Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor β

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We have analysed the interactions of three proteoglycans of the decorin family, decorin, biglycan and fibromodulin, with transforming growth factor β (TGF-β). The proteoglycan core proteins, expressed from human cDNAs as fusion proteins with *Escherichia coli* maltose-binding protein, each bound TGF-β1. They showed only negligible binding to several other growth factors. Intact decorin, biglycan and fibromodulin isolated from bovine tissues competed with the fusion proteins for the TGF-β binding. Affinity measurements suggest a two-site binding model with K_d values ranging from 1 to 20 nM for a high-affinity binding site and 50 to 200 nM for the lower-affinity binding site. The stoichiometry indicated that the high-affinity binding site was present in one of ten proteoglycan core molecules and that each molecule contained a low-affinity binding site. Tissue-derived biglycan and decorin were less effective competitors for TGF-β binding than fibromodulin or the non-glycosylated fusion proteins; removal of the chondroitin/dermatan sulphate chains of decorin and biglycan (fibromodulin is a keratan sulphate proteoglycan) increased the activities of decorin and biglycan, suggesting that the glycosaminoglycan chains may hinder the interaction of the core proteins with TGF-β. The fusion proteins competed for the binding of radiolabelled TGF-β to Mv 1 Lu cells and endothelial cells. Affinity labelling showed that the binding of TGF-β to betaglycan and the type-I receptors in Mv 1 Lu cells and to endoglin in endothelial cells was reduced, but the binding to the type-II receptors was unaffected. TGF-β2 and 3 also bound to all three fusion proteins. Latent recombinant TGF-β1 precursor bound slightly to fibromodulin and not at all to decorin and biglycan. The results show that the three decorin-type proteoglycans each bind TGF-β isoforms and that slight differences exist in their binding properties. They may regulate TGF-β activities by sequestering TGF-β into extracellular matrix.

INTRODUCTION

One of the key functions of the extracellular matrix is the storage and presentation of growth factors to cells. Proteoglycans are important mediators of growth-factor binding, and they have been shown to modulate the biological activities of a variety of growth factors through interaction via their glycosaminoglycan moieties as well as their core proteins (Ruoslahti, 1989; Ruoslahti and Yamaguchi, 1991).

Growth factors that bind to glycosaminoglycans include acidic and basic fibroblast growth factor (Burgess and Maciag, 1989), granulocyte–macrophage colony-stimulating factor, interleukin 3 (Roberts et al., 1988), pleiotrophin (Li et al., 1990), amphiregulin (Shoyab et al., 1988), heparin-binding epidermal growth factor (EGF) (Higashiyama et al., 1991) and platelet factor 4 (Huang et al., 1982), each of which binds avidly to heparin and heparan sulphate. The binding of fibroblast growth factors to heparin or heparan sulphate proteoglycans protects the growth factors from proteolytic degradation and is thought to create a matrix-bound growth-factor reservoir (Saksela et al., 1988; Gospodarowicz et al., 1990) from which the growth factor can be released in an active form by partial proteolysis of the proteoglycan core protein or through degradation of the heparan sulphate moiety of the proteoglycans (Saksela and Rifkin, 1990; Ishai-Michaeli et al., 1990). Basic fibroblast growth factor has to be bound to glycosaminoglycan to be able to interact with its signal-transduction receptor (Yayon et al., 1991; Rapraeger et al., 1991).

The binding of transforming growth factor β (TGF-β) to proteoglycans represents a different type of growth-factor–proteoglycan interaction. TGF-β has been demonstrated to bind to the core proteins of at least two proteoglycans. One of these proteoglycans is the type-III TGF-β receptor, betaglycan (Segarini and Seyedin, 1988; Andres et al., 1989). Betaglycan is a cell-membrane proteoglycan (López-Casillas et al., 1991; Wang et al., 1991) which apparently is not involved in the TGF-β signal-transduction pathway but may function as a cell-surface TGF-β reservoir presenting TGF-β to its signal-transduction receptors.

The second type of TGF-β-binding proteoglycan is decorin, a small interstitial extracellular matrix proteoglycan that can interact with TGF-β via its core protein (Yamaguchi et al., 1990). Decorin (Krusius and Ruoslahti, 1986) is the prototype of a group of proteoglycans characterized by core proteins of ~ 40 kDa which consist mainly of leucine-rich repeats of 20–24 amino acids (Patthy, 1987). So far, four members of this group

Abbreviations used: TGF-β, transforming growth factor β; IPTG, isopropyl β-D-thiogalactopyranoside; DMEM, Dulbecco's modified Eagle's medium; MBP, maltose-binding protein; FCS, fetal calf serum; EGF, epidermal growth factor; NTA, nitrilotriacetate; NGF, nerve growth factor.

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of proteoglycans have been cloned; in addition to decorin, these are biglycan (Fisher et al., 1989), fibromodulin (Oldberg et al., 1989) and lumican (Blochberger et al., 1992). Decorin and biglycan are ubiquitous, although they show a quite divergent localization within tissues, with decorin found more in the extracellular matrix of tissues where it is bound to type-I collagen (Vogel et al., 1984; Scott, 1986; Brown and Vogel, 1989) and biglycan localized more closely around cells (Bianco et al., 1990). Fibromodulin has a somewhat more restricted distribution with high concentrations in cartilage, tendon and sclera, and low concentrations in skin and mineralized bone (Heinegård et al., 1986). Lumican is found mainly in the cornea (Blochberger et al., 1992).

In the present study we have investigated the abilities of recombinant decorin, biglycan and fibromodulin fusion proteins, as well as the corresponding proteoglycans purified from bovine tissues, to interact with TGF-β. We show that all three proteoglycans interact with the three mammalian isoforms of TGF-β but that they display slightly different binding properties.

