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Transforming Growth Factor Beta Stimulation of Biglycan Gene Expression Is Potentially Mediated by Sp1 Binding Factors

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Abstract Biglycan is a small leucine-rich proteoglycan which is localized in the extracellular matrix of bone and other specialized connective tissues. Both biglycan mRNA and protein are up-regulated by transforming growth factor-β_1 (TGF-β_1) and biglycan appears to influence TGF-β_1 activity. In this study, we have investigated the mechanism by which TGF-β_1, TGF-β_2 and TGF-β_3 stimulate biglycan mRNA expression in the osteoblastic cell line MG-63. The cells were transfected with a series of deletional human biglycan promoter constructs and a region in the biglycan 5′ DNA was found to respond to TGF-β_1 with increased transcriptional activity in a dose-dependent manner. Also TGF-β_2 and TGF-β_3, two structurally highly related TGF-β isoforms stimulated biglycan transcription. A TGF-β responsive region was identified within the first 218 bp of the human biglycan promoter upstream from the transcriptional start site, which contained several binding sites for the transcription factor Sp1. Electrophoretic mobility shift assays with nuclear extracts from MG-63 cells showed binding of both Sp1 and Sp3 to a site at −216 to −208. When the biglycan promoter construct was co-transfected with Sp1 and Sp3 expression vectors in Sp1-deficient *Drosophila* Schneider-2 cells, Sp1 induced the transcriptional activity of biglycan. Addition of Sp3 augmented the effect of Sp1 on biglycan gene expression. Induction of biglycan mRNA expression in response to TGF-β in MG-63 cells was abrogated by mithramycin, an inhibitor of Sp1 binding to GC-rich DNA sequences. A mutation in the Sp1 site at −216 to −208 within the −218 biglycan promoter construct substantially diminished the transcriptional up-regulation by TGF-β_1. Taken together this data shows for the first time that TGF-β_1 stimulation of human biglycan mRNA expression relies on increased transcription of the biglycan gene, and is mediated by members of the Sp1 family of transcription factors. J. Cell. Biochem. 93: 463–475, 2004.

Key words: biglycan, Sp1, Sp3; TGF-β; transforming growth factor-β

Biglycan belongs to the family of small leucine-rich proteoglycans (SLRPs) where it is closely related to decorin and asporin [Ameye et al., 2002]. It consists of a core protein of approximately 38 kDa and in most cases two glycosaminoglycan chains [Fisher et al., 1989]. Biglycan is expressed in the extracellular matrix of many different tissues such as in the kidney [Pyke et al., 1997; Schaefer et al., 2000], lung [Romaris et al., 1991; Veness-Meehan et al., 1994], uterus [San Martin et al., 2003], arteries [Yeo et al., 1995], skin, cartilage, and is highly expressed in bone [Bianco et al., 1990; Corsi et al., 2002]. The biological function of biglycan has long been obscure, however, the generation of transgenic mice with a targeted disruption of biglycan has provided new insight. Biglycan deficient mice (*bgn* KO mice) suffer from various connective tissue disorders characterized by low trabecular bone mass, collagen fibril abnormalities in skin, tendon, and bone, and osteoarthritis and ectopic tendon ossification [Xu et al., 1998; Ameye and Young, 2002; Corsi et al., 2002]. The low trabecular bone mass

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of the *bgn* KO mice becomes more severe with age compared to wild type mice and suggests an osteoporosis-like phenomenon. Fluorescent double-labelling of the bone in vivo revealed that the mice have a low bone formation rate, and when bone marrow stromal cells (BMSCs) were isolated from *bgn* KO and wild type mice there was an age-dependent loss in the colony-forming capacity of the BMSCs from *bgn* KO mice [Chen et al., 2002], and indications of a diminished response of the BMSCs from *bgn* KO mice to transforming growth factor-β (TGF-β) stimulation. Biglycan and the homologous small proteoglycan decorin have previous been implicated in the regulation of TGF-β activity. Originally, Yamaguchi et al. [1990] reported that decorin could bind TGF-β and inhibit its activity, and that biglycan acted as a competitor for decorin binding to TGF-β. Several groups have since addressed this question and found that: (1) biglycan and decorin recombinantly expressed or isolated from bovine tissues would bind TGF-β_1, TGF-β_2, and TGF-β_3 with a high and a low affinity binding site [Hildebrand et al., 1994]; (2) that the inhibition of TGF-β by decorin was selective such that the TGF-β induced expression of biglycan protein was inhibited, but other functions of TGF-β were intact [Hausser et al., 1994]; (3) that the inhibitory effect of decorin was only present when decorin was part of the extracellular matrix, but not when it was added together with TGF-β in culture [Markmann et al., 2000]; (4) that decorin and biglycan could significantly and dose-dependently reduce TGF-β induced gene expression in vitro [Kolb et al., 2001; Abdel-Wahab et al., 2002], and finally that pre-treatment of TGF-β with biglycan inhibited TGF-β-mediated proliferation of renal interstitial fibroblast cells [Kobayashi et al., 2003]. Thus there is mounting evidence for an inhibitory interaction of decorin and biglycan with TGF-β, and considering that TGF-β enhances biglycan mRNA and protein expression in numerous cell types it supports the hypothesis that biglycan might be part of a regulatory loop of TGF-β activity.

Eludating the mechanism by which TGF-β upregulates biglycan mRNA expression is important for understanding the complex interactions of the extracellular matrix and TGF-β. However, it is still unclear how the effect of TGF-β stimulation results in increased biglycan expression [Ungefroren and Krull, 1996; Chen et al., 2002; Ungefroren et al., 2003]. Therefore, we have in this study investigated the effect of TGF-β on biglycan gene regulation, and we present data suggesting that TGF-β induces biglycan transcription and that this is dependent on members of the Sp1 family of transcription factors.

