Positive and Negative Modulation of Vitamin D Receptor Function by Transforming Growth Factor-β Signaling through Smad Proteins*

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Several lines of experiments demonstrated the interplay between the transforming growth factor (TGF)-β/bone morphogenetic protein signaling pathway, potentiates ligand-induced transactivation function of vitamin D receptor (VDR), vitamin D receptor; VDRE, vitamin D response element; SRC-1, steroidal hormone receptor coactivator 1; BMP, bone morphogenetic protein; 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; HA, hemagglutinin; TpRI(TD), TGF-β type I receptor; RXR, retinoid X receptor.

Cell growth, differentiation, and function are tightly regulated by orchestrated functions of extracellular signals, i.e., growth regulatory factors, such as transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMPs), and lipophilic hormones, such as vitamin D and retinoic acids. Lipophilic hormones transcriptionally control gene expression by binding to cognate nuclear receptors (1), which act as ligand-inducible transcription factors with transcriptional coactivators such as the SRC-1/TIF2 family and CBP/p300 (2–5). The cell membrane receptors for TGF-β/BMP are activated by ligand binding and phosphorylate and activate certain members of the Smad protein family (Smad1–Smad8) as intracellular signal transducers (6, 7). The signals for TGF-β are mediated by Smad2 and Smad3 (8, 9), whereas Smad1, Smad5, and Smad8 are specific signal transducers for the BMP signals (10, 11). In addition to these pathway-restricted Smads, Smad4, a common partner Smad, is required for the functional heterooligomerization with the pathway-restricted Smads (12, 13). These complexes translocate into the nucleus, where they activate transcription as coactivators and/or DNA-binding transcription factors (14–18). In contrast to these positive transducers of TGF-β/Ri/BMP signalings, inhibitory Smad proteins (Smad6 and Smad7) were identified (19–22). These Smad proteins directly interact with BMP type I receptors, consequently inhibiting the regulation of the pathway-restricted Smads. TGF-β/BMP signaling is regulated and Smad7 are up-regulating pathways by antagonizing the feedback regulation (23–24).

The interplay between TGF-β and vitamin D signaling pathways is, at least in part, mediated by two classes of Smad proteins, which modulate VDR signaling interactions. Extensive studies have been directed toward the interplay between the two factors, TGF-β/BMP, they are considered as negative feedback regulators of the TGF-β/BMP signalings, inhibitory Smad proteins (Smad6 and Smad7) are up-regulating pathways by antagonizing the feedback regulation (23–24).

EXPERIMENTAL PROCEDURES

Plasmid Construction—N-terminally FLAG or HA tagged mouse Smad3, mouse Smad6, and human Smad7 were inserted between the EcoRI and XhoI sites of the mammalian expression vector pcDNA3 (Invitrogen) (FLAG-Smad3, -6, and -7 and HA-Smad6 and -7, respectively). The original construction of constitutively active TGF-β type I receptor was as described (TpRI(TD)) (13). Full-length human SRC-1a was inserted between the XhoI and XbaI sites of pcDNA3 (pcDNA3-SRC-1) (28). Full-length rat VDR was inserted between the EcoRI and BamHI sites of the mammalian expression vector pSG5 (pSG5-VDR) (28). DEF domians of rat VDR and mouse retinoid X receptor (RXXR) were inserted between the EcoRI and BamHI sites of the pM vector (CLONTECH) (GAL4-VD-AF2 (25) and GAL4-RXR-I).
AP-2). Full-length mouse Smad3 was inserted between the EcoRI and SalI sites of the pVP vector (CLONTECH) (VP-Smad3). Synthetic oligonucleotides containing eight tandem copies of the 17-mer of GAL4-DNA-binding site followed by the adenovirus E1A TATA sequence were inserted between the HindIII and ClaI sites of pGL3-basic (Promega) (17m8-luc).

Transfection and CAT Assay—COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium without phenol red with 5% dextran-coated charcoal-stripped fetal bovine serum. Cells were transfected at 40–50% confluence using a total of 20 μg of indicated plasmids. All transfections were performed in the presence of 3 μg of CAT reporter, 0.5 μg of VP-Smad3, 10 ng of pSG5-VDR, 100 ng of pRL-tk, and 200 ng of pCMV-β-gal. In all assays, 200 ng of pCMV-β-gal was transfected for the internal control. 6–8 h after transfection, cells were washed with fresh medium, ligand was added to the medium, and cells were incubated for an additional 24–36 h. Cell extract preparations and dual luciferase assays were performed following the manufacturer’s protocols (Promega).

RESULTS AND DISCUSSION

Smad7, but Not Smad6, Abrogates the Smad3-mediated Potentiation of VDR Function—In an attempt to clarify the roles of Smad proteins for VDR, we found that Smad3 acts as a coactivator for the VDR (28). We extended our studies to another Smad class, inhibitory Smad proteins, Smad6 and Smad7, which were shown to inhibit VDR function. Consistent with the report on the suppressive effect of Smad3 on VDR, we found that Smad3 inhibits VDR-AF2 function in a dose-dependent manner (28).

