

# Dual Role for the Latent Transforming Growth Factor- $\beta$ Binding Protein in Storage of Latent TGF- $\beta$ in the Extracellular Matrix and as a Structural Matrix Protein

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**Abstract.** The role of the latent TGF- $\beta$  binding protein (LTBP) is unclear. In cultures of fetal rat calvarial cells, which form mineralized bonelike nodules, both LTBP and the TGF- $\beta$ 1 precursor localized to large fibrillar structures in the extracellular matrix. The appearance of these fibrillar structures preceded the appearance of type I collagen fibers. Plasmin treatment abolished the fibrillar staining pattern for LTBP and released a complex containing both LTBP and TGF- $\beta$ . Antibodies and

antisense oligonucleotides against LTBP inhibited the formation of mineralized bonelike nodules in long-term fetal rat calvarial cultures. Immunohistochemistry of fetal and adult rat bone confirmed a fibrillar staining pattern for LTBP in vivo. These findings, together with the known homology of LTBP to the fibrillin family of proteins, suggest a novel function for LTBP, in addition to its role in matrix storage of latent TGF- $\beta$ , as a structural matrix protein that may play a role in bone formation.

TGF- $\beta$  is secreted by virtually all cells in one or more latent forms that are biologically inert (Miyazono et al., 1988; Wakefield et al., 1988). Most cells secrete TGF- $\beta$  as a 290-kD high molecular mass latent TGF- $\beta$  complex that contains a 190-kD latent TGF- $\beta$  binding protein (LTBP)<sup>1</sup> linked by a disulfide bridge to the TGF- $\beta$ 1 precursor (also known as latency-associated peptide) (see Fig. 1) (Kanzaki et al., 1990; Tsuji et al., 1990). Platelets secrete a latent complex with a 130-kD truncated form of LTBP. We have shown previously that osteosarcoma cells and bone organ cultures secrete at least three forms of latent TGF- $\beta$ , including the 290-kD high molecular mass form (Bonewald et al., 1991; Dallas et al., 1994). However, bone cells are unique in that at least 50% of their latent TGF- $\beta$  is secreted as a 100-kD latent complex that lacks LTBP and consists only of the mature 25-kD TGF- $\beta$  homodimer noncovalently associated with a 70-kD portion of the precursor homodimer.

Mature (or active) TGF- $\beta$  exerts effects in a variety of cell types. These can be broadly grouped into effects on matrix formation, inhibition of cell growth, and immunosuppression (for reviews see Roberts and Sporn, 1990;

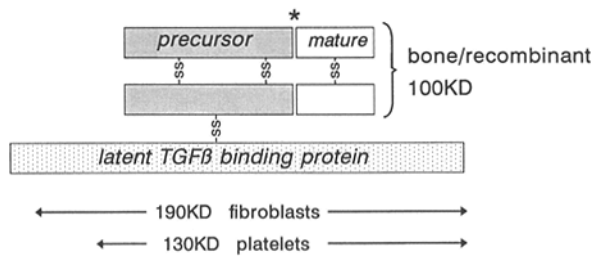
Border and Ruoslahti, 1992; Sporn and Roberts, 1992; Bonewald and Dallas, 1994; Centrella et al., 1994). Whereas many activities have been ascribed to active TGF- $\beta$ , the functions of the different latent TGF- $\beta$  complexes are less clear. In particular, the function of the latent TGF- $\beta$  binding protein has not yet been defined. Although it was assumed initially to confer latency to the TGF- $\beta$  complex, it is now known that the 100-kD precursor complex that lacks LTBP is also latent, indicating that LTBP is not essential for latency (Gentry et al., 1987; Bonewald et al., 1991). LTBP appears to play a role in assembly and secretion of the latent TGF- $\beta$  complex in human erythroleukemia cells (Miyazono et al., 1991). However, this does not seem to be the case in bone cells, where the 100-kD complex that lacks LTBP is efficiently secreted. Indeed, the rat osteosarcoma cell line UMR-106 secretes high amounts of latent TGF- $\beta$ , and produces exclusively the 100-kD latent TGF- $\beta$  complex lacking LTBP (Dallas et al., 1994). Although it has been shown that LTBP may facilitate activation of latent TGF- $\beta$  in co-cultures of endothelial and smooth muscle cells (Flaumenhaft et al., 1993), the possible mechanism for this remains unclear.

Sequence analysis of LTBP reveals a number of features characteristic of matrix or adhesion molecules, which include 16 EGF-like repeats, an RGD sequence, a putative calcium-binding domain, and an eight-amino acid motif identical to the cell-binding domain of  $\beta$ 2-laminin (Kanzaki et al., 1990; Tsuji et al., 1990). Several of these fea-

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1. *Abbreviation used in this paper:* LTBP, latent TGF- $\beta$  binding protein.

## Latent Forms of TGF $\beta$



**Figure 1.** Schematic diagram showing latent TGF- $\beta$  complexes that have been characterized in various cell types. The 100-kD recombinant latent TGF- $\beta$  complex consists of the mature 25-kD TGF- $\beta$  homodimer noncovalently associated with a 70-kD portion of the precursor homodimer (Gentry et al., 1987). Bone cells secrete ~50% of their latent TGF- $\beta$  in a similar 100-kD precursor form (Bonewald et al., 1990). Most other cell types produce a high molecular weight latent TGF- $\beta$  complex in which a third protein, the LTBP, is linked to the precursor by a disulfide linkage (Miyazono et al., 1988; Wakefield et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990). LTBP is 190 kD in fibroblasts and 130 kD in platelets, making latent TGF- $\beta$  complexes of 290 kD and 230 kD respectively. \*, internal cleavage site.

tures are shared with the fibrillin family of extracellular matrix proteins (Sakai et al., 1986, 1991; Maddox et al., 1989; Rosenbloom et al., 1993; Zhang et al., 1994). LTBP also contains a novel eight-cysteine repeat motif unique to the fibrillin family. Protein studies have confirmed that a large amount of LTBP, a proportion of which has TGF- $\beta$  associated with it, is found in the extracellular matrix of fibroblasts (Taipale et al., 1994). We hypothesized that in bone the 290-kD latent TGF- $\beta$  complex containing LTBP targets latent TGF- $\beta$  to bone matrix for storage, whereas the 100-kD form may be a secretory form, which is more readily available for cellular activation. Consistent with this hypothesis, Sato et al. (1993) have demonstrated that the 100-kD latent TGF- $\beta$  is able to bind to the cell surface by an unknown mechanism.

To determine whether LTBP could target the latent TGF- $\beta$  complex for matrix storage in bone cells, we used fetal rat calvarial cell cultures as a representative *in vitro* model of bone formation. These cells differentiate *in vitro* and form mineralized nodules with the characteristics of woven bone (Bellows et al., 1986; Harris et al., 1994). Morphologically distinct stages can be identified during the differentiation process (Dworetzky et al., 1990). In this study these were defined as the proliferating stage, the early (confluent) stage, the middle stage, where multilayered cellular nodules are beginning to form and the extracellular matrix is maturing, and the late stage, where the cellular nodules have become mineralized. We performed immunocytochemistry for LTBP, TGF- $\beta$ 1 precursor, and type I collagen in long-term fetal rat calvarial cell cultures to examine the expression and possible colocalization of these molecules. LTBP was also localized *in vivo* by immunohistochemistry in frozen sections of long bones from young adult and fetal rats. We then determined the effect of plasmin digestion on the release of latent TGF- $\beta$  complexes from the matrix of fetal rat calvarial cells as exam-

ined by Western blotting and immunohistochemistry. Finally, the effect of addition of antibodies and antisense oligonucleotides against LTBP was examined in long-term fetal rat calvarial cell cultures. These studies show that LTBP may be a structural fibrillar-type extracellular matrix protein that plays an important role in the bone formation process. These findings may have implications for the role of LTBP in normal and pathological matrix formation in other tissues.

## Materials and Methods

### Materials

Antibody against LTBP (Ab39) was a rabbit antiserum raised against native LTBP purified from human platelets (Kanzaki et al., 1990). Antibody against the TGF- $\beta$ 1 precursor (LT-2) was a rabbit antiserum raised against recombinant TGF- $\beta$ 1 (Mizoi et al., 1993). Antibody against mature TGF- $\beta$  was a polyclonal rabbit antibody raised against porcine TGF- $\beta$ 1.2 obtained from R & D Systems, Inc. (Minneapolis, MN). Antibody against rat type I collagen was a rabbit polyclonal antiserum obtained from BIODSIGN International (Kennebunkport, ME). Recombinant human LTBP was generously donated by K. Takemura (Kirin Brewery Co. Ltd., Maebashi Gunma, Japan). Partially purified platelet latent TGF- $\beta$  complex was prepared in our laboratory as described previously (Bonewald et al., 1991). Bovine plasmin was obtained from Sigma Chemical Co. (St. Louis, MO). Oligonucleotides were synthesized on a DNA/RNA synthesizer (Model 392; Applied Biosystems, Inc., Foster City, CA), and purified using Poly Pak cartridges (Glen Research Corp., Sterling, VA) according to manufacturer's instructions. Sense and antisense nucleotides were made for the sequence GAGACTGCCCGCATGG of rat LTBP, which includes the translation start site (Tsuji et al., 1990).