MATERIALS AND METHODS

Materials

MG-63 cells were from American Type Culture Collection (ATCC). TGF-β_1 was obtained from Austral Biological, San Ramon and from R&D Systems Inc., Minneapolis, MN. TGF-β_2 and TGF-β_3 were kindly donated by Dr. Seong-Jin Kim, National Institutes of Health and later purchased from R&D Systems Inc. Mithramycin was from Sigma. Sp1 recombinant protein/1 footprinting unit/μl) was purchased from Promega, Madison, WI. Antibody against Sp1 was obtained from Santa Cruz Biotechnologies, Ltd., and the antibodies specific for Sp3 and Sp4 were a generous gift from Dr. G. Suske [Hagen et al., 1994]. The control antibody Z-0271 was from DakoCytomation A/S, Denmark.

Cell Culture

MG-63 cells were grown at 37°C, 5% CO_2 in α-Minimum essential medium (α-MEM) with 10% fetal calf serum (FCS) and with penicillin and streptomycin. For TGF-β induction, MG-63 cells were grown to 80% confluence, rinsed twice with phosphate-buffered saline (PBS), and then cultivated in α-MEM with 0.1% bovine serum albumin (BSA) and 5 ng/ml TGF-β for 24 h. For the mithramycin experiments, MG-63 cells were seeded at 20,000 cells per cm^2, the following day the cells were rinsed twice with PBS and then cultured in 0.1% BSA/α-MEM with either 100 nM Mithramycin/H_2O or 5 μl H_2O per 25 ml medium. After 24 h of culture, the medium was replaced with 0.5% FCS/α-MEM and 100 nM mithramycin or vehicle and 5 ng/ml TGF-β1 or vehicle were added 24 h before harvesting. *Drosophila* S-2 cells (kindly donated by Dr. Leif Søndergaard) were grown at 25–27°C in Schneider's insect medium (Sigma) supplemented with 10% FCS.

Northern Blot Analysis

The cells were rinsed twice with PBS and harvested with GSSM (4 M guanidinium thio-

cyanate, 25 mM sodium citrate pH 7, 0.5% sacrosyl, 0.1 M 2-mercaptoethanol), and the RNA was extracted with 0.1 volume of 2 M sodium acetate, 1 volume of water saturated phenol, 0.3 volume of chloroform:isoamyl alcohol (24:1), incubated on wet ice for 30 min, and separated by centrifugation 10,000*g* for 30 min at 4°C. The supernatant was collected and precipitated with isopropanol and the pellet was resuspended in GSSM and equal volume of isopropanol was added. After 1 h incubation at 4°C, the pellet was recovered by centrifugation, washed in 80% ethanol, air-dried, and resuspended in deionised water. Twenty micrograms of total RNA was loaded in each lane of a denaturing formaldehyde agarose gel and separated by electrophoresis. The RNA was transferred to Hybond-N filters (Amersham, Uppsala, Sweden) and immobilized by UV-crosslinking (Stratalinker; Stratagene, La Jolla, California). The filters were then prehybridized for 3 h at 42°C in 5 × SSC, 5 × Denhardt's solution, 20 μg/ml carrier DNA, 50% formamide, and 10% Dextran Sulphate and hybridized in the same solution with a [α-^{32}P]dCTP labeled (Random Prime-IT, II labelling kit, Stratagene) 1.6 kbp human biglycan cDNA [Fisher et al., 1989] or a 1.04 kbp human TGF-β_1 cDNA probe (Genentech, Inc.). Following hybridization at 42°C overnight, the blots were washed repeatedly in 1× SSC, 0.1% SDS, and 0.2× SSC, 0.1% SDS at room temperature and at 65°C, and after exposure the expression levels were quantified by a PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA). The biglycan mRNA levels were normalized to the levels of 28S rRNA in the same RNA blot as determined by rehybridization of the filter with a ^{32}P-end labeled 28S rRNA oligonucleotide probe (5′-AAC GAT GAG AGT AGT GGT ATT TCA CC-3′) together with excess amount unlabelled 28S oligonucleotide. The unlabelled 28S oligonucleotide was added to avoid saturation of the labeled probe.

Construction of Expression Vectors

The construction of the deletional biglycan promoter-chloramphenicol-acetyltransferase (CAT) vectors has previously been described [Heegaard et al., 1997]. For this study, constructs spanning up to 713 bp upstream from the transcriptional start site were used. The biglycan promoter construct with the mutated Sp1 site (−218 Sp1 mut) was obtained by splicing by overlap extension (SOE). The 218 biglycan promoter CAT construct was used as a template for two separate polymerase chain reactions (PCR) using primers A: 5′ gcg ggt gtt ggc ggg tgt cgg ggc 3′ B: 5′ gct gag ccc ccg *aa*c cct cgc ccc c 3′ C: 5′ ggg ggc gag gg*t t*cg ggg gct cag c 3′ D: 5′ gct cct gaa aat ctc gcc aag ctg atc ctc 3′. Two 20 μl reactions were set up with primers A + B and C + D (3.75 μM of each primer), respectively, together with 1 mM dNTP and pfu buffer. Ampliwax PCR Gem (Ampliwax PCR Gem-100, Perkin Elmer) was added to each mix and incubated for 5 min at 80°C. The upper layer was composed of 1 μl pfu polymerase, 5 μl pfu buffer, 50 ng template (−218 BGN CAT construct) in a total of 80 μl. The PCR was performed for 14 cycles with denaturing at 97°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 2 min, followed by 1 cycle with 97°C for 1 min, 65°C for 1 min, and 72°C for 7 min. The products were purified with QIAgen spin PCR purification kit (Qiagen, Hilden, Germany). The following PCR reaction was set up and run under exactly the same conditions as the first two reactions but with the use of primer A and D, and the template being 1 μl from each of the purified products. Analysis of the product revealed one clear band with the expected size of 868 bp. The band was isolated by electrophoresis onto NA 45 paper and eluted [Ribaudo, 1994]. The PCR product was digested with the restriction enzymes *Bgl* II (Promega) and *BamH*1 (Promega) and the insert was ligated into the POBCAT7 vector [Baker, 1990]. The sequence and orientation of the insert was verified by DNA sequencing using Sequenase Version 2.0 DNA Sequencing Kit (Amersham). The final construct was cesium chloride purified and resuspended in deionized H_2O. A vector (pRL-TK, Promega Corporation) with *Renilla* luciferase and the herpes simplex virus thymidine kinase (HSV-TK) promoter was used as a control for transfection efficiency. *Drosophila* expression vectors: pPacSp1 was a kind gift from Dr. R. Tjian [Courey and Tjian, 1988], PacUSp3 was a generous gift from Dr. G. Suske [Hagen et al., 1994], and an expression vector with the Ac5C promoter and luciferase cDNA was a generous gift from Dr. L. Søndergaard. As a control, a vector without insert (pPac0) was generated by digestion of pPacSp1 with *Xho*I and then re-ligation of the vector without the 2.1 kb Sp1 cDNA.

