Stimulation of Osteoprotegerin (OPG) Gene Expression by Transforming Growth Factor-β (TGF-β)

MAPPING OF THE OPG PROMOTER REGION THAT MEDIATES TGF-β EFFECTS*

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Transforming growth factor-β (TGF-β) regulates osteoclastogenesis and osteoclast survival, in part through the induction of osteoprotegerin (OPG), a protein known to inhibit osteoclast formation and function. To explore the molecular basis of TGF-β regulation of OPG expression, we evaluated the effects of TGF-β on osteoclast formation, OPG protein secretion, mRNA expression, and gene transcription. The marked inhibitory effect of TGF-β on osteoclast differentiation was confirmed in a co-culture model utilizing murine stromal/osteoblastic BALC cells and bone marrow hematopoietic precursors. This inhibition in osteoclast differentiation was preceded by a decrease in RANKL mRNA expression (5-fold) and a reciprocal increase in OPG mRNA (6.1-fold) and protein (7.1-fold) expression in BALC cells. At the promoter/transcriptional level, TGF-β treatment resulted in a 3–10-fold increase in reporter gene activity directed by a 5.9-kilobase fragment of the human OPG promoter in transfection assays performed in UMR106 cells. The effect of TGF-β was mimicked by TGF-β2 and -β3 but not by BMP-4, suggesting a TGF-β signal-specific effect. Deletion analysis revealed that a 183-base pair region (−372 to −190) in the promoter was required for TGF-β responsiveness, and this region was sufficient to confer TGF-β inducibility to a heterologous (osteocalcin) minimal promoter. Substitution mutations that disrupted the Cbfa1- and/or Smad-binding elements present in the 183-base pair region resulted in a decrease in co-transfected Cbfa1 expression and in the responsiveness to TGF-β and Cbfa1. Collectively, these studies indicate the involvement and possible interaction of Cbfa1 and Smad proteins in mediating the effects of TGF-β on OPG transcription.

The interaction of osteoclast precursors with cells of the osteoblast lineage is essential for their differentiation to form mature, bone-resorbing osteoclasts. Molecules mediating this interaction include RANK ligand (RANKL)† (also known as nuclear factor-κB ligand; AP1, activator protein-1; BMP, bone morphogenetic protein; bp, base pair(s); Cbfa1, core binding factor a1; kb, kilobase(s); NF-κB, osteoclast differentiation factor, tumor necrosis factor-related activation-induced cytokine, and OPG ligand) (1, 2) that is expressed on the osteoblast/stromal cell surface and the cognate receptor, receptor activator of NF-κB (RANK) (3, 4), expressed on hematopoietic precursor cells. The interaction of RANKL with RANK initiates a cascade of signaling events (4–7) that result in the differentiation of these precursors to form tartrate-resistant acid phosphatase-positive multinucleated osteoclasts that are capable of resorbing bone. OPG, a secreted glycoprotein of the tumor necrosis factor receptor superfamily, acts as a decoy receptor and blocks the interaction between RANKL and RANK, thus inhibiting osteoclast differentiation. OPG has also been shown to inhibit the activity and survival of osteoclasts in vitro and bone resorption in vivo (8–13).

The effects of a number of hormones, growth factors, and cytokines that modulate osteoclast differentiation are mediated by the osteoblasts. Many of these agents have now been shown to exert their actions by regulating OPG and/or RANKL expression in osteoblasts (1, 14–20). TGF-β is one such cytokine that plays a major role in the regulation of bone formation and resorption (21). It has been shown to inhibit the formation of osteoclast-like cells in long term human marrow cultures (22) and to inhibit bone resorption in fetal rat long bone cultures (23). More recently, TGF-β has been shown to induce OPG expression in osteoblastic cells and to inhibit osteoclast differentiation and survival (17, 19). However it is not known whether TGF-β directly affects the transcriptional activity of the OPG gene. To further investigate the molecular basis of TGF-β effects on OPG production, we analyzed the effect of TGF-β on osteoclast formation, OPG protein secretion, mRNA expression, and gene transcription. In order to analyze the mechanism by which TGF-β stimulates OPG gene transcription, we have characterized the TGF-β-responsiveness of a 5.9-kb fragment of the human OPG promoter that we recently cloned (24). We provide evidence that TGF-β inhibition of osteoclast formation is preceded by reciprocal up-regulation of OPG mRNA and protein and down-regulation of RANKL. TGF-β effects on OPG expression occurred by direct activation of the OPG promoter as demonstrated in transient and stable transfection analyses. Additionally, we demonstrate that a 183-bp proximal region (−372 to −190) of the promoter is necessary and sufficient for mediating TGF-β effects. Mutation of a Cbfa1-binding element (OSE₂) (25) and/or a Smad-binding element (OSE₃) (26) are necessary for the inducible expression of OPG. Mutation of Smad-binding element (OSE₃) is sufficient for the TGF-β regulation of OPG expression. The TGF-β effects are mimicked by TGF-β2 and -β3 but not by BMP-4, suggesting a TGF-β signal-specific effect. Deletion analysis revealed that a 183-base pair region (−372 to −190) in the promoter was required for TGF-β responsiveness, and this region was sufficient to confer TGF-β inducibility to a heterologous (osteocalcin) minimal promoter. Substitution mutations that disrupted the Cbfa1- and/or Smad-binding elements present in the 183-base pair region resulted in a decrease in co-transfected Cbfa1 expression and in the responsiveness to TGF-β and Cbfa1. Collectively, these studies indicate the involvement and possible interaction of Cbfa1 and Smad proteins in mediating the effects of TGF-β on OPG transcription.
element (SBE) (26) that reside in this promoter region resulted in a reduction in base-line expression and in responsiveness to TGF-β and Cbfa1. Taken together, our studies suggest the involvement and possible interaction of Cbfa1 and Smad proteins in mediating the effects of TGF-β on the OPG promoter.