EXPERIMENTAL

Materials

pBluescript SK(+) vector and *Escherichia coli* XL1-Blue host cells were from Stratagene (La Jolla, CA, U.S.A.). The bacterial expression vector pQE-8, *E. coli* M15 host cells and Ni–nitrilotriacetate (NTA)–agarose were purchased from Qiagen (Chatsworth, CA, U.S.A.). Restriction endonucleases and other DNA-modifying enzymes were from Gibco Bethesda Research Laboratories if not indicated otherwise. Vent-DNA polymerase was from New England Biolabs (Beverly, MA, U.S.A.), isopropyl β-D-thiogalactopyranoside (IPTG) from Boehringer-Mannheim (Indianapolis, IN, U.S.A.), $Na^{125}I$ from Dupont–NEN (Burbank, CA, U.S.A.) and TGF-β3 from R&D Systems (Minneapolis, MN, U.S.A.). Protease-free chondroitinase ABC was purchased from Seikagaku Corp. (Tokyo, Japan). Precast SDS/polyacrylamide gels (4–20 %) were from Novex (San Diego, CA, U.S.A.). All chemicals were from Sigma and were of the highest quality available. Mv 1 Lu cells were obtained from American Type Culture Collection (ATCC CCL 64). Human umbilical vein endothelial cells were obtained from Clonetics (San Diego, CA, U.S.A.). Fetal calf serum (FCS) was purchased from Tissue Culture Biologicals (Tulare, CA, U.S.A.), and L-glutamine, antibiotics and antimycotic agents were from Irvine Scientific (Santa Ana, CA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco Bethesda Research Laboratories. Iodogen was from Pierce Chemical Co., Immulon-2 Removawell microtitre wells from Dynatech Laboratories (Chantilly, VA, U.S.A.) and PD-10 columns from Pharmacia LKB Biotechnology. Platelet factor 4 was from Calbiochem (La Jolla, CA, U.S.A.), 7S-nerve growth factor (NGF) from Boehringer-Mannheim, EGF from ICN (Cleveland, OH, U.S.A.) and insulin from Novo Nordisk (Copenhagen, Denmark). Simian TGF-β1 and TGF-β1 precursor were purified from culture supernatants of stable Chinese hamster ovary-cell transfectants as described previously (Gentry et al., 1987). Bovine TGF-β2 was purified from demineralized bone (Seyedin et al., 1985). Decorin, biglycan and fibromodulin were purified from bovine tissues as described previously (Heinegård et al., 1985).

Cloning of human biglycan and fibromodulin cDNAs

Total cellular RNA was extracted by using guanidinium isothiocyanate (Sambrook et al., 1989) from subconfluent cultures of WI-38 human lung fibroblasts that had been exposed to TGF-β1 (3 ng/ml) for 12 h. Total cellular RNA (1 μg) was reverse-transcribed with MoMuLV reverse transcriptase using random hexanucleotides for cDNA priming (Kawasaki, 1989). Double-stranded cDNAs encoding the full-length coding sequences of human biglycan or human fibromodulin were generated by amplification of the reverse-transcribed WI-38 RNAs using amplimers based on the published sequences of human biglycan (Fisher et al., 1989) or bovine fibromodulin (Oldberg et al., 1989). The PCR products were subcloned into pBluescript and analysed by DNA sequencing (Sanger et al., 1977). Our biglycan clone differs from the published biglycan sequence in five bases. Two of these could be reconciled by resequencing of clone p16 of Fisher et al. (1989), which was kindly provided by Dr. L. Fisher (NIDR, Bethesda, MD, U.S.A.). The remaining differences resulted in one amino acid exchange (Lys^{176} to Asn^{176}).

Human fibromodulin was found to have 92 % identity with the previously published bovine fibromodulin sequence. DNA sequencing analysis of the 1.2 kb PCR product revealed a 1128 bp open-reading frame that codes for a 376-amino-acid protein (EMBL accession number X75546).

Construction of expression vector

The pQE-8/MBP expression vector was generated by subcloning a *Bgl*II–*Bam*HI DNA fragment coding for maltose-binding protein (MBP) and a factor Xa protease cleavage site into the *Bam*HI site of pQE-8. *Bam*HI fragments coding for the core proteins of human biglycan, decorin or fibromodulin were subsequently cloned into the resulting single *Bam*HI site in pQE-8/MBP.

Expression and purification of fusion proteins

Biglycan, decorin and fibromodulin were prepared according to the instructions provided by Qiagen. Briefly, recombinant bacteria were grown in Luria–Bertani medium containing ampicillin (100 μg/ml) and kanamycin (25 μg/ml) at 37 °C to a density of $A_{600} \sim 0.6$–0.8. IPTG was then added to a final concentration of 2 mM and protein expression was allowed to proceed for 3 h. The bacteria were then collected by centrifugation (5000 *g*, 15 min) and lysed for 45–60 min in buffer A (0.1 M $NaHPO_4$, 0.01 M Tris, 6 M guanidinium chloride, pH 8.0). The lysates were centrifuged for 20 min at 20000 *g*. Imidazole was added to the supernatants to a final concentration of 10 mM and the mixtures were loaded on Ni–NTA columns (Hochuli et al., 1987). The columns were washed with several column volumes of buffer B (0.1 M $NaHPO_4$, 0.01 M Tris, 8 M urea, pH 8.0) and eluted with buffer C (buffer B adjusted to pH 5.9). Protein-containing fractions were adjusted to pH 8 by adding 1 M Tris/HCl in 8 M urea. After reduction of the proteins with dithiothreitol and carboxymethylation with iodoacetamide (Charbonneau, 1989), they were separated from the reagents and buffers exchanged for the respective binding buffers by gel filtration using PD-10 columns.