Transfection of MG-63 Cells

MG-63 cells (5×10^5) per well were seeded in 6 well plates with α-MEM, 10% FCS 24 h prior to transfection. For transfection, cells were washed twice in PBS and cultured for 6 h in serum-free α-MEM before the medium was changed to α-MEM, 0.5% FCS, and the DNA constructs were added with either Effectene (Qiagen Inc.) or Lipofectamine 2000 (Invitrogen Lifetechnologies) according to the instructions of the manufacturers. Briefly, 1 μg (with Effectene) or 5 μg (with Lipofectamine, 2000) biglycan promoter-CAT-vector DNA and 0.1 μg (with Effectene) or 0.5 μg (with Lipofectamin) pRL-TK vector (Promega Corporation) were added to the cells with the transfection agent and incubated at 37°C, 5% CO_2 for 24 h. TGF-β or vehicle was added at the time of transfection. Before harvesting, the cells were washed twice in phosphate buffered saline and scraped of with Passive Lysis Buffer (Dual-Luciferase® reporter Assay System, Promega Corporation). A CAT Enzyme Assay System (Promega Corporation) was used for measuring CAT activity; the CAT enzyme assays were performed as described in the Promega protocol using the standard reaction enzyme assay, xylene extraction, and liquid scintillation counting. For each standard reaction 50 μl cell extract, 5 μl ^{14}C-chloramphenicol, 5 μl *n*-butyryl Coenzyme A and 65 μl 0.25 M TRIS-HCl, pH 8.0 were used, the samples were incubated for 16–18 h at 37°C. The luciferase activity was measured using a TD-20/20 luminometer (Turner Designs) according to the instructions in the protocol for the Dual-Luciferase® reporter Assay System. Briefly, 20 μl cell extract was added to 100 μl LAR II buffer, and the *Renilla* luciferase activity measured when 100 μl of Stop & Glo® was added. Each sample was measured in duplicate, and CAT activity was normalized to luciferase activity.

Transfection of *Drosophila* Cells

The −218 biglycan promoter CAT construct (3 μg) described earlier was co-transfected with the Sp1 (0.2 μg) and Sp3 (0.02 μg) expression vectors together with 0.1 μg the Ac5c-luciferase vector. The Sp1 vector without the Sp1 cDNA insert (empty vector) was added to make the total amount of vector equal in each individual transfection reaction. Lipofectin (Invitrogen lifetechnologies) was used as the transfection agent as recommended by the manufacturer. Briefly, 2×10^6 *Drosophila* S-2 cells were seeded in 60 mm tissue culture dishes. A total of 3.5 μg of DNA in 20 μl was mixed with 30 μl of lipofectin, incubated at room temperature for 15 min, and then 1 ml of Schneider's insect medium (Sigma) supplemented with 10% FCS was added. The transfection mixture was then added to the cells and incubated for 48 h at 25–27°C. The cells were harvested by rinsing with PBS, and the cells were recovered by centrifugation at 15,000 rpm for 2 min. For analysis of CAT and luciferase activities, the pellet was resuspended in Passive Lysis Buffer and processed as described above except that the Stop & Glo® buffer was excluded.

Nuclear Extract Preparation

A modification of the method of Dignam et al. [Abmayr and Workman, 1993] was used to prepare nuclear extracts from subconfluent cultures of human MG-63 osteosarcoma cells. The following changes in the method were made: One Complete™ protease inhibitor cocktail tablet (Boehringer Mannheim) per 50 ml buffer and 1 mM sodium orthovanadate was used in all the buffers. Additionally, dialysis was omitted, but the extracts were concentrated using Microcon™-30-microconcentrators from Amicon according to the manufacturer's instructions. The protein concentration in the extracts was determined using a Bio-Rad Protein Assay Kit I (Bio-Rad 500-0001). A confluent cell culture in a 150 mm dish resulted in 35 μl nuclear extract with a protein concentration of ~10 μg/μl.

Electrophoretic Mobility Shift Assay (EMSA)

Double-stranded, polyacrylamide gel purified oligonucleotide probes (custom-made, DNA technology, Denmark) were end-labelled with [γ-^{32}P]ATP and then for each EMSA reaction (total volume 15–17 μl) 50,000 cpm was mixed with 2 μg POLY(dI-dC) (Pharmacia 27–7880), 10 μl buffer (20 mM HEPES pH 7.9, 20% glycerol, 50 mM KCl, 0.5 mM DTT), H_2O, and approximately 20 μg nuclear protein. After 25 min incubation at 30°C, 1 μl 0.25% xylene cyanol-bromophenol blue was added to each sample which was then resolved on a 6% non-denaturing polyacrylamide gel (6% polyacrylamide, gelshift running buffer, 2.8% glycerol, 0.1% ammonium persulfate and TEMED) in gelshift running buffer (50 mM TRIS, 380 mM

glycine, 2 mM EDTA, pH 8.5). The gel was dried and the bands were visualized by autoradiography. For the experiments that included antibodies 1 μl of the antibody solution was added to the EMSA reaction and incubated on wet ice for 30 min before the DNA probe was added. The sequences of the double-stranded oligonucleotides used in the EMSA was: wild type biglycan Sp1 oligonucleotide: 5′gag ggg gc ggg ggc tca gct agt cca gcc gtc tac aag aaa att g 3′; mutated biglycan Sp1 oligonucleotide: 5′ gag ggt tcg ggg gct cag cta gtc cag ccg tct aca aga aaa ttg 3′; Sp1 consensus oligonucleotide: 5′ att cga tcg ggg cgg ggc gag c 3′; non-specific oligonucleotide: 5′ tag gt ccg aat gca tgc 3′.

