Smad1 Domains Interacting with Hoxc-8 Induce Osteoblast Differentiation*

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Bone morphogenetic proteins are potent osteotropic agents that induce osteoblast differentiation and bone formation. The signal transduction of bone morphogenetic proteins has recently been discovered to involve Smad proteins. Smad1 is an essential intracellular component that is specifically phosphorylated by bone morphogenetic protein receptors and translocated into the nucleus upon ligand stimulation. Previously, we have reported that Smad1 activates osteopontin gene expression in response to bone morphogenetic protein stimulation through an interaction with a homeodomain transcription factor, Hoxc-8. In the present study, the interaction domains between the two proteins were characterized by deleterional analysis in both yeast two-hybrid and gel shift assays. Two regions within the amino-terminal 87 amino acid residues of Smad1 were mapped to interact with Hoxc-8, one of which binds to the homeodomain. Overexpression of recombinant cDNAs encoding the Hoxc-8 interaction domains of Smad1 effectively activated osteopontin gene transcription in transient transfection assays. Furthermore, stable expression of these Smad1 fragments in 2T3 osteoblast precursor cells stimulated osteoblast differentiation-related gene expression and led to mineralized bone matrix formation. Our data suggest that the interaction of amino-terminal Smad1 with Hoxc-8 mimics bone morphogenetic protein signaling and is sufficient to induce osteoblast differentiation and bone cell formation.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF-β) superfamily, which plays a vital role in regulating cell proliferation, differentiation, and apoptosis, and which supports the formation, patterning, and repair of particular morphological features (1, 2). BMPs induce de novo bone formation in post-fetal life through the process of intramembranous and endochondral ossification. BMP-2, -4, and -7 are the most potent osteotropic factors that promote new cartilage and bone formation both in vitro and in vivo (3–5).

Smad1 is the downstream effector of BMP signaling and is phosphorylated by BMP type I receptors (6–8). The phosphorylation of Smad1 induces its accumulation in the nucleus where it regulates gene transcription by associating with a nuclear transcription factor (9, 10) or by binding directly to DNA (11). Smad1 consists of three distinct domains: two highly conserved amino- (NH2-) and carboxyl- (COOH-) terminal domains, referred to as MH1 (mad homology 1) and MH2, respectively, and a more divergent intervening linker region. MH1 has DNA binding activity when MH2 is removed. MH2 contains a conserved receptor phosphorylation motif, SSX5, and has transactivation activity. Cross-talk between Smad and mitogen-activated protein kinase signaling pathways is conferred by the linker region in which the serine residues can be phosphorylated by mitogen-activated protein kinases, leading to an inhibition of Smad1 translocation into the nucleus (12). In the inactive state, MH1 and MH2 bind to one another, mutually inhibiting the function of each domain. In the active state, the phosphorylation of Smad1 opens up this structure to allow association with Smad4 or with other DNA-binding proteins via the MH2 domain (13–15).

Studies on the mechanism by which Smads mediate TGF-β/activin-regulated gene transcription have led to the discovery of several Smad-interacting nuclear transcription factors and their cis-acting DNA elements. In particular, the Xenopus FAST-1 (forkhead activin signal transducer-1) binds to an activin response element upstream of the homeobox gene Mix.2. The transcription activation requires the presence of activin and assembly of a FAST-1-Smad2-Smad4 complex (16). The mammalian homolog FAST-2 activates the hox gene goosecoid where formation of a higher order complex of FAST-2-Smad2-Smad4 is also essential for transactivation (17). Transcription factor μF3 binds to the E-box of plasminogen activator inhibitor-1 promoter, whereas Smad3 and Smad4 bind to a sequence adjacent to the transcription factor μF3 binding site to cooperatively activate plasminogen activator inhibitor-1 gene transcription (18).

