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Cross-talk between 1,25-Dihydroxyvitamin D_3 and Transforming Growth Factor-β Signaling Requires Binding of VDR and Smad3 Proteins to Their Cognate DNA Recognition Elements*

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1,25-Dihydroxyvitamin D_3 (vitamin D) and transforming growth factor-β (TGF-β) regulate diverse biological processes including cell proliferation and differentiation through modulation of the expression of target genes. Members of the Smad family of proteins function as effectors of TGF-β signaling pathways whereas the vitamin D receptor (VDR) confers vitamin D signaling. We investigated the molecular mechanisms by which TGF-β and vitamin D signaling pathways interact in the regulation of the human osteocalcin promoter. Synergistic activation of the osteocalcin gene promoter by TGF-β and vitamin D was observed in transient transfection experiments. However, in contrast to a previous report by Yanagisawa, J., Yanagi, Y., Masuhiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., Toriyabe, T., Kawabata, M., Miyazono, K., and Kato, S. (1999) *Science*, 283, 1317–1321, synergistic activation was not detectable when the osteocalcin vitamin D response element (VDRE) alone was linked to a heterologous promoter. Inclusion of the Smad binding elements (SBEs) with the VDRE in the heterologous promoter restored synergistic activation. Furthermore, this synergy was dependent on the spacing between VDRE and SBEs. The Smad3-Smad4 heterodimer was found to bind in gel shift assay to two distinct DNA segments of the osteocalcin promoter: −1030 to −989 (SBE3) and −418 to −349 (SBE1). Deletion of SBE1, which is proximal to the VDRE, but not the distal SBE3 in this promoter reporter abolished TGF-β responsiveness and eliminated synergistic co-activation with vitamin D. Thus the molecular mechanism, whereby Smad3 and VDR mediate cross-talk between the TGF-β and vitamin D signaling pathways, requires both a VDRE and a SBE located in close proximity to the target promoter.

The biological actions of 1,25-dihydroxyvitamin D_3 (vitamin D)[1] are mediated through the vitamin D receptor (VDR), a member of the nuclear receptor superfamily (1). Although VDR homodimers may play some role, most VDR actions are thought to be mediated via VDR heterodimerization with the retinoid X receptor (RXR) (2). The VDR/RXR heterodimers bind to vitamin D response elements (VDREs) in the promoters of vitamin D target genes (3, 4). VDREs typically consist of at least two copies of the consensus motif, PuG(G/T)TCA, arranged as direct repeats with a three-nucleotide spacing (DR3) (5). In addition to spacing, small differences in the half-site sequence, and the sequence of the flanking extension of the response elements also appear to be important in determining receptor binding efficiency (Ref. 6 and references therein). Upon binding, these receptors can either stimulate or repress transcription of target genes (7), assisted by direct and indirect interaction with other transcriptional regulatory proteins. Such additional factors may be recruited by other regulatory signals, such as TGF-β, which has been shown to act in synergy with vitamin D (8, 9).

Transforming growth factor-β (TGF-β), like activin and bone morphogenetic protein (BMP), signals through the Smad family of intracellular transducing proteins (10). Among the Smads, Smad2 and Smad3 are known to permit signaling by TGF-β and activin ligands (11). Once phosphorylated, these Smad proteins heterodimerize with Smad4, the common mediator of TGF-β pathways. These complexes translocate to the nucleus where they regulate target genes either by interacting with DNA or with other transcription factors (11, 12). Though a palindromic Smad binding element (SBE), GTCTAGAC, was shown to interact with the Smad3 and Smad4 complex, the smaller 4-bp half-site of this SBE DNA sequence, GTCT or AGAC, has been shown to be sufficient to bind these proteins (12, 13).

Although Smads can bind to DNA, Smad-DNA binding alone is not always sufficient for gene activation. There are now a growing number of examples in which Smads cooperate with DNA-binding partners to regulate transcription (14). This type of interaction has been demonstrated for the *Mix.2, goosecoid* and *Xvent2* promoters where Smads interact with FAST-1, FAST-2, mixer, milk and OAZ transcription factors (12). DNA binding by Smad3, in conjunction with TFE3, PEBP2, and ATF-2 to their cognate sites, has been reported to be essential for TGF-β-dependent activation of the *PAI-1*, *IgA*, and collagenase A promoters respectively (12). Some of these cooperativities may be involved in mediating signals for other signaling pathways, as suggested by recent studies that described a physical interaction of liganded VDR, Smad3, and SRC-1/TIF2 mediating cross-talk between vitamin D and TGF-β signaling pathways (8, 9).