Experimental Procedures
Co-culture of Bone Marrow Cells and BALC Cells
For analyzing the role of TGF-β on osteoclast differentiation, cocultures of bone marrow cells and BALC cells (a murine calvarial-derived cell line) were performed in the presence or absence of TGF-β as described previously (27, 28). Bone marrow cells from the femora and tibiae of 10-week-old mice (mixed-breed inbred, C57BL/6, backcrossed into C57BL/6J for five generations) were seeded into 24-well cluster dishes (Costar, Cambridge, MA) at a density of 5 × 10⁴ mononuclear cells/cm² in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 5% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 1% antibiotic/antimycotic solution (Life Technologies). BALC cells (1.5 × 10⁴ cells/cm²) were co-cultured with the bone marrow cells. The cultures were treated either with vehicle or with 10⁻⁶ M 1,25-(OH)₂ D₃ vitamin D₃ (Biomed, Plymouth Meeting, PA) in the presence of vehicle or recombinant human TGF-β (Hyclone, Logan, UT) and 1% antibiotic/antimycotic solution (Life Technologies).

The proximal OSE element (SBE) (26) that reside in this promoter region resulted in a reduction in base-line expression and in responsiveness to TGF-β and Cbfa1, and ligating them to pŋŋ Basal digested with the same enzymes. Double mutants (OSEₑ and SBE) were utilized utilizing the same two-step PCR strategy, with a template containing the OSEₑ mutation and primers containing the SBE mutation.

Cloning of the Human OPG Promoter
Cloning of the 5.9-kb fragment of the human OPG promoter (p.OPG.9/gal) as well as sequential 5'-deletions of the promoter (p.OPG.3.6/gal, p.OPG1.9/gal, p.OPG1.5/gal, p.OPG0.9/gal, p.OPG0.4/gal, and p.OPG0.2/gal) linked to the β-galactosidase (β-gal) reporter gene in pŋŋ Basal-reporter vector (CLONTECH, Palo Alto, CA) were performed using standard cloning procedures, as described previously (24).

Site-directed mutagenesis of the proximal OSEₑ element, the AP1-like element, and the SBE was performed using the two-step PCR strategy (29). The proximal OSEₑ core element (AACCTCA, at positions 343 to 352 of total) using the two first step PCR products as template. To generate the β-gal reporter plasmid (p.OPG.9/gal), the 0.4-kb region (OSEmutRev, 5'-gct gtc tcc gcg ggg ctc gat atc ttc ccg gcc cct tcc cgc c-3′ (H11032)) oligonucleotides, digesting the double-stranded oligonucleotide with Bgl II and BstII, and ligating them to pŋŋ Basal digested with the same enzymes. Double mutants (OSEₑ and SBE) were created by annealing OGG2For (5'-tatt agc gct gcc gct cta cta gat gtt ggg tgc gac gaa aag aca caa gct gta ttg ggt tcc ggc ggt ggt tcg aga gca gcc gcc ttg ttc ctc ag-3′) and OGG2Rev (5'-ata gtc tcc ggc gct cta cta gat gtt ggg tgc gac gaa aag aca caa gct gta ttg ggt tcc ggc ggt ggt tcg aga gca gcc gcc ttg ttc ctc ag-3′) oligonucleotides (24).

The integrity of all plasmid constructs was confirmed by restriction mapping and automated DNA sequencing.

Cell Culture and DNA Transfection
BALC cells were grown as described above (27). The UMR106 rat osteosarcoma cell line was maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium (3:1) (Life Technologies), supplemented with 10% fetal bovine serum, 50 μM HEPES, and 2 mM glutamine. All cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Experiments were initiated when cells were ~70–80% confluent.