In contrast to the TGF-β/activin pathway, little progress has been made in the identification of factors involved in Smad1-mediated transcriptional regulation in response to BMP signaling. We have reported that Smad1 interacts with homeodomain transcription factor Hoxc-8 (9). Hoxc-8 belongs to a highly conserved hox gene family and is expressed in limbs, backbone rudiments, neural tube of mouse midgestation embryos, and in the cartilage and skeleton of newborns (19–21). Its expression is also found in the mouse hematopoietic organs, fetal liver, and adult bone marrow (22). Hoxc-8 knockout mice displayed skeletal abnormalities in ribs, sternum and lumbar vertebra, and neuronal tissues (23, 24). Similar alterations of axial skeletal structures also occurred in Hoxc-8 transgenic mice (25, 26). Overexpression of the Hoxc-8 transgene in mice demonstrated that Hoxc-8 could regulate chondrocyte differentiation at the...
level of the proliferating chondrocyte or its immediate precursor (20).

The present study is aimed at investigating the mechanism by which the Smad1-Hoxc-8 interaction mediates induction of osteoblast differentiation. We show that two domains, one within MH1 and the other at the MH1-linker boundary of Smad1, interact with the Hoxc-8 and are functionally sufficient to activate bone marker gene transcription. More importantly, permanent expression of these Hoxc-8 interaction domains in 2T3 osteoblast precursor cells is able to mimic BMP signaling and induce osteoblast terminal differentiation.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Interactions—**cDNAs encoding full-length forms of Smad1 and Hoxc-8 were fused in-frame to the GAL4 DNA binding domain of pGBT9 vector and to the GAL4 activation domain of pACT2 vector, respectively, to obtain the bait and prey plasmids as described previously (9). All of the Smad1 or Hoxc-8 deletion constructs as indicated in figures were generated by Pfu (Stratagene) polymerase chain reaction-based strategy and inserted into their respective bait (pGBT9) or prey (pACT2) vectors. Resultant constructs were sequenced and subjected to yeast two-hybrid assays. To facilitate the interaction test, we first transformed a yeast reporter strain Y190 with the bait or prey plasmids and selected on SD-Trp plate for pGall9/Smad1 and SD-Leu plate for the pACT2/Hoxc-8. Various deletions of Hoxc-8 were transformed individually into Y190 bearing pGBT9/Smad1, whereas the different truncated constructs of Smad1 were transformed into Y190 containing pACT2/Hoxc-8.

In **Vitro Binding Assays—**the methods of constructing the bacterial expression plasmids for GST-Smad1 and GST-Hoxc-8 have been described previously (9). GST fusion constructs of all of the Smad1 and Hoxc-8 deletion mutants were made by the polymerase chain reaction-based strategy with the pGEX-KG vector. Bacterially expressed GST fusion proteins were purified with glutathione substrate-affinity agarose beads (Sigma) as described (27) and analyzed for their purity by 10% SDS-polyacrylamide gel electrophoresis. In **in vitro** binding was studied using a gel shift assay (28) with constant amounts (1.5 μg for all forms of GST-Smad, 0.2 μg for GST-Hoxc-8, and 10 ng for deletions of GST-HDC and GST-HD) of purified GST fusion proteins. A double-stranded oligomer corresponding to the osteopontin promoter, −206 to −180 relative to the transcription start site (OPN-5) labeled using T4 polynucleotide kinase and [γ-32P]ATP, was used as probe.

**Construction of pGBT9/Smad1 C1(1) cDNA—**cDNA encoding a full-length form of Smad1 was kindly provided by D. Chen (University of Texas). The single Hoxc-8 binding site and its flanking region derived from osteopontin promoter −290 to −166 were inserted in the pGL3 control vector (Promega) that uses luciferase as reporter (Hoxc-pGL3) (9). The plasmids encoding various forms of Smad1 fused with a nuclear localization signal were constructed by inserting the polymerase chain reaction-amplified fragments into the cytomegalovirus (CMV) promoter-based mammalian expression vector pCMV5. Each construct contained one of the following regions: Smad1-NL (amino acids aa 3–278), Smad1-L (aa 145–278), and Smad1-M (aa 101–191). The expression plasmid for Hoxc-8 was made by subcloning the cDNA from pACT2/Hoxc-8 into pcDNA3 (Invitrogen). C3H10T1/2 cells (5 × 10⁴ cells/well in a 12-well culture dish) were transfected with 0.5 μg of Hoxc-pGL3 plasmid together with 200 ng of the indicated constructs using Tfx-60 as instructed (Promega). Luciferase activity was determined 16 h after the start of transfection, and values were normalized with protein content. The luciferase activity shown in the figures is representative of transfections performed in triplicate in at least three independent experiments.