RESULTS

Transforming Growth Factor (TGF)-β_1, TGF-β_2, and TGF-B_3 Induce Biglycan Promoter Activity in MG-63 Cells

When 5 ng/ml of transforming growth factor-β_1 (TGF-β_1) was added to MG-63 cells for 24 h a 2–3 fold increase in biglycan mRNA expression was observed. This is similar to previously published observations [Breuer et al., 1990], however, in this study TGF-β_2 and TGF-β_3 was also found to stimulate biglycan expression (Fig. 1). To investigate whether the TGF-β induced biglycan mRNA expression reflected increased transcriptional activity the biglycan promoter was transfected into MG-63 cells using multiple deletions of the promoter. Several of the constructs (−218, −262, −428, and −713) reacted to TGF-β stimulation with a significant increase in promoter activity (Fig. 2), and TGF-β_2 and TGF-β_3 also increased the transcriptional activity of the human biglycan promoter constructs (Fig. 3) to a level comparable with the effect of the growth factors on biglycan mRNA expression. A similar increase in activity of the biglycan promoter constructs was also observed when the biglycan CAT constructs were transiently transfected into TGF-β stimulated non-transformed human skin fibroblasts, a cell type known to respond to TGF-β stimulation with an increase in biglycan mRNA (data not shown).

The Transcription Factors Sp1 and Sp3 Bind to a Functional Sp1 Binding Site within the TGf-β Responsive Region of the Biglycan Promoter

The shortest deletional promoter construct that was responsive to TGF-β_1 contained 218 bp of human biglycan promoter upstream from the transcriptional start site. Within this DNA there are several putative binding sites for the transcription factor Sp1 [Ungefroren and Krull, 1996; Heegaard et al., 1997], and we have previously, by DNase footprinting found that nuclear proteins bind the Sp1 site at position −216 to −208 (GGGGCGGGG, [Heegaard et al., 1997]). To confirm specific Sp1 binding to this site, electrophoretic mobility shift assays (EMSA) with nuclear extracts from MG-63 cells and a synthetic oligonucleotide encompassing this region (−219 to −175) were performed. The EMSA showed three DNA-protein complexes (I, II, III, Fig. 4c). The two slowest migrating bands (I, II, Fig. 4c) represented specific binding to the Sp1 site; 100-fold molar excess of the cold oligonucleotide could compete for binding whereas 100-fold excess of an oligonucleotide with a mutated Sp1 site (Fig. 4a,b) did not prevent binding. Several transcription factors are known to act through an Sp1 binding site.

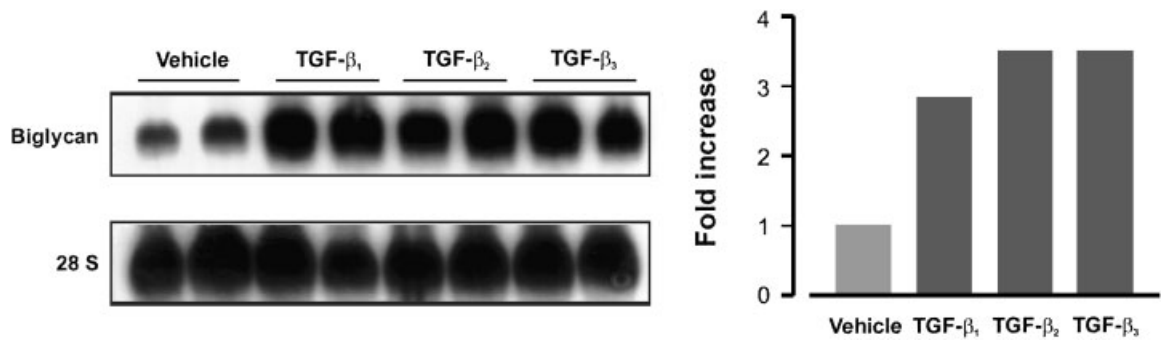

Fig. 1. TGF-β_2 and TGF-β_3 up-regulate biglycan mRNA expression in MG-63 cells at a level similar to that of TGF-β_1. Subconfluent MG-63 cells were cultured with 5 ng/ml of either TGF-β1, TGF-β2, or TGF-β3 in α-MEM, 0.1% BSA for 24 h. Total RNA was prepared, separated by denaturing formamide agarose gel electrophoresis, and analysed by Northern blot. The level of biglycan mRNA was quantified by PhosphorImager measurements and normalized to 28S expression.