### Fetal Rat Calvarial Cell Cultures

Unless stated otherwise, all tissue culture reagents were purchased from Life Technologies Inc. (Gaithersburg, MD) or JRH Biosciences (Lenexa, KS). The isolation of fetal rat calvarial cells was performed as described previously (Harris et al., 1994), using a modification of the method of Bellows et al. (1986). Briefly, frontal and parietal bones from ICR-Swiss rat fetuses (19-d gestation) were digested in 0.2% collagenase/0.05% trypsin in HBSS to obtain six sequential 20-min digests. The third through sixth digests were combined and grown to confluence in MEM, alpha modification ( $\alpha$ -MEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin. At confluence the cells were trypsinized and plated for experiments at 10,000 cells/cm<sup>2</sup> growth area. Long-term mineralizing fetal rat calvarial cell cultures were maintained in  $\alpha$ -MEM supplemented with 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 5 mM  $\beta$ -glycerophosphate, and 100  $\mu$ g/ml L-ascorbic acid with or without test factors. Culture medium (including test factors) was changed every 3 d. For sense and antisense experiments, fresh oligonucleotides were added to the cultures every 24 h.

For quantitation of mineralized nodules in the fetal rat calvarial cell cultures, the cultures were fixed in neutral buffered formalin and stained by the Von Kossa method with Van Gieson's picric acid-acid fuchsin counterstain (Harris et al., 1994). The number and area of Von Kossa-stained nodules was then quantified by automated image analysis using a video analysis program (Jandel Scientific, San Rafael, CA) linked up to a videorecorder camera (CCD/RGB; Sony Corp., Park Ridge, NJ) and microscope (BH2; Olympus Corp., Precision Instruments Division, Lake Success, NY) equipped with metallurgical lenses.

### Immunocytochemistry

Immunocytochemistry was performed on cells grown in chamber slides (Lab-tek; Nunc Inc., Naperville, IL). These preparations were fixed for 10 min in 95% ethanol. Immunocytochemistry was also performed on frozen sections of bone from young-adult Wistar rats (body wts 85–150 g) and fetal rats (19-d gestation, ICR-Swiss). Frozen tissue sections were obtained as described previously (Skerry et al., 1989). Briefly, fresh, undecalcified tissue was frozen by precipitate immersion in hexane cooled in a slurry bath of ethanol and dry ice. 8  $\mu$ m cryostat sections were cut using a heavy-duty cryostat fitted with a tungsten carbide-edged blade (Bright Instru-

ments, Huntingdon, UK). The sections were collected onto slides coated with Vectabond (Vector Laboratories, Burlingame, CA) and were then fixed in chilled acetone for 5 min before immunohistochemical staining.

Endogenous peroxidase activity was blocked by preincubation in 3% hydrogen peroxide in PBS for 30 min at room temperature. Background blocking was performed by incubating specimens in 1% normal goat serum in PBS for 3 h at room temperature. Specimens were incubated in primary antibody or control preimmune serum diluted in PBS/1% normal goat serum for 2 h at 37°C (1:2,000 dilution for Ab39, and 1:1,000 dilution for LT-2 and type I collagen antibodies). For detection an immunocytochemistry kit (Vectastain ABC-elite; Vector Laboratories, Inc., Burlingame, CA) for rabbit IgG was used in combination with a DAB peroxidase substrate kit (brown reaction product) according to manufacturer's instructions (Vector Laboratories, Inc.). Some preparations were counterstained for 2 min with 1% methyl green before mounting. Other preparations were also counterstained with alkaline phosphatase using an alkaline phosphatase substrate kit (red reaction product) according to manufacturer's instructions (Vector Laboratories, Inc.). Cell culture preparations were mounted in aqueous mounting medium according to manufacturer's instructions (Crystal mount; Biomedica Corp., Foster City, CA). Cryostat tissue sections were dehydrated through graded alcohols and mounted in mounting medium (DPX; Fluka Chemika, Buchs, Switzerland). Specificity of immunostaining for LTBP was confirmed by preincubating the immune serum for 30 min with partially purified human platelet latent TGF- $\beta$  (230 kD) or recombinant human LTBP (kindly donated by K. Takemura).

For immunofluorescent staining a biotinylated goat anti-rabbit second antibody (Vector Laboratories, Inc.) was used in conjunction with streptavidin-FITC (Pierce, Rockford, IL). The preparations were then viewed on a confocal laser scanning microscope (LSM 10; Carl Zeiss Inc., Thornwood, NY). Fiber sizes were measured by image analysis.

### **Treatment of Fetal Rat Calvarial Cell Preparations with Plasmin**

For immunohistochemical analysis of fetal rat calvarial cell cultures after plasmin treatment, the cells were grown on Lab-tek chamber slides as described above until the early stage of differentiation, where the cells had reached confluence and LTBP fibrillar staining was well developed (see Fig. 2). The cells were washed twice in PBS and incubated with plasmin (0.1 U/ml) in serum-free  $\alpha$ -MEM medium for 1 h at 37°C. After incubation with plasmin the cell cultures were fixed and stained for LTBP by immunocytochemistry as described above.

For Western blot analysis of plasmin-released latent TGF- $\beta$  complexes, fetal rat calvarial cells were grown in six-well plates until the early stage of differentiation. The cells were then washed twice in PBS and lysed in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate). The deoxycholate-insoluble pellet (which consists predominantly of matrix proteins) was collected by centrifugation at 14,000 rpm for 10 min. The pellet was washed twice in PBS, and then digested with plasmin (0.1 U/ml) in serum-free  $\alpha$ -MEM medium for 1 h. Proteins released by plasmin digestion were concentrated by precipitation in 80% ethanol. The protein pellet was then dissolved in nonreducing sample buffer, heated at 95°C for 5 min, and analyzed by SDS-PAGE followed by Western blotting.

### **Western Blot Analysis of Plasmin-released Latent TGF- $\beta$ Complexes**

Proteins released by plasmin digestion of extracellular matrices were analyzed by SDS-PAGE using 5–12% gradient polyacrylamide gels as described previously (Bonewald et al., 1991; Dallas et al., 1994). The samples were run under nonreducing conditions (to keep the latent complexes intact). Proteins were then transferred onto a nitrocellulose membrane by electroblotting as described previously (Bonewald et al., 1991; Dallas et al., 1994). Membranes were blocked in 5% nonfat milk in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 2 h at room temperature, and then incubated with either a rabbit polyclonal antiserum against LTBP (Ab39), a rabbit polyclonal antibody against mature TGF- $\beta$  (R & D Systems, Inc.), or control nonimmune antiserum. Antibody incubations were carried out in TBS buffer + 5% BSA. Immunostained bands were visualized using peroxidase-conjugated protein-A (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) in conjunction with the enhanced chemiluminescence detection system used according to manufacturer's instructions (Amersham Corp., Arlington Heights, IL).

### **Measurement of LTBP by ELISA**

A single-sided ELISA was used to measure LTBP concentrations in conditioned media from fetal rat calvarial cell cultures treated with sense and antisense oligonucleotides against LTBP. This ELISA has a detection limit of 10–20 ng/ml LTBP. High protein-binding EIA/RIA 96-well plates (Costar Corp., Cambridge, MA) were coated overnight with test samples or recombinant LTBP standards diluted in PBS. Background blocking was performed for 2 h at 37°C in PBS/5% BSA. The plates were then incubated with antiserum against LTBP (Ab39, 1:1,000 dilution in PBS/0.5% BSA) for 1 h at 37°C. The detection antibody was an HRP-conjugated goat anti-rabbit antibody (Sigma Chemical Co.). *O*-phenylenediamine dihydrochloride tablets (Sigma Chemical Co.) were used as the reaction substrate according to manufacturer's instructions. The reaction was stopped by the addition of 3 M HCl (25  $\mu$ l/100  $\mu$ l reaction vol). The absorbance at 492 nm was read, and LTBP concentrations in test samples calculated by linear regression using the Immunofit EIA/RIA program, version 3.00 (Beckman Instruments Inc., Fullerton, CA).

## **Results**

### **Immunolocalization of LTBP in Fetal Rat Calvarial Cells**

LTBP staining was cytoplasmic in subconfluent, proliferating fetal rat calvarial cultures (Fig. 2 A). Two days after the cells became confluent, LTBP staining was no longer cytoplasmic, but was localized in large fibrillar structures in the extracellular matrix, which appeared to form an organized network throughout the culture (Fig. 2 B). As the cultures began to differentiate and cellular nodules began to form by days five through eight of culture, the LTBP fibrillar structures appeared organized around and between the nodules and actually appeared to span between nodules, linking them together (Fig. 2 C). This pattern of staining was maintained in the cultures when the nodules had become mineralized after 9–16 d of culture (Fig. 2 D). Specificity of the antibody was confirmed by preincubating the antibody with an excess of partially purified platelet-derived latent TGF- $\beta$  or recombinant LTBP (data not shown). These controls appeared identical to the preimmune controls shown in Fig. 2, E–H.

