 monoclonal antibody 1919 (5 μg/mL); (K), I and J merged; (O) M and N merged; (Q) M, N and P merged. (R) mouse IgG (10 μg/mL) control for E and M; (S) mouse IgG (5 μg/mL) control for J; (T) rabbit IgG (10 μg/mL) control for I; (U) rabbit IgG (500 μg/mL) control for F and N. Magnification bars = 50 μm. (a) adventitia; (e) elastic lamellae; (i) intima; (m) media.

strongly. Neither LTBP showed strong signal in the adventitia. Overall, both proteins showed significant localization to the outer regions of the medial layer.

To determine the extent of co-localization of each LTBP with elastic fiber components, particularly fibrillin, higher power examination of the outer media region was conducted using

confocal microscopy with anti-fibrillin-1 antibodies and elastin autofluorescence. Where staining for LTBP-1 occurred (Fig. 8E), it coincided with that for fibrillin-1 (Fig. 8F), (visualized as orange/yellow areas in Fig. 8G) and elastin autofluorescence (Fig. 8H). This result is consistent with most of the LTBP-1 in the outer media being localized on fibrillin-containing microfibrils associated with elastic lamellae. LTBP-2 was also co-localized with fibrillin-1 and elastin in this region (Fig. 8I–L). Neither LTBP showed extensive localization to the fibrillin-containing microfibrillar bundles associated with the adjacent adventitia, consistent with the lower power immunofluorescence images (Fig. 8A and B).

To determine the extent of co-localization of LTBP-2 with LTBP-1 in the outer media, sections were dual immunostained for the two LTBPs. The confocal images showed extensive co-localization of LTBP-2 and LTBP-1 to the same elastic lamellae (Fig. 8 M–O). On the same section anti-LTBP-1 antibodies were visualized as green (Fig. 8M), anti-LTBP-2 were visualized as red (Fig. 8N) and elastin autofluorescence was visualized as blue (Fig. 8P). The merged images of LTBP-2 and LTBP-1 localizations are shown in Fig. 8O. Extensive regions of balanced co-localization were evident, visualized as yellow. However the relative signal distributions of the two LTBPs were not identical within a given field with some localized regions of each elastic lamella staining more strongly for LTBP-1 (green patches) or LTBP-2 (orange–red). The co-localization of the LTBPs with the surfaces of elastic lamellae was confirmed by merging the LTBP images with the elastin autofluorescence (Fig. 8Q). Control sections incubated appropriately with pre-immune mouse or rabbit immunoglobulin-G showed only minimal non-specific cellular staining and elastin autofluorescence (Fig. 8R–U).

3. Discussion

LTBP-2 is a matrix protein of unknown function. The domain structure of LTBP-2 clearly places the molecule in the LTBP family of proteins despite evidence that LTBP-2 does not form covalent complexes with latent TGF-β in contrast to LTBP-1, LTBP-3 and LTBP-4 (Gibson et al., 1995; Rifkin, 2005). Indeed, LTBP-2 lacks the necessary consensus sequence in the third 8-Cys motif for attachment to LAP (Saharinen and Keski-Oja, 2000). LTBP-2 null mice have proved to be uninformative as they die in early embryogenesis (Shipley et al., 2000). This phenotype contrasts markedly with those of gene knock-out animals for other LTBPs (Sterner-Kock et al., 2002; Koli et al., 2004; Colarossi et al., 2005; Dabovic et al., 2005), TGF-βs (22, 28, 41 Kulkarni et al., 1993; Kaartinen et al., 1995; Sanford et al., 1997) and fibrillins (Carta et al., 2006; Chaudhry et al., 2001) which become evident at much later stages of development.