Transfection Assay—For studies on TGF-β induction, UMR106 cells (2 × 10⁵ cells/well) were plated in six-well plates and incubated for 24 h. Cells were then serum-starved for 12–16 h (in medium containing 0.1% serum) and then transfected with 1 μg of either the OPG promoter-reporter constructs (p.OPG.9/gal constructs) or the negative control promoterless plasmid pŋŋ Basal, using Fugene™ 6 transfection reagent (Roche Molecular Biochemicals) as recommended by the manufacturer. Approximately 5.5 h after transfection, the medium was replaced and replaced with complete medium. Four hours later, the cells were subjected to serum starvation (in medium containing 0.1% serum) overnight. Cells were then treated with either vehicle or TGF-β (10 ng/ml) for the indicated times. After the treatments, the cells lysed in 100 μl of lysis buffer, and β-gal activity was assayed in a fixed amount of the extracts (one-fifth of total) using the β-gal reporter gene assay kit (Roche Molecular Biochemicals). β-gal enzyme activity was determined and expressed as relative light units or as the percentage change over control activity (serum-free controls, with no TGF-β addition). The results from a representative experiment are shown as the mean ± S.E. of 4–12 separate wells.

As a positive control and to verify transfection efficiency, separate plates were transfected with a β-gal expression plasmid (pŋŋ Basal-Promot-ER; CLONTECH, Palo Alto, CA), that has the β-gal reporter gene coding region under the control of the SV40 early promoter. This was done to avoid possible squelching of factors that could arise when cotransfecting multiple plasmids (31, 32). β-gal expression was quantified either by histochemical staining or by β-gal enzymatic assay. The transfection efficiency was 85–90% and comparable across plates. Additionally, all of our experiments were repeated many times (~2–6 times) using multiple clones of the same construct and different preparations of the plasmids. The results under these conditions were similar or identical in nature. Furthermore, the luciferase reporter vector was not used to normalize for transfection efficiency because of our recent finding that cryptic enhancer elements in the promoterless luciferase reporter vector p,GL-Basic could mediate transactivation by Cbfa1, leading to spuriously elevated luciferase activity.

For Cbfa1 transactivation studies, 2 × 10⁵ cells were plated per well in six-well plates and incubated for 24 h. Cells were transfected with 1 μg of each of the reporter plasmid (OPG promoter-β-gal constructs or the negative control promoterless plasmid pŋŋ Basal) and the effector plasmid (pĚF-Cbfa1 or the control expression vector, pĚF/myc/cyto) and incubated for an additional 48 h.

The GCG Wisconsin Package (Genetics Computer Group, Inc., Madison, WI) was used to analyze the 5.9-kb OPG promoter for the presence of consensus transcription factor binding sites, including promoter sites, SBEs, OSEₑ (Cbfa1-binding element), and other elements.

The use of mouse or rat cells in this study. The OPG promoter is a highly conserved sequence throughout evolution, with the human and mouse promoters being 99.7% identical at the nucleotide level. This conservation suggests that the OPG promoter elements have an important role in its expression.
**Stable Transfection**—The OPG promoter construct pOPG5.9βgal (−5917 to +19), was stably transfected into UMR106 cells using Fugene™ 6 reagent (Roche Molecular Biochemicals) as recommended by the manufacturer. A second plasmid, pRzCMV (Invitrogen, San Diego, CA), encoding the neomycin gene was cotransfected for selection. Forty-eight hours after transfection in T25 flasks (Corning Glass), cells were reseeded (1:10) into T75 flasks (Corning Glass) and selected in medium containing 1 mg/ml G418 for 10 days. Regular medium changes were made at 3–4-day intervals. On day 10, randomly selected G418-resistant colonies (containing more than 50 cells) were picked and expanded in fresh medium containing 1 mg/ml G418. For analysis of gene expression, selected colonies were plated in 96-well plates (50,000 cells/well) for 24 h, and experiments were initiated following serum withdrawal for 12–16 h. Cells were stimulated with TGF-β1, -β2, -β3, or BMP-4 (R & D Systems), as indicated and analyzed as described above for transient transfection assays.

**β-gal Assays**

Cell extracts were assayed for β-gal activity using the β-gal reporter gene assay kit (Roche Molecular Biochemicals) as recommended by the manufacturer. Luminescence was measured in a Dynatech MLX luminometer, and light integration was measured at 2 s (relative luminescence units summed). Results were analyzed using Student’s t test, and probability (p) values of less than 0.05 were considered statistically significant.