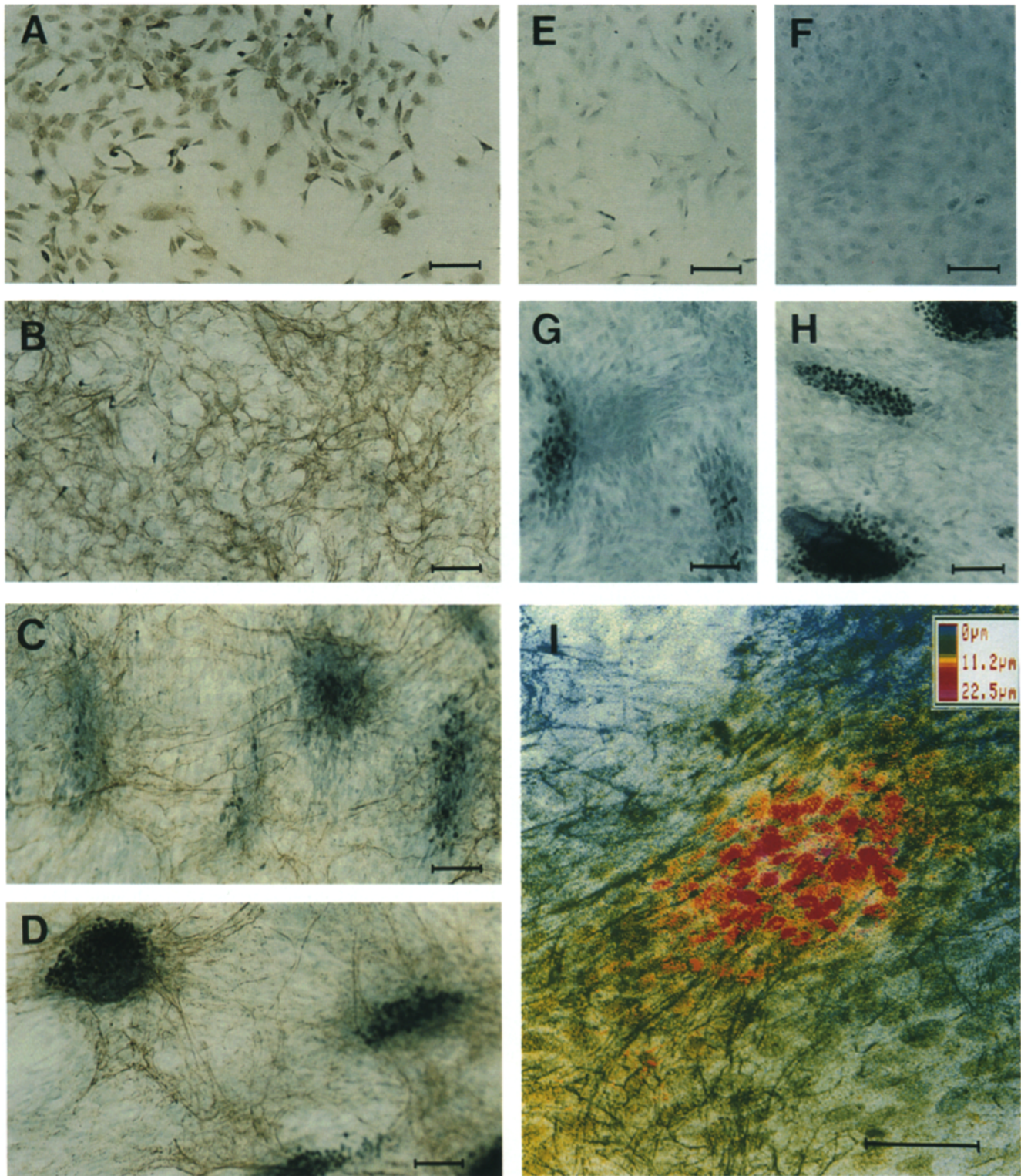
Confocal immunofluorescent depth profiles through the developing nodules, such as the example shown in Fig. 2 I, revealed that the LTBP fibers were located almost exclusively in the cell layers at the bottom of the nodule, and did not penetrate the cell layers higher in the nodule. Thus it appeared that the bone nodule formed on top of the cell layer containing an organized network of LTBP fibers.

### **LTBP Immunolocalization in Adult Rat Bone**

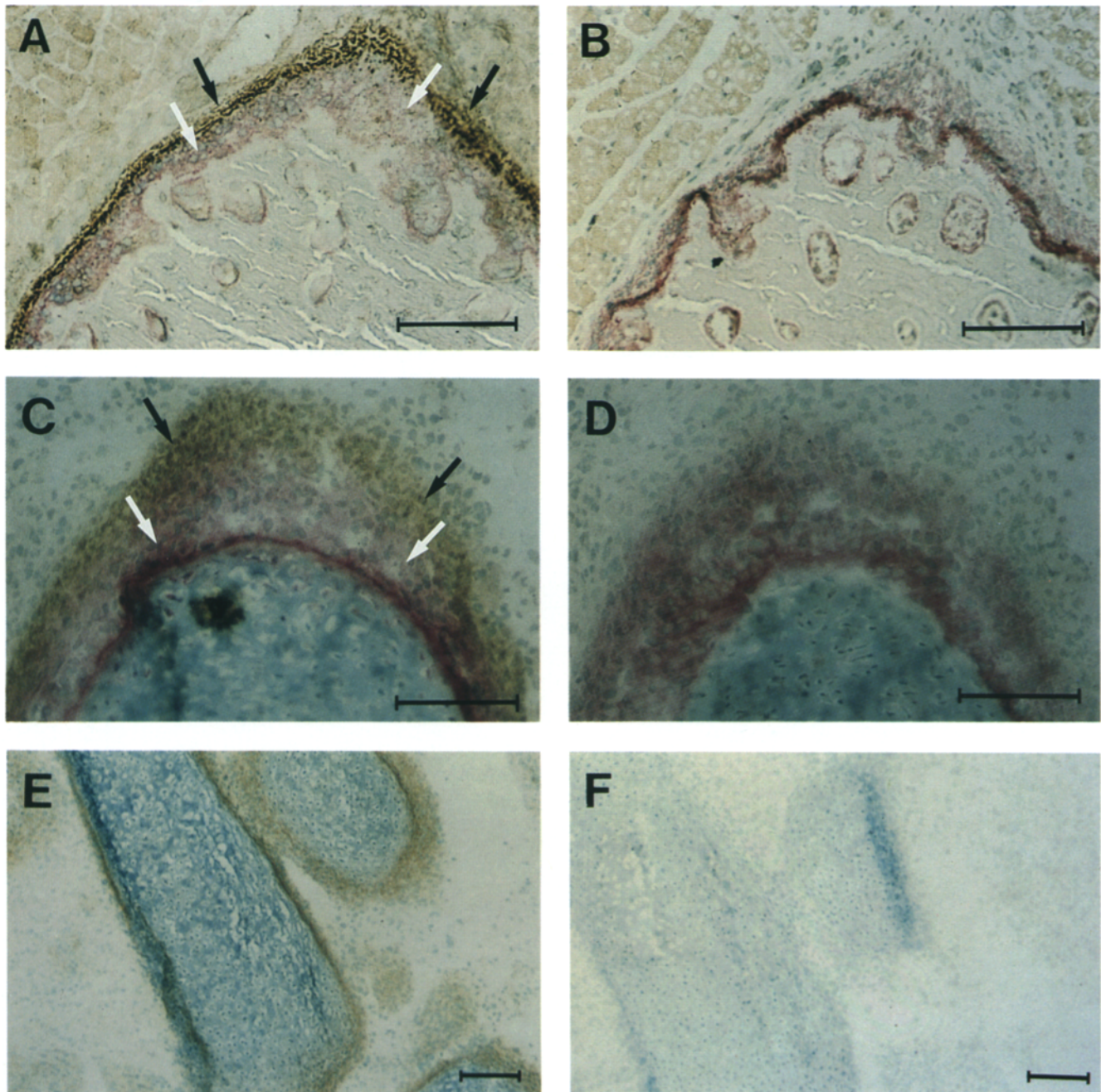
In the young adult rat ulna, LTBP expression was found in a zone encircling the whole circumference of the bone. This corresponds to the fibrous periosteum, the layer of cells peripheral to the alkaline phosphatase-positive osteoblastic layer (Fig. 3 A). The staining was fibrillar, although here the fibers are in cross section. The fibrillar nature of the LTBP staining is more clearly demonstrated in the connective tissue of the interosseous ligament that runs between the radius and ulna (Fig. 4, B and C) from which measurements of the fiber sizes were made using image analysis.