We have previously shown that LTBP-2 is abundant in developing bovine elastic tissues such as aorta and nuchal ligament where it was specifically immunolocalized to fibrillin-containing microfibrils (Gibson et al., 1995). These findings indicated that LTBP-2 must have some function in the biology of fibrillin-containing microfibrils and/or elastic fibers. Analysis of microfibrils purified by density gradient ultracentrifugation showed an absence of immunolabeling for LTBP-2 indicating that the protein is not an integral, covalently attached structural component of these elements (Isogai et al., 2003; Kielty et al., 1998). In another study, both LTBP-1 and LTBP-2 showed partial co-immunolocalization with fibrillin-1 in the wall of human coronary arteries. The intensity of staining increased following angioplasty-induced injury to the arteries suggesting some role for LTBP-2 in tissue repair processes (Sinha et al., 1998). Other evidence indicates that LTBP-2 can function in cell adhesion, being anti-adhesive for embryonic lung fibroblasts (Hyytiainen and Keski-Oja, 2003) and pro-adhesive for melanoma cells (Vehvilainen et al., 2003). The latter interaction appears to involve a non-RGD mediated mechanism possibly via α3β1 integrin resulting in focal contact and actin stress fiber formation (Vehvilainen et al., 2003).

To learn more about LTBP-2 function we have investigated the interactions of full-length recombinant LTBP-2 with fibrillins and other elastic fiber components. LTBP-2 was found to specifically bind the amino-terminal recombinant fragment of fibrillin-1, Fib1(H)NT but not to fibrillin-2, tropoelastin, MAGPs-1 and -2, decorin and biglycan. This region of fibrillin-1 contains the LTBP-1 binding site reported by Isogai et al. (2003) and the result suggested that the two LTBPs may share the same binding site. However, the interaction of LTBP-2 with fibrillins appears to have some differences with the previously documented fibrillin binding properties of LTBP-1. (Isogai et al., 2003). These workers reported that LTBP-1 bound both fibrillins, although the interaction was found to be much stronger with fibrillin-1 than fibrillin-2. In addition, the LTBP-1/fibrillin-1 interaction was reported to be insensitive to treatment with EDTA whereas our results showed that the LTBP-2 interaction with fibrillin-1 was enhanced by Ca^{2+} ions and could be blocked by the addition of EDTA. Isogai et al. (2003) also reported that the fibrillin binding sites were located in the carboxyl-terminal regions of LTBPs-1 and -4. To determine if LTBP-2 had a similar fibrillin-1 binding site, we investigated the interaction of the analogous carboxyl-terminal region of LTBP-2 with fragment Fib1(H)NT. The carboxyl-terminal fragment of LTBP-2 was found not only to bind fibrillin-1 but it also blocked the interaction of fibrillin-1 with full length LTBP-2 and with the analogous LTBP-1 fragment. These findings indicated that the LTBP-2 binding site on fibrillin-1 is located close to that for LTBP-1 and that the two LTBPs compete for binding to the fibrillin *in vitro*. The binding affinities of the two LTBPs for fibrillin-1 were found to be of similar strengths, suggesting that the proteins may share a similar binding motif. Sequence comparison of the carboxyl-terminal regions of LTBPs-1 and -2 showed that there were few unusual similarities between the sequences of the final 8-Cys motif, the cys-free region and the final EGF-like motif. However there was extensive sequence homology of the penultimate EGF-like motif between LTBPs-1 and -2. Moreover, this EGF-like motif in LTBP-4 had strong sequence similarities with LTBPs-1 and -2 but the analogous motif of LTBP-3 showed much less homology. Since fibrillin-1 binds LTBP-4 but not LTBP-3

(Isogai et al., 2003) these observations point to the penultimate EGF-like motif of LTBPs-1, -2 and -4 as a strong candidate for the fibrillin-1 binding site.

The competitive binding studies raise the possibility that LTBP-2 may compete with LTBP-1 for binding to fibrillin-containing microfibrils. It has already been established by immunoelectron microscopy that LTBP-2 is extensively co-localized with these microfibrils in developing bovine aorta (Gibson et al., 1995) and that LTBP-1 is located on at least some of these structures in fetal human aorta (Isogai et al., 2003). However, it was unclear whether the distributions of the two proteins overlapped extensively within aortic tissue at the same stage of development. To investigate this possibility we undertook a detailed immunohistochemical study of fetal human thoracic aorta, a tissue which is also of particular importance to the Marfan phenotype (Judge and Dietz, 2005). Both LTBPs appeared to co-localize specifically with fibrillin-1 but LTBP-2 showed strong and relatively uniform distribution to the elastic tissue of the medial layer whereas LTBP-1 had a strong signal in the outer media but had decreasing signal across the vessel wall such that the inner media appeared to contain little LTBP-1.