**mRNA Isolation and Northern Blot Analysis**

BALC and UMR106 cells were plated in T150 flasks, allowed to grow to 70–80% confluence, transferred to medium containing 0.1% serum for 12–16 h, and then treated with TGF-β (10 ng/ml) for the indicated periods of time. Total RNA was extracted using Ultraspec™ II reagent, as recommended by the manufacturer (Biotex, Houston, TX). Poly(A)+ RNA was isolated from total RNA using Oligotex resin (Qiagen, Santa Clarita, CA) according to the manufacturer’s protocol and quantified by spectrophotometry. OPG, RANKL, Cbfa1, and GAPDH cDNAs were used to generate radioactive probes using the Random Primer DNA labeling kit (Life Technologies). 25 ng of cDNA were labeled using [α-32P]dCTP (Amersham Pharmacia Biotech), and free nucleotides were removed by centrifugation through a Centricon-50 column (Amicon). OPG and RANKL mRNA expression was analyzed by Northern blot. GAPDH was used as a control for RNA integrity and to normalize for variations in loading and transfer efficiency. Prehybridization and hybridization were carried out at 48 °C in NorthernMax buffers (Ambion, Inc., Austin, TX). After hybridization, the membranes were washed for 30 min at room temperature in buffer containing 2× SSC and 0.1% SDS and then for 30 min at 48 °C in 0.2× SSC and exposed to Biomax MS x-ray film (Eastman Kodak Co.) at 70 °C. Autoradiograms were quantified by scanning laser densitometry (LKB 2400 Gel Scan XL; Amer- sham Pharmacia Biotech).

**OPG Enzyme-linked Immunosorbent Assay**

In order to quantify the amount of OPG secreted into the cell culture medium, BALC cells (4800 cells/well) were plated in 96-well plates, treated with TGF-β (10 ng/ml), and incubated for 72 h. The amount of OPG secreted into the culture medium was analyzed using a sandwich enzyme-linked immunosorbent assay procedure, utilizing rabbit polyclonal antisera directed against recombinant human OPG, as described previously (18, 24).

**RESULTS**

**TGF-β Stimulates the Expression of Endogenous OPG in Stromal (BALC) and Osteoblastic (UMR106) Cell Lines**—We reported earlier that co-culture of the murine calvaria-derived stromal/osteoblastic cell line BALC along with murine bone marrow cells in the presence of 1,25(OH)2 vitamin D3 resulted in the differentiation of hematopoietic osteoclast progenitors in the bone marrow to form tartrate-resistant acid phosphatase-positive multinucleated osteoclasts (27, 34). These osteoclasts expressed calcitonin receptor and were fully capable of resorbing bone in pit formation assays performed on bovine cortical bone slices. The addition of TGF-β to these co-cultures resulted in a dose-dependent decrease in the number of osteoclasts formed (Fig. 1A). At a concentration of 10 ng/ml, TGF-β completely inhibited the differentiation of osteoclast precursors.

Since a reciprocal expression of OPG and RANKL in osteo-blasts/stromal cells is essential for supporting osteoclast for- mation (35), we analyzed the effect of TGF-β on OPG and RANKL expression in BALC cells. As shown in Fig. 1, B and C, treatment of BALC cells with TGF-β (10 ng/ml) led to a time-dependent increase in the steady state levels of OPG mRNA, with a maximum of 6.1-fold stimulation observed after 12 h of treatment, and a parallel decrease (−5-fold) in RANKL mRNA levels. Consistent with the increase in OPG mRNA levels, treatment of BALC cells with TGF-β led to a 7.1-fold increase in the amount of OPG protein secreted into the culture medium (Fig. 1D). TGF-β had a very similar effect in stimulating OPG mRNA (3.7-fold increase at 24 h) (Fig. 1E) and protein levels (data not shown) in the rat osteosarcoma cell line UMR106.

**TGF-β Stimulates OPG Promoter Activity in Vitro**—To better understand the mechanism by which TGF-β regulates OPG expression, we investigated its effect on OPG gene transcription. We analyzed the human OPG promoter (−5917 to +19) fused to the β-gal reporter gene (24) in both transient and stable transfections in UMR106 cells. UMR106 cells were chosen for these studies because of their clonal stability and their receptiveness to transfection. To demonstrate that the promoter is functional, we first analyzed its ability to drive β-gal expression in transient transfection assays. The 5.9-kb promoter resulted in a 3.7-fold increase in β-gal expression compared with the promoterless reporter vector (β-gal-Basic) (Fig. 2A), and treatment with TGF-β led to a 3.6-fold increase in promoter activity directed by the 5.9-kb promoter (Fig. 2A). To further confirm the functionality and TGF-β-responsiveness of the promoter, we generated stable transfectants. We isolated three randomly picked clones that either had a low, medium, or high basal expression of β-gal activity. Treatment of these clones with TGF-β led to a dose-dependent (optimal 10 ng/ml) and time-dependent (optimal 24–48 h) increase in OPG promoter activity in all three clones. The data from a representative clone that showed a 5-fold increase in promoter activity upon treatment with varying concentrations of TGF-β for 48 h is depicted in Fig. 2B. In the time course assay (Fig. 2C), TGF-β treatment points beyond 48 h were not tested because the cells do not tolerate low serum conditions (0.1% serum) for prolonged periods.