### **LTBP Staining in Fetal Rat Long Bones**

Fig. 3 C shows an oblique cryostat section through the de-



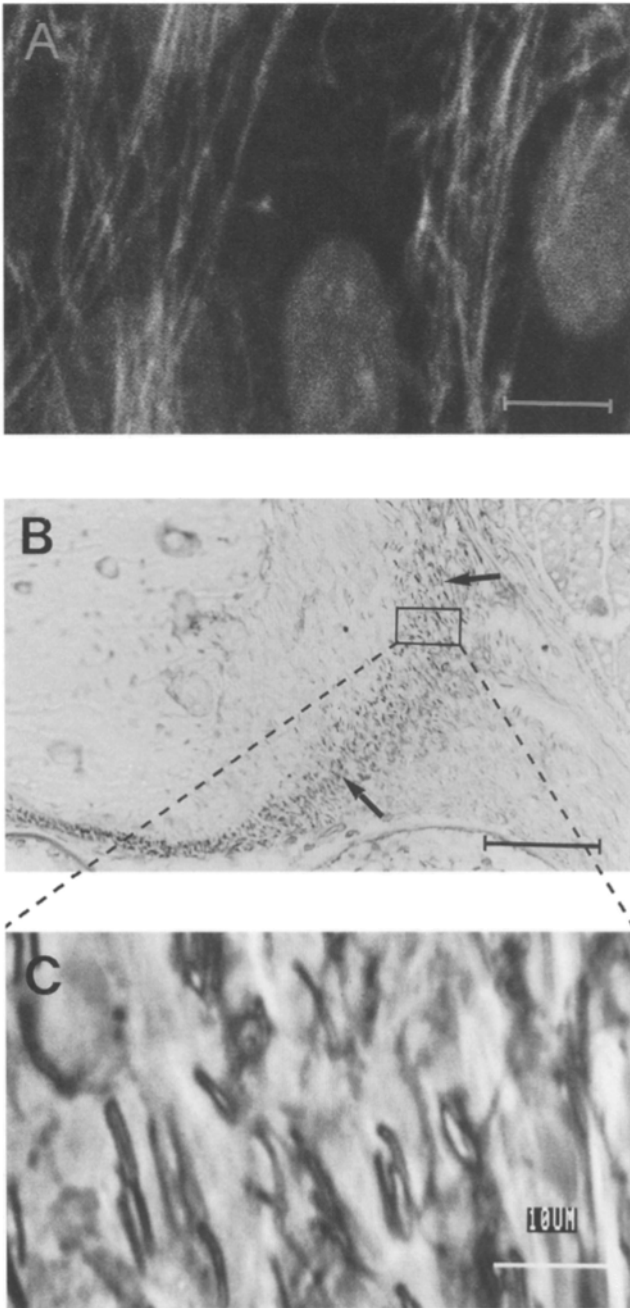
**Figure 2.** Immunolocalization of LTBP (brown immunoperoxidase staining with methyl green counterstain) in long-term cultures of fetal rat calvarial cells using a polyclonal antiserum against LTBP (*A–D*) with preimmune serum controls (*E–H*). Bar, 100  $\mu\text{m}$ . (*A*) Proliferating fetal rat calvarial cell cultures showing cytoplasmic localization of LTBP. (*B*) 2 d after reaching confluence (early stage) LTBP is localized to large fibrillar structures which form an organized network throughout the cultures. (*C*) 8 d after reaching confluence (middle stage) the fetal rat calvarial cell cultures are forming multilayered cellular nodules. The large fibrillar structures which stain positively for LTBP are organized around and between the developing nodules. (*D*) 15 d after reaching confluence the cellular nodules have become mineralized (late stage). The organization of LTBP-positive fibers spanning between mineralized nodules is maintained. (*E–H*) Preimmune serum controls for (*A–D*), respectively. (*I*) Confocal immunofluorescence depth profile through a developing nodule from a fetal rat calvarial cell culture at the middle stage stained for LTBP. Color scale indicates the distance of each structure from the surface of the culture plate (*blue*) to the top of the nodule (*red*). Bar, 50  $\mu\text{m}$ .



**Figure 3.** Immunolocalization of LTBP in the ulnae of young adult rats and the tibiae and metatarsals of 19-d fetal rats using a polyclonal antiserum against LTBP (A, C, E) with preimmune serum controls (B, D, F). Immunoperoxidase staining for LTBP (brown) with alkaline phosphatase histochemical stain (red) and methyl green nuclear counterstain. Bar, 100  $\mu$ m. (A) LTBP staining (black arrows) surrounding a transverse section of a young adult rat ulna. Staining is present in the fibrous periosteum peripheral to the alkaline phosphatase-positive osteoblast layer (white arrows). Staining is fibrillar, although here the fibers are in cross section. (B) Preimmune serum control, serial section to A. (C) oblique section of a 19 d fetal rat tibia showing LTBP staining (black arrows) adjacent to the alkaline phosphatase positive layer (white arrows) of the developing periosteum. (D) Preimmune serum control, serial section to C. (E) LTBP staining completely surrounds and delineates the developing metatarsals in this section of a 19-d fetal rat limb and extends into the cartilaginous matrix. (F) Preimmune control, serial section to E.

veloping tibia of a 19-d fetal rat. LTBP staining was localized in the developing periosteum in the layer of cells adjacent to the alkaline phosphatase-positive osteoblast layer, similar to the staining pattern in the young adult rat. There was a small zone of overlap where the cells were positive for both LTBP and alkaline phosphatase. In developing

fetal rat metatarsals (Fig. 3 E) that had not yet begun to mineralize, LTBP staining completely surrounded and delineated the developing cartilaginous bone rudiments. At higher magnification it could be seen that LTBP staining also extended partway into the cartilage matrix in certain areas (data not shown). There was evidence of fibrillar



**Figure 4.** (A) Confocal immunofluorescence micrograph showing examples of LTBP fibrillar structures in a confluent fetal rat calvarial cell culture which were measured using image analysis. Fiber sizes ranged from 0.6–1.2  $\mu\text{m}$  in diameter. Bar, 10  $\mu\text{m}$ . (B) Immunoperoxidase staining of LTBP fibrillar structures (arrows) in the interosseous ligament between the radius and ulna of a young adult rat. Bar, 100  $\mu\text{m}$ . (C) Higher power computer enhanced image showing examples of fibers measured by image analysis. Fiber sizes ranged from 0.7–1.3  $\mu\text{m}$  in diameter. Bar, 10  $\mu\text{m}$ .

staining in some areas, but the fibrillar staining pattern was not as well developed as in the young adult rat.

#### **Size Measurement of LTBP Fibrillar Structures**

The LTBP fibrillar structures in fetal rat calvarial cell cultures were measured by image analysis using confocal immunofluorescence microscopy (Fig. 4 A). Fibers ranged

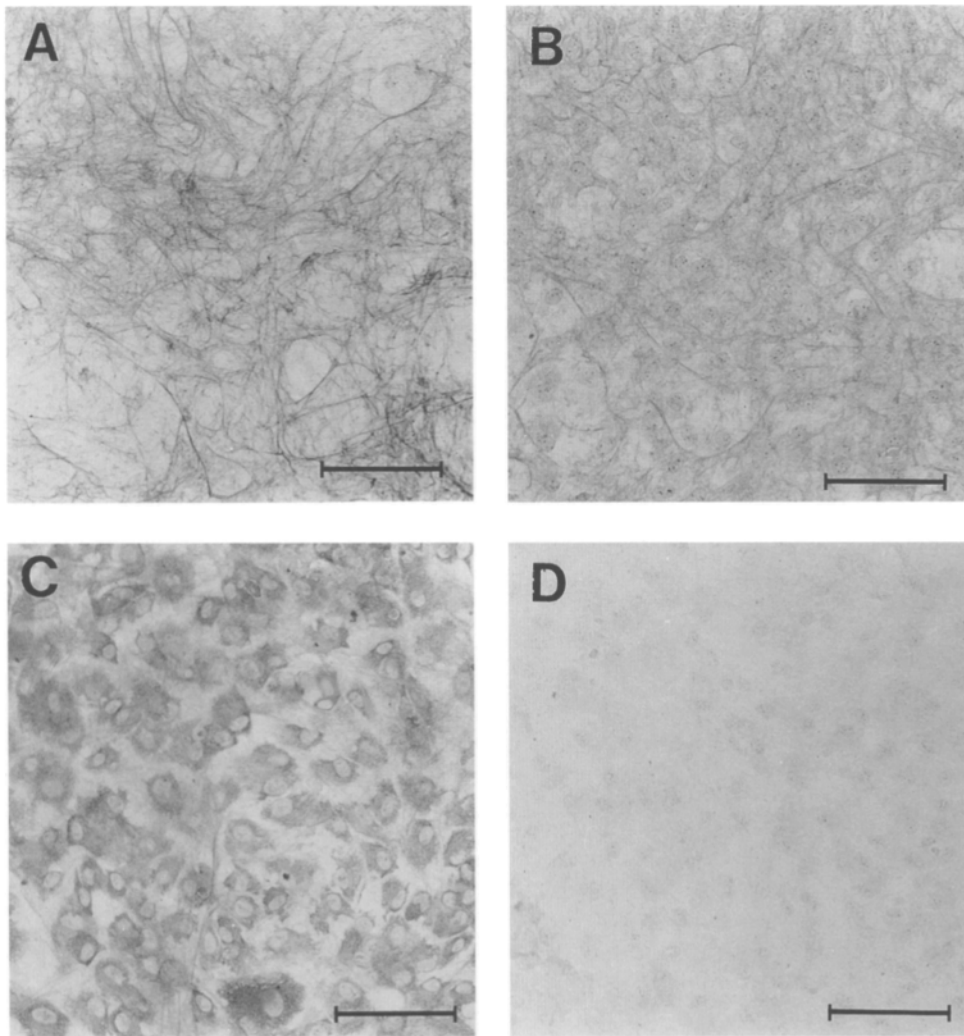
from 0.6 to 1.2  $\mu\text{m}$  in diameter. In growing adult rat bones, the fibrillar nature of the LTBP staining was most evident in the interosseous ligament that connects the radius and ulna (Fig. 4 B). Fiber measurements made from this region indicated that the fibers ranged from 0.7 to 1.3  $\mu\text{m}$  in diameter, which was almost an identical size to the fiber sizes measured in vitro.