Confocal imaging showed extensive co-localization of the two LTBPs to the elastic lamellae of the outer media. Thus LTBP-2 appears to be available to compete with LTBP-1 for binding to fibrillin-containing microfibrils in the LTBP-1-rich outer medial region and throughout the aortic wall. It is therefore possible that LTBP-2, although not covalently binding to the latent TGF-β complex, may play some indirect role in modulation of storage of the complex on microfibrils containing predominantly fibrillin-1 in aorta and other tissues. LTBP-2 does not appear to bind to fibrillin-2, and thus it follows that LTBP-2 will not interfere with the docking of the latent TGF-β complexes onto fibrillin-2 rich microfibrils. Therefore it is an interesting possibility that in developmental situations where a tissue is rich in LTBP-2, fibrillin-2-rich microfibrils may assume greater importance for storage of the latent growth factor. Obviously further experiments using appropriate cell culture model systems are needed to establish whether LTBP-2 can modulate TGF-β storage on microfibrils and influence the activation of the latent growth factor.

Although we have established that LTBP-2 specifically binds fibrillin-1 and appears to compete for binding with other LTBPs, the function of LTBP-2 remains unclear. From its structural similarities to fibrillins and other LTBPs, LTBP-2 is likely to share other functional characteristics with these proteins. Although LTBP-2 does not form a covalent complex with latent TGF-β (Gibson et al., 1995; Saharinen and Keski-Oja, 2000), non-covalent interactions with members of the TGF-β family cannot yet be ruled out. Interestingly fibrillin-1 has recently been reported to bind non-covalently to the prodomain of growth factor BMP-7 and thus it may be that fibrillin-containing microfibrils act as latent stores for this and other BMP molecules (Gregory et al., 2005). BMP-7 is structurally similar to TGF-β but its prodomain lacks cysteine ruling out covalent interaction with structural elements of the extracellular matrix (Gregory et al., 2005). It is possible that LTBP-2 functions as a storage molecule for an unidentified growth factor(s) on the surface of fibrillin-containing microfibrils. Alternatively LTBP-2 may be involved in other aspects of cytokine biology. Recent evidence has indicated that several matrix proteins are modulators of latent TGF-β processing and activation. Thrombospondin is a direct activator of the growth factor (Murphy-Ullrich and Poczatek, 2000). Fibronectin has recently been shown to be required for integrin-mediated latent TGF-β activation (Fontana et al., 2005). Most recently the elastic fiber-associated protein emilin-1 has been demonstrated to regulate the processing of the pro-TGF-β polypeptides by furin convertases to the mature latent TGF-β complex. From analysis of *Emilin1* null mouse phenotype, the action of emilin on TGF-β processing appears to be particularly important in blood vessel development and for blood pressure homeostasis (Zacchigna et al., 2006). Since LTBP-2 has been immunolocalized in close proximity to LTBP-1 (and thus the large latent complex) in some tissues, the potential role of LTBP-2 in the modulation of processing and activation of the TGF-β family of growth factors is worthy of investigation.

4. Experimental procedures

4.1. Production of recombinant polypeptides

Recombinant human fibrillin fragments (see Fig. 1) were produced in 293-EBNA cells and purified as described previously (Hanssen et al., 2004). A cDNA for full-length human LTBP-2, corresponding to bases 212–6331 (Genbank™ accession number NM_000428), was obtained by RT-PCR from human fibroblast RNA using superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) using primer 5′-TCAGGGCCCAGAACA-GATTG-3′ followed by RNaseH digestion and by amplification with sense primer 5′-CGCGCAGCCCTCGTTCCG-3′, antisense primer 5′-CAGTCCAAGCTTCCCCAAATCCT-3′ and *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA). PCR was conducted for 35 cycles with annealing temperature of 61 °C as described previously (Kitahama et al., 2000). The PCR product (6119 bp) was purified by 1% w/v agarose gel electrophoresis, A-tailed by incubation with dATP and platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) at 70 °C for 30 min and cloned into pGEM-T-easy vector (Promega, Madison, WI) following the manufacturers instructions.