Iodination of proteins

Fusion proteins expressed in bacteria were iodinated using Iodogen according to the manufacturer's instructions. Briefly, 50 μl of an Iodogen solution (1 mg/25 ml of chloroform) was dried to the bottom of a borosilicate glass tube. Protein (10–20 μg) dissolved in iodination buffer (0.1 M $NaHPO_4$, 1 mM EGTA, 150 mM NaCl, pH 7.4) and carrier-free $Na^{125}I$ were added to the

tube. After incubation for 12 min at room temperature, 200 μl of iodination buffer and the mixture were loaded on to a PD-10 column for radiochemical purification of the labelled protein. The specific radioactivities and radiochemical purities of the labelled fusion proteins were calculated by determination of the picric acid-precipitable radioactive protein fraction in the labelling mixture before and after the purification step. The specific radioactivities ranged from 2300 to 2800 Ci/mmol, with radiochemical purities greater than 95%. TGF-β1, 2 and 3 (1–5 μg) were labelled as described above using 0.25 M $NaHPO_4$/2 M urea, pH 7.4, as iodination buffer.

Chondroitinase ABC treatment of proteoglycans

Enzymic removal of glycosaminoglycan side chains from native biglycan and decorin was performed by treating 0.5 mg/ml proteoglycan with 50 μl of 20 units/ml chondroitinase ABC (Seikagaku 100330; 110 units/mg) in 0.1 M Tris/HCl, pH 8.0, containing 0.03 M sodium acetate for 5 h at 37 °C. The efficiency of the enzyme digestion was confirmed by SDS/PAGE, where samples of treated biglycan and decorin migrated with the expected core protein molecular sizes of about 40 kDa.

Solid-phase binding assays

Immulon-2 microtitre wells were coated with TGF-β1 (75 μl, 1 μg/ml) or other proteins dissolved in 0.1 M $NaHCO_3$ buffer, pH 9.5, overnight at 4 °C. The coated wells were then flicked dry and incubated with 200 μl of binding buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2% BSA, 0.05% Tween 20, 0.01% NaN_3) for 3 h at 37 °C to block non-specific binding sites. Plates were either used immediately or stored for future use at −20 °C for up to 2 weeks. For binding assays, the blocked wells were washed once, then labelled, and unlabelled proteins were added in a total volume of 100 μl of binding buffer and incubated for 6 h at 37 °C if not indicated otherwise. Thereafter the well contents were removed by aspiration and the wells were washed three times with ice-cold binding buffer. Binding to the surface-immobilized proteins was determined by counting the entire wells in a γ counter. Non-specific binding to the wells was less than 5% of the total radioactivity added. The coating efficiency for TGF-β1 was 58.7 ± 0.5% ($n = 3$), giving approximately 44 ng of TGF-β1 per well. The coating efficiency was calculated by adding a small amount of ^{125}I-labelled TGF-β1 to the coating solution and determining the surface-associated radioactivity after the overnight incubation period and the subsequent washing step. All experiments were performed in duplicate or triplicate.

Analyses of equilibrium binding data

Data from equilibrium binding experiments were analysed using the LIGAND computer program (Munson and Rodbard, 1980) on an Apple Macintosh II computer.

Cell-binding experiments

Cell-binding experiments were performed as described by Massagué (1987). Briefly, subconfluent monolayers of Mv 1 Lu cells in 48- or 24-well cell culture dishes (Costar, Cambridge, MA, U.S.A.) maintained in DMEM containing 10% FCS were used in binding experiments. The cells were washed twice with ice-cold cell-binding buffer (128 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.2 mM $CaCl_2$, 50 mM Hepes, pH 7.5, 2 mg/ml BSA) and then incubated with cell-binding buffer for 30 min at 4 °C to remove endogenous receptor-associated TGF-β. Samples containing labelled and unlabelled proteins were added to the wells in a total volume of 100 μl for 48-well or 200 μl for 24-well dishes and incubated at 4 °C with gentle agitation on a rotary platform. After incubation for 4 h, the cells were washed three times with binding buffer. Then 100 μl (200 μl for 24-well dishes) of solubilization buffer (25 mM Hepes, pH 7.5, 10% glycerol, 1% Triton X-100, 1 mg/ml BSA) was added to each well and incubated for 30 min at 4 °C. Cell-associated radioactivity from triplicate samples was determined by counting a portion of the solubilized cells in a γ counter.

Cross-linking of TGF-β to receptors

For receptor cross-linking, cells were grown in 24-well culture dishes and processed as described above. After incubation with labelled and unlabelled ligands, the cells were washed once with binding buffer and three times with binding buffer without BSA. Then 100 μl of binding buffer (without BSA) containing disuccinimidyl suberate (final concn. 0.2 mM) was added, and the cells were incubated for 15 min at 4 °C. After the cross-linking reaction, the cells were lysed in 100 μl of solubilization buffer (125 mM NaCl, 10 mM Tris/HCl, pH 7, 1 mM EDTA, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml antipain, 50 mg/ml aprotinin, 100 μg/ml benzamidine hydrochloride, 10 μg/ml pepstatin). The lysates were mixed with sample buffer and analysed by SDS/PAGE under reducing conditions using precast 4–12% Novex gels. After electrophoresis the gels were dried and exposed to XAR-100 X-ray film for several days at −70 °C.

RESULTS

Bacterial fusion proteins

Attempts to express the decorin, biglycan and fibromodulin core proteins for use in functional assays as non-fusion proteins in bacteria or insect cells, or as bacterially expressed fusions with glutathione S-transferase or dihydrofolate reductase, failed mainly because of poor solubility in non-denaturing buffers of at least one of the proteins produced by any one of these methods. Soluble products were obtained when the core proteins were expressed as C-terminal fusions of *E. coli* MBP in a vector that also encoded an affinity tag consisting of a cassette of six histidines. The MBP part did not retain sufficient maltose-binding activity for affinity purification of the fusion proteins, but the histidine affinity tag allowed their purification to more than 95% purity by metal–chelate affinity chromatography. The purified MBP–fusion proteins displayed electrophoretic mobilities in SDS/PAGE compatible with the predicted amino acid sequences (Figure 1). These proteins were relatively soluble in physiological buffers, although some precipitation occurred during prolonged storage at 4 °C.