#### **Immunolocalization of TGF- $\beta$ 1 Precursor and Type I Collagen in Fetal Rat Calvarial Cells**

Immunostaining for TGF- $\beta$ 1 precursor also revealed fibrillar structures in the extracellular matrix of postconfluent cultures of fetal rat calvarial cells (Fig. 5 B). Although the fibrillar staining with anti-TGF- $\beta$ 1 precursor was not as pronounced as with anti-LTBP (Fig. 5 A), the fibers were of similar size, suggesting that the LTBP fibrillar structures probably had TGF- $\beta$ 1 associated with them. In contrast, staining for type I collagen was distinctly different from that seen with LTBP. At 2 d after confluence, when a well-organized network of LTBP fibers was already formed (Fig. 5 A), staining for type I collagen was still predominantly cytoplasmic, with only a few collagen fibers visible in the extracellular matrix (Fig. 5 C). Collagen fibers were not abundant in the extracellular matrix until the middle stage of differentiation at  $\sim$ 5–6 d after confluence. Thus the appearance of LTBP fibrillar structures in the extracellular matrix preceded the appearance of type I collagen fibers.

#### **Effect of Plasmin Treatment on LTBP Immunostaining and Release of Latent TGF- $\beta$ Complexes from the Extracellular Matrix of Fetal Rat Calvarial Cells**

Taipale et al. (1994) have reported previously that LTBP is covalently linked to the extracellular matrix of fibroblasts, but that latent TGF- $\beta$  complexes containing proteolytic fragments of LTBP are released by digestion with plasmin. In the present study, treatment of fetal rat calvarial cell cultures with plasmin (0.1 U/ml) abolished the fibrillar staining for LTBP in the extracellular matrix (see Fig. 6, A and B). Disappearance of the fibrillar staining coincided with the release of both LTBP and TGF- $\beta$  from the extracellular matrix of fetal rat calvarial cells. These were present in a high molecular weight latent TGF- $\beta$  complex which migrated slightly greater than 200-kD under nonreducing conditions (see Fig. 6 C). This agrees well with the band sizes reported by Taipale et al. (1994), and probably represents a high molecular weight latent TGF- $\beta$  complex consisting of the 100-kD precursor form of latent TGF- $\beta$  complexed to an  $\sim$ 120-kD proteolytic fragment of LTBP. A higher molecular weight doublet was also seen with anti-LTBP antibodies which may represent aggregates of LTBP molecules. Antibodies to TGF- $\beta$  appeared to recognize only the lower band of the high molecular weight doublet, indicating that this form also had TGF- $\beta$  associated with it. Consistent with the Western blot data, plasmin treatment of fetal rat calvarial cell cultures also resulted in an increase in the amount of latent TGF- $\beta$  detectable in the conditioned medium by bioassay (data not shown).

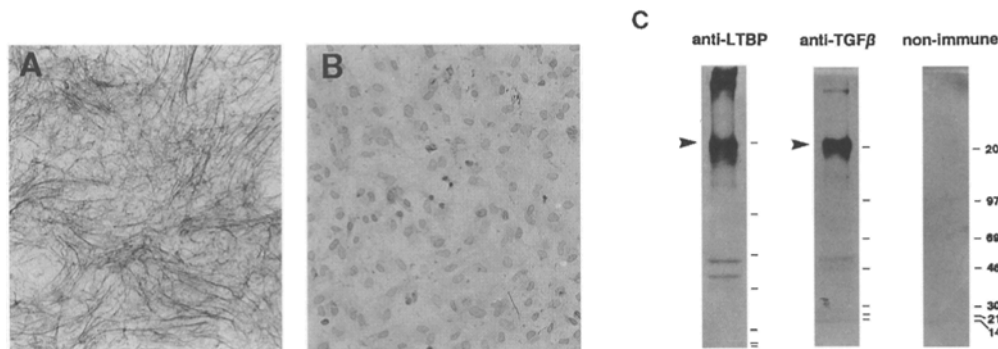


**Figure 5.** Immunolocalization of (A) LTBP, (B) TGF- $\beta$ 1-precursor, (C) type I collagen in fetal rat calvaria cell cultures compared with (D) nonimmune serum control. Immunoperoxidase staining was performed with no counterstain. Bar, 100  $\mu$ m. (A) LTBP fibrillar structures are present in a well-formed network by 2 d after confluence in early fetal rat calvarial cell cultures. (B) TGF- $\beta$ 1 precursor localizes to similar fibrillar structures in the extracellular matrix. (C) type I collagen staining is still predominantly cytoplasmic at this stage of culture, with only a few collagen fibers present in the extracellular matrix. Collagen fibers are not abundant in the extracellular matrix until day 5–6 after confluence. (D) Non-immune control.

**Effect of Antibodies and Antisense Oligonucleotides to LTBP on Bone Nodule Formation in Fetal Rat Calvarial Cell Cultures**

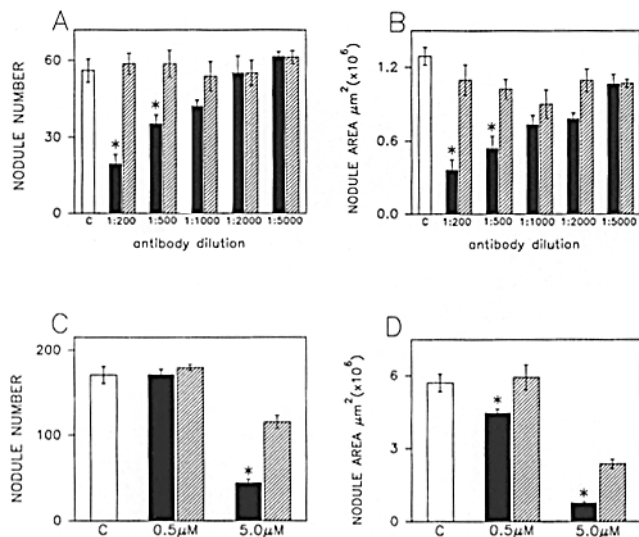
To determine whether the LTBP fibrillar structures played any role in the formation of mineralized bone nodules in fetal rat calvarial cell cultures, the cultures were treated with antibodies or antisense oligonucleotides directed against LTBP. When antibodies to LTBP were added

throughout the 12-d culture period they inhibited bone nodule formation in a dose-dependent manner (Fig. 7, A and B). Both the number and size of the bone nodules was inhibited. Antisense oligonucleotides against LTBP also inhibited bone nodule formation moderately, but significantly, compared with control sense oligonucleotides (Fig. 7, C and D). When conditioned media samples were collected after 3 d of treatment, antisense oligonucleotides to



**Figure 6.** (A and B) Immunolocalization of LTBP and (C) Western blot analysis of LTBP and TGF- $\beta$  after treatment of fetal rat calvarial cell cultures with 0.1 U/ml plasmin. Immunoperoxidase staining was performed with no counterstain. Bar, 100  $\mu$ m. (A) Control culture stained with anti-LTBP showing fibrillar staining pattern, (B) culture treated with 0.1 U/ml plasmin for 1 h then stained with anti-

LTBP. Fibrillar staining is abolished, (C) Western blot analysis of latent TGF- $\beta$  complexes released by plasmin treatment of the extracellular matrix from fetal rat calvarial cells (nonreducing gel). A complex slightly greater than 200 kD is seen (arrow) which reacts with both anti-LTBP and anti-TGF- $\beta$  antibodies.