A cDNA encoding the BM-40 signal peptide was obtained by RT-PCR from human trabecular bone mRNA using superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers followed by amplification with primers 5 ′-GCCC-GGAGAGCGCGCTCTG-3′ and 5′-CCACCACCTCTGTCT-CATCAGGC-3′. PCR was conducted for 35 cycles with an annealing temperature of 58 °C as described previously (Kitahama et al., 2000) and cloned into pGEM-T-easy as described above. This cDNA was added to the 5′ end of the LTBP-2 cDNA (replacing bases 212–494) using the splicing by overlap extension technique (Horton et al., 1993). The LTBP-2 cDNA template was modified using PCR (30 cycles with an annealing temperature of 68 °C) and the primer pair 5′-TGGCCGGGAGGGCTCTGG-CAGCCCCAAGCTTCCAAAGGGACCCCGTAGGGAG-3′ and 5′-CTAATGGTGATGGTGATGGTGAAGCTTGGCA-

GTGCAGTGGGGGGG-3′. The cDNA for the BM40 peptide signal was modified by PCR (30 cycles, annealing temperature 68 °C) using the primer pair 5′-GCCCGGAGAGCGCGCTCTG-3′ and 5′-CTCCCTACGGGGTCCCTTTGGAAGCTTGGGCT-GCCAGAGCCCTCCCGGA-3′. The PCR products were gel purified using a Bresaspin kit (Geneworks, Adelaide, Australia) and mixed together with primer pair 5′-GCCCGGAGAGCG-CGCTCTG-3′ and 5′-CTAATGGTGATGGTGATGGTGAA-GCTTGGCAGTGCAGTGGGGGGG-3′ for further PCR amplification of 10 cycles with an annealing temperature of 68 °C. The PCR product of the correct size was A-tailed and cloned into pGEM T-easy as described above. The insert was excised with *Not*I and subcloned into the pCEP-4Δ*Hin*dIII expression vector (Hanssen and Gibson, unpublished).

To produce a recombinant carboxyl-terminal fragment of LTBP-2 (LTBP-2CT(H)), bases 5089-5848 of LTBP-2 cDNA were PCR amplified from the full length clone using sense 5′-CCCCAAGCTTGAACAGCACCAGCAGCACGG-3′ and antisense 5′-TGGTGATGGTGAAGCTTGGCAGTGC-3′ primers and *Pfu* turbo DNA polymerase for 29 cycles with an annealing temperature of 61 °C. The 756 bp product was purified by agarose gel electrophoresis, digested with *Hin*dIII and subcloned into the *Hin*dIII site of the modified pGEM-T-easy vector encoding an amino-terminal BM-40 signal peptide and a carboxyl-terminal His6 tag (Hanssen and Gibson, unpublished). An error-free clone was subcloned into the *Not*I site of the modified pCEP-4 vector described above.