In the present study, we identified functional TGF-β-re-

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[1] The abbreviations used are: vitamin D or VD3, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; RXR, retinoid X receptor; VDRE, vitamin D response element; DR, direct repeat; TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; SBE, Smad binding element; luc, luciferase; GR, glucocorticoid receptor; hOC, human osteocalcin; PAI-1, plasminogen activator inhibitor-1; bp, base pair; kb, kilobase pair.

sponse elements in the human osteocalcin (*hOC*) gene promoter; demonstrated cross-talk between vitamin D and TGF-β signaling pathways in regulation of this promoter; and determined the molecular mechanism for synergistic activation of the *hOC* promoter by vitamin D and TGF-β.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Reporter plasmids pOSLUC2 (−1.3 kb) and pOSLUC1 (−344 bp), of the human osteocalcin gene promoter linked upstream of the luciferase reporter of pGL-3basic have been described previously (15). pOSLUC3 (−1.3 kb to −1111 plus −905 to −833) was created by deletion of a *Stu*I fragment from pOSLUC2. Deletion of the *Pst*I and *Sac*I fragment from pOSLUC2 resulted in pOSLUC4 (−1.3 kb to −833 plus −341). pOSLUC5 (−1.3 kb to −341 plus −191) was constructed by deletion of the *Sac*I and *Apa*I fragment from pOSLUC2. pOSLUC6 was created by cloning in an osteocalcin promoter PCR fragment (forward primer −1337 to −1311 with *Mlu*I 5′ overhang and reverse primer −475 to −500 with *Xho*I 5′ overhang) into the pGL-3 promoter vector. The p3TP-Lux reporter, which contains SBEs from the plasminogen activator inhibitor-1 (*PAI-1*) gene in front of the luciferase reporter, was a kind gift from Dr. J. Massague (16). Double-stranded oligonucleotides corresponding to human osteocalcin VDRE (−507 to −470 or −515 to −484) or rat osteocalcin VDRE (−515 to −484) were cloned upstream of the SV40 promoter of pGL-3 promoter vector to produce hVDREluc and rVDREluc respectively. hVDRE-3TPlux was created by inserting the hVDREs in front of the SBEs from the p3TP-Lux plasmid. Insertion of 450 bp fragment, which do not contain known SBEs from pSG5 expression vector, between VDRE and SBEs of hVDRE-3TPlux into *Xho*I site resulted in the V450T reporter plasmid. VDR expression vector, mVDR-pSG5 and FLAG-Smad2,3,4/pcDNA3 plasmids that express Smad2, 3, or 4 with N-terminal FLAG tags have been described elsewhere (15, 17). FLAG-S3-(41–435) that expresses Smad3 with an N-terminal 41 amino acid deletion was a kind gift from Dr S. Kato (8).

Cell Culture and Transfections—COS-1 cells were grown in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from Life Technologies, Inc.) at 37 °C in 5% CO_2. Cells were plated at ~30% confluence in 24-well plates and the following day were transfected using FuGENE-6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) with 250 ng/well of reporter plasmid and 50 ng of the VDR or Smad expression plasmid. After a 6-h incubation at 37 °C in 5% CO_2, 2% charcoal stripped fetal calf serum containing medium was added having either 10 nM 1,25-dihydroxy vitamin D_3 (Hoffman-LaRoche) or vehicle (isopropyl alcohol, final conc. 0.01%) and/or TGF-β (1 ng/ml) (Sigma, Saint Louis, MI) or vehicle (4 nM HCl in 1 mg/ml bovine serum albumin). Following incubation for 16 h, cells were lysed with 2× Promega lysis buffer, and luciferase assays were performed in triplicate with the firefly luciferase assay kit (Promega, Madison, WI) and measured using a Berthold LB953 Autolumat luminometer (Berthold, Bad Wildbad, Germany). Each experimental condition was measured in triplicates, and the values given represent the mean ± S.D. from two to three experiments.

Gel Shift Assays—Oligonucleotides were endlabeled by T4 polynucleotide kinase using [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham Pharmacia Biotech). DNA binding reactions of 20 μl were carried out in buffer containing 20 mM Tris-Cl, pH 8.0, 10% (W/V) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 400 ng of poly(dI-dC) (Amersham Pharmacia Biotech), 75 mM KCl, 3% bovine serum albumin, 0.1–0.3 ng of radiolabeled oligonucleotide, and 2 μl of Smad3 and Smad4 proteins transcribed and translated *in vitro*, in rabbit reticulocyte lysate (Promega, Madison, WI). Binding reactions were performed in room temperature for 20 min. Free and bound DNA were separated on 4% polyacrylamide (acrylamide/bisacrylamide, 29:0.5) gels, which were run at a constant voltage of 250 V in 22 mM Tris-borate, 0.5 mM EDTA (6).