To verify the suppressive effect of Smad7, we further investigated the effects of Smad6 and Smad7 on full-length VDR by a CAT assay using CAT reporters containing various vitamin D response elements (VDREs). First, we used synthetic VDRE (DR 3). As with VDR-AF2, we found that full-length VDR transactivation activated by Smad3 or constitutively active TGF-β type I receptor was remarkably abrogated when Smad7 was co-transfected. This suppression was dependent on the dose of the transfected Smad7 (Fig. 1B). At the maximum dose we employed, Smad7 suppressed the enhancement of VDR function by Smad3 nearly to the control level (i.e. the level in the absence of Smad3). In contrast, Smad6 did not alter Smad3-activated responses. Similar suppressive effects of Smad7 were also observed with mouse osteopontin VDRE and rat osteocalcin VDRE (30), suggesting that this suppressive effect of Smad7 on Smad3-activated VDR transactivation does not depend on the promoter context but is derived from the decreased transactivation function of VDR.

Smad7 Inhibits the Formation of the VDR-Smad3 Complex—Because some coactivators of VDR directly interact with VDR, we thought that a possible molecular mechanism underlying this inhibitory effect could be competition between Smad3 and inhibitory Smad proteins for binding to VDR. Alternatively,
We then determined whether the interaction between Smad3 and VDR is affected in the presence of these inhibitory Smad proteins. First we employed a mammalian two-hybrid assay system. When Smad7 was cotransfected, this interaction was completely abolished. However, Smad6 did not affect this interaction (Fig. 2B). Next, to determine whether Smad3 also binds to the functional VDR unit, VDR-RXR heterodimer, we employed a mammalian three-hybrid assay system. As described previously, Smad3 bound VDR-RXR heterodimer in a ligand-dependent manner. Similar to the result of the mammalian two-hybrid assay, this interaction was strongly inhibited by Smad7 but not by Smad6 (Fig. 2B).

To further confirm the observations that Smad3-VDR complex formation is abolished in the presence of Smad7, we examined the effects of these inhibitory Smad proteins on the interaction of Smad3 with VDR using a communoprecipitation assay. Coimmunoprecipitations were performed using antibody against the N-terminal FLAG tag of Smad3 and followed by Western blotting using anti-VDR antibody. Smad3 weakly bound VDR by itself. However, in the presence of SRC-1, Smad3 efficiently bound to the VDR-SRC-1 complex as described previously (28). The presence of Smad7 caused almost complete abolishment of this VDR-Smad3 complex formation.

Inhibitory Smads have been shown to act in vivo and in vitro to modulate Smad3-VDR complex formation indirectly. We employed the GST pull-down assay to test the former possibility. As described elsewhere (28), we have previously shown that Smad3 bound VDR by itself. However, under the same conditions, we could detect no interaction of Smad3 with VDR weakly but significantly in the presence of SRC-1, which presumably inhibits the nuclear accumulation of Smad3. This effect is opposed by Smad7, which presumably inhibits the nuclear accumulation of Smad3. Because Smad7 was shown to localize also in the nucleus (31), it is possible that Smad7 abrogates the Smad3-mediated potentiation of VDR function in the nucleus through unknown molecular mechanism. Our results demonstrate that relative expression levels of Smad7 to Smad3 determine the extent of vitamin D signaling may be due to the differences in the expression levels of the Smad3 and Smad7 proteins in the tested cells.

Considering the complex modulations of nuclear receptor transactivation functions by TGF-β/BMP signaling, together with the possibility that there are more unknown Smad proteins, our results demonstrate that the vitamin D signaling pathway is positively regulated by the nuclear accumulation of Smad7, which presumably inhibits the nuclear accumulation of Smad3. Because Smad7 was shown to localize also in the nucleus (31), it is possible that Smad7 abrogates the Smad3-mediated potentiation of VDR function in the nucleus through unknown molecular mechanism. Our results demonstrate that relative expression levels of Smad7 to Smad3 determine the extent of the potentiation of VDR function by TGF-β signaling. Because TGF-β/BMP treatments are reported to alter the gene expression of Smad3 (32) and Smad7 (23) in a cell-specific manner, inconsistent previous studies about the effects of TGF-β or vitamin D signaling may be due to differences in the expression levels of the Smad3 and Smad7 proteins in the tested cells.

We demonstrated here that Smad7, as well as Smad3, is strongly involved in the interplay between the TGF-β and vitamin D signaling pathways by modulating VDR transactivation. We propose the model that TGF-β signaling regulates the vitamin D signaling pathway positively by the nuclear accumulation of Smad3, and this effect is opposed by Smad7, which presumably inhibits the nuclear accumulation of Smad3. Because Smad3 was shown to localize also in the nucleus (31), it is possible that Smad7 abrogates the Smad3-mediated potentiation of VDR function in the nucleus through unknown molecular mechanism. Our results demonstrate that relative expression levels of Smad7 to Smad3 determine the extent of the potentiation of VDR function by TGF-β signaling. Because TGF-β/BMP treatments are reported to alter the gene expression of Smad3 (32) and Smad7 (23) in a cell-specific manner, inconsistent previous studies about the effects of TGF-β or vitamin D signaling may be due to the differences in the expression levels of the Smad3 and Smad7 proteins in the tested cells.

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teins, such Smad proteins could be involved in the cross-talk between the signaling pathways mediated by nuclear receptors and by the TGF-β/BMP cell membrane receptors.

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