**Establishment of Permanent Cell Lines—**the cDNA encoding Smad1-Hoxc-8 fusion protein interacted with Smad1 expressing 2T3 osteoblast precursor cells (29). Smad1-NL, -L, or -M linked to a nuclear localization signal was subcloned from pCMV5 into pTet-Splice vector (Life Technologies, Inc.). 2T3 cells (10⁵ cells/60-mm dish) were transfected with a mixture of 2 μg of either empty pTet-Splice vector (control), recombinant pTet-Splice/Smad1-NL, -L, or -M along with 2 μg of pTet-tTAk, and 12 μl of Triticase-0 (Promega) containing an α-minimal essential medium containing 0.5 μg/ml tetracycline, and 400 μg/ml G418 was added to exclude nontransfected cells. 2–3 weeks later, drug-resistant colonies developed from single cells were isolated and maintained in an α-minimal essential medium containing 10% fetal bovine serum, 0.2 mg/ml G418, and 0.5 μg/ml tetracycline. To induce the expression of Smad1 fragments, tetracycline was omitted from the growth medium. The expression was analyzed with Slot-Blot (Bio-Rad) by Northern hybridization using 5 μg of total RNA and 5 × 10⁶ cpm/ml [α-32P]-CTP-labeled probe. Total RNA was prepared with STAT-66 (Tel-Test) from G418-resistant clones grown in a medium with or without tetracycline. 10–20 clones of each indicated construct were analyzed. The tetracycline-regulated Smad1 fragment-expressing clones were kept for further characterization of the osteoblastic phenotype as described below.

**Marker Gene Expression—**2T3 and its derivative cell lines containing empty pTet-Splice (vector control) and those displaying regulated expression of indicated Smad1 fragments were grown in the absence of tetracycline to reach confluence. Then the medium was replaced with a medium consisting of 5% fetal bovine serum, 0.1 mg/ml ascorbic acid, and 5 mM β-glycerolphosphate with or without 50 ng/ml recombinant human (rh) BMP-2 in a α-minimal essential medium. mRNA was isolated at day 4 with MicroPoly(A)/Pure (Ambion), and 3 μg of poly(A)− RNA was used for Northern blot using Rapid-Hyb buffer (Amersham Pharmacia Biotech) according to the manufacturer’s direction. Probes for osteopontin and osteocalcin were polymerase chain reaction amplified using cDNA prepared from rhBMP-2-treated C3H10T1/2 cells as template. Type I procollagen a1(1) cDNA was kindly provided by D. Chen (University of Texas).

**Alkaline Phosphatase and Mineralized Bone Matrix Formation Assays—**Bone cell differentiation was monitored by alkaline phosphatase (ALP) assay (30) and von Kossa stain of mineralized bone matrix (31). In brief, cells were prepared in a fashion similar to that described in the Northern analysis except that the cell density was 5 × 10⁴ cells/well in 12-well culture plates for ALP assay and 2 × 10⁴ cells/well in 24-well plates for mineralization staining. Cells were lysed with 0.05% Triton 2 days after reaching confluence. ALP activity was assayed using p-nitrophenol phosphate (Sigma) as the substrate. The A405 was normalized to protein content, and the data shown are representative of positive clones. For von Kossa staining, cells were washed with phosphate-buffered saline, fixed with formalin, and stained with 2% silver nitrate and with 1% acid fuchsin as described (31) with the exception of the dehydration and rehydration steps, which were omitted.