RESULTS

Vitamin D and TGF-β Synergistically Activated Human Osteocalcin Promoter Activity—The modulatory effects of 1,25-dihydroxyvitamin D_3 and TGF-β signaling on the human osteocalcin promoter were analyzed by transient transfections into COS1 cells using the pOSLUC2 reporter and VDR expression plasmid. 1,25-Dihydroxyvitamin D_3 transactivated the pOSLUC2 reporter activity by 6-fold whereas TGF-β alone augmented the reporter activity 2-fold (Fig. 1). Both ligands, produced a synergistic effect (10-fold induction). This synergy was specific to the pOSLUC2 reporter, because these two ligands together or singly did not significantly alter transcriptional activity of pOSLUC1, which lacks 960 bp of distal 5′ DNA sequence from the human osteocalcin promoter including the VDRE (15). Smad3 and Smad2, but not BMP Smads (Smad1 or Smad5), activated the osteocalcin promoter reporter (data not shown), both in the absence and presence of TGF-β. Augmentation of reporter activity was greater with Smad3, suggesting a putative Smad3 binding site conferred the TGF-β signal for this promoter. Overexpression of Smads 3 and 4 constitutively activated basal promoter activity mimicking TGF-β signaling, as has been reported for other well characterized TGF-β responsive promoters (11, 12).

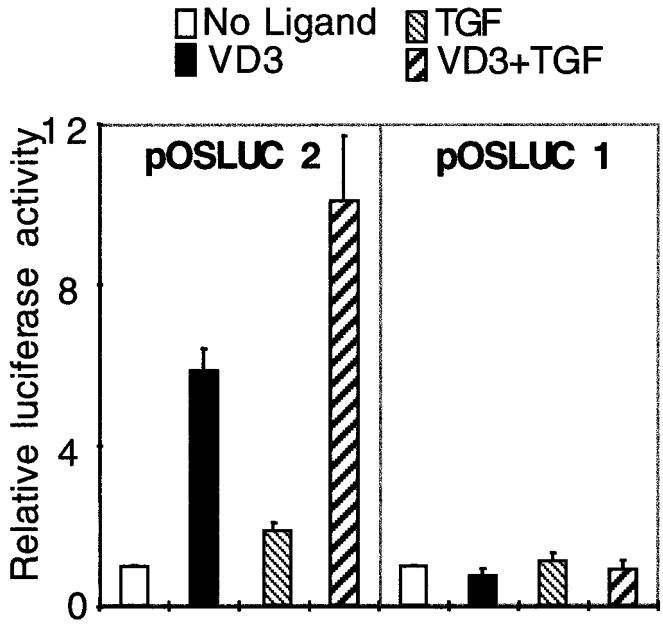

FIG. 1. **1,25-dihydroxyvitamin D_3 (*VD3*) and TGF-β-activated *OC* promoter.** The human osteocalcin promoter conferred both 1,25-dihydroxyvitamin D_3 and TGF-β signaling to a luciferase reporter. The pOSLUC2 or pOSLUC1 reporter was transiently co-transfected with VDR expression plasmid into COS1 cells, and 1,25-dihydroxyvitamin D_3 (*VD3*) and/or TGF-β-induced luciferase activity was measured and related to the uninduced reporter activity.

TGF-β Did Not Augment 1,25-Dihydroxyvitamin D_3 Activation of VDRE-dependent Reporters—To investigate whether the synergistic activity observed in our previous experiment was in fact mediated through the VDRE (4), the hVDREluc reporter was co-transfected with a VDR expression plasmid into COS1 cells. Whereas 1,25-dihydroxyvitamin D_3 increased the reporter activity 10-fold, TGF-β did not affect reporter activity (Fig. 2*A*), nor was there any synergy between 1,25-dihydroxyvitamin D_3 and TGF-β, as observed in the case of the pOSLUC2 reporter. As the DR3 VDRE in the human osteocalcin promoter overlaps a DR6 VDRE and an AP1 site, testing of these overlapping elements (−515 to −484) was carried out in a luciferase reporter construct. This construct also responded to 1,25-dihydroxyvitamin D_3 but did not respond to TGF-β, and there was no synergy between 1,25-dihydroxyvitamin D_3 and TGF-β (Fig. 2*B*). Comparable experiments performed with the rVDREluc reporter had a similar 1,25-dihydroxyvitamin D_3 but no response to TGF-β and no synergy between 1,25-dihydroxyvitamin D_3 and TGF-β signals, irrespective of nucleotide differences in human and rat VDREs (Fig. 2*C*). These results suggest that VDRE alone is not sufficient to mediate the synergy.