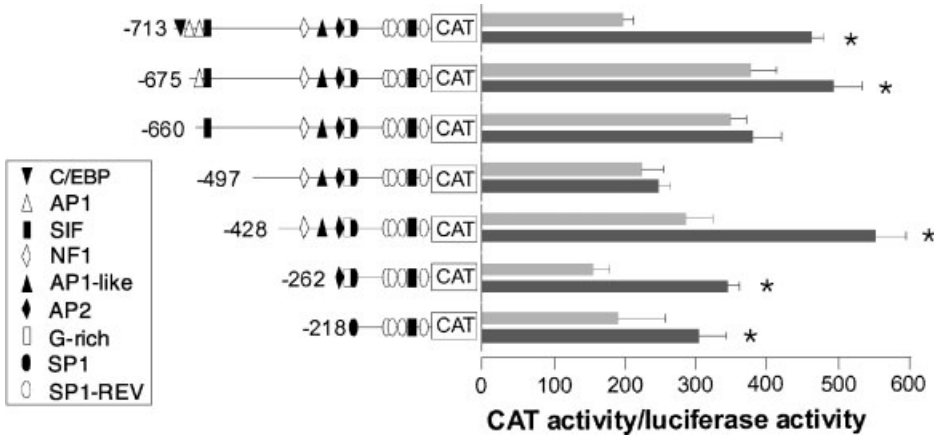

Fig. 2. TGF-β_1 increases human biglycan promoter activity in MG-63 cells. To the left is shown a schematic diagram of the biglycan-5′-DNA-CAT constructs used for the transient transfections. Deletional human biglycan promoter CAT constructs were co-transcfected with a HSV-TK-*renilla* luciferase vector into MG-63 cells. The cells were stimulated for 24 h with 5 ng/ml TGF-β_1 (dark gray columns) or vehicle (light gray columns), and then harvested and the CAT and luciferase activities were measured. The CAT activity was normalized to luciferase activity. At least three independent experiments in triplicate were performed. The columns represent the mean of results obtained from triplicate samples in one representative experiment, and the bars represent the standard deviation. Untreated was compared to treated for each promoter construct. *$P < 0.05$ by *t*-test.

Therefore, an EMSA using MG-63 nuclear extracts and antibodies against Sp1, Sp3, and Sp4 was performed. The results showed that the anti-Sp1 antibody induced a supershift of the probe (band IV, Fig. 4d) and a specific decrease in the intensities of band I and II. Addition of the anti-Sp3 antibody resulted in a specific decrease in the intensity of band II (Fig. 4d). In contrast, addition of an anti-Sp4 antibody or a control antibody did not affect the formation of the DNA–protein complexes. This strongly suggests binding of both Sp1 and Sp3 to the Sp1 site in the biglycan promoter. To investigate whether this binding has a functional role, *Drosophila* S-2 cells where co-transfected with the −218 biglycan construct and Sp1 and/or Sp3 expression vectors. A constitutively expressed luciferase reporter construct was used as a control for transfection efficiency, and an expression vector without an open reading frame was used as a control. Minimal biglycan promoter activity was observed when the control vector was added with the −218 biglycan CAT promoter construct (Fig. 5). Addition of 200 ng of the Sp1 expression vector resulted in a more than tenfold increase in biglycan promoter activity. When the Sp3 expression vector was added alone with the biglycan promoter it had a small, positive effect on biglycan transcription, however when the Sp1 and Sp3 expression vectors where added together to the biglycan promoter construct a synergistic effect was observed (Fig. 5).

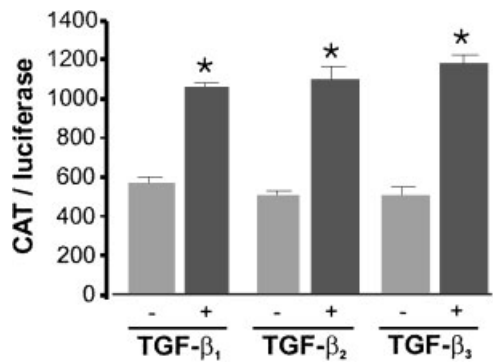

Fig. 3. TGF-β_2 and TGF-β_3 up-regulate biglycan promoter activity in MG-63 cells at a level similar to that of TGF-β_1. The −713 biglycan promoter CAT construct was co-transfected with a HSV-TK-*renilla* luciferase vector into MG-63 cells. The cells were stimulated for 24 h with 5 ng/ml of either TGF-β_1, TGF-β_2, or TGF-β_3 and then harvested and the CAT and luciferase activities were measured. The CAT activity was normalized to luciferase activity. The columns represent the mean of results obtained from three culture dishes, and the bars represent the standard deviation. *$P < 0.05$ by *t*-test.

TGF-β_1 Induction of Biglycan mRNA Expression in MG-63 Cells Requires Binding of the Transcription Factor Sp1 to the Biglycan Promoter

Sp1 binding has previously been shown to be important for TGF-β induced transcriptional regulation of several genes [Inagaki et al., 1994; Li et al., 1995; Greenwel et al., 1997; Botella et al., 2001]. Considering that there is an active Sp1 binding element within the TGFβ responsive region in the biglycan promoter we hypothesized that Sp1 might have a role in the

