A Nuclear Antagonistic Mechanism of Inhibitory Smads in Transforming Growth Factor-β Signaling*

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Inhibitory Smads (I-Smads), including Smad6 and Smad7, were initially characterized as cytoplasmic antagonists in the transforming growth factor-β signaling pathway. However, I-Smads are also localized in the nucleus. Previously, we have shown that Smad6 can function as a transcriptional co-repressor. In this study, we found both Smad6 and Smad7 interact with histone deacetylases (HDACs). Acetylation state of core histones plays a critical role in gene transcription regulation. An HDAC inhibitor, trichostatin A, released Smad6-mediated transcription repression. Moreover, class I HDACs (HDAC-1 and -3), not class II HDACs (HDAC-4, -5, and -6), were co-immunoprecipitated with Smad6. Endogenous HDAC-1 was also shown to interact with both Smad6 and Hoxc-8. Mapping of the interaction domain indicates Smad6 MIH2 domain is mainly involved in recruiting HDAC-1. Most interestingly, Smad6 also binds to DNA through its MIH1 domain, and the MIH2 domain of Smad6 masks this binding activity, indicating that Smad6 MIH1 and MIH2 domains associate reciprocally and inhibit each other’s function. Hoxc-8 induces Smad6 binding to DNA as a transcriptional complex. Our findings revealed that I-Smads act as antagonists in the nucleus by recruiting HDACs.

Transforming growth factor β (TGF-β)† superfamily members, which include TGF-βs, activins, and bone morphogenetic proteins (BMPs), play a very important role during embryonic development and maintaining adult tissue homeostasis (1). TGF-β signaling is mediated by two transmembrane serine-threonine kinase receptors, type II and type I receptors (2). Upon ligand binding, the constitutively active type II receptors phosphorylate and activate type I receptors, leading to the propagation of signaling by recruiting and phosphorylating a group of specific proteins, Smads (3). Smads are pivotal intracellular nuclear effectors of TGF-β family members, which transduce the signal from the cell membrane to the nucleus (3).

Smads contain two highly conserved domains: MH1 and MH2 domains (1). Commonly, MH1 domain binds to DNA, whereas MH2 domain is the protein-protein interaction and transactivation domain (1). These two domains interact reciprocally and inhibit each other’s function (1, 4). Based on their function and sequence similarity, Smads are divided into three subgroups. 1) The receptor-regulated Smads (R-Smads) are the targets of the activated type I receptors. Smad1, Smad5, and Smad8 mediate BMP signaling (3), whereas Smad2 and Smad3 mediate TGF-β signaling (5). 2) The common partner Smads (Co-Smads), Smad4 being the only one identified in mammals thus far, are shared by all of the R-Smads (1). 3) Inhibitory Smads (I-Smads), including Smad6 and Smad7, stably bind to activated type I receptors and block phosphorylation of R-Smads (6, 7). Both TGF-β and BMP induce I-Smad expression, indicating their negative feedback function in TGF-β signaling (8, 9).

Smad6 preferably inhibits BMP signaling, whereas Smad7 is more a general inhibitor (10). Smad6 and Smad7 are expressed at the earliest stage during embryo development and highly expressed in the developing cardiovascular system, eyes, bones, and other tissues (11, 12). The expression of Smad6 overlaps BMP-2, -4, and -7 expression, which orchestrates BMP-mediated cardiac development (11). Aortic ossification and elevated blood pressure were reported in viable Smad6 mutants (13). BMP induces the ventral mesoderm formation (14, 15), and the overlapped expression of Smad6 and BMP indicates that Smad6 is a key protein in balancing the function of BMP during embryo development. Smad6 inhibits BMP-induced osteoblast differentiation (16). It is also reported that Smad6 inhibits adipogenesis (17). Smad7, not Smad6, was identified as inhibiting TGF-β function during embryonic vasculogenesis (18) and lung development, injury, and repair (19, 20).