**RESULTS**

**MH1 and Liniker of Smad1 Contribute to the Interaction with Hoxc-8—**the interaction of Smad1 and Hoxc-8 in yeast by two-hybrid assay, in mammalian cells by coimmunoprecipitation, and in vitro by pull-down analysis has been demonstrated previously (9). To resolve region(s) mediating the protein-protein interaction, we first constructed five Smad1 deletions by removing various amino acids from either the NH2 terminus, COOH terminus, or both. All of these deletion forms of Smad1, pGBT9/Smad1 (positive control), and empty bait plasmid pGBT9 (negative control) were transformed individually into yeast reporter strain Y190 carrying a prey plasmid pACT2/Hoxc-8 to test their associations with full-length Hoxc-8. Transfomants were plated on a medium deficient in His, Trp, and Leu, Fig. 1A shows that all forms of Smad1 containing MH1 (5–169) and/or linker domains (146–278) were able to grow. In contrast, yeast containing empty pGBT9 or pGBT9/MH2 (279–465) failed to grow. These results suggest that the Hoxc-8 interaction domains of Smad1 may locate within the MH1 and linker domains.

To localize further the domains involved in the protein-protein interaction, we took advantage of our previous observation from gel shift assays in which GST-Smad1 fusion protein inhibited Hoxc-8 binding to OPN-5, a 27-base pair element derived from the osteopontin promoter from 206 to 180 (9). A set of Smad1 fragments fused with GST was expressed in bacteria and purified to homogeneity as shown in Fig. 1A. An equal amount (1.5 μg) of each purified GST-Smad1 or its deletion mutants was incubated with 0.2 μg of GST-Hoxc-8 and OPN-5 probe for gel shift assays. Fig. 1B shows that the binding of Hoxc-8 (lane 4) was reduced by the addition of wild type Smad1 (lane 5) or mutant Smad1 containing either MH1 (3–
Smad1-Hoxc-8 Interaction Domains in Bone Cell Formation

Homeodomain Is Responsible for Hoxc-8 Association with Smad1—Hox proteins have in common a similar homeodomain (HD) consisting of a highly conserved DNA binding motif of 60 amino acid residues (32). In addition to the HD that lies from aa 149 to 209, Hoxc-8 contains two other conserved regions (CR), CR1 (aa 1–8) and a hexapeptide (aa 137–142) located upstream from the homeodomain (21) (Fig. 2). The hexapeptide of Leu-Met-Phe-Pro-Trp-Met is presumably involved in the interaction with Hox-assisting cofactors such as the Pbx family (33). The regions outside of the HD may determine the functional specificity of Hox proteins (32, 34).

To determine region(s) that are involved in the association with Smad1, we constructed five deletion mutants of Hoxc-8 in addition to the originally identified truncated form of Hoxc-8 clone 19 (Fig. 2), pACT2 prey plasmid containing nonrelated cDNA (randomly chosen from cDNA library (9) as a control), the full-length, and the deletion mutants of Hoxc-8 were transformed separately into Y190 harboring pGBT9/Smad1 bait plasmid, and β-galactosidase activity was assayed. Fig. 2 shows that the full-length Hoxc-8 (1–242) and all four HD-containing deletions (67–237, 137–242, 151–242, and 151–212) interacted with Smad1 as indicated by higher β-galactosidase activity compared with the negative control. The association was stronger with full-length Hoxc-8 and clone 19 compared with HD alone (151–212), indicating that the NH2-terminal region of Hoxc-8 contributes to the interaction. However, two constructs containing only the NH2-terminal region (1–137 and 1–151) failed to bind Smad1, showing negligible β-galactosidase activity (Fig. 2). This suggests that the NH2 terminus does not participate directly in the protein-protein interaction between Hoxc-8 and Smad1. The NH2-terminal region may help to stabilize the proper configuration of the homeodomain increasing its interaction with Smad1. Elimination of conserved region-1 (compare 1–242 with clone 19) and the hexapeptide (compare 137–242 with 151–242) appeared to produce no significant reduction in the association (Fig. 2). In fact, HD alone is sufficient to support the interaction (Fig. 2, 151–212), suggesting that HD is involved directly in Hoxc-8-Smad1 interaction.