**Effect of TGF-β Isoforms and Members of the TGF-β Superfamily on OPG Promoter Activity**—Members of the TGF-β superfamily exert their influences on cell growth, development, and differentiation by binding to their cognate receptors on the cell surface, followed by the utilization of related, yet distinct signaling pathways involving various members of the Smad protein family (36, 37). In order to distinguish the putative signal transduction pathways(s) involved, we tested the effects of different isoforms of TGF-β (TGF-β1, -2, and -3) as well as BMP-4 on OPG promoter activity in the UMR106 stable clone. As shown in Fig. 3, all three isoforms of TGF-β led to an almost identical level of stimulation of the promoter. However, BMP-4 did not stimulate promoter activity even at the highest concentration tested (100 ng/ml).

**Identification of the Region in the OPG Promoter That Is Responsible for Mediating TGF-β Effects**—In order to map the region of the OPG promoter that confers responsiveness to TGF-β, we made sequential 5′-deletions of the promoter in the context of the β-gal reporter construct pOPG5.9βgal (24) and obtained seven different deletion constructs that are shown schematically in Fig. 4A. These constructs were transiently transfected into UMR106 cells that were then treated with vehicle or TGF-β (10 ng/ml) for 48 h. Assay for β-gal activity in cell extracts showed that sequential deletions of the promoter up to the 0.4-kb region resulted in a slight increase in base-line
expression. However, deletion of the region between 0.4 and 0.2 kb (−372 to −190) resulted in a substantial drop in base-line promoter activity, and the removal of the entire proximal promoter region (pβgal-Basic) resulted in an almost complete loss of base-line activity. The SV40 promoter-driven βgal construct (SV40-βgal) was used as a positive control, and it directed high
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The results represent the mean ± S.E. of 4–8 separate experiments done in triplicate. H11006

In order to delineate the DNA element(s) in the 183-bp region (−372 to −190) that may be involved in mediating TGF-β effects, we analyzed the sequence of this region for the presence of consensus transcription factor binding sites. In addition to the Cbfa1-binding element (OSE2) (the deletion or mutation of which leads to a 50% decrease in Cbfa1-mediated transactivation (24)), we have noted the presence of an AP1-like element and a consensus SBE (Fig. 6A) in this region of the promoter. An AP1

levels of βgal expression compared with all of the OPG promoter-βgal constructs (Fig. 4A).

In terms of TGF-β responsiveness, the longer promoter sequences (5.9–1.9 kb) gave the greatest response to TGF-β (300–400% of control) (Fig. 4B). Deletion of the sequence between 1.9 and 1.5 kb (−1855 to −1455) resulted in a strong decline in TGF-β responsiveness. Further 5’ deletions up to 0.9 and 0.4 kb did not significantly decrease TGF-β responsiveness, while deletion of the region between 0.4 and 0.2 kb (−372 to −190) completely abolished the response to TGF-β (Fig. 4B). Thus, there are proximal (−372 to −190) and distal (−1855 to −1455) regions in the 5.9-kb OPG promoter that contribute to majority of the TGF-β responsiveness, and the proximal region is required for responsiveness.

The −372 to −190 Nucleotide Region of the OPG Promoter Confers TGF-β Responsiveness to a Heterologous Minimal Promoter—Since the deletion of the 183-bp region between −372 and −190 led to a complete loss of response to TGF-β, we focused on this region and tested whether this fragment could function as a TGF-β response region in the context of a heterologous minimal promoter. We generated a reporter construct containing the 183-bp region linked to the osteocalcin minimal promoter (25) upstream of the β-gal reporter gene in pβgal-Basic (Fig. 5) and performed transient transfection assays. The osteocalcin minimal promoter by itself directed very low base-line β-gal expression and did not respond significantly to TGF-β treatment (Fig. 5). In contrast, the construct containing the 183-bp fragment had a high base-line expression. Furthermore, treatment with TGF-β resulted in a 5-fold increase in β-gal activity directed by this construct (Fig. 5), suggesting that the 183-bp region is sufficient to confer TGF-β responsiveness to an otherwise unresponsive heterologous minimal promoter.