The biological activities of Smads are closely associated to their cellular localization. R-Smads are located in the cytoplasm in the absence of signaling. Upon ligand stimulation, they are phosphorylated at their extreme carboxyl end SSSS motif and recruit the common partner Smad4 into the nucleus, where they act as transcriptional activators (21). I-Smads are located in both the cytosol and the nucleus (22–25). Smad6 cellular distribution is not affected by TGF-β or BMP treatment (24, 25) whereas TGF-β induces nuclear export of Smad7 (22). The inhibitory mechanisms of I-Smads have been characterized in the cytoplasm. I-Smads interact with the activated type I receptors, which then block the phosphorylation of the R-Smads (6, 7). Smad6 was also demonstrated to interact with the phosphorylated Smad1 in the cytoplasm, competing with Smad4 to form an inactive Smad1-Smad6 complex (26). We have shown that, in the nucleus, Smad6 acts as a transcriptional co-repressor on osteopontin promoter by interacting with Hoxc-8 (25), a transcriptional repressor in BMP signaling (27, 28). The mechanism of Smad6 repressive function in the nucleus remains unclear.

* This work was supported in part by National Institutes of Health Grants DK 53757 and DK 57501 (to X. C.) The costs of publication of this article were defrayed in part by the payment of page charges. This paper is available on line at http://www.jbc.org

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‡ The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; OPN, osteopontin; HAT, histone acetyltransferase; HDAC, histone deacetylase; CBP, cAMP-responsive element-binding protein-binding protein; GST, glutathione S-transferase; Hox, homeobox gene; TSA, trichostatin A; aa, amino acids; I-Smad, inhibitory Smad; R-Smad, receptor-regulated Smad; Co-Smad, common partner Smad; HA, hemagglutinin.
Signaling to chromatin through histone modification is demonstrated as a major step for regulating target gene transcription (29). One of the modifications of histones is acetylation, where specific lysine residues are functional targets for histone acetyltransferases (HATs) and histone deacetylases (HDACs) (30–32). The acetylation of chromosomal histones loosens the structure of the target gene promoter and results in increased accessibility of the chromatin for transcription factors (30). Conversely, hypoacetylation of a gene regulatory region is strongly related to silencing gene expression (30). Several transcription represors and co-repressors have been demonstrated to recruit HDACs to specific genes for silencing gene expression (33). For example, TGIF, a Smad transcriptional co-repressor, was identified as interacting with HDAC-1 and inhibiting TGF-β-induced gene transcription (34). In addition to HDAC-1, eight other HDACs have been identified thus far (35, 36).

Given that Smad6 displays similar activity as TGIF and other transcriptional repressors, all of which appear to recruit different HDAC complexes, we considered whether histone deacetylase activity might extend to Smad6 and affect its transcriptional activity in the nucleus. To address this issue, we characterized the mechanism of Smad6-mediated gene transcription. We demonstrated that an HDAC activity inhibitor, trichostatin A (TSA), rescued the repressive function of Smad6 in BMP signaling, indicating that HDACs engaged in the inhibition of Smad6 in the nucleus. Furthermore, Smad6 was shown to interact with HDAC-1 and -3 in an immunoprecipitation assay. HDAC activity was detected in HDAC assay with an immunoprecipitated Smad6 protein complex. These data suggest that Smad6 represses gene transcription by recruiting class I HDACs and modifying chromatin conformation.