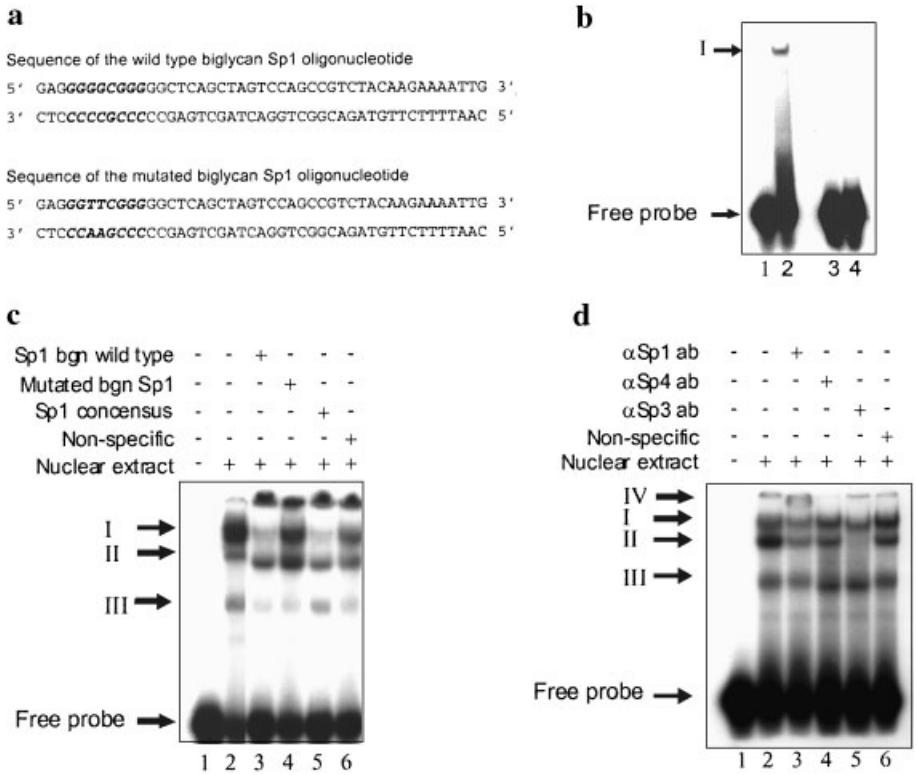

Fig. 4. Sp1 and Sp3 bind to the Sp1 site in the TGF-β responsive region of the biglycan promoter. **a**: The wild type biglycan Sp1 oligonucleotide represents −219 to −175 bp of the human biglycan promoter. In the mutated biglycan Sp1 oligonucleotide a mutation has been introduced in the Sp1 site. **b**: Electrophoretic mobility shift assay (EMSA) showed that Sp1 does not bind to the mutated biglycan Sp1 oligonucleotide. Radioactive labelled wild type (**lanes 1–2**) or mutated biglycan Sp1 oligonucleotide (**lanes 3–4**) was incubated without (lanes 1 and 3) or with 2 μl recombinant Sp1 protein (lanes 2 and 4) and resolved by non-denaturing gel electrophoresis. One DNA–protein complex (I), as indicated by the arrow, was observed when the wild type probe was incubated with the Sp1 protein. **c**: EMSA. Radioactive labelled wild type biglycan Sp1 oligonucleotide was incubated without (**lane 1**) or with MG-63 nuclear extract (**lanes 2–6**) and with 100-fold molar excess of either unlabelled wild type biglycan Sp1 oligonucleotide (lane 3), the mutated biglycan Sp1 oligonucleotide (lane 4), an Sp1 consensus oligonucleotide (lane 5), or a non-specific oligonucleotide (lane 6). Three protein–DNA complexes (I, II, and III), as indicated by arrows, were observed when the probe was incubated with nuclear protein. **d**: EMSA. MG-63 nuclear extract was incubated with the antibodies and EMSA buffer before the radioactive labelled wild type biglycan Sp1 oligonucleotide was added. The DNA–protein complexes were resolved by non-denaturing gel electrophoresis. Three protein–DNA complexes (I, II, and III), as indicated by arrows, were observed when the probe was incubated with nuclear protein, and one super-shifted band (IV) was observed when the nuclear extract was pre-incubated with an Sp1 antibody. The labelled probe was incubated without (**lane 1**) or with nuclear extract (**lanes 2–6**) and with antibodies directed against the transcription factors Sp1 (lane 3), Sp4 (lane 4), Sp3 (lane 5) or a non-specific antibody against alkaline phosphastase.

regulation of biglycan gene expression by TGF-β_1. To test this hypothesis, mithramycin A, which is an inhibitor of GC-binding [Blume et al., 1991], was added together with 5 ng/ml TGF-β_1 to MG-63 cells, and biglycan mRNA expression was analysed by Northern blot. The result showed that mithramycin A completely blocked the stimulation of biglycan mRNA expression by TGF-β_1 without affecting the basal level of biglycan expression (Fig. 6a), and the effect of mithramycin was specific for biglycan; mithramycin did not affect the auto-regulation of TGF-β_1 (Fig. 6b) which is not considered to involve Sp1 activity but rather the transcription factors AP1 and NF1 [Kim et al., 1990].

A role for Sp1 in the stimulation of biglycan transcription by TGF-β was further supported by transfection experiments with a mutated DNA construct. A mutation (GG → TT, Fig. 4a) that abolished all binding activity in vitro was introduced into the Sp1 site (−216 to −208) in the −218 biglycan promoter CAT expression vector. MG-63 cells were then transfected with either the native −218 or the mutated −218 biglycan promoter construct. The mutation in

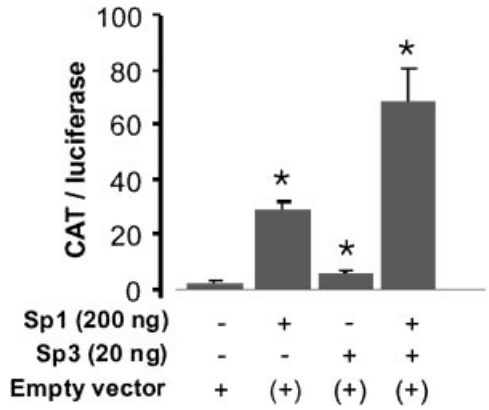

Fig. 5. Both Sp1 and Sp3 stimulate biglycan promoter activity. *Drosophila* S-2 cells were transfected with the −218 biglycan promoter CAT construct (3 μg) together with 0.1 μg Ac5c-luciferase vector and with Sp1 (0.2 μg) and Sp3 (0.02 μg) expression vectors. An empty vector was added to make the total amount of added DNA equal in each individual transfection reaction. The transfected cells were incubated for 48 h before they were harvested and the CAT and luciferase activities were measured. The CAT activity was normalized to luciferase activity. The columns represent the mean of results obtained from triplicate samples, and the bars represent the standard deviation. $^*P < 0.05$ by *t*-test.

the Sp1 site in the biglycan promoter significantly blocked the transcriptional response to TGF-β_1 and, as with the mithramycin assay without affecting the basal level of transcription (Fig. 7).