Binding of proteoglycan core proteins to TGF-β in equilibrium binding experiments

Solid-phase binding assays were performed by incubating radiolabelled MBP–proteoglycan fusion proteins in microtitre wells coated with increasing amounts of TGF-β1. Radiolabelled MBP–biglycan, MBP–decorin and MBP–fibromodulin bound to solid-phase-associated TGF-β1 in a concentration-dependent manner, displaying maximum binding of 50, 20 and 55%

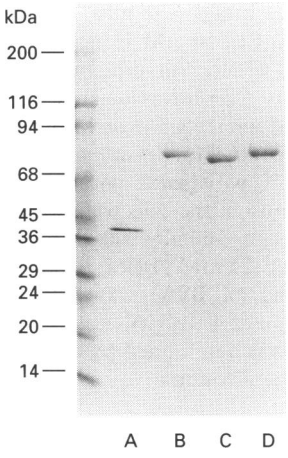

Figure 1 Gel-electrophoretic analysis of purified recombinant proteoglycan core fusion proteins

Each purified protein (1 μg/well) was loaded on to a 4–20% SDS/polyacrylamide gel. After electrophoresis under non-reducing conditions, the gel was stained with Coomassie Blue R-250. Lane A, MBP; lane B, MBP–biglycan; lane C, MBP–decorin; lane D, MBP–fibromodulin. The sizes (kDa) of molecular-mass marker proteins are indicated.

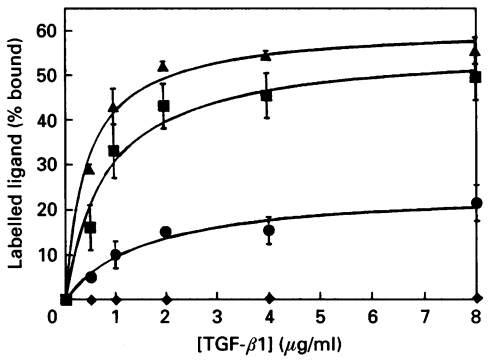

Figure 2 Binding of radiolabelled proteoglycan fusion proteins and MBP to microtitre wells coated with TGF-β1

TGF-β1 was used at the indicated concentrations (75 μl/well) to coat microtitre wells. The wells were incubated with ^{125}I-labelled MBP–biglycan (■), MBP–decorin (●), MBP–fibromodulin (▲) or MBP (◆). Constant amounts (~ 50000 c.p.m./well, specific radioactivities 2300–2800 Ci/mmol) of the labelled proteins were added to the TGF-β1-coated wells (total volume 100 μl). After incubation for 6 h at 37 °C, the wells were washed four times. TGF-β1 binding was determined by counting the entire wells in a γ counter and is expressed (±S.D.) as percentage of the total amount of labelled proteins added to the wells.

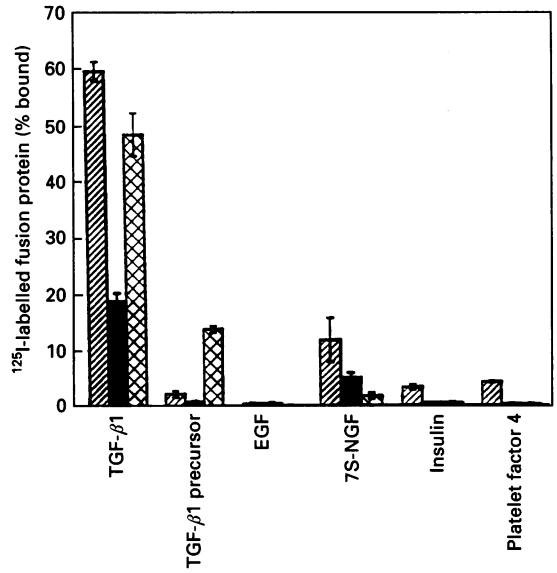

Figure 3 Specificity of proteoglycan core protein binding to TGF-β1

Microtitre wells were coated with the indicated proteins (75 μl/well, 3 μg/ml). ^{125}I-MBP–biglycan (▨), ^{125}I-MBP–decorin (■) or ^{125}I-MBP–fibromodulin (⊠) were added to the wells (total volume 100 μl). After incubation for 6 h at 37 °C, the wells were washed three times and counted in a γ counter. Binding (±S.D.) is expressed as percentage of the total amount of labelled proteins added to the wells.

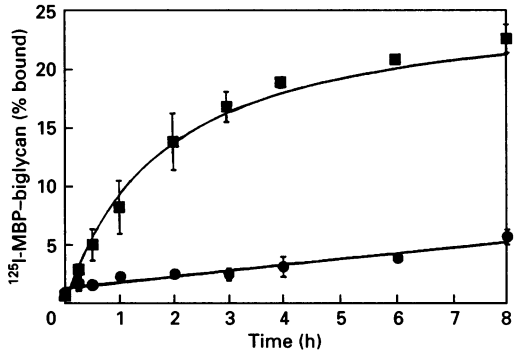

Figure 4 Time course of MBP–biglycan binding to TGF-β1

^{125}I-MBP–biglycan was added to TGF-β1-coated wells (75 μl, 1 μg/ml) at 4 °C or 37 °C in the presence or absence of excess unlabelled MBP–biglycan (1 μM). After the indicated time periods, the wells were washed three times and counted in a γ counter. Specific ^{125}I-MBP–biglycan binding (±S.D.), i.e. total binding minus binding in the presence of excess unlabelled MBP–biglycan, is expressed as percentage of the total amount of ^{125}I-MBP–biglycan added to the wells for the 4 °C (●) and 37 °C (■) incubations.

respectively (Figure 2). Radiolabelled MBP alone did not bind to the TGF-β1-coated wells.