**EXPERIMENTAL PROCEDURES**

**Constructs and Cell Culture**—The FLAG-tagged HDAC-1, HDAC-4, HDAC-5, and HDAC-6 were gifts from Dr. S. L. Schreiber (Harvard University, Cambridge, MA). The HA-tagged HDAC-3 was a gift from Dr. M. S. Featherstone (McGill University, Montreal, Quebec, Canada). HDAC-3 was cloned into pBJS vector linked with FLAG at the carboxyl terminus by PCR. To get HA-tagged HDAC-1 and Smad6, we cloned HDAC-1 and pDNA3 vector tagged with the carboxyl end and Smad6 was cloned into pDNA3 vector tagged with HA at the NH2 terminus by PCR method. GST-CBP (1099–1758), which contains the CBP HAT domain, was a gift from Dr. T. Kovarides (University of Cambridge, Cambridge, United Kingdom). Osteopontin promoter-reporter plasmid (Hox-pGL3), Smad1B, Smad6, Hox-o, and Smad-7 have been described previously (25, 27). Mv1Lu cells and COS-1 cells were incubated in Dulbecco’s modified Eagle’s medium supplemented with antibiotics and 10% fetal bovine serum at 37 °C modified Eagle’s medium supplemented with antibiotics and 10% fetal bovine serum at 37 °C. Osteopontin promoter-reporter plasmid (Hox-pGL3), Smad1B, Smad6, and Hox-o, respectively, in the absence or presence of the HDAC inhibitor TSA (100 nM) as indicated in Fig. 1 using Tfx-50 according to the manufacturer’s instructions (Promega). Using the same reporter plasmid, HDAC-1 was co-transfected in Mv1Lu cells with Smad1B, Smad6, and Hox-o, respectively, and the absence or presence of the HDAC inhibitor TSA (100 nM) as indicated in Fig. 1 using Tfx-50 according to the manufacturer’s instructions (Promega). Using the same reporter plasmid, HDAC-1 was co-transfected in Mv1Lu cells with Smad1B, Smad6, and Hox-o, respectively, and the absence or presence of the HDAC inhibitor TSA (100 nM) as indicated in Fig. 1 using Tfx-50 according to the manufacturer’s instructions (Promega). The cells were lysed 48 h after transfection. Luciferase activity was measured and normalized by Renilla luciferase activity expressed from pRL-SV40 reporter plasmid using the Dual-Luciferase® assay kit (Promega) according to the manufacturer’s directions (Promega). Luciferase values shown in the figures are representative of transfection experiments performed in triplicate in at least three independent experiments.

**Immunoprecipitation Assay**—HA-tagged Smad6 was co-transfected with FLAG-tagged HDAC-1, HDAC-4, HDAC-5, and HDAC-6 into COS-1 cells using Tfx-50 according to the manufacturer’s instructions (Promega). The cells were lysed 48 h after transfection. Anti-HA polyclonal antibody (poly-HA, Babco) was added into the same amount of protein samples and rotated at 4 °C for 3 h. The protein complexes were immunoprecipitated by protein G-Sepharose beads (Amersham Biosciences, Inc.), and the samples were loaded and run in a 12% SDS-PAGE. The precipitates were transferred to nitrocellulose membrane and immunoblotted by anti-FLAG M2 monoclonal antibody (Sigma). FLAG-tagged Smad6, Smad6 truncated proteins, and Smad7 were co-transfected in COS-1 cells with HA-tagged HDAC-1 using Tfx-50 according to the manufacturer’s instructions (Promega). Anti-FLAG M2 monoclonal antibody was added into the same amount of protein samples. Immunoprecipitation, and the precipitate was used for immunoblotting the precipitated protein complex. To determine the interaction of Hox-8 and HDAC-1, the HA-tagged Hox-8 and FLAG-tagged HDAC-1 were co-transfected into COS-1 cells. The cell lysates were immunoprecipitated by poly-HA, and the protein complexes were immunoblotted by anti-FLAG M2 monoclonal antibody. For the interaction of HDACs effect on osteopontin promoter, the poly-HA was used for immunoprecipitation, and anti-HDAC-1 polyclonal antibody (Upstate Biotechnology, Inc.) was used for immunoblotting the precipitated protein complex.

**HAT Assay**—GST and GST-CBP (aa 1099–1758) (each for 3 μg) were incubated with crude core histones (25 μg) (Sigma) in a final volume of 30 μl in buffer IPI (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% (by volume) Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride) (38). The reactions were initiated by the addition of 3H-labeled acetyl-CoA (3.7 kBq) (American Radiolabeled Chemicals, Inc.), and then incubated at 30 °C for 45 min. The GST and GST-CBP (aa 1099–1758) precipitates were harvested by pre-prepared poly-HA-coated beads. The supernatants were spotted on Whatman filter and soaked in 0.05 mM NaHCO3-Na2CO3 (pH 9.2) at 37 °C for 30 min. The Whatman filters were washed with acetone, methanol, and chloroform (1:2) and dried at room temperature. 3H-Labeled acetyl-CoA incorporation into histones was determined by liquid scintillation counting (39).

**HDAC Assay**—HDAC-1, HDAC-6, and Hox-8 were co-transfected in COS-1 cells, as indicated in Fig. 3B, using Tfx-50 according to the manufacturer’s description (Promega). Anti-FLAG M2 monoclonal antibody or poly-HA was used to immunoprecipitate the protein complexes. The 3H-acetylated histones (1.5 μg/reaction) were mixed with these protein complexes and incubated at 30 °C for 45 min (38, 39). The reactions were stopped by adding 15 μl of 1N HCl, and the released 3H was measured by addition of 1N NaOH and acetic acid. The samples were centrifuged, and the remaining 3H-acetylated histones were remained in the inorganic layer. Histone acetylation degree was measured using the Whatman filter assay described under “HAT Assay” (39).