Identification of the TGF-β Response Element(s) in the Proximal (183-bp) Region of the OPG Promoter—In order to delineate the DNA element(s) in the 183-bp region (−372 to −190) that may be involved in mediating TGF-β effects, we analyzed the sequence of this region for the presence of consensus transcription factor binding sites. In addition to the Cbfa1-binding element (OSE2) (the deletion or mutation of which leads to a ~50% decrease in Cbfa1-mediated transactivation (24)), we have noted the presence of an AP1-like element and a consensus SBE (Fig. 6A) in this region of the promoter. An AP1

FIG. 2. TGF-β stimulates OPG promoter activity in UMR 106 cells. The 5.9-kb human OPG promoter that was linked to the β-gal reporter gene (in pβgal-Basic reporter vector) was used for functional studies. A, basal and TGF-β-stimulated expression (mean ± S.E.) of the OPG promoter in UMR106 cells transiently transfected with pOPG5.9[βgal and subsequently treated with 10 ng/ml TGF-β for 48 h. B, TGF-β results in a dose-dependent increase in OPG promoter activity in a randomly selected stable clone of UMR106 cells that harbor the pOPG5.9[βgal construct. β-gal activity (mean ± S.E.) directed by the OPG promoter was measured after 48 h of TGF-β treatment and is expressed as relative light units. C, time course of TGF-β responsiveness of the OPG promoter. β-gal activity is expressed as percentage change over control activity (serum-free control, with no TGF-β addition). The results represent the mean ± S.E. of 4–8 separate treatments.

FIG. 3. TGF-β1, -β2, and -β3 but not BMP-4 stimulate OPG promoter activity. UMR106 cells that were stably transfected with the OPG promoter were treated with increasing amounts of TGF-β1, -β2, -β3, or BMP-4 and incubated for 48 h. The β-gal activity (mean ± S.E.) measured in cell extracts from a representative experiment (of three experiments done in triplicate) is shown.

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element has been shown to mediate TGF-$
\beta$ induction of the TGF-$
\beta$ and c-jun gene promoters (38), and the Smad binding element has been shown to mediate TGF-$
\beta$ effects on the jun-B promoter (26) via inducible binding of the Smad3 and Smad4 proteins. The SBE sequence (CAGACA) (26) as well as artificial elements containing this sequence (39, 40) have been shown to function as Smad-binding sites using both gel shift and transfection assays. However, it should be noted that the AP1-like element (GGAGACA) in the human OPG promoter does not match the consensus (TGAg/cTCA) as well as nonconsensus binding site sequences that are known to mediate AP1 effects on various genes (41).

To assess the role of these DNA elements in mediating TGF-$
\beta$ responsiveness of the 183-bp sequence, we performed substitution mutagenesis of the elements (either alone or in combination) in the context of the native (OPG) promoter (Fig. 6A) and in the context of the heterologous (osteocalcin) minimal promoter (Fig. 6B). These constructs were then transiently transfected into UMR106 cells that were subsequently treated with either vehicle or TGF-$
\beta$ (10 ng/ml) for 48 h. The $\beta$-gal activity (mean ± S.E.) in TGF-$
\beta$-treated cell extracts (open circles) is expressed as percentage increase over its own control (no TGF-$
\beta$ added; shaded circles). As an additional control, cells were also transfected with the promoterless $\beta$-gal vector (p$\beta$gal-Basic).

**Fig. 4.** Mapping the region of the OPG promoter that is responsible for mediating TGF-$
\beta$ effects. A, schematic representation of the OPG promoter deletion constructs. Deletions were made using suitable restriction sites and subcloned into p$\beta$gal-Basic vector. The basal levels of $\beta$-gal expression (mean ± S.E.) directed by the constructs are shown as percentage of activity directed by the 5.9-kb promoter fragment. The SV40-$\beta$gal construct served as a positive control and also enabled us to compare the relative strength of the OPG promoter. B, analysis of OPG deletion constructs for responsiveness to TGF-$
\beta$. The deletion constructs were transiently transfected into UMR106 cells, followed by treatment with vehicle or TGF-$
\beta$ (10 ng/ml) for 48 h. The $\beta$-gal activity (mean ± S.E.) in TGF-$
\beta$-treated cell extracts (open circles) is expressed as percentage increase over its own control (no TGF-$
\beta$ added; shaded circles). As an additional control, cells were also transfected with the promoterless $\beta$-gal vector (p$\beta$gal-Basic).
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The region of the OPG promoter between −372 and −190 nucleotides (183-bp fragment) imparts TGF-β responsiveness to a heterologous (osteocalcin) minimal promoter. The 183-bp fragment of the OPG promoter was linked to the −34/+13 fragment of the osteocalcin minimal promoter using PCR and ligated upstream of the β-gal coding sequence (−372 to −190 OCβgal). This construct was transiently transfected into UMR106 cells that were subsequently treated with vehicle or TGF-β (10 ng/ml) for 48 h. As a control, a construct containing the osteocalcin minimal promoter (−34/+13 OCβgal) linked to β-gal was used. The fold induction in β-gal activity (mean ± S.E.) from a representative experiment, done in triplicate wells, is shown.

**β-gal activity:**

|        | − TGF-β | + TGF-β | fold induction by TGF-β |
|--------|---------|---------|------------------------|
| −34/+13| 1.5 ± 0.0 | 2.3 ± 0.5 | 1.8 ± 0.3 |

**Fig. 5.** The region of the OPG promoter between −372 and −190 nucleotides (183-bp fragment) imparts TGF-β responsiveness to a heterologous (osteocalcin) minimal promoter.