1,25-Dihydroxyvitamin D_3 Did Not Augment TGF-β Induction of a TRE-dependent Reporter—Possible effects of 1,25-

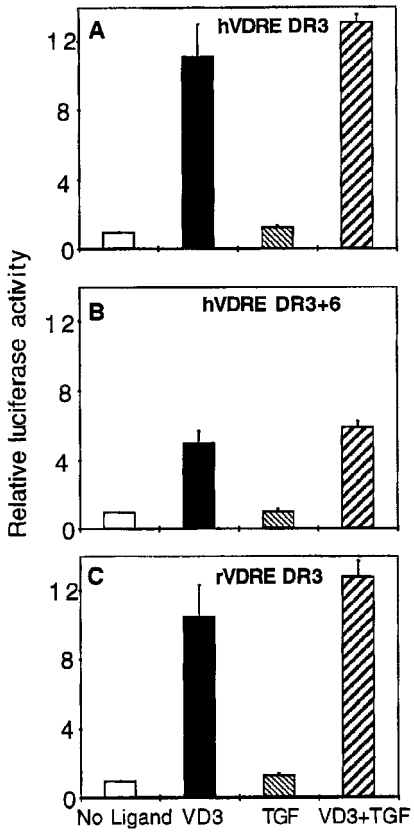

FIG. 2. **VDRE-linked heterologous promoters were not sufficient to convey TGF-β signaling to a luciferase reporter.** hVDRE-luc (*DR3*, *A*), hVDRElluc (*DR3+6*, *B*), and rVDRElluc (*C*) reporters were transiently co-transfected with VDR expression plasmid into COS1 cells, and 1,25-dihydroxyvitamin D_3 (*VD3*) and/or TGF-β-induced luciferase activity was measured and related to each uninduced reporter activity.

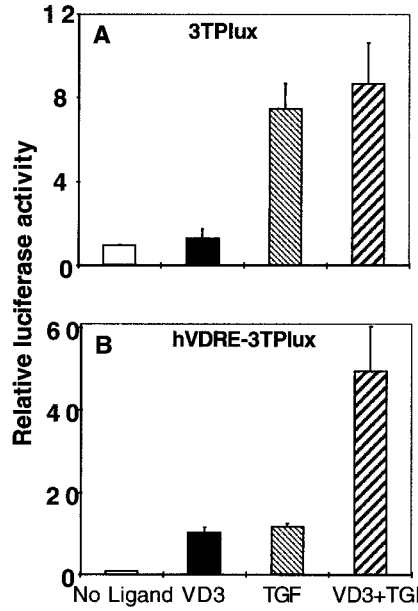

FIG. 3. **Presence of a TRE sequence was essential to convey TGF-β signaling through VDRE-linked heterologous promoters.** p3TP-Lux (*A*) and hVDRE-3Tplux (*B*) reporters were transiently co-transfected with VDR expression vector into COS1 cells, and 1,25-dihydroxyvitamin D_3 (*VD3*) and/or TGF-β-induced luciferase activity were measured in luciferase units. All luciferase activities were related to uninduced reporter activity.

dihydroxyvitamin D_3 on TGF-β transcriptional activation were tested on a heterologous reporter construct carrying the well characterized TRE from the *PAI-1* gene (p3TP-Lux) in COS1 cells (16). Whereas TGF-β activated the reporter in these cells 7-fold, 1,25-dihydroxyvitamin D_3 had no effect on the basal activity of the reporter (Fig. 3*A*). Moreover, TGF-β-activated transcription was not augmented by 1,25-dihydroxyvitamin D_3 (Fig. 3*A*). This result suggests that the TRE alone is incapable of converging vitamin D and TGF-β signaling pathways.

VDRE Coupled to the TRE Sequence Restored Synergy by Vitamin D and TGF-β Signaling Pathways—To explore whether the synergy by 1,25-dihydroxyvitamin D_3 and TGF-β pathways required concomitant binding of both VDR and Smad to their separate cognate elements, the hVDRE-3TPlux reporter plasmid, which contains both respective response elements, was co-transfected with VDR expression plasmid in COS1 cells. 1,25-Dihydroxyvitamin D_3 or TGF-β alone activated this reporter approximately by 10-fold (Fig. 3*B*). Importantly, hVDRE-3TPlux reporter responded synergistically to 1,25-dihydroxyvitamin D_3 and TGF-β (45-fold), suggesting both binding sites are needed to mediate the synergy (Fig. 3*B*).