**Gel Shift Assay**—Gel shift assays were performed as described previously (40). GST fusion constructs of Smad6, Smad7, and Hox-8 were generated in our previous study (25, 27). Smad6 truncated fragments were generated by PCR and cloned into pGEX vectors. These constructs were transformed into BL21 Escherichia coli. The proteins were extracted as described previously following induction with isopropyl-β-D-thiogalactopyranoside (27). Osteopontin Hox binding element (OPN-5) (27) was used as the probe to test the interaction between Hox-8 and Smad6. This probe was also used for testing whether Smad6 binds to DNA. We randomly mutated some base pairs at the flanking regions of the Hox binding site for identifying the Smad6 binding site. The upstream strain of the wild type probe is 5′-GGGATA GGTAATTAGATCATGTCTACTCAG-3′, mutant probe 1 is 5′-GGCAAGTG TAATTGACCTGTTCATCAG-3′, and mutant probe 2 is 5′-GGGATCTTG GTGATCCACTGATC-3′.

**RESULTS**

**An HDAC Inhibitor Releases Both Smad6- and Hox-8-mediated Transcriptional Repression**—We have shown that Hox-8 functions as a transcriptional repressor in the BMP signaling pathway (27, 28). Smad6 interacts with Hox-8 as a transcriptional co-repressor in response to BMP stimulation (25). These findings revealed Smad6 antagonistic function in the nucleus. Therefore, it is important to understand the mechanism of Smad6-repressed gene transcription. Histone acetylation is one of the major chromatin modifications in gene transcription regulation, and hypoacetylation of histone H4 and H3 may play a role in repression of gene expression, which is mediated by HDACs (30–32). TSA, an HDAC inhibitor, was used to examine if HDACs affect Smad6 repressive activity in the nucleus. Smad1B, the interaction domain of Smad1 with Hox-8, dislodges Hox-8 binding from its binding sites and activates BMP downstream gene transcription (25, 28, 37). BMP-inducible
reporter construct, Hox-pGL3 (27), was co-transfected with Smad1B and/or Smad6, Hoxc-8 in mink lung epithelial cells (Mv1Lu) treated with or without TSA (100 nM). As shown in Fig. 1, Smad1B stimulated the transcriptional activity of osteopontin promoter, whereas Smad6 and Hoxc-8 inhibited it. Importantly, TSA released the transcription repression by Smad6 and Hoxc-8, suggesting that HDACs are involved in transcription repression of the osteopontin promoter mediated by Smad6 and Hoxc-8.

I-Smads and Hoxc-8 Interact with HDAC-1—There are two classes of HDACs including nine members: HDACs 1–3 and 8 in class I, and HDACs 4–7 and 9 in class II (35, 36). To characterize the molecular mechanisms of HDAC-mediated I-Smads and Hoxc-8 transcription repression, we examined whether HDACs interact with I-Smad and Hoxc-8 in an immunoprecipitation assay. HA-tagged Smad6 expression plasmid was co-transfected with FLAG-tagged HDAC-1, -3, -4, -5, and -8 plasmids in COS-1 cells. The cell extracts were immunoprecipitated by an anti-HA polyclonal antibody. The immunoprecipitates were then examined by Western blot analysis with an anti-FLAG monoclonal antibody. As shown in Fig. 2A, HDAC-1 and -3, not HDAC-4, -5, and -8, were co-immunoprecipitated with Smad6, indicating that Smad6 recruits class I HDACs to inhibit transcriptional activity.

Smad7 is the other inhibitory Smad located in the nucleus in the absence of TGF-β stimulation (22). Unlike Smad6, it does not interact with Hoxc-8 or other Hox proteins (25). Nevertheless, the MH1 and MH2 domains are highly conserved between Smad6 and Smad7 (26). Smad7 may also interact with HDACs. Smad6 interacts with class I HDACs; therefore, we examined the interaction between Smad7 and HDAC-1. FLAG-tagged Smad7 was co-transfected with HA-tagged HDAC-1 in COS-1 cells. As expected, Smad7 was co-immunoprecipitated with HDAC-1 (Fig. 2B), indicating interaction of Smad7 with HDAC-1. This finding suggests that I-Smads are transcriptional repressors or co-repressors in the nucleus.