To delineate whether the COOH-terminal extension of HD contributes to the protein-protein interaction by gel shift assay, cDNAs encoding HD (151–212) or HDC (151–242) were cloned into pGEX-KG vector to make truncated forms of GST-Hoxc-8 fusion proteins. Purified GST-HDC and GST-HD (10 ng) bound to the OPN-5 probe. Reaction with full-length GST-Hoxc-8 (0.2 μg) was run alongside for comparison (Fig. 3A). The affinity of GST-HDC and GST-HD to the DNA probe is at least 20 times higher, suggesting that the NH2 terminus not only assists the Hoxc-8 to interact with Smad1 (Fig. 2) but also modulates the protein binding to its cognate element. Both deletions of Hoxc-8 were also tested for their interaction with either wild type or mutant Smad1. As shown in Fig. 3, B and C, the binding pattern of HDC and HD to the OPN-5 is nearly identical and is also comparable to that of full-length Hoxc-8 (Fig. 1B). Similarly, Smad1 and all other MH1 or linker-containing mutants inhibited the binding of both HDC and HD to the probe. Interestingly, the MH1 domain showed the strongest inhibition of HDC and HD domains, which is different from the pattern seen earlier with full-length Hoxc-8 binding (compare Fig. 3, B and C, 3–169 and 3–144, with Fig. 1B, 3–278). Note that the linker region alone (148–278) and its smaller deletion (148–191) had no effect on the HDC and HD binding (Fig. 3, B and C). The binding of both HDC and HD to DNA was reduced by the smaller domain (aa 101–144), but it required a higher amount (6 μg; Fig. 3, B and C). As a control, the same amount of GST (6 μg) had no effect on the binding (lane 14 in Fig. 3, B and C).

**Fig. 1.** NH2-terminal domains of Smad1 interact with Hoxc-8. A, left panel, SDS-polyacrylamide gel electrophoresis profile of purified GST-Smad1 fragments used in the gel shift assays. Bacterially expressed GST recombinant Smad1 proteins were purified on glutathione-agarose. Glutathione elutions were subjected to 10% SDS-polyacrylamide gel electrophoresis, and proteins were visualized by Coomassie Blue staining. Each deletion is marked with the end point amino acid residues. Molecular mass markers in kDa are shown on the left panel of the gel. Middle panel, schematic presentation of Smad1 deletion constructs. Right panel, the growth property of yeast clones. Yeast strain Y190 containing the plasmid pACT2/Hoxc-8 was transformed with pGBT9 (control, not shown) or pGBT9 harboring various Smad1 deletions as indicated. Transformants were plated on SD plates with 30 μg 3-amino-1,2,4-Triazole and without His, Trp, and Leu. ND, not determined. B, two regions of Smad1 confer an inhibitory effect on Hoxc-8 binding to OPN-5. Gel shift assay was performed using purified GST fusion proteins and 32P-labeled probe derived from osteopontin (OPN) cDNAs encoding HD (151–212) or HDC (151–242) were cloned into pGEX-KG vector to make truncated forms of GST-Hoxc-8 fusion proteins. Purified GST-HDC and GST-HD (10 ng) bound to the OPN-5 probe. Reaction with full-length GST-Hoxc-8 (0.2 μg) was run alongside for comparison (Fig. 3A). The affinity of GST-HDC and GST-HD to the DNA probe is at least 20 times higher, suggesting that the NH2 terminus not only assists the Hoxc-8 to interact with Smad1 (Fig. 2) but also modulates the protein binding to its cognate element. Both deletions of Hoxc-8 were also tested for their interaction with either wild type or mutant Smad1. As shown in Fig. 3, B and C, the binding pattern of HDC and HD to the OPN-5 is nearly identical and is also comparable to that of full-length Hoxc-8 (Fig. 1B). Similarly, Smad1 and all other MH1 or linker-containing mutants inhibited the binding of both HDC and HD to the probe. Interestingly, the MH1 domain showed the strongest inhibition of HDC and HD domains, which is different from the pattern seen earlier with full-length Hoxc-8 binding (compare Fig. 3, B and C, 3–169 and 3–144, with Fig. 1B, 3–278). Note that the linker region alone (148–278) and its smaller deletion (148–191) had no effect on the HDC and HD binding (Fig. 3, B and C). The binding of both HDC and HD to DNA was reduced by the smaller domain (aa 101–144), but it required a higher amount (6 μg; Fig. 3, B and C). As a control, the same amount of GST (6 μg) had no effect on the binding (lane 14 in Fig. 3, B and C).
Collectively, these data suggest that the HD of Hoxc-8 is sufficient for the interaction with Smad1 possibly by making direct contact with amino acid residues within MH1 domain.