**Figure 7.** Histograms showing the effects of treatment with anti-serum (A and B) and antisense oligonucleotides (C and D) against LTBP on the number and area of mineralized nodules formed in long term cultures of fetal rat calvarial cells. \*, significantly decreased compared with preimmune serum control or sense oligonucleotide control.  $P < 0.05$  (ANOVA/student-Newman-Keul's method of multiple comparisons). (A and B) □, control; ■, anti-LTBP; and ▨, nonimmune. (C and D) □, control; ■, LTBP-antisense; and ▨, LTBP-sense.

LTBP at the 5- $\mu\text{M}$  dose were found to reduce significantly the amount of LTBP protein secreted by the cells as measured by ELISA (see Table I). Other supportive data for a role for LTBP in bone formation in vitro includes reduction of the number and size of mineralized bone nodules formed in cultures of fetal rat calvarial cells treated with plasmin, which releases LTBP from the extracellular matrix (data not shown).

To determine whether active TGF- $\beta$  was also playing a role in bone nodule formation in vitro, fetal rat calvarial cell cultures were treated with antibodies to TGF- $\beta$ 1 and TGF- $\beta$ 2. These antibodies had essentially no effect on either the number or size of the nodules (data not shown). This suggests that, in contrast to LTBP, active TGF- $\beta$  does not play a significant role in bone formation in vitro.

**Table I. Concentrations of LTBP in Conditioned Media from Fetal Rat Calvarial Cell Cultures Treated with Sense and Antisense Oligonucleotides against LTBP (as shown in Fig. 7, C and D)**

		LTBP concentration
		ng/ml
Control		196.1 $\pm$ 11.2
LTBP sense	5.0 $\mu\text{M}$	176.9 $\pm$ 1.3
	0.5 $\mu\text{M}$	165.0 $\pm$ 2.0
LTBP antisense	5.0 $\mu\text{M}$	81.1 $\pm$ 3.9*
	0.5 $\mu\text{M}$	186.9 $\pm$ 7.6

Data are mean  $\pm$  SEM from triplicate wells.

\*Significantly decreased compared with sense control,  $P < 0.05$  (ANOVA/student-Newman-Keul's method of multiple comparisons).

## Discussion

In this study we have clearly demonstrated that LTBP localizes to large (0.6–1.3  $\mu\text{m}$ ) fibrillar structures in the extracellular matrix of bone cells in vitro and in vivo. Although these observations are entirely consistent with the hypothesis that LTBP targets latent TGF- $\beta$  to the extracellular matrix for storage, they also suggest a previously undescribed role for this protein as a structural extracellular matrix protein. The fibrillar localization of LTBP is particularly interesting in view of the homology of LTBP to the fibrillins. Fibrillins 1 and 2 have been identified, and a fibrillin-like protein has also been reported (Sakai et al., 1986, 1991; Rosenbloom et al., 1993; Zhang et al., 1994). These proteins are a major component of connective tissue microfibrils and share a number of features with LTBP including multiple EGF-like repeats, an RGD sequence, a putative calcium-binding domain, and a novel eight-cysteine repeat motif which is unique to this family of proteins. This suggests that there may be a whole gene family of fibrillin-like proteins that are involved in the formation of microfibrils and that have important structural roles in maintaining tissue integrity and organization.

A number of other unrelated proteins have been shown to be constituents of microfibrils. These include a 78-kD microfibrillar protein (MP78) and a 31-kD protein called microfibril-associated glycoprotein (Gibson et al., 1989). In some tissues, microfibrils may be associated with elastin to form elastic fibers (for review see Rosenbloom et al., 1993) and a recent report has shown that the proteoglycan versican is associated with elastic fibers in the dermis (Zimmerman et al., 1994). Thus it appears that microfibrils may be composed of a number of constituent proteins, whose relative proportions vary in different tissues, thus defining structural and functional characteristics specific for each tissue (Davis, 1994). A second latent TGF- $\beta$  binding protein (LTBP-2) recently has been isolated which has 41% homology of LTBP (Morén et al., 1994) and it seems probable that the LTBPs will be classified as new members of the growing family of fibrillin-like proteins involved in the formation of connective tissue microfibrils. This hypothesis is currently under investigation in our laboratory and preliminary data indicate that, like fibrillin, some of the LTBP fibrillar structures colocalize with elastic fibers in the young adult rat ulna, but that other LTBP fibers exist independently of any elastin (Dallas, S. L., unpublished observations).

The fibrillar localization of LTBP in the extracellular matrix of bone cells is clearly consistent with a role for this molecule in storing latent TGF- $\beta$  in the matrix. This idea is supported by the finding that immunostaining for TGF- $\beta$ 1-precursor reveals similar fibrillar structures in the extracellular matrix of fetal rat calvarial cells. Furthermore, the demonstration that loss of fibrillar LTBP staining after treatment with plasmin is associated with release of both LTBP and TGF- $\beta$  in a high molecular weight complex provides further evidence that the LTBP fibers do have TGF- $\beta$  associated with them.

Our data support those of Taipale et al. (1994) showing that LTBP complexed to TGF- $\beta$  can be released from the extracellular matrix of fibroblasts by plasmin treatment. This finding may be very significant in view of the poten-



tial role of plasmin in activation of latent TGF- $\beta$ . It is well established that plasmin is able to activate the precursor form of latent TGF- $\beta$  in the test tube (Lyons et al., 1988; 1990). The plasminogen-plasminogen activator system may also be involved in the activation of latent TGF- $\beta$  observed in co-cultures of endothelial and smooth muscle cells (Sato and Rifkin, 1989) and in osteoblasts stimulated by parathyroid hormone (Yee et al., 1993). Expression of plasminogen activator is regulated by numerous agents, including osteotropic factors such as parathyroid hormone (Hamilton et al., 1985; Pfeilschifter et al., 1990; Yee et al., 1994). Therefore the plasminogen-plasminogen activator system may mediate the effects of agents such as parathyroid hormone on TGF- $\beta$  activity. The observations of the present study together with those of Taipale et al. (1994) indicate that plasmin is able not only to activate soluble forms of latent TGF- $\beta$ , but is also able to access matrix-bound stores of latent TGF- $\beta$  through cleavage of LTBP. This may have important consequences for tissue repair and wound healing, and release of matrix-bound latent TGF- $\beta$  may be one of the mechanisms through which plasmin exerts its effects on matrix remodeling.

In nodule-forming fetal rat calvarial cell cultures the LTBP fibrillar structures were located in the cell layers underneath the bone nodule. In vivo LTBP was found in an analogous distribution, surrounding the bone and adjacent to the alkaline phosphatase-positive bone forming osteoblasts. These observations, together with the evidence that antibodies and antisense oligonucleotides against LTBP inhibited nodule formation, indicate that formation of the LTBP fibrillar structures may be an important event for subsequent bone formation to occur. It seems unlikely that this is a process specific to bone cells, but rather that the LTBP fibrillar structures are involved in maintaining the structural integrity of the tissue or cell monolayer, which may be a prerequisite for bone formation to occur. Thus, the LTBP fibers may form a "scaffolding" upon which the bone subsequently forms. An alternative possibility is that the LTBP fibrillar structures are involved in cell migration and organization, and that the arrangement of LTBP fibers may determine where bone will form. These hypotheses are currently under investigation.

It is well documented that TGF- $\beta$  autoinduces its own mRNA expression in several cell types (Van Obberghen-Schilling et al., 1988; Bascom et al., 1989; Kim et al., 1989, 1990). TGF- $\beta$  has also been shown to stimulate expression of numerous matrix proteins that include fibronectin and the fibronectin receptor, collagen, elastin, tenascin, thrombospondin, biglycan, and vitronectin (Ignatz and Massagué, 1986; Penttinen et al., 1988; Roberts et al., 1988; Rossi et al., 1988; McKay et al., 1993; Marigo et al., 1994). We have shown previously that TGF- $\beta$  is able to stimulate expression of mRNA and protein for both TGF- $\beta$  and LTBP in osteosarcoma cells (Dallas et al., 1994) and have recently confirmed a similar effect in subconfluent fetal rat calvarial cells (Dallas, S. L., unpublished observations). The addition of LTBP to the list of extracellular matrix proteins whose expression is stimulated by TGF- $\beta$  further emphasizes the importance of this molecule in inducing new extracellular matrix formation. TGF- $\beta$  also promotes matrix accumulation by inhibiting the expression of the proteases that might degrade matrix proteins while stimu-

lating expression of protease inhibitors (Edwards et al., 1987; Laiho et al., 1987; Kubota et al., 1991; Tomooka et al., 1992). Thus TGF- $\beta$  promotes extracellular matrix accumulation by exerting control at multiple levels. The incorporation of latent TGF- $\beta$  into the matrix through binding to LTBP may represent another level of control for TGF- $\beta$ , whereby it can exert effects on cells at a later date after release from the matrix and subsequent activation. This may be particularly important in tissue repair, where TGF- $\beta$  appears to play a pivotal role in determining whether healing will be normal as in wound healing, or pathological as in scarring and fibrotic diseases.