Osteoclast differentiation involves the interaction between adherent stromal cells and nonadherent hematopoietic progenitor cells via specific cell surface molecules. Proteins involved in this interaction include RANKL, a membrane-bound ligand that is expressed on the surface of stromal/osteoblastic cells, and the cognate receptor RANK, found on the surface of hematopoietic cells. OPG, a soluble factor that is secreted by a variety of cell types, functions as a decoy receptor for RANKL, thereby competing for the interaction between RANKL and RANK and leading to the inhibition of osteoclast differentiation. Cytokines and hormones that regulate osteoclast differentiation exert their effects by modulating the expression and/or activity of one or more of these molecules.

In the present study, we evaluated the effect of TGF-β on osteoclast formation and OPG expression. Our results confirm that TGF-β inhibits osteoclast differentiation in a dose-dependent manner in co-culture assays involving mouse bone marrow cells and BALC stromal/osteoblastic cells. Further, concentrations of TGF-β that inhibit osteoclast formation resulted in an increase in steady state levels of OPG mRNA and a concomitant decrease in the levels of RANKL mRNA in BALC cells. The expression of mRNA for OPG and RANKL was reciprocal and temporally preceded TGF-β effects on osteoclast formation, reminiscent of the established roles of OPG and RANKL in regulating osteoclast formation. These results are in agreement with previous observations showing that TGF-β regulates OPG and RANKL expression in ST2 stromal cells (19) and mouse calvaria-derived primary osteoblasts (17). We have shown that TGF-β treatment increases secretion of OPG protein in BALC cells and provide evidence that TGF-β directly stimulates OPG promoter activity in experiments using the UMR106 osteosarcoma cells. TGF-β treatment resulted in a dose- and time-dependent stimulation of OPG promoter activity. The effects were mimicked by two of the isoforms of TGF-β (TGF-β2 and TGF-β3) but not by BMP-4. Even at a 10-fold higher concentration (100 ng/ml), BMP-4 could not induce OPG promoter activity, suggesting a TGF-β signal-specific effect. These results suggest that TGF-β signal-specific Smad proteins (Smad2 and -3), along with the common Smad (Smad4) are involved in mediating TGF-β induction of the OPG promoter.

It has recently been reported that BMPs stimulate OPG promoter expression (45) based on the evidence that overexpression of Smad1 and a constitutively active type IA BMP receptor (ALK3) in C3H10T1/2 cells results in an increase in OPG promoter activity. Two Hox binding sites in the OPG promoter...
have been shown to mediate this effect. However, this conclusion is based only on transient overexpression studies, and the authors have not directly shown that treatment with BMPs results in an increase in OPG promoter construct activity in the cell lines that were studied.

Extensive deletion analysis of the 5.9-kb OPG promoter allowed us to delimit a proximal 183-bp region (H11002 to H11002) that is required for imparting TGF-β responsiveness to the promoter. This region of the promoter, when linked to the osteocalcin minimal promoter, resulted in an increase in base-line expression, consistent with the loss in base-line expression observed upon deletion of the region from the native promoter (Fig. 4A; compare the activities of the 0.4- and 0.2-kb fragments). Furthermore, the 183-bp region was sufficient to confer TGF-β responsiveness to the otherwise nonresponsive osteocalcin minimal promoter. This region includes, among other binding sites, a Cbfa1-binding element (OSE2), an AP1-like binding element (an AP1 element has been shown to mediate TGF-β effects on the TGF-β and c-jun gene promoters (38)), and an SBE that mediates TGF-β stimulation of the Jun-B promoter (26). Mutational analyses of these elements revealed that the SBE and OSE2 elements are involved in directing base-line expression, but the AP1-like element had no effect on base-line expression.