**Hoxc-8 Interaction Domains of Smad1 Are Sufficient to Induce Osteopontin Promoter Activation**—The C3H10T1/2 (C3H) mesenchymal cell line provides an ideal model system for exploring the mechanism of Smad1-mediated BMP signaling. In response to BMP stimulation, C3H expresses bone markers, including osteopontin, ALP, collagen type I, and sialoprotein genes, leading to mineral deposition, the terminal stage of osteoblast differentiation (4, 35). Previously, we have reported that Hoxc-8 binds to a 266-base pair osteopontin promoter fragment and represses reporter gene transcription. Cotransfection of Smad1, Smad4, and a constitutively active form of the BMP type I receptor ALK3 (Q233D) in C3H cells induces osteopontin promoter activity in a dose-dependent manner (Fig. 4A). The same reporter Hox-pGL3 containing a Hoxc-8 binding site in front of the luciferase gene and SV-40 promoter (9) was used for the transfections. Cotransfection was performed with Hox-pGL3 or its mutant (mHox-pGL3 (9)) and plasmids for various Smad1 fragments separately. Results showed that Smad1-L (1–276), Smad1-NL (1–276), -M (1–191), and -H (1–191) stimulated the luciferase activity of the Hox-pGL3 reporter in a dose-dependent manner (Fig. 4A). In contrast, none of these Smad1 fragments affected the luciferase activity of the mHox-pGL3 reporter in which the Hox binding site was mutated.
increased level of expressing clones after tetracycline withdrawal for 4 days. An analysis was performed using mRNAs from 2T3 cells or HID-HIDs of Smad1 on the expression of such genes, Northern hybridization using corresponding cDNA probes. Fig. 5 demonstrates tetracycline-regulated expression of the three different Smad1 fragments of such clones.

ALP activity is a hallmark of bone formation, and induction of its activity in progenitor cells marks the entry of a cell into the osteoblastic lineage. BMP and its constitutively active receptors have been shown to induce effectively 2T3 cells to express high level of ALP activity (30, 36). The effect of the HID-containing fragments of Smad1 on ALP activity was examined in these stable cell lines. The expression of Smad1 fragments was induced by withdrawal of tetracycline from the growth medium. ALP activity was determined in 5–10 stable clones (for details, see “Experimental Procedures”), all of which showed increased ALP activity after tetracycline withdrawal. Fig. 5B shows the results for one of each of the Smad1-NL, -L, or -M clones. ALP activity remained at a basal level in all cells that were kept in tetracycline-containing medium. 2T3 cells that were permanently transfected with pTet-Splice vector showed little or no increased ALP activity upon tetracycline removal. These data indicate that the ALP activity in the stable clones is induced by the expression of HID-containing fragments of Smad1.

The progression of osteoblastic differentiation can also be monitored by the temporal expression of other bone cell phenotypic genes, such as *osf-2*, collagen type I, osteopontin, osteocalcin, and bone sialoprotein genes. To investigate the effect HIDs of Smad1 on the expression of such genes, Northern analysis was performed using mRNAs from 2T3 cells or HID-expressing clones after tetracycline withdrawal for 4 days. An increased level of *osf-2* expression is not detectable. However, consistent with ALP activity, the expression of type I procollagen α1(1) and osteopontin genes is elevated upon tetracycline withdrawal. The levels of the marker gene expression are compatible with positive controls (BMP-treated, Fig. 5C). A much higher expression of osteopontin gene was observed in both 2T3 cells that were treated with rhBMP-2 and clones that express HIDs at 12 days (data not shown). These data indicate that the Hoxc-8 interaction domains of Smad1 induce bone marker gene transcription in 2T3 cells and thus mimic BMP signaling.