The binding of the radiolabelled fusion proteins was specific for TGF-β, as little or no binding was observed to immobilized NGF, EGF, insulin or platelet factor 4. MBP–fibromodulin bound slightly to immobilized TGF-β1 precursor protein, but MBP–biglycan and MBP–decorin did not (Figure 3).

As the biglycan fusion protein, MBP–biglycan, showed high binding activity toward TGF-β1, we used it to characterize further the proteoglycan–TGF-β1 interactions. The binding of MBP–biglycan to TGF-β1 was dependent on time and temperature (Figure 4). Binding increased rapidly at 37 °C but very slowly at 4 °C, reaching at 4 °C only about 20% of the maximum binding seen at 37 °C. Prolonged incubation for up to 16 h revealed saturable binding also at 4 °C (results not shown).

Unlabelled MBP–biglycan competed for the binding of ^{125}I-labelled MBP–biglycan to TGF-β1 in a concentration-dependent manner (Figure 5a). MBP–decorin and MBP–fibromodulin also competed for the binding of labelled MBP–biglycan to TGF-β1. They competed about as effectively as MBP–biglycan, yielding half-maximal inhibitory concentrations of about 30–40 nM, whereas MBP alone was inactive. When purified bovine proteo-

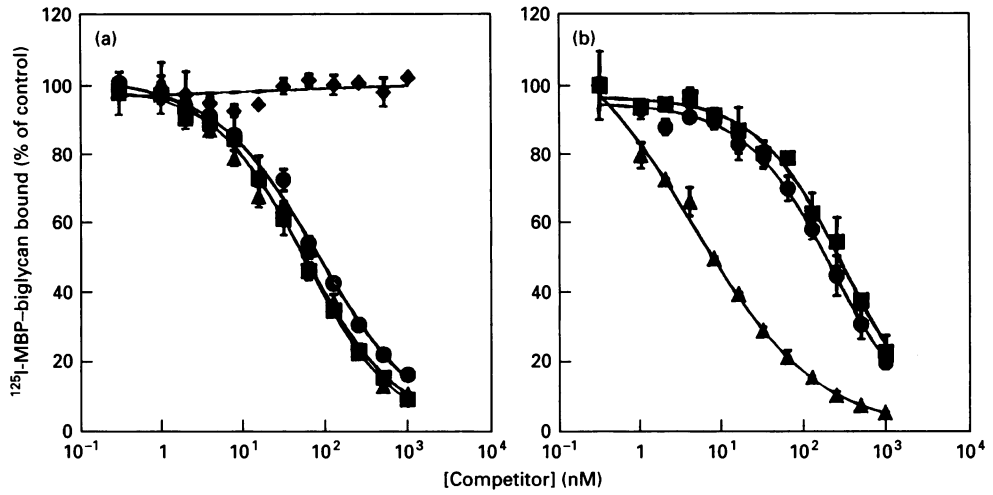

Figure 5 Inhibition of the binding of biglycan fusion protein to TGF-β1 by proteoglycan fusion proteins and intact proteoglycans

Binding of ^{125}I-MBP–biglycan to TGF-β1 was measured in the presence of the indicated concentrations of (**a**) unlabelled MBP–biglycan (■), MBP–decorin (●), MBP–fibromodulin (▲) or MBP (◆) or (**b**) purified biglycan (■), decorin (●) or fibromodulin (▲). After incubation for 6 h at 37 °C, the wells were washed three times and counted in a γ counter. Binding (±S.D.) is expressed as percentage of radiolabel bound in the absence of competitor.

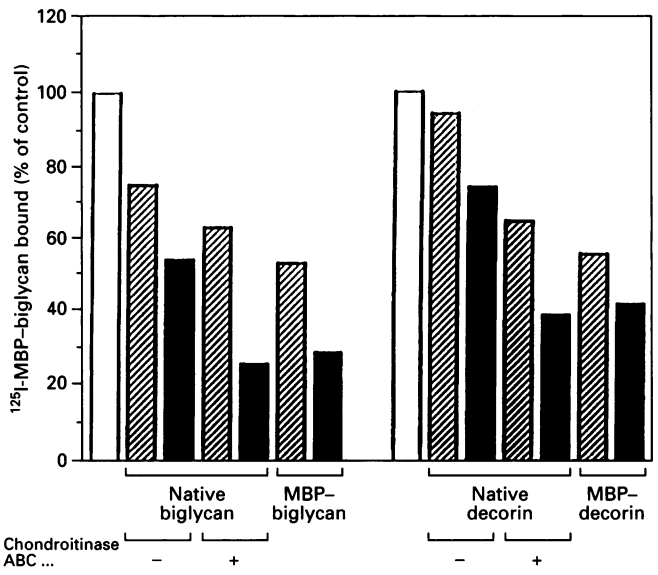

Figure 6 Effect of glycosaminoglycan removal on TGF-β1 binding by biglycan

Binding of ^{125}I-MBP–biglycan to TGF-β1 in the absence (□) or presence of MBP–biglycan, native bovine biglycan or chondroitinase ABC-treated bovine biglycan and MBP–decorin, native bovine decorin or chondroitinase ABC-treated decorin at 100 nM (▨) or 500 nM (■) is shown. After incubation for 6 h at 37 °C, the wells were washed three times and counted in a γ counter. Binding was corrected for non-specific binding and is expressed as percentage of the total amount of labelled ^{125}I-MBP–biglycan added to the wells.

glycans were used as competitors (Figure 5b), biglycan and decorin were found to be less potent than fibromodulin; the half-maximal inhibitory concentrations were 150, 200 and 10 nM respectively.