Similarly a cDNA for the carboxyl-terminal region of human LTBP-1CT(H), corresponding to bases 3681–4317 (Genbank™ accession number NM_000627), was obtained by RT-PCR from human trabecular bone RNA using primers sense 5′-GTA-TAAGCTTAGCTGAGTCAAACGAA CAAA-3′ and antisense 5′-GTGAAGCTTCTCC AGGTCACTGTCTTTCTC-3′. Cycle number was 25 and annealing temperature was 60 °C. The 636 bp product was cloned using GATEWAY cloning technology following the manufacturer's protocol (Invitrogen, Carlsbad, CA). Briefly the gateway consensus sequences were added to the cDNA by 12 further cycles of PCR using sense 5′-GGGGACAAGTTTGTACAAAAAAGCAGGC-TAAGCTTAGCTGAGTCAAACGAACAAA-3′ and antisense 5′-GGGGACCACTTTGTACAAG AAAGCTGGG-TAAGCTTCTCCAGGTCACTGTCTTTCTC-3′ primers, and an annealing temperature of 55 °C. The cDNA was then incubated with BP clonase and vector pDONR201 for 3 h at 25 °C. The product was transfected into DH5α cells. An error-free clone was selected and the LTBP-1CT(H) cDNA was excised with *Hin*dIII and subcloned into the *Hin*dIII site of the modified pGEM-T-easy vector encoding the N-terminal BM-40 sequence and C-terminal His6 tag. The modified cDNA was then excised with *Not*1 and subcloned into the modified pCEP-4 vector as described above. The error-free pCEP-4 constructs encoding full-length LTBP-2, LTBP-2CT(H) and LTBP-1CT(H) (see Fig. 1) were then stably transfected into 293-EBNA cells and recombinant protein was purified from serum-free culture medium (DMEM or Excell 293 [JRH Biosciences, Lenexa, KS]) using nickel affinity chromatography with methods described previously (Hanssen et al., 2003). Purified recombinant proteins were dialyzed into 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl, and stored frozen at −20 °C.

4.2. Antibodies and immunoassays

Rabbit polyclonal anti-LTBP-2 peptide antibody, FLP-E has been described previously (Gibson et al., 1995). Using similar protocols an additional polyclonal anti[LTBP-2 peptide] antibody, 3504, was raised to a synthetic peptide DDLHY-SIYGPDGAC from the carboxyl-terminal 8-Cys domain of LTBP-2. Rabbit polyclonal anti-fibrillin-1 antibody Fib1A was raised to a unique fibrillin-1 sequence VPRPPVEYLYPS-REPPRV present in fibrillin-1 fragment Fib1(H)NT. The specificities of 3504 and Fib1A were confirmed by enzyme-linked immunosorbent assay (ELISA) and immunoblotting against the recombinant polypeptides of LTBP-2, LTBP-2CT (H), LTBP-1CT(H) and our range of fibrillin-1 and -2 fragments (data not shown). Monoclonal anti-fibrillin-1 antibodies 2502 and 1919 were purchased from Chemicon International (Temecula, CA). Monoclonal anti-fibrillin-2 antibody, 16E12, has been described previously (Hanssen et al., 2004). Monoclonal anti-His5 antibody was purchased from Qiagen (Valencia, CA). Anti-LTBP-1 mouse monoclonal antibody 388 was purchased from R&D systems (Minneapolis, MN).

4.3. Radiolabeling of recombinant polypeptides

Each recombinant fibrillin fragment (100 μg) was reacted with a washed IODO-BEAD (Pierce) and 1 mCi of $Na^{125}I$ (Amersham Biosciences, Sydney, Australia) for 15 min in 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl as described previously (Finnis and Gibson, 1997). Free radiolabel was removed by gel filtration through Sephadex PD-10 desalting columns (Amersham). The specific activities were 1×10^7 dpm/μg unless stated otherwise.

4.4. Molecular binding assays

Solid phase binding was detected using previously described methods (Finnis and Gibson, 1997). Briefly, plastic flat-bottomed multiwell microtiter plates (immuno-maxisorb modules (Nalge–Nunc International, Roskilde, Denmark)) were coated with the purified recombinant LTBP (usually 400 ng in 100 μL of Tris–HCl, pH 7.4, 0.13 M NaCl, (TBS) per well) at 4 °C overnight. Control wells were coated with molar equivalent of BSA. The wells were rinsed with TBS, blocked with 3% low-fat dried milk in TBS and then extensively washed with TBS. For radioactive detection, a ^{125}I-labeled recombinant fibrillin fragment (100 ng in 100 μL of TBS, 2 mM $CaCl_2$), was added to triplicate wells and incubated for 3 h at 37 °C.