In a similar experiment, FLAG-tagged HDAC-1 plasmids were co-transfected with HA-tagged Hoxc-8 into COS-1 cells. Hoxc-8 was co-immunoprecipitated with HDAC-1 (Fig. 2C). These results further support our model of BMP-induced gene transcription, in which Hoxc-8 is a transcription repressor. Phosphorylated Smad1 removes Hoxc-8 and, perhaps, other Hox proteins such as Hoxa-9 and activates BMP downstream transcription (27, 28, 37). Smad6 interacts with Hoxc-8 as a transcriptional co-repressor as part of the BMP negative feedback loop.

We also mapped the interaction domain of Smad6 with HDAC-1. A series of truncated Smad6 expression plasmids linked to FLAG (25) were co-transfected with HA-tagged HDAC-1 in COS-1 cells. Immunoprecipitation assay results in Fig. 2D showed that the Smad6 MH2 domain was mainly involved in interaction with HDAC-1, whereas the NH2-terminal domain and linker region had a very weak interaction. The linker region alone has no interaction with HDAC-1.

HDAC Activity in the Smad6 and Hoxc-8 Complex—Transcription repressors and co-repressors have been demonstrated to recruit HDACs to specific target gene promoters to deacetylate chromosomal histones in silencing gene transcription. Therefore, we examined the HDAC enzymatic activity in the Smad6 transcriptional complex because I-Smads and Hoxc-8 have been shown to interact with HDACs. FLAG-tagged Smad6 or HA-tagged Hoxc-8 plasmids were co-transfected with HDAC-1 expression plasmids in COS-1 cells as indicated in Fig. 3B. Cell lysates were immunoprecipitated with anti-FLAG antibody or anti-HA antibody for measuring HDAC activity (Fig. 3B). The acetylated histones, the substrate for HDAC assays, were prepared with GST-CBP (aa 1099–1758), which contains a HAT domain, as described previously (38) (Fig. 3A). The resulted acetylated histones were incubated with the immunoprecipitated complexes at 30 °C for 45 min. The degree of histone acetylation was measured by liquid scintillation counting (Fig. 3A). Both Smad6-HDAC-1 and Hoxc-8-HDAC-1 complexes deacetylated about one third of3H-labeled histones in comparison with controls, indicating HDAC activity in the Smad6 or Hoxc-8 complex. Furthermore, we examined endogenous HDAC activity in the Smad6 and Hoxc-8 immunoprecipitated complex. In this case, FLAG-Smad6 or HA-Hoxc-8 plasmids were transfected alone into COS-1 cells, and the cell lysates were immunoprecipitated with anti-FLAG antibody or anti-HA antibody. As expected, the Smad6 and Hoxc-8 immunoprecipitated complex exhibited significant endogenous HDAC activities (Fig. 3B). These results clearly suggest that Smad6 and Hoxc-8 inhibit gene transcription by recruiting HDACs.

To examine whether endogenous HDAC-1 is involved in Smad6- and Hoxc-8-induced transcription repression, we attempted to co-immunoprecipitate endogenous HDAC-1 with HA-tagged Smad6 and Hoxc-8. Fig. 3C demonstrated that both Smad6 and Hoxc-8 interact with endogenous HDAC-1. Both Smad6 and Hoxc-8 inhibit BMP-induced osteopontin promoter activity (25). We then examined the function of HDAC-1 in this process. HDAC-1 expression plasmid was co-transfected in Mv1Lu cells with Smad6, Hoxc-8, and other necessary expression vectors (Fig. 3D). As expected, Smad1B stimulated osteopontin promoter transcriptional activity. Expression of HDAC-1 alone partially inhibited Smad1B-induced transcription. However, co-transfection of HDAC-1 with Smad6 and Hoxc-8 completely abolished the Smad1B-induced transcriptional activity. These results indicate that HDAC-1 mediates the repressive activity of Smad6 and Hoxc-8 in BMP signaling pathway.