An interesting finding was that in fetal rat calvarial cell cultures, a well-organized network of LTBP fibers was clearly visible by two days after confluence, which is well before type I collagen fibers became abundant in the extracellular matrix at five to six days after confluence. Although this does not rule out the possibility that the fibrillar LTBP staining occurs as a result of its binding to other fibrillar matrix protein(s), such as the fibrillins or fibronectin, it does indicate that LTBP is not colocalizing with type I collagen, the most abundant matrix protein in bone. The fact that the LTBP fibrillar staining precedes the appearance of collagen fibers in the extracellular matrix suggests that it may be one of the earliest proteins to appear in the matrix. Preliminary data in our laboratory indicate that LTBP expression is increased in certain fibrotic disease states. Therefore an understanding of the mechanisms for regulation of LTBP expression may be of great clinical importance in treating fibrotic disease states, particularly since excess TGF- $\beta$  may play a major causative role in these conditions (Border et al., 1992).

In conclusion, this study is the first demonstration that the latent TGF- $\beta$  binding protein is a structural extracellular matrix protein involved in the formation of large fibrillar structures in the extracellular matrix of bone cells. LTBP appears to play a role in bone formation in vitro and its localization in vivo is also suggestive of a role in bone development and formation. Future studies are required to elucidate further the role of this protein in bone formation, particularly in view of recent reports that Marfan syndrome and related disorders of the cardiovascular and skeletal systems have been linked to mutations in genes for the fibrillin family of extracellular matrix proteins of which LTBP may be a new member (Dietz et al., 1991; Lee et al., 1991; Maslen et al., 1991; Milewicz et al., 1992; Lönnqvist et al., 1994). TGF- $\beta$  may be the first example of a growth factor whose latent form is complexed to a protein which actually appears to function as a structural matrix protein, and may play a role in both storage of the molecule in the matrix and its subsequent release and activation. This may be a precedent for other growth factors, and could represent an exquisite system for the fine regulation of growth factor actions.

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## References

- Bascom, C. B., J. R. Wolfshohl, R. J. Coffey, L. Madisen, N. R. Webb, A. R. Purchio, R. Derynck, and H. L. Moses. 1989. Complex regulation of transforming growth factor  $\beta$  1,  $\beta$  2,  $\beta$  3 mRNA expression in mouse fibroblasts and keratinocytes by transforming growth factors  $\beta$  1 and  $\beta$  2. *Mol. Cell. Biol.* 9: 5508-5515.
- Bellows, C. G., J. E. Aubin, J. N. M. Heersche, and M. E. Antosz. 1986. Mineralized bone nodules formed in vivo from enzymatically released rat calvaria cell population. *Calcif. Tissue Int.* 38:143-154.
- Bonewald, L. F., and S. L. Dallas. 1994. The role of active and latent TGF- $\beta$  in bone formation. *J. Cell. Biochem.* 55:350-357.
- Bonewald, L. F., L. Wakefield, R. O. C. Oreffo, A. Escobedo, D. R. Twardzik, and G. R. Mundy. 1991. Latent forms of transforming growth factor-beta (TGF- $\beta$ ) derived from bone cultures. Identification of a naturally occurring 100-kDa complex with similarity to recombinant latent TGF- $\beta$ . *Mol. Endocrinol.* 5:741-751.
- Border, W. A., and E. Ruoslahti. 1992. Transforming growth factor- $\beta$  in disease: the dark side of tissue repair. *J. Clin. Invest.* 90:1-7.
- Border, W. A., N. A. Nole, T. Yamamoto, J. R. Harper, Y. Yamaguchi, M. D. Pierschbacher, and E. Ruoslahti. 1992. Natural inhibitor of TGF-beta protects against scarring in experimental kidney disease. *Nature (Lond.)* 360: 361-364.
- Centrella, M., M. C. Horowitz, J. M. Wozney, and T. L. McCarthy. 1994. Transforming growth factor- $\beta$  gene family members and bone. *Endocr. Rev.* 15: 27-39.
- Dallas, S. L., S. Park-Snyder, K. Miyazono, D. Twardzik, G. R. Mundy, and L. F. Bonewald. 1994. Characterization and autoregulation of latent transforming growth factor  $\beta$  (TGF- $\beta$ ) complexes in osteoblast-like cell lines. *J. Biol. Chem.* 269:6815-6822.
- Davis, E. C. 1994. Immunolocalization of microfibril and microfibril-associated proteins in the subendothelial matrix of the developing mouse aorta. *J. Cell Sci.* 107:727-736.
- Dietz, H. C., G. R. Cutting, R. E. Pyeritz, C. L. Maslen, L. Y. Sakai, G. M. Corson, E. G. Puffenberger, A. Hamosh, E. J. Nathakumar, S. M. Curristin, et al. 1991. Marfan syndrome caused by a recurrent de novo missense mutation in the fibrillin gene. *Nature (Lond.)* 352:337-339.
- Dwartzky, S. I., E. G. Fey, S. Penman, J. B. Lian, J. L. Stein, and G. S. Stein. 1990. Progressive changes in the protein composition of the nuclear matrix during rat osteoblast differentiation. *Proc. Natl. Acad. Sci. USA.* 87:4605-4609.
- Edwards, D. R., G. Murphy, J. J. Reynolds, S. E. Whitham, A. J. P. Docherty, P. Angel, and J. K. Heath. 1987. Transforming growth factor beta modulates the expression of collagenase and metalloprotease inhibitor. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1899-1904.
- Flaumenhaft, R., M. Abe, Y. Sato, K. Miyazono, J. Harper, C.-H. Heldin, and D. B. Rifkin. 1993. Role of the latent TGF- $\beta$  binding protein in the activation of latent TGF- $\beta$  by co-cultures of endothelial and smooth muscle cells. *J. Cell Biol.* 120:995-1002.
- Gentry, L. E., N. R. Webb, J. G. Lim, A. M. Brunner, J. E. Ranchalis, D. R. Twardzik, M. N. Lioubin, H. Marquardt, and A. F. Purchio. 1987. Type I transforming growth factor beta: amplified expression and secretion of mature and precursor polypeptides in Chinese hamster ovary cells. *Mol. Cell. Biol.* 7:3418-3427.
- Gibson, M. A., J. S. Kumaratilake, and E. G. Cleary. 1989. The protein components of the 12-nanometer microfibrils of elastic and nonelastic tissues. *J. Biol. Chem.* 264:4590-4598.
- Hamilton, J. A., S. Lingelbach, N. C. Partridge, and T. J. Martin. 1985. Regulation of plasminogen activator production by bone-resorbing hormones in normal and malignant osteoblasts. *Endocrinology.* 116:2186-2191.
- Harris, S. E., L. F. Bonewald, M. A. Harris, M. Sabatini, S. L. Dallas, J. Feng, N. Ghosh-Choudhury, J. Wozney, and G. R. Mundy. 1994. Effects of TGF- $\beta$  on bone nodule formation and expression of bone morphogenetic protein-2, osteocalcin, osteopontin, alkaline phosphatase and type I collagen mRNA in prolonged cultures of fetal rat calvarial osteoblasts. *J. Bone Miner. Res.* 9: 855-863.
- Ignatz, R. A., and J. Massagué. 1986. Transforming growth factor- $\beta$  stimulates expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J. Biol. Chem.* 261:4337-4345.
- Kanzaki, T., A. Olofsson, A. Morén, C. Wernstedt, U. Hellman, K. Miyazono, L. Claesson-Welsh, and C. H. Heldin. 1990. TGF- $\beta$  1 binding protein: a component of the large latent complex of TGF- $\beta$  1, with multiple repeat sequences. *Cell.* 61:1051-1061.
- Kim, S.-J., A. Glick, M. B. Sporn, and A. B. Roberts. 1989. Characterization of the promoter region of the human transforming growth factor- $\beta$  1 gene. *J. Biol. Chem.* 264:402-408.
- Kim, S. J., P. Angel, R. Lafyatis, K. Hattori, K. Y. Kim, M. B. Sporn, M. Karin, and A. B. Roberts. 1990. Autoinduction of TGF- $\beta$  1 is mediated by the AP-1 complex. *Mol. Cell. Biol.* 10:1492-1497.
- Kubota, S., R. Fridman, and Y. Yamada. 1991. Transforming growth factor- $\beta$  suppresses the invasiveness of human fibrosarcoma cells in vitro by increasing expression of tissue inhibitor of metalloprotease. *Biochem. Biophys. Res. Commun.* 176:129-136.
- Laiho, M., O. Saksela, and J. Keski-Oja. 1987. Transforming growth factor- $\beta$  induction of type-I plasminogen activator inhibitor. *J. Biol. Chem.* 262:17467-17474.
- Lee, B., M. Godfrey, E. Vitale, H. Hori, M. G. Mattei, M. Sarfarazi, P. Tsiouras, F. Ramirez, and D. W. Hollister. 1991. Linkage of Marfan syndrome and a phenotypically related disorder to two different fibrillin genes. *Nature (Lond.)* 352:330-334.
- Lönnqvist, L., A. Child, K. Kainulainen, R. Davidson, L. Puhakka, and L. Peltonen. 1994. A novel mutation of the fibrillin gene causing ectopia lentis. *Genomics.* 19:573-576.
- Lyons, R. M., J. Keski-Oja, and H. L. Moses. 1988. Proteolytic activation of latent transforming growth factor- $\beta$  from fibroblast-conditioned medium. *J. Cell Biol.* 106:1659-1665.
- Lyons, R. M., L. E. Gentry, A. F. Purchio, and H. L. Moses. 1990. Mechanism of activation of latent recombinant transforming growth factor  $\beta$  1 by plasmin. *J. Cell Biol.* 110:1361-1367.
- Maddox, B. K., L. Y. Sakai, D. R. Keene, and R. W. Glanville. 1989. Connective tissue microfibrils. Isolation and characterization of three large pepsin-resistant domains of fibrillin. *J. Biol. Chem.* 264:21381-21385.
- Marigo, V., D. Volphin, G. Vitale, and G. M. Bressn. 1994. Identification of a TGF- $\beta$  responsive element in the human elastin promoter. *Biochem. Biophys. Res. Commun.* 199:1049-1056.
- Maslen, C. L., G. M. Corson, B. K. Maddox, R. W. Glanville, and L. Y. Sakai. 1991. Partial sequence of a candidate gene for the Marfan syndrome. *Nature (Lond.)* 352:334-336.
- McKay, N. G., T. F. Khong, N. E. Haites, and D. A. Power. 1993. The effect of transforming growth factor beta 1 on mesangial cell fibronectin synthesis: increased incorporation into the extracellular matrix and reduced pI but no effect on alternative splicing. *Exp. Mol. Pathol.* 59:211-224.
- Milewicz, D. M., R. E. Pyeritz, E. S. Crawford, and P. H. Byers. 1992. Marfan syndrome: defective synthesis, secretion, and extracellular matrix formation of fibrillin by cultured dermal fibroblasts. *J. Clin. Invest.* 89:79-86.
- Miyazono, K., U. Hellman, C. Wernstedt, and C. H. Heldin. 1988. Latent high molecular weight complex of transforming growth factor beta. *J. Biol. Chem.* 263:6407-6415.
- Miyazono, K., A. Olofsson, P. Colosetti, and C. H. Heldin. 1991. A role of the latent TGF- $\beta$  1-binding protein in the assembly and secretion of TGF- $\beta$  1. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1091-1101.
- Mizoi, T., H. Ohtani, K. Miyazono, M. Miyazawa, S. Matsuno, and H. Nagura. 1993. Immunoelectron microscopic localization of transforming growth factor  $\beta$  1 and latent transforming growth factor  $\beta$  1 binding protein in human gastrointestinal carcinomas: qualitative difference between cancer cells and stromal cells. *Cancer Res.* 53:183-190.
- Morén, A., A. Olofsson, G. Stenman, P. Sahlin, T. Kanzaki, L. Claesson-Welsh, P. Ten Dijke, K. Miyazono, and C. H. Heldin. 1994. Identification and characterization of LTBP-2, a novel latent transforming growth factor- $\beta$  binding protein. *J. Biol. Chem.* 269:32469-32478.
- Penttinen, R. P., S. Kobayashi, and P. Bornstein. 1988. Transforming growth factor  $\beta$  increased mRNA for matrix proteins both in the presence and absence of changes in mRNA stability. *Proc. Natl. Acad. Sci. USA.* 85:1105-1108.
- Pfeilschifter, J., J. Erdman, W. Schmidt, A. Naumann, H. W. Minne, and R. Ziegler. 1990. Differential regulation of plasminogen activator and plasminogen and activator inhibitor by osteotropic factors in primary cultures of mature osteoblasts and osteoblast precursors. *Endocrinology.* 126:703-711.
- Roberts, A. B., and M. B. Sporn. 1990. The transforming growth factor- $\beta$ s. In *Peptide Growth Factors and their receptors I, Handbook of Experimental Pharmacology.* M. B. Sporn and A. B. Roberts, editors. Springer-Verlag, New York Inc., New York. 419-472.
- Roberts, C. J., T. M. Birkenmeier, J. J. McQuillan, S. K. Akiyama, S. S. Yamada, W. T. Chen, K. M. Yamada, and J. A. McDonald. 1988. Transforming growth factor  $\beta$  stimulates expression of fibronectin and both subunits of the human fibronectin receptor by cultures of human lung fibroblasts. *J. Biol. Chem.* 263:4586-4592.
- Rosenbloom, J., W. R. Abrams, and R. Mecham. 1993. Extracellular matrix 4: the elastic fiber. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 7:1208-1218.
- Rossi, P., G. Karsenty, A. B. Roberts, N. S. Roche, M. B. Sporn, and B. De Crombrughe. 1988. A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor- $\beta$ . *Cell.* 52:405-414.
- Sakai, L. Y., D. R. Keene, and E. Engvall. 1986. Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils. *J. Cell Biol.* 103:2499-2509.
- Sakai, L. Y., D. R. Keene, R. W. Glanville, and H. P. Bächinger. 1991. Purification and partial characterization of fibrillin, a cysteine-rich structural component of connective tissue microfibrils. *J. Biol. Chem.* 266:14763-14770.
- Sato, Y., and D. B. Rifkin. 1989. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor- $\beta$  1-like molecule by plasmin during co-culture. *J. Cell Biol.* 109:309-315.
- Sato, Y., F. Okada, M. Abe, T. Seguchi, M. Kuwano, S. Sato, A. Furuya, N. Hanai, and T. Tamaoki. 1993. The mechanism for the activation of latent TGF- $\beta$  during co-culture of endothelial cells and smooth muscle cells: Cell-type spe-