Smad3-Smad4 Heterodimer Binding to Osteocalcin Promoter—Sequence analysis of the human osteocalcin promoter identified three regions: −415 to −353 (SBE1); −313 to −228 (SBE2); and −1027 to −997 (SBE3); each of which contains three copies of GTCT or its reverse complement AGAC, an optimal target/binding site for Smad3 and Smad4 (13). Gel shift assays using Smad3 and Smad4 proteins, transcribed and translated *in vitro* in rabbit reticulocyte lysate, were used to examine DNA binding activity of these putative SBEs as well as the PE2 fragment of the *PAI-1* promoter (13) as a positive Smad binding control. SBE1 and SBE3 formed complexes similar to PE2 (Fig. 4) whereas there was no complex formation with SBE2 (data not shown). Rabbit reticulocyte lysate alone did not form any complexes with these DNA probes (data not shown). DNA binding specificity of complexes formed with DNA probes, was examined using cold DNA probes as competitors. Both SBE1 and SBE3 inhibited complex formation. However the PE2 element (−586 to −551) from the *PAI-1* promoter, which contained three AGAC motifs, moderately inhibited the complex formation with either SBE1 or SBE3.

Smad3-Smad4-mediated TGF-β-responsive Regions in the Osteocalcin Promoter—To determine functional significance of the Smad binding to the potential SBEs in the promoter, we made deletions of larger regions from the pOSLUC2 (−1.3 kb) that removed SBE2, SBE1, and/or SBE3 (pOSLUC3–6). Cotransfection of Smad3 and Smad4 expression vectors with reporter plasmids pOSLUC1-6 augmented basal and TGF-β-dependent activity (Fig. 5). The highest activation was observed with the wild-type promoter reporter, pOSLUC2. Deletion of SBE1 (pOSLUC4) greatly affected Smad3-mediated augmentation whereas a moderate reduction was observed with the deletion of SBE3 (pOSLUC3), both of which reflected the previous binding studies that SBE1 and SBE3 were capable of binding to Smad3 and Smad4. Deletion of SBE2 (pOSLUC5), which did not bind with Smad3 and Smad4 in the gel shift assay (data not shown), did not affect the reporter activity. Combined deletion of SBE1 and SBE3 (pOSLUC1) or SBE1 and SBE2 (pOSLUC6) also resulted in marked loss of reporter activity. These results suggest that the Smad-mediated activity was largely through the SBE1.

VDRE and TRE Spacing Crucial for 1,25-Dihydroxyvitamin D_3 and TGF-β Synergy—The role of the spacing between VDRE and TRE in the 1,25-dihydroxyvitamin D_3 and TGF-β synergy was examined using V450T reporter plasmid. This construct, containing both VDRE and TRE separated by 450 bp, was co-transfected with VDR expression plasmid in COS1 cells. This reporter activation by 1,25-dihydroxyvitamin D_3 or TGF-β

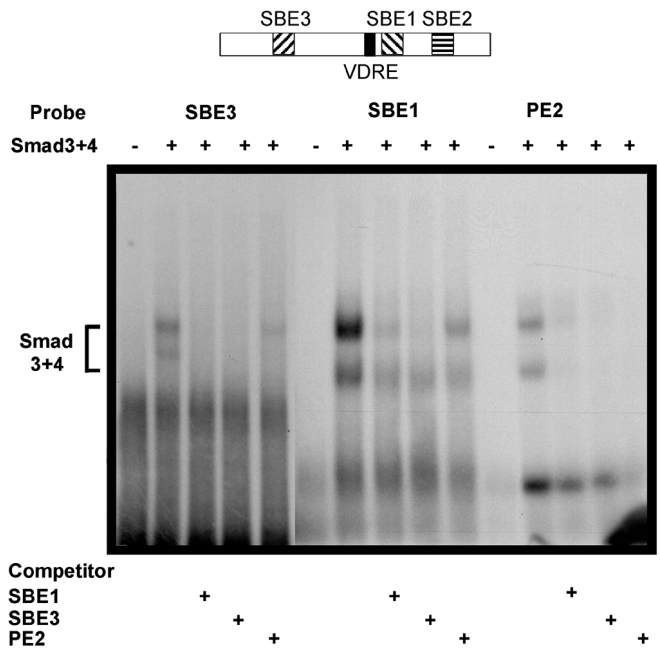

FIG. 4. **Smad3 and Smad4 binding to SBE1 and SBE3 *in vitro*.** *In vitro*-produced Smad3 and Smad4 proteins (2 μl) were incubated with radiolabeled SBE1, SBE2, SBE3, and PE2 and analyzed by gel shift assay. A 50-fold excess of unlabeled SBE1, SBE3, or PE2, a fragment of the *PAI-1* promoter (13), were included in the incubation reaction before the assay.