Smad6 MH1 Domain Binds to DNA—We have shown that Hoxc-8 binds to osteopontin promoter, Smad6 and Hoxc-8 form a complex on the DNA element, although Smad6 alone does not bind to this DNA element (25). Both Smad6 and Hoxc-8 interact with HDAC-1, which implies that Smad6 may also be a DNA-binding protein. Smad proteins contain two highly con-
were then Western blotted by anti-FLAG M2 monoclonal antibody. Blanes 2 FLAG-tagged Smad6 (lane 2) were co-transfected in COS-1 cells with HA-tagged HDAC-1 (lane 3), HDAC-3 (lane 4), HDAC-4 (lane 5), HDAC-5 (lane 6), and HDAC-6 (lane 7). Cell lysates were immunoprecipitated by poly-HA 48 h after transfection. The precipitates were then Western blotted by anti-FLAG M2 monoclonal antibody. Smad7 interacts with HDAC-1. Empty pcDNA3 vector (lanes 1 and 4), HDAC-5 (lane 6), and HDAC-6 (lane 7) formed shifted bands with Smad6. However, the full length Smad6 does not bind to DNA, the homeodomain of Hoxc-8 was maintained for all the truncated Hoxc-8 proteins. The results were shown in Fig. 4D. All the proteins containing the hexapeptide motif, such as Hoxc-8NH, Hoxc-8hh, and full-length Hoxc-8, form shifted bands with Smad6. However, Hoxc-8HC, which does not contain the hexapeptide motif, failed to form a protein complex with Smad6. These results suggest that the hexapeptide motif of Hoxc-8 is important in interacting with Smad6.

Thus far, we know the interaction domains of both Smad6 and Hoxc-8. Incubation of these two interacting domains together with the same probe (OPN-5) should yield a supershift band. Unexpectedly, Smad6 MH2 domain did not form a complex with Hoxc-8HH. However, the full length of Smad6 formed a shifted band with Hoxc-8HH (Fig. 4E). These results strongly
suggest that the MH1 domain of Smad6 is required for Hoxc-8-Smad6 complex formation. The DNA binding activity of Smad6 MH1 domain may stabilize the complex. Taken together, our results suggest that Smad6 MH1 and MH2 domains interact reciprocally and inhibit each other’s function. Upon interaction of Smad6 with Hoxc-8, the MH2 domain interacts with Hoxc-8, allowing the MH1 domain to bind to DNA.

**DISCUSSION**

I-Smads are antagonistic mediators in TGF-β signaling (1). Both Smad6 and Smad7 expressions are induced by TGF-β or BMPs as negative feedback responses (8, 9). I-Smad interacts with the activated type I receptors, blocking the phosphorylation of R-Smads (6, 7). Particularly, Smad6 competes with Smad4 to form an inactive complex with phosphorylated Smad1 (26). However, antagonistic function of I-Smads is not well characterized in the nucleus. I-Smads are localized in both the cytosol and the nucleus, and TGF-β or BMP stimulation does not change the cellular distribution of Smad6. Initially, we reported that Smad6 functions as a transcriptional co-repressor through interacting with Hoxc-8 (25). In this report, we demonstrated that both Smad6 and Smad7 were co-immunoprecipitated with HDAC-1, which is normally recruited by transcriptional repressors or co-repressors in inhibition of gene transcription. Importantly, we also find that Smad6 is a DNA-binding protein as a partner of Smad6/Hoxc-8 complex. Like other Smads, Smad6 binds to DNA through its MH1 domain, whereas Smad6 MH2 domain recruits HDAC-1. These findings revealed an important functional aspect of I-Smads in the nucleus.