- cific targeting of latent TGF- $\beta$  to smooth muscle cells. *J. Cell Biol.* 123:1249–1254.
- Skerry, T. M., L. Bitensky, J. Chayen, and L. E. Lanyon. 1989. Early strain-related changes in enzyme activity in osteocytes following bone loading in vivo. *J. Bone Miner. Res.* 4:783–788.
- Sporn, M. B., and A. B. Roberts. 1992. Transforming growth factor- $\beta$ : recent progress and new challenges. *J. Cell Biol.* 119:1017–1021.
- Taipale, J., K. Miyazono, C.-H. Heldin, and J. Keski-Oja. 1994. Latent transforming growth factor- $\beta$ 1 associates to fibroblast extracellular matrix via latent TGF- $\beta$  binding protein. *J. Cell Biol.* 124:171–181.
- Tomooka, S., W. A. Border, B. O. Marshall, and W. A. Noble. 1992. Glomerular matrix accumulation is linked to inhibition of the plasmin protease system. *Kidney Int.* 42:1462–1469.
- Tsuji, T., F. Okada, K. Yamaguchi, and T. Nakamura. 1990. Molecular cloning of the large subunit of transforming growth factor type  $\beta$  masking protein and expression of the mRNA in various rat tissues. *Proc. Natl. Acad. Sci. USA.* 87:8835–8839.
- Van Obberghen-Schilling, E., N. S. Roche, K. C. Flanders, M. B. Sporn, and A. B. Roberts. 1988. Transforming growth factor- $\beta$ 1 positively regulates its own expression in normal and transformed cells. *J. Biol. Chem.* 263:7741–7746.
- Wakefield, L. M., D. M. Smith, K. C. Flanders, and M. B. Sporn. 1988. Latent transforming growth factor-beta from human platelets. A high molecular weight complex containing precursor sequences. *J. Biol. Chem.* 263:7646–7654.
- Yee, J. A., L. Yan, J. C. Diminguez, E. H. Allan, and T. J. Martin. 1993. Plasminogen-dependent activation of latent transforming growth factor beta (TGF- $\beta$ ) by growing cultures of osteoblast-like cells. *J. Cell. Physiol.* 157:528–534.
- Zhang, H., S. D. Apfelroth, W. Hu, E. C. Davis, C. Sanguinetti, J. Bonadio, R. P. Mecham, and F. Ramirez. 1994. Structure and expression of fibrillin-2, a novel microfibrillar component preferentially located in elastic matrices. *J. Cell Biol.* 124:855–863.
- Zimmermann, D. R., M. T. Dours-Zimmermann, M. Schubert, and L. Bruckner-Tuderman. 1994. Versican is expressed in the proliferating zone in the epidermis and in association with the elastic network of the dermis. *J. Cell Biol.* 124:817–825.