**FIG. 6.** Functional analysis of the role of consensus DNA elements (OSE2, AP1-like, and SBE) in mediating TGF-β effects on the OPG promoter. A, mutational analysis of the role of the OSE2 (AACCTCA), AP1-like (GGAGACA), and SBE (CAGACA) elements in the context of the native (OPG) promoter. The spatial arrangement of OSE2, AP1-like element, and SBE in the region between –372 and –190 nucleotides in the OPG promoter are shown. The nucleotide numbers represent the location of the elements corresponding to the transcription start site (H11001). Substitution mutations were made to disrupt the sequence of one or more of the DNA elements (either individually or in combination) (OSE2, AACCTCA → AGATATC; AP1-like, GGAGACA → TCGGACA; SBE, CAGACA → GAATTC). The wild type and mutant constructs were transiently transfected into UMR106 cells that were then treated with TGF-β (10 ng/ml) for 48 h. Three independent transfection experiments were performed in triplicate, and the β-gal activities (mean ± S.E.) from a representative experiment are shown. B, mutational analysis of the role of the elements in the context of a heterologous (osteocalcin) promoter. To assess the function of the elements in the context of the osteocalcin minimal promoter, the same substitution mutations were created in (H11002 to H11002 OCgal) construct. The constructs were transfected into UMR106 cells that were then treated with TGF-β (10 ng/ml) for 48 h. The β-gal activity (mean ± S.E.) in cell extracts from one of three independent experiments done in triplicate is shown. C, stimulation of Cbfa1 mRNA expression by TGF-β. UMR106 cells were treated with either vehicle or 10 ng/ml TGF-β for 24 h, and poly(A)+ RNA was isolated. Northern blot analysis was performed with 2 μg of RNA and probed with Cbfa1 and GAPDH probes. The Cbfa1 band intensities were quantified and then normalized to GAPDH levels. The -fold induction in Cbfa1 expression in TGF-β-treated cells compared with untreated control cells is shown. Two independent analyses were performed, and identical results were obtained.
expression. The data also show that in addition to the SBE, the OSE2 element is also needed for maximal TGF-β inducibility, but the AP1-like element is dispensable (Fig. 6A). Similar results were observed with constructs containing the heterologous osteocalcin minimal promoter (Fig. 6B), except that the OSE2 mutation resulted in an increase in base-line expression, the reason for which is not clear. Consistent with these functional results, use of the AP1-like element as a probe did not result in a specific DNA-protein complex in gel shift assays, suggesting that the AP1-like element is not a functional binding site for AP1 (data not shown). In contrast, a specific DNA-binding complex was observed for the OSE or SBE (24, 26, 39, 40). We have not been able to show an increase in Cbfa1 or Smad binding intensity upon TGF-β treatment, suggestive of the moderate effects of the OSE2 and SBE in mediating TGF-β responsiveness of the OPG promoter (data not shown). However, we could detect a consistent 2.5-fold increase in Cbfa1 mRNA levels upon TGF-β treatment (Fig. 6C).

We have shown previously (24) that deletion of this 183-bp region resulted in a complete loss of transactivation of the OPG promoter by Cbfa1, with the proximal OSE2 element being necessary for maximal induction. Interestingly, disruption of the SBE also resulted in a ~45% decrease in Cbfa1 mediated transactivation, while deletion of both of the elements resulted in a 75% decrease (Fig. 7). This suggests that there may be an interaction between Cbfa1 and Smad proteins in mediating the stimulatory effects of Cbfa1 and TGF-β on the OPG promoter. There is precedence for a synergistic interaction between Smad and AML proteins (Cbfa homologs) in mediating TGF-β stimulation of the human (44) and mouse (42) germ line IgA gene promoters, and it has recently been shown that a truncated Cbfa1 protein that displays impaired transactivation and Smad interaction results in cleidocranial dysplasia (46). In addition, a region containing two copies of the core-binding element (to which all Cbfa homologs are capable of binding) also functions as a TGF-β response element in the mouse IgA promoter (42). Furthermore, Cbfa1 has recently been shown to be a major TGF-β1-responsive element-binding protein that is induced by TGF-β1 and BMP-2 in C2C12 mesenchymal precur- sors (47).

It is important to note that the deletion of both elements does not completely abolish TGF-β effects. Since TGF-β has been shown to regulate the expression of various target genes through a variety of different response elements (48), it is possible that other known or novel elements in the 183-bp region, either alone or in combination with factors binding to the OSE and SBE sites, could also be involved in mediating TGF-β effects on the promoter. Also, in the context of the 5.9-kb promoter, elements in the region between ~1855 and ~1455 are expected to play a role in mediating TGF-β effects, since the deletion of the region leads to a decrease in TGF-β responsiveness. Further studies are needed to get a more complete understanding of the molecular interactions involved in mediating TGF-β stimulation of OPG promoter activity.

TGF-β is produced by a number of cell types, including osteoblasts and stromal cells and plays a major role in the regulation of bone formation and resorption (21). It is stored in abundant amounts in bone and is released from the bone matrix during osteoclastic bone resorption. The released TGF-β can then induce OPG expression by osteoblasts in the local bone microenvironment and thereby inhibit osteoclast formation/activity and stop bone resorption. However, in vitro studies have shown that anti-OPG antibodies only partially reverse the inhibitory effects of TGF-β on osteoclast differentiation in co-culture assays (17, 19). Furthermore, in co-cultures established from mice rendered null for the OPG gene, TGF-β re-
Stimulation of Osteoprotegerin (OPG) Gene Expression by Transforming Growth Factor- β (TGF-β): MAPPING OF THE OPG PROMOTER REGION THAT MEDIATES TGF- β EFFECTS
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