Chromatin modification plays a critical role in regulating gene transcription. Acetylation is one of the major modification processes. HATs acetylate lysine residues on histone N-tails, leading to loosening of the chromatin structure and increasing the accessibility of transcriptional factors to the chromosome, whereas HDACs mediate silencing of gene transcription. R-Smads translocate to the nucleus upon BMP or TGF-β stimu-
lation, where they function as transcriptional activators through interacting and recruiting p300/CBP to specific gene promoters (34, 41). TGIF, a Smad co-repressor in the TGF-β signaling pathway, interacts with HDAC-1 and inhibits target gene expression (34). HDACs inhibit gene transcription by tightening the chromosome structure. TSA, an HDAC inhibitor, rescued Smad6- and Hoxc-8-mediated transcription repression. Interaction of Smad6 and Smad7 with HDAC-1 and recruitment of endogenous HDAC activity clearly indicate the nuclear function of I-Smads as transcription repressors or corepressors. Smad7 does not interact with Hox proteins (25). Therefore, Smad7 was not shown as a transcriptional repressor or co-repressor. However, the interaction of Smad7 with HDAC-1 suggests its potential function as a transcriptional repressor or co-repressor in different transcription mechanisms because other cytokines such as TNF-α induce Smad7 expression (49). Thus, Smad7 could also mediate cross-talk between TGF-β and other signaling pathways (49, 50). The detailed mechanism of Smad7 in the nucleus remains to be characterized.
The finding of Smad6 binding to DNA suggests that all of the Smads are DNA binding proteins. I-Smads contain both MH1 and MH2 domains, which are necessary for full activities of Xenopus Smad6 and Smad7 (51). The MH2 domain is highly conserved with other Smads, but the MH1 domain is distinct (44). MH1 domains of R-Smads and Smad4 bind DNA Smad box (GTCT) (41–43). Like other Smads, Smad6 binds to DNA also through its MH1 domain. The full length of Smad6 does not bind to DNA, whereas, in the presence of Hoxc-8, Smad6 binds to DNA and forms a heterodimer. Importantly, the MH1 domain of Smad6 is required for the Hoxc-8-Smad6 complex formation although it is the MH2 domain of Smad6 that interacts with Hoxc-8. It appears that the MH2 domain of Smad6 masks its MH1 domain DNA binding activity, and that the MH1 and MH2 domains of Smad6 interact reciprocally and inhibit each other’s function. Indeed, Smad6 MH1 DNA binding activity decreased when the length of Smad6 truncated MH1 domain extended to MH2 domain. This observation suggests that Hoxc-8 induces Smad6 conformation change, in which Smad6 MH1 domain becomes available to bind to DNA. Phosphorylation of Smad6 could be another way to change its conformation for DNA binding activity, which could also serve as a cross-talk with other signaling pathways.

Taken together, we demonstrated a specific interaction of I-Smads with HDACs. As a model, we describe a transcription repression mechanism of Smad6. In response to BMP or TGF-β stimulation, Smad6 interacts with Hoxc-8 and binds to DNA as a heterodimer, which inhibits Smad6-induced gene transcription by recruiting HDACs. Importantly, Smad6 is also a DNA-binding protein. Like other Smads, Smad6 binds to DNA through its MH1 domain, and Smad6 MH2 domain interacts with HDAC-1 and Hoxc-8. Consistent with the observation of I-Smads cellular distribution, our data indicate that I-Smads can function as transcription repressors or co-repressors in the nucleus as antagonistic feedback loop of the TGF-β signaling pathway.

Acknowledgments—We are grateful to Dr. S. L. Schreiber for kindly providing the HDAC-1, HDAC-4, HDAC-5, and HDAC-6 expression vectors; Dr. M. S. Featherstone for HDAC-3 expression plasmid; Dr. T. Kozuizides for GST-CBP (aa 1099–1758) cDNA clone; Dr. Ali H. Brivanlou for Smad6 cDNA; and Dr. Peter ten Dijke for Smad7 cDNA. We thank Manjunath T. K. and Dr. J. Kouzarides for GST-CBP (aa 1099–1758) cDNA clone; Dr. Ali H. Brivanlou for Smad6 cDNA; and Dr. Peter ten Dijke for Smad7 cDNA. We are grateful to Dr. S. L. Schreiber for kindly providing the HDAC-1, HDAC-4, HDAC-5, and HDAC-6 expression vectors; Dr. M. S. Featherstone for HDAC-3 expression plasmid; Dr. T. Kozuizides for GST-CBP (aa 1099–1758) cDNA clone; Dr. Ali H. Brivanlou for Smad6 cDNA; and Dr. Peter ten Dijke for Smad7 cDNA. We thank Manjunath T. K. and Dr. J. Kouzarides for GST-CBP (aa 1099–1758) cDNA clone; Dr. Ali H. Brivanlou for Smad6 cDNA; and Dr. Peter ten Dijke for Smad7 cDNA.
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J. Biol. Chem. 2002, 277:4176-4182.
doi: 10.1074/jbc.M105105200 originally published online November 15, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105105200

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