Calculation of dissociation constants from the data shown in Figure 5(a) indicated the presence of two types of binding site, with K_d values ranging from 1 to 17 nM for the high-affinity binding site and 47 to 200 nM for the low-affinity binding site. The $B_{max.}$ values were approx. 10^{-13} mol per well for the high-affinity binding site and 1.6×10^{-12} mol per well for the low-affinity binding site. Given a TGF-β1 coating concentration of 1 μg/ml, a coating volume of 75 μl and a coating efficiency of about 60%, these values indicate a molar ratio between proteoglycan fusion protein and TGF-β1 of 1:10 for the high-affinity binding site and 1:1 for the low-affinity binding site.

To investigate whether the glycosaminoglycan chains on native proteoglycans influenced the binding of TGF-β1, we tested proteoglycans treated with chondroitinase ABC to remove the glycosaminoglycan chains. Such treatment of native biglycan and decorin (Figure 6) increased their ability to compete for ^{125}I-MBP–biglycan binding to TGF-β1.

We also tested the proteoglycans for their ability to bind TGF-β2 and TGF-β3, the other known mammalian isoforms of TGF-β. Binding of TGF-β1, 2 and 3 to immobilized MBP–biglycan was inhibited by all three fusion proteins and all three intact proteoglycans (Figure 7). Binding of TGF-β3 to MBP–biglycan was more effectively inhibited by decorin and biglycan than fibromodulin.

The ability of the proteoglycan fusion proteins to compete for TGF-β1 binding to cells was examined in cell-binding experiments. Mv 1 Lu cells were incubated with labelled TGF-β1 in the presence or absence of proteoglycan fusion proteins or with unlabelled TGF-β1 as a control. Half-maximal competition was achieved at proteoglycan fusion protein concentrations averaging about 3 μM (Figure 8a). Cross-linking experiments revealed that TGF-β1 binding to betaglycan (type-III receptor) and type-I receptor was reduced by all three proteoglycan fusion proteins, whereas binding to the type-II receptors was essentially unchanged (Figure 8b). Laser-densitometry analysis of the respective autoradiograms showed that the binding of labelled TGF-β1 to betaglycan and to the type-I receptor was decreased by approx. 50% and 75% respectively at the proteoglycan concentration used. Cell-binding experiments using human umbilical vein endothelial cells, which express the TGF-β-binding protein endoglin but not betaglycan, showed that all proteoglycan

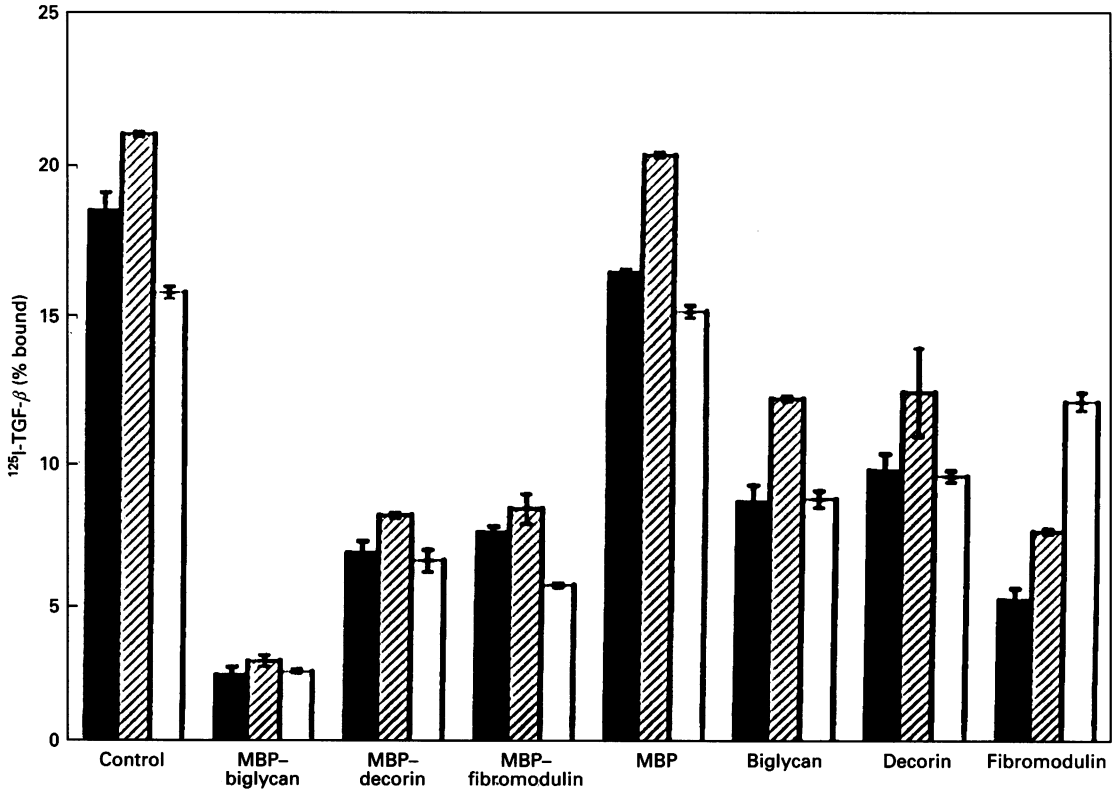

Figure 7 Competition for the binding of radiolabelled TGF-β1, -β2 and -β3 to microtitre wells coated with biglycan fusion protein

The binding of ^{125}I-labelled TGF-β1 (■), TGF-β2 (▨) or TGF-β3 (□) (50000 c.p.m./well; specific radioactivities 5000 to 7000 Ci/mmol) to surface-bound MBP–biglycan (coating concentration 10 μg/ml, 75 μl/well) was studied in the absence (control) or presence of unlabelled MBP–biglycan, MBP–decorin, MBP–fibromodulin, MBP, biglycan, decorin or fibromodulin (1 μM). Binding was corrected for non-specific binding and is expressed as percentage (±S.D.) of the total amount of labelled TGF-β1, -β2 or -β3 added to the wells.