The terminal differentiation of bone cells is characterized by the onset of extracellular matrix mineralization, which can be visualized by van Kossa staining. To confirm the role of Smad1 HIDs in induction of the final stage of osteoblast differentiation, we determined the formation of mineralized bone matrix in prolonged cultures of 2T3 and its derivative cell lines in the presence of rhBMP-2 or under indicated conditions. Stable cell lines were cultured in parallel in the presence or absence of tetracycline to modulate the expression of the Smad1 fragments. As expected, rhBMP-2 treated 2T3 cells and permanent 2T3 cell lines that expressed Smad1-NL, -L, or -M (+Tet) underwent mineralization at day 12 showing black stained spots. However, no mineralized bone matrix was observed in cells that were stably transfected with the empty vector or in cells that did not express Smad1-NL, -L, or -M (-Tet) (Fig. 5D). Together, these data indicate that the interaction between Smad1 and Hoxc-8 is mediated through H1D1 and H1D2 of Smad1 and HD of Hoxc-8. The HID of Smad1 mimics BMP signaling by inducing ALP activity, osteoblast marker gene transcription, and mineralization in 2T3 cells.

**DISCUSSION**

This study focused on mapping functional domains that are involved in the interaction between Smad1 and Hoxc-8 and subsequently on investigating the role of these domains in the induction of osteoblast differentiation and bone cell formation. From both yeast two-hybrid and gel shift assays with a series of deletion forms of the two proteins, we identified two regions of Smad1, namely H1D1 and H1D2, which interact with Hoxc-8, one of which (H1D1) interacts specifically with Hoxc-8 at the homeodomain.
HID1 lies within the MH1 domain between aa 101 and 144 of Smad1, and it inhibits both the full-length and the HD of Hoxc-8 binding to DNA (Figs. 1 and 3). The MH1 domain of Drosophila Mad (a homolog of mammalian Smad1), Smad3, and Smad4 binds to the DNA (11, 37, 38). Knowing that MH1 is highly conserved among Smads, one would wonder whether the inhibition of Hoxc-8 binding by Smad1 to osteopontin promoter proximal sequence was caused by competition for a cognate site. To address this issue, we tested all forms of GST-Smad1, none of which bound to the OPN-5 probe except for HID1, which showed a very weakly shifted band when 2 µg of the protein was used. Increasing the amount of HID1 had no effect on binding (data not shown). Thus, the HID1 is unlikely to have an intrinsic DNA binding activity on the OPN-5 of osteopontin promoter. This conclusion is in agreement with the yeast two-hybrid assay data in which clone 19 and full-length Hoxc-8 interacted with Smad1 more strongly than did the HD alone or any other HD-containing deletions (Fig. 2). Binding of HID2 to Hoxc-8 may mask the accessibility of HD to DNA, thereby inhibiting the protein-DNA interaction.

Our previous data showed that Smad2 and Smad3 have no effect on the Hoxc-8 binding to OPN-5 probe (9). Sequence alignment analysis revealed that several residues within the two HIDs are highly conserved, the actual aa differ between BMP and TGF-β pathway-restricted Smads (Smad1, -5, and -8 versus Smad2 and -3 in Fig. 6, boxed amino acids). Interestingly, a 7-aa insertion in BMP pathway-restricted Smad1, -5, and -8 is absent in both TGF-β pathway-restricted Smad2 and -3. It is also noteworthy that high homology exists between Smad2 and Smad3 in HID2 but is relatively less conserved among Smad1, -5, and -8 (Fig. 6). This is appealing because the subtle differences of HID2 among the three BMP pathway-restricted Smads may be implicated in the specificity of each.

The well conserved homeodomain consists of three α helices and a flexible NH2-terminal arm, which makes contact with the DNA (32). Residues that contact the DNA directly are usually conserved among multiple Hox paralog groups and appear to

Fig. 5. Smad1 domains induce bone cell differentiation. A, inducible expression of Smad1 fragments. Constructs shown in Fig. 4A were subcloned into pTet-Splice vector to make tetracycline (Tet)-regulated expression plasmids for Smad1-NL, -L, and -M. These were transfected permanently into 2T3 osteoblast precursor cells, and the total RNA was extracted from 2-day cultures grown in the absence or presence of tetracycline. Slot-blot hybridization assay (Bio-Rad) was carried out using 5 µg of total RNA from indicated clones and 32P-labeled corresponding cDNA probes. B, ALP activity is induced by the HIDs of Smad1. Stable 2T3 cell lines bearing pTet-Splice vector (Vector) or pTet-Splice recombinant constructs containing each of the HIDs of Smad1 were cultured in an osteoblastic promotion medium with or without tetracycline. 2T3 cells were cultured in the absence or presence of rhBMP-2 (50 ng/ml) as controls. ALP activity was determined as described under “Experimental Procedures,” and the A50 was normalized to protein content. Data shown are representative of positive clones.