DISCUSSION

There is ample evidence that TGF-β induces biglycan expression in many different human cell types including human skin, kidney, and lung fibroblasts [Bassols and Massague, 1988; Kahari et al., 1991; Romaris et al., 1991; Westergren-Thorsson et al., 1991], and human chondrocytes and osteoblast-like cells [Breuer et al., 1990; Roughley et al., 1994]. Several groups have shown that the TGF-β induced biglycan expression can be abrogated by actinomycin D, an inhibitor of transcription [Bassols and Massague, 1988; Romaris et al., 1991]. These observations suggest that the increase in biglycan expression is due to increased biglycan transcription, and indeed, in this study we showed that TGF-β significantly increased biglycan promoter activity. The biglycan 5′-DNA included in the deletional biglycan promoter constructs ranged from −713 to −218 bp upstream from the transcriptional start site. Two of the constructs (−497, and −660) were not induced by TGF-β, indicating the presence of dominating inhibitory elements upstream in these biglycan promoter constructs. The fact that mithramycin A blocked TGF-β1-induced biglycan mRNA expression supported the notion of a transcription-dependent mechanism. Ungefroren and Krull [1996] have previously studied the possible mechanisms by which TGF-β induces biglycan expression in MG-63 cells, and they came to the conclusion that it is not caused by increased biglycan transcription. In their study, two different biglycan promoter constructs containing either 1,218 bp or 686 bp of the biglycan DNA upstream from the transcriptional start site were used for transient transfections in MG-63

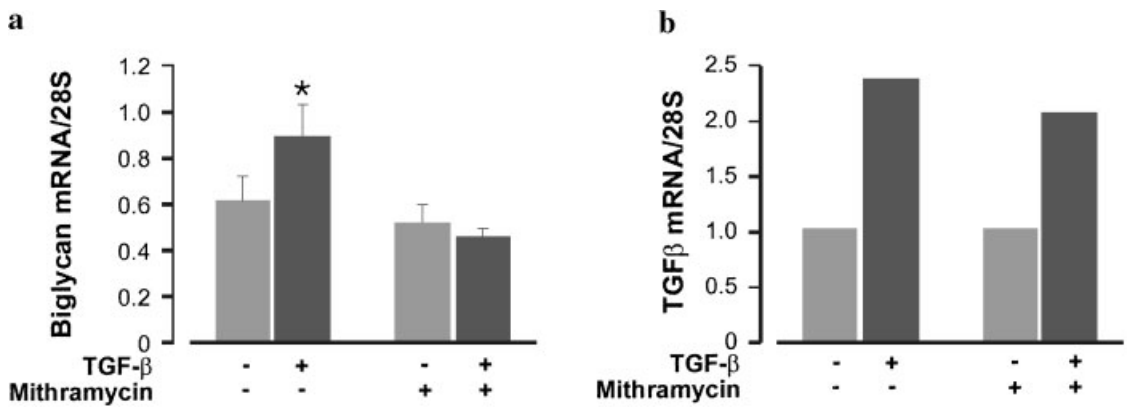

Fig. 6. TGF-β_1 induction of biglycan mRNA requires binding of the transcription factor Sp1. After pre-treatment with 100 nM mithramycin or vehicle, MG-63 cells were stimulated with 5 ng/ml TGF-β_1 or vehicle for 24 h, and total RNA was isolated and analysed for either biglycan (**a**) or TGF-β_1 (**b**) expression by Northern blot. As a control the RNA blots were stripped and rehybridized with a 28S oligonucleotide. The level of expression was measured using a PhosphorImager and levels were normalized to 28S expression. The columns represent the average of 3–4 (biglycan) or 2 (TGF-β) samples. The bars represent standard deviation. $^*P < 0.05$ by *t*-test.

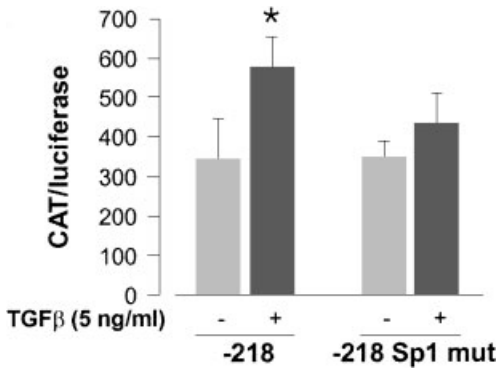

Fig. 7. TGF-β_1 stimulates biglycan promoter activity via the Sp1 binding site at position −216 to −208 in the biglycan promoter. The −218 biglycan promoter CAT vector or the −218 biglycan promoter CAT vector with a GG → TT mutation in the Sp1 site at −216 to −208 (Fig. 4a) was co-transcfected with a HSV-TK-*renilla* luciferase vector into MG-63 cells. The cells were stimulated for 24 h with 5 ng/ml TGF-β_1, and then harvested and the CAT and luciferase activities were measured. The CAT activity was normalized to luciferase activity. At least three independent experiments in triplicate were performed. The columns represent the mean of results obtained from one representative experiment, and the bars represent the standard deviation. *$P < 0.05$ by *t*-test.

cells. No induction by TGF-β was found for either construct, and since TGF-β did not affect mRNA stability it was concluded that the regulation of biglycan by TGF-β might involve a nuclear post-transcriptional mechanism. Our results suggest another explanation. When we used a biglycan promoter CAT construct corresponding to the −686 construct of Ungefroren and Krull, we as well could not detect an induction by TGF-β, and on the basis of our previous work showing that this region is characterized by low transcriptional activity, it is likely that negative regulatory elements are governing the activity of this construct. The same could be the case for the −1218 construct, which in our hands also contains very low activity [Heegaard et al., 1997] and is not induced by TGF-β (data not shown). It is possible that transient transfections are insufficient to reveal the entire regulatory pattern of biglycan promoter activity, since only part of the regulatory DNA is tested, and since the promoter is not in the proper in vivo setting but instead removed from its normal context in the nuclear structure [Stein et al., 1999, 2000].