The wells were then washed three times in TBS, 0.05% Tween-20. Binding of ^{125}I-labeled protein was directly measured using a Wallac 1261 Multi-γ counter. For binding of unlabeled recombinant fibrillin fragments, a peroxide–ELISA system was used (Finnis and Gibson, 1997) with an appropriate dilution of anti-His_5 antibody or specific anti-fibrillin antibody. The dissociation constant (K_d) was calculated

from non-linear regression analysis of the binding curve using the Prism 4.0 program (Graphpad software, San Diego, CA). Briefly, maxisorb modules coated with recombinant LTBP-2 or BSA (control) were incubated with serial dilutions of ^{125}I-labeled Fib-1(H)NT for 3 h at 37 °C. Bound and unbound ^{125}I-labeled Fib1(H)NT was measured in each well and, following subtraction of background binding to the corresponding BSA coated wells, a specific binding curve was plotted (see Fig. 4 legend for more details).

Overlay blotting was conducted using a previously described method (Finnis and Gibson, 1997). 1 μg samples of purified LTBP-2(H) were analyzed by SDS-PAGE on an 8% gel and transferred onto polyvinylidene difluoride membrane. The membrane was then blocked with 3% low-fat dried milk in TBS for 1 h. Duplicate lanes were incubated at 4 °C for 18 h with Fib-1(H)NT (5 μg/mL in TBS/0.05% BSA), in the presence of 2 mM $CaCl_2$. Specific binding was detected with anti-fibrillin monoclonal antibody 2502 diluted 1:2000 in TBS/3% milk followed by anti-mouse IgG antibody conjugated to alkaline phosphatase (Finnis and Gibson, 1997).

4.5. Competitive binding assays

Wells of flat-bottomed multiwell microtiter plates [immunomaxisorb modules (Nalge–Nunc International, Roskilde, Denmark)] were coated with 400 ng of fragment LTBP-1CT(H) or LTBP-2CT(H) as described above. Control wells were coated with a molar equivalent of BSA. Fibrillin fragment Fib1(H)NT (280 ng/well) was pre-treated with 10-fold molar excess of LTBP-1CT(H), LTBP-2CT(H) or BSA for 18 h at 4 °C, then added to triplicate wells and incubated for a further 3 h at 37 °C. Binding of Fib1(H)NT was detected using antibody 2502 (1:2000 dilution in TBS) followed by peroxidase-conjugated secondary antibody and color development using 100 μL of 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich, St Louis, MO) as described previously (Finnis and Gibson, 1997).

4.6. Immunofluorescence

For immunofluorescence microscopy cryostat sections (5 μm) of third trimester human fetal aorta were incubated overnight at 4 °C with mouse anti-LTBP-1 monoclonal antibody 388 (10 μg/ml) or affinity-purified rabbit anti[LTBP-2 peptide] antibody 3504 (10 μg/ml). Control sections were incubated with mouse IgG and rabbit IgG (10 μg/ml) respectively. Sections were washed with phosphate buffered saline and incubated with the appropriate secondary antibody-fluorescein conjugate for 2 h at room temperature. Sections were washed then mounted in 90% glycerol/10% phosphate buffered saline containing anti-fade reagent *p*-phenylenediamine (Sigma-Aldrich). Sections were examined using a Nikon microphot FX-a microscope, a 470/490-nm excitation filter, a 520-nm/560-nm barrier filter and CF epifluorescence fluor objectives. Photographs were taken using an FX-35DX digital camera (Nikon) set on automatic exposure with an ISO setting of 3000. Control sections were photographed using manual exposure times matching those of the appropriate antibodies.

For confocal microscopy similar sections were incubated with antibody 3504, antibody 388, mouse anti-fibrillin-1 monoclonal antibody 1919 (5 μg/ml) or affinity-purified rabbit anti-[fibrillin-1 peptide] antibody Fib1A (500 μg/ml) followed by the appropriate secondary antibody conjugated to fluorophore-Alexa 488 (Invitrogen) or Cy3 (Jackson immunoresearch, West Grove, PA). The specimens were examined using a Bio-Rad MRC-1000 (Bio-Rad) scanning confocal microscope equipped with a 40× objective and krypton–argon laser using emission filters 522/35, 585LP and 680DF32. Images were processed using Confocal Assistant software version 4.02 and assimilated into figures using Photoshop version 6.0 (Adobe Software Ltd).

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