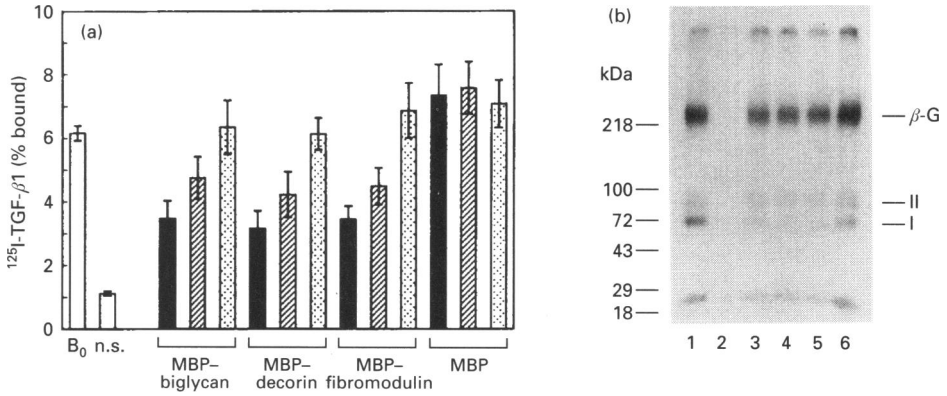

Figure 8 Competition for the binding of labelled TGF-β1 to Mv 1 Lu cells by proteoglycan fusion proteins

(**a**) Subconfluent cultures of Mv 1 Lu mink lung cells cultured in 48-well plates were incubated with ^{125}I-TGF-β1 (100 pM) in the presence (n.s.) or absence (B_0) of unlabelled TGF-β1 (20 nM) or 3 (■), 1 (▨) or 0.1 (▩) μM proteoglycan fusion proteins in a total volume of 100 μl. After incubation for 4 h at 4 °C, the cells were washed four times. The cells were then solubilized for 40 min in 1% Triton X-100 and assayed for radioactivity in a γ counter. Binding (±S.D., $n = 3$) is expressed as percentage of the total amount of ^{125}I-TGF-β1 added. (**b**) Mink lung cells were incubated with ^{125}I-TGF-β1 (100 pM) in the absence or presence of unlabelled TGF-β (20 nM) or MBP–fusion proteins (3 μM) in 24-well plates. After incubation for 4 h at 4 °C, the cells were treated with the cross-linker disuccinimidyl suberate and analysed by SDS/PAGE and autoradiography as described in the Experimental section. Binding was in the absence of competitor (lane 1), with TGF-β1 (lane 2), MBP–biglycan (lane 3), MBP–decorin (lane 4), MBP–fibromodulin (lane 5), or MBP (lane 6). The positions of prestained marker proteins are indicated on the left. The positions of the TGF-β type I and type II receptors and of betaglycan (β-G) are indicated on the right.

fusion proteins competed with total binding of TGF-β1 (Figure 9a), biglycan and fibromodulin being the most efficient. Cell-surface-affinity labelling with TGF-β showed that the proteoglycan fusion proteins competed with endoglin for TGF-β binding; fibromodulin was particularly effective in this regard (Figure 9b). The type-I receptor band was too weak in these cells

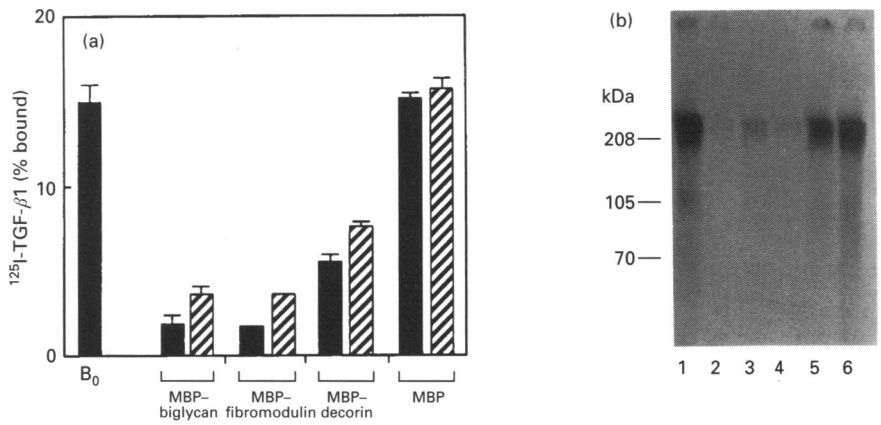

Figure 9 Competition for the binding of labelled TGF-β1 to human endothelial cells by proteoglycan fusion proteins

(**a**) Confluent cultures of human endothelial cells grown in 48-well plates were incubated in the absence (B_0) or presence of ^{125}I-TGF-β1 (100 pM) and 1 (■) or 3 (▨) μM proteoglycan fusion proteins in a total volume of 100 μl as described in Figure 8, and the radioactivity was measured. (**b**) Receptor affinity labelling of human endothelial cells incubated with ^{125}I-TGF-β1 (100 pM) in the presence of unlabelled TGF-β (20 nM) or 3 μM proteoglycan fusion proteins in 24-well plates as described in the legend of Figure 8. The lanes represent binding in the absence of competitor (1), with TGF-β1 (2), MBP–biglycan (3), MBP–fibromodulin (4), MBP–decorin (5) or MBP (6). The positions of prestained marker proteins are indicated.

to allow any conclusion about the effect of proteoglycan on that receptor.

DISCUSSION

The data presented here extend the previously published findings that decorin can bind TGF-β1 and that biglycan can block that binding (Yamaguchi et al., 1990). We demonstrate that at least three members of the family of small interstitial decorin-like tissue proteoglycans, decorin, biglycan and fibromodulin, show similar but not identical TGF-β-binding activities.