The biglycan promoter DNA contained within the −218 biglycan CAT construct induced by TGF-β contains no obvious Smad responsive elements [Shi et al., 1998; Zawel et al., 1998], but does have an Sp1 consensus sequence at −216 to −208 [Heegaard et al., 1997]. In addition, binding of Sp1 and Sp3 to the Sp1 site was demonstrated by EMSAs. Both Sp1 and Sp3 bind to the same DNA sequence [Hagen et al., 1992], however where Sp1 is always a transcriptional activator [Suske, 1999], Sp3 can work either as an activator or as a transcriptional inactive molecule that represses Sp1-mediated transcription [Hagen et al., 1994; Suske, 1999]. One of the determining factors of whether Sp3 acts as a repressor appears to be the structure and arrangement of the recognition site in the DNA. Promoters containing a single binding site are activated and promoters with multiple binding sites are often not activated or respond weakly to Sp3 [Suske, 1999], however, in a recent report Ammanamanchi et al. [2003] showed that the acetylation status of Sp3 can determine its activity. For the biglycan promoter, both Sp1 and Sp3, separate and together, acted as transcriptional activators in Sp1-deficient *Drosophila* Schneider-2 cells.

TGF-β induction mediated by the transcription factor Sp1 has been shown for several genes [Inagaki et al., 1994; Li et al., 1995; Botella et al., 2001], including type I collagen [Greenwel et al., 1997] which, like biglycan is present in the extracellular matrix in bone and skin. When MG-63 cells were treated with a combination of TGF-β and mithramycin A, an inhibitor of GC-binding [Blume et al., 1991; Datta et al., 2000], mithramycin A attenuated the effect of TGF-β thus indicating that similar to the collagen α1(I) and α2(I) genes Sp1 binding is also important for TGF-β induced biglycan expression. Finally, a 2 base pair mutation in the Sp1 site in the biglycan promoter abolished the TGF-β induced activity. Neither incubation with mithramycin nor the mutation at the Sp1 site affected the basal expression of the biglycan gene. This was somewhat surprising since the biglycan 5′ flanking region does not contain a CCAAT or TATA box, and possibly Sp1 could be involved in the basal transcription of the gene [Heegaard et al., 1997]. Explanations for this could be that the level of mithramycin A was not high enough to affect the basal transcription and that since the mutation in the Sp1 site does not affect the basal level of transcription that this specific Sp1 binding site is only important for the TGF-β induced expression of biglycan. In a recent paper, Verrecchia et al. showed that NIH 3T3 fibroblasts stably transfected with an antisense Sp1 expression vector have reduced levels

of Sp1 and simultaneously reduced expression of several extracellular matrix genes including biglycan [Verrecchia et al., 2001]. In addition, Ungefroren et al. have described a pyrimidine-rich sequence between −59 and −49 that seems to be involved in the basal transcription of biglycan [Ungefroren et al., 1998], and that binds Sp1/Sp3-like proteins, also, there are several potential Sp1 binding sites within the first 200 base pairs upstream from the transcriptional start site of the biglycan promoter [Ungefroren and Krull, 1996; Heegaard et al., 1997].

The signaling from TGF-β receptors to the cell nucleus is predominantly mediated by Smad proteins. When TGF-β binds TGF-β type II receptor, TGF-β type I receptor is recruited, and the receptor-activated Smad proteins (R-Smad), Smad2 and Smad3 are phosphorylated and subsequently activated. The R-Smads then form complexes with the Co-mediator (Co-Smad; Smad4) before being translocated into the nucleus [Verrecchia and Mauviel, 2002; Shi and Massague, 2003]. In the nucleus, Smad3 and Smad4 can act as transcription factors, either by direct binding to DNA and/or by associating with other DNA binding proteins such as Sp1 [Moustakas et al., 2002]. In response to TGF-β, functional interactions between Sp1 and Smad family members lead to the activation of the p21, p15, COL1A2, the beta 5 integrin subunit, and plasminogen activator inhibitor-1 genes [Datta et al., 2000; Feng et al., 2000; Lai et al., 2000; Pardali et al., 2000; Zhang et al., 2000; Inagaki et al., 2001; Poncelet and Schnaper, 2001; Koutsodontis et al., 2002], and it is tempting to speculate that the same would be the case for biglycan. Recently it was demonstrated that in a pancreatic carcinoma cell line PANC-1 up-regulation of biglycan expression by TGF-β was dependent on both Smad4 [Chen et al., 2002] and on MKK6-p38 MAP kinase signaling [Ungefroren et al., 2003] suggesting a cross-talk between the MAP kinase and Smad pathways. In support of this, Ohshima and Shimotohno have recently shown that Smad4 is a substrate for SUMO-1 (small ubiquitin-like modifier), and that this sumoylation of Smad4 enhanced Smad dependent transcription and was dependent on p38 MAP kinase activity [Ohshima and Shimotohno, 2003].

Interestingly, decorin which is highly homologous to biglycan appears to modulate TGF-β activity in human mesangial cells though a mechanism that involves phosphorylation of Smad2 [Abdel-Wahab et al., 2002]. This indicates that the interference with TGF-β activity in some cases involves more than just high-affinity binding of decorin to TGF-β, but rather an effect on the signaling pathway. Although biglycan and decorin are closely related in structure, the mechanisms involved in transcriptional regulation of the two proteoglycans differ. Contrary to biglycan, the decorin gene has two promoters and contains a negative TGF-β responsive region [Santra et al., 1994; Mauviel et al., 1995; Wahab et al., 2000; Demoor-Fossard et al., 2001].

In conclusion, our results suggest that TGF-β regulates biglycan expression via increased biglycan transcription and mediated by Sp1 and Sp3, and we propose that this could be via an interaction with Smad4. However, the transcriptional regulation of human biglycan is complex and to get a true picture of the in vivo situation, it will be necessary to design experiments where the biglycan gene is placed in the context of the nuclear matrix.

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