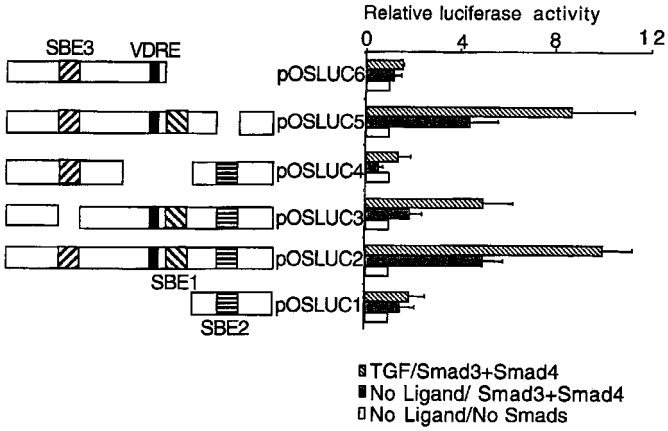

FIG. 5. **Smad3 and Smad4 activated the osteocalcin promoter mainly through SBE1.** pOSLUC1, pOSLUC2, pOSLUC3, pOSLUC4, pOSLUC5, and pOSLUC6 reporters were co-transfected with empty vector or Smad3 and Smad4 expression plasmids into COS-1 cells, and cells were treated with or without TGF-β. All luciferase activities were related to individual uninduced reporter activity.

alone or in combination was remarkably reduced (Fig. 6) in comparision to the parent hVDRE-3TPlux reporter plasmid (Fig. 3*B*). The level of reduced synergy conferred by V450T was comparable with pOSLUC6 reporter that has the VDRE and SBE3 at a similar 450-bp distance. This result demonstrated that synergy by 1,25-dihydroxyvitamin D_3 and TGF-β signaling, conferred via their respective transducers VDR and Smad3, only occurred through binding to VDRE and TRE located in the close proximity in the target promoter.

Direct Interaction between VDR and Smad3 Also Required for Synergy—It was previously demonstrated that the 40-amino acid N-terminally truncated Smad3 did not physically interact with VDR in either GST pull-down assays or the mammalian two-hybrid system, whereas the 20-amino acid N-terminally truncated Smad3 retained such abilities (8). We examined whether these Smad3 mutants together with VDR

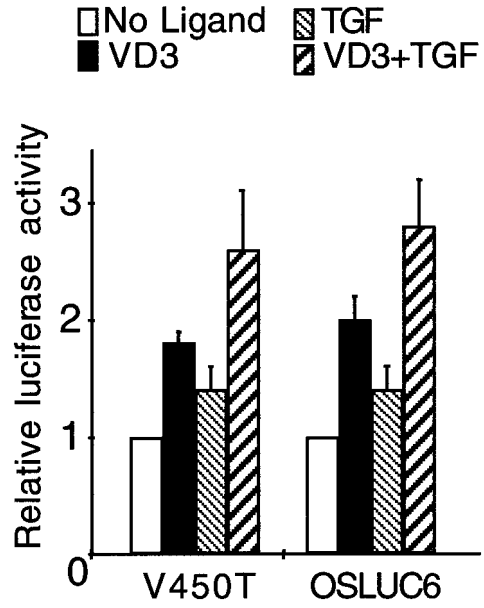

FIG. 6. **Spacing between VDRE and TRE affects synergy.** The V620T, V450T, or pOSLUC6 reporters were transiently co-transfected with VDR expression plasmid into COS1 cells, and 1,25-dihydroxyvitamin D_3 (*VD3*) and/or TGF-β-induced luciferase activity were measured and related to individual uninduced reporter activity.

were capable of synergistically activating the hVDRE-3TPlux reporter. The wild-type Smad3 and Smad3-(21–435), capable of transducing TGF-β signal, together with VDR synergistically transactivated this reporter (Fig. 7). Smad3-(41–345) failed to mediate TGF-β signaling, and with VDR, was unable to transactivate synergistically as Smad3, suggesting that a competent Smad3 in addition to separate and distinct binding sites is also required for the synergy.