Solid-phase radioligand-binding studies demonstrated that recombinant fusion proteins containing the core protein sequences of human biglycan, decorin and fibromodulin compete for binding of labelled MBP–biglycan to TGF-β1 with similar affinities, indicating that functionally highly conserved regions of the core proteins are involved in this binding. The fact that bacterially produced recombinant proteoglycan core proteins had similar activities to recombinant decorin produced by mammalian cells (Yamaguchi et al., 1990) and tissue-derived proteoglycans definitively established the presence of TGF-β-binding activity in the core proteins of these proteoglycans. It is not yet known which regions of the core protein are involved, but the highest homology scores are found within the leucine-rich repeat regions. As these repeats are thought to represent a general motif for protein–protein interaction (Ruoslahti, 1988), it seems likely that the repeat regions are involved in TGF-β binding.

The interaction of TGF-β with the proteoglycans appears to be more complex than binding to a single isolated peptide sequence. The competition experiments indicated the existence of two classes of binding site of differing affinity. It is not clear whether this is the result of a heterogeneous presentation of binding sites, which can be assumed for surface-adsorbed ligands, but it may be that more than one class of binding site on the proteoglycans is involved in the TGF-β binding and that in some molecules only one is available.

Whereas the fusion proteins showed little difference in their abilities to bind TGF-β1, the intact proteoglycans were different in this regard. In an assay that measured the binding of a biglycan fusion protein to TGF-β1, bovine biglycan and decorin were less active competitors than fibromodulin. Decorin and biglycan were also less active than the corresponding recombinant fusion proteins. Fibromodulin was also the only one of the proteoglycans that seemed to be able to bind the latent TGF-β1 precursor to some extent. As biglycan and decorin carry long chondroitin or dermatan sulphate glycosaminoglycan side chains at their N-termini, whereas bovine fibromodulin contains keratan sulphate chains linked to the leucine-rich repeat region (Plaas et al., 1990), it appears that the chondroitin sulphate chains may interfere with the TGF-β binding. This assumption was supported by our finding that chondroitinase treatment increased the TGF-β-binding activities of both biglycan and decorin.

Each of the three proteoglycans was able to bind all three isoforms of TGF-β, but fibromodulin appeared to be a less effective binder of TGF-β3 than decorin or biglycan. The availability of TGF-β2 and TGF-β3 in only small quantities precluded detailed studies of the affinities of the proteoglycans for these isoforms, but our results suggest that subtle differences in the binding properties of the decorin-type proteoglycans exist.

The proteoglycan fusion proteins competed for binding of labelled TGF-β to Mv 1 Lu and endothelial cells. Under appropriate conditions, decorin can also neutralize TGF-β in bioassays performed with cultured cells (Yamaguchi et al., 1990; Border et al., 1992). In this study, we were unable to consistently neutralize TGF-β in the Mv 1 Lu bioassay with the proteoglycan fusion proteins or the natural proteoglycans (results not shown). The decorin-type proteoglycans and their core proteins competed with betaglycan for TGF-β binding in our receptor affinity-labelling experiments. These core proteins also compete with a TGF-β-binding fragment of betaglycan for TGF-β binding in microtitre assays (Fukushima et al., 1993).

Affinity labelling of the type-I receptor by TGF-β also appeared to be affected by the decorin-type proteoglycans. However, workers in our laboratory have recently identified a 60 kDa TGF-β-binding protein that is superimposed on the type-I receptor in gel analysis of TGF-β-affinity-labelled components but that is bound to the cell surface via glycosaminoglycans rather than being a receptor (Bützow et al., 1993). The Mv 1 Lu cells used in the assay do not appear to possess significant amounts of the 60 kDa protein (Bützow et al., 1993); nevertheless, the apparent effect of the proteoglycans on the type-I

receptor labelling should be interpreted with some caution at this time. The proteoglycans had no detectable effect on the labelling of the type-II signal-transduction receptor, and, because of this, it may be that secondary factors such as matrix binding or internalization of the proteoglycan (Hausser et al., 1989) determine whether or not TGF-β activity is inhibited. In fact, the experimental conditions used so far may not adequately reflect the situation *in vivo*; *in vivo* the proteoglycan–TGF-β interaction is unlikely to take place in the fluid phase. It is more likely that TGF-β would encounter extracellular matrix-associated proteoglycans; decorin and fibromodulin would be associated with type-I collagen in tissues. Although it has not been shown yet that collagen-associated decorin or fibromodulin can interact with TGF-β, it is possible that proteoglycans bound to the extracellular matrix compete with the receptors by sequestering TGF-β into the extracellular matrix, away from the receptors. Work is underway to test this hypothesis.

Increased TGF-β production has been found to be an important element in a number of fibrotic diseases that are characterized by an accumulation of extracellular matrix components (Border and Ruoslahti, 1992). For example, TGF-β plays a pivotal role in the pathogenesis of experimentally induced glomerulonephritis, the most critical manifestation of which is the accumulation of extracellular matrix in the glomeruli (Border et al., 1990b). In addition to increasing the synthesis of fibronectin, collagens and tenascin (Ignotz and Massagué, 1986; Varga et al., 1987; Pearson et al., 1988), TGF-β also up-regulates the expression of proteoglycans (Bassols and Massagué, 1988). In mesangial cells both decorin and biglycan can increase as much as 50-fold after induction by TGF-β (Border et al., 1990a), whereas in fibroblasts only biglycan seems to be increased (Romarís et al., 1991; Kähäri et al., 1991). Fibromodulin has not been studied in this regard. A recent study shows that injection of recombinant decorin into glomerulonephritic rats can suppress matrix accumulation (Border et al., 1992). It may be significant that the type-I receptors, which appear to be affected in their TGF-β binding by the decorin-type proteoglycans, have been found to be responsible for the effects of TGF-β on matrix accumulation (Arrick et al., 1992; Chen et al., 1993). Our results suggest that each of the decorin-type proteoglycans may be able to suppress *in vivo* matrix accumulation and that fibromodulin could be particularly effective in this regard.

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