C, NH2-terminal domains of Smad1 induce osteoblast differentiation-related gene expression. mRNA was extracted from the indicated cell clones grown in the presence or absence of tetracycline for 4 days, and 3 µg of each was used for Northern analysis with indicated probes. Bone marker gene expression in 2T3 cells that were grown in the presence or absence of rhBMP-2 shown in the figure serves as a positive control. D, HIDs of Smad1 induce mineralized bone matrix formation. 2T3 cells and the indicated stable clones were cultured for 12 days in osteoblastic promotion medium with/without tetracycline or with/without 50 ng/ml rhBMP-2. Cells were then fixed and stained by silver nitrate and acid fuchsin as described under “Experimental Procedures” to visualize mineralized bone matrix (black spots).
provide a general means of binding. The functional specificity of Hox proteins may be determined by ‘characteristic residues’ within or outside of the HD which are likely to contact other partners, such as Hox, Pbx, Extradenticle, or Engrailed homeo-proteins (33, 40–42). A recent crystallographic study has revealed that a hexapeptide of Hoxb-1 binds to its DNA-binding partner at a pocket formed partly by a three-amino acid insertion in the Pbx1 homeodomain (43). HD helix-1 of Hox-8 also mediates a direct contact with Hoxc-9 and inhibits the latter’s binding to DNA (44). We found previously that two Hox proteins, Hoxc-8 and Hoxa-9, are able to interact with Smad1, yet the only homology between the two is the HD. Here, we show that the binding of HD to OPN-5 is reduced by the HID1 of Smad1 (Fig. 3), indicating that the HD is also responsible for the protein-protein interaction.

Both Smad1 and Smad5 have been shown to induce ALP activity (45) and osteocalcin production (46) in a pluripotent mesenchymal cell line C2C12. Our data demonstrate that the HIDs interact with Hoxc-8, preventing binding of Hoxc-8 to osteopontin promoter (Figs. 1and 3). Thus, HIDs release the repression of Hoxc-8 leading to activation of the gene transcription and mimic the Smad1 activity. HIDs appear to be sufficient to activate bone cell phenotypic gene transcription and, subsequently, to cause mineralized bone matrix formation (Figs. 4 and 5).

Three Hox proteins, namely Hoxa-7, Hoxc-8, and Hoxb-4, are all found to repress gene transcription (47), and previous work from our laboratory showed that Hox-8 functions as a transcriptional repressor of osteopontin gene (Fig. 5) (9). It has been suggested that repression may be a general mode of action for Hox proteins, which may be required for maintaining cells in an undifferentiated state during development to prevent premature differentiation of precursor cells (47–49). Overexpression of Hoxc-8 in skeletal tissue results in an accumulation of progenitors in the hypertrophic area (20). It is likely that the involvement of Hoxc-8 in both osteo- and chondrogenic processes is the prevention of switch from proliferation to differentiation.

BMPs as important growth factors participate in many processes during embryonic development. In addition to the well-known function that BMPs induce bone and cartilage formation in ectopic sites in vitro, BMP-2 induces the undifferentiated mesenchymal progenitors to differentiate into osteoblasts, chondrocytes, and adipocytes in vitro (4, 5). BMP-2 also inhibits myogenic cells from differentiating into myotubes (50). Given the fact that both BMP and Hox genes play a fundamental role in directing cell fate, Smad-mediated BMP signaling through the interaction with Hox proteins might be also involved in some of the above processes. Various functional domains of both Smad and Hox proteins may be utilized selectively for mediating protein-protein or protein-DNA interaction and for repression or activation of gene transcription, depending upon developmental stage, cell type, and promoter context. Clearly, a detailed study of the structural and functional properties of Smad and Hox proteins will provide important insights into deciphering the complexity of their roles in embryogenesis and cell differentiation.

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