DISCUSSION

DNA-binding nuclear factors interact with one another and with cofactors to form complex networks of DNA-protein-protein interactions for transcriptional regulation in eukaryotic cells (5, 8). Transcription regulatory regions of eukaryotic gene promoters generally consist of clusters of transcription factor-binding sites that coordinate responses to combinations of signaling molecules and different transcription factors utilize a variety of means to promote or repress target genes. (19). Osteocalcin gene expression is facilitated by overlapping and contiguous regulatory elements including a functional VDRE in the distal promoter region (4, 20, 21). Regulatory factors, including VDR, Cbfa-1, AP-1 factors, and homeodomain proteins act directly, and BMPs and TGF-β act indirectly to mediate commitment to the osteoblastic cell lineage and regulation of the osteocalcin promoter (22, 23).

Only two reports to date describe transcriptional regulation through protein-protein interaction involving Smad3 and nuclear hormone receptors (8, 24). Whereas Yanagisawa *et al.* (8) reported that activation by VDR, but not other receptors (oestrogen receptor α, androgen receptor, GR, retinoic acid receptor, and RXR), was affected by the treatment with TGF-β, Song *et al.* (24) found that Smad3 was the direct target for repression by the GR on a type-1 *PAI-1* gene promoter. They provided evidence for GR-Smad3 interaction *in vivo* and *in vitro*, although they could not rule out the possibility that other proteins might have mediated the interaction.

A structure/function analysis of the *hOC* promoter was initiated to explore its synergistic activation by vitamin D and TGF-β (Fig. 1). Recently it has been reported that Smad3, a downstream component of the TGF-β signaling pathway, acts

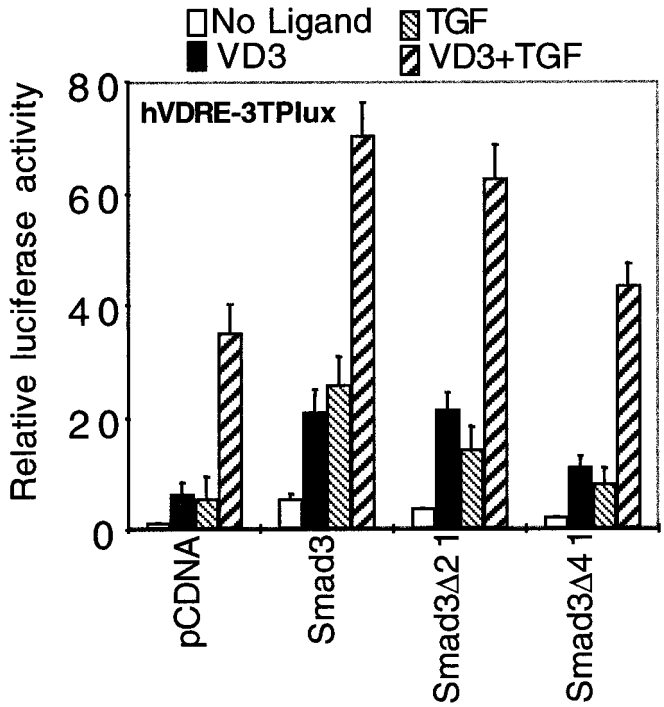

FIG. 7. **Competent Smad3 required for synergy *in vivo*.** hVDRE-3TPlux reporter was transiently co-transfected with VDR and in the absence or presence of Smad3 or S3-(21–435), S3-(41–435) expression vector into COS-1 cells, and 1, 25-dihydroxyvitamin D_3 (*VD3*) and/or TGF-β-induced luciferase activity was measured and related to uninduced reporter activity.

as a coactivator of VDR potentiating its ligand-induced transactivation (8, 9). Hence, we tested the VDRE from the *hOC* promoter as a separate response element in the context of a minimal heterologous promoter. There was no synergy between 1,25-dihydroxyvitamin D_3 and TGF-β signals through this VDRE (Fig. 2*A*). In addition, the slightly longer VDRE, which contained both DR6 and DR3 spacing, exhibited no synergy in transient transfection studies in COS-1 cells by these signals (Fig. 2*B*). A rat osteocalcin DR3-type of VDRE placed in heterologous context also did not mediate synergistic effects of 1,25-dihydroxyvitamin D_3 and TGF-β (Fig. 2*C*). These results suggested that nucleotide differences in different minimal VDREs cannot explain the failure to observe TGF-β-mediated reporter activity. In addition, similar results were obtained in three separate cell lineages, suggesting differences in cellular context could not be responsible for the disparity of the results. These data are not consistent with direct protein-protein interaction as the sole mode of action to explain the synergy, and therefore are in sharp contrast to the findings of Yanagisawa *et al.*(8, 9) who reported that the VDRE alone was sufficient for convergence of 1,25-dihydroxyvitamin D_3 and TGF-β signaling pathways.

Disparity between our results and previously reported results may be attributed to the design of the reporter construct used in the transient transfection studies. Constructs in Ref. 8 derived from pGCAT (25) and contained a synthetic oligonucleotide insert with *Hin*dIII and *Xba*I sites. This *Xba*I site follows GT nucleotide of the parental plasmid sequence and creates a DNA sequence 5′-GTCT-3′, which has been defined as a Smad box (12) and could form part of a cryptic SBE in close proximity to the VDRE. Potentially, the presence of these two closely spaced sites could be mediating the synergy between 1,25-dihydroxyvitamin D_3 and TGF-β signals in their reporter construct. Irrespective of these reasons, in our studies, convergence of vitamin D and TGF-β signaling required both specific DNA response elements in both heterologous promoter model constructs and in the human osteocalcin promoter.

The ability of the Smad3-Smad4 complex alone or in the presence of TGF-β to transactivate the OSLUC2 reporter is consistent with the existence of Smad binding sites in this promoter. This led us to test the activation of reporters containing both a VDRE (human) and a cluster of TGF-β response elements from the *PAI-1* gene, upstream of the SV40 promoter. Transient transfection studies with these reporter constructs recreated the synergy between vitamin D and TGF-β signals, with activation by vitamin D comparable with the induction of hVDREluc reporter (Fig. 2*A*), and TGF-β stimulated activation comparable with that of the standard TGF-β responsive reporter p3TP-Lux (Fig. 3*A*). Of three potential SBE clusters in the osteocalcin promoter, two (SBE1 and SBE3) bound to Smad3 and Smad4 proteins in a gel shift assay (Fig. 4). Deletion of these two putative regions separately or together reduced TGF-β-mediated reporter activity confirming their functional role (Fig. 5). Taken together, these results suggest that the convergence of TGF-β and vitamin D signaling does not take place in the absence of either VDRE or TRE. In agreement with these data, a Smad3-Smad4 complex and an AP-1 complex have been reported to synergize in transcriptional activation of the c-*jun* promoter by binding to separate sites 120 bp apart (26). Similarly, transcriptional cooperativity between c-Jun and Smad3 at the AP-1 binding site of the collagenase I promoter also required the interaction of c-Jun and Smad3 with DNA (27). Several other recent reports point to cross-talk between Smad proteins and transcription factors such as TFE3, FAST-1, SP1, CREB, and Tinman in the expression of various TGF-β-inducible genes (Ref. 13 and references therein). It has been proposed that the number of SBEs as well as discrete spacer segments between SBEs and/or Smad-partner transcription factor binding site(s) will also be critical for the specificity of TGF-β-induced transcription of these promoters (13). Our study also found that the synergy by vitamin D and TGF-β was reduced when the spacing between VDRE and TRE was extended (Fig. 6).

The promoter studies further indicate that simultaneous binding of VDR and Smad3 to their cognate sequences (Fig. 3*B*) and possibly a direct interaction between these two proteins (Fig. 7), are both required for the convergence of the vitamin D and TGF-β signals. The Smad3-(41–345) construct failed to mediate TGF-β signaling through the 3TPlux reporter (data not shown) suggesting that this Smad3 mutant is functionally inert. The same N-terminal deletion mutant failed to interact with VDR (8). Our study also found that this mutant was unable to transactivate synergistically with VDR through hVDRE-3TPlux reporter, therefore we could not positively conclude that a direct VDR-Smad3 interaction was involved in the cross-talk between these regulatory pathways.

In the present study we demonstrated that the VDRE-containing *hOC* gene promoter contains two functional Smad3 binding elements through which the TGF-β and vitamin D signaling pathways converge. Our data thus strongly suggest that cross-talk between these two signaling pathways requires the presence in the promoter of both respective response elements in close proximity. These data support the concept of specific response elements being required to transduce both single agents and to integrate the synergistic activation of hormone responsive genes.

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Cross-talk between 1,25-Dihydroxyvitamin D_3 and Transforming Growth Factor-β Signaling Requires Binding of VDR and Smad3 Proteins to Their Cognate DNA Recognition Elements

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