Smad6 as a Transcriptional Corepressor

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Smad6 and Smad7, a subgroup of Smad proteins, antagonize the signals elicited by transforming growth factor-β. These two Smads, induced by transforming growth factor-β or bone morphogenetic protein (BMP) stimulation, form stable associations with their activated type I receptors, blocking phosphorylation of receptor-regulated Smads in the cytoplasm. Here we show that Smad6 interacts with homeobox (Hox) c-8 as a transcriptional corepressor, inhibiting BMP signaling in the nucleus. The interaction between Smad6 and Hoxc-8 was identified by a yeast two-hybrid approach and further demonstrated by co-immunoprecipitation assays in cells. Gel shift assays show that Smad6, but not Smad7, interacts with both Hoxc-8 and Hoxa-9 as a heterodimer when binding to DNA. More importantly, the Smad6-Hoxc-8 complex inhibits interaction of Smad1 with Hoxc-8- and Smad1-induced transcription activity. These data indicate that Smad6 interacts with Hox transcription factors as part of the negative feedback circuit in the BMP signaling pathway.

Members of TGF-β superfamily transduce their signals into the cell through a family of mediator proteins called Smads. Receptor-regulated Smad1, Smad5, and Smad8 mediate BMP signaling, whereas Smad2 and Smad3 respond to TGF-β (1–4). Upon phosphorylation by their type I receptors, the receptor-regulated Smads interact with the common partner, Smad4, and translocate to the nucleus where the complex recruits DNA-binding protein(s) to activate specific gene transcription (5–9). Smad6 and Smad7 are structurally divergent Smads as antagonists of TGF-β family signaling (5, 6). They can associate with activated TGF-β and BMP type I receptors, thereby preventing phosphorylation of receptor-regulated Smads (11–13). In addition, Smad6 has also been demonstrated to interact with phosphorylated Smad1 to prevent the formation of an active signaling complex of Smad1 and Smad4, preferentially inhibiting the signaling pathways activated by BMPs (14, 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 that play critical roles in the transduction of Smad-mediated signals into gene expression. Two of these factors, Smad4 and Smad6, are essential for the activation of TGF-β and activin signaling pathways. Smad4 has been implicated in the regulation of a wide range of target genes, including the homeobox gene goosecoid (go) in Xenopus. These findings suggest that Smad4 may play a key role in the transduction of TGF-β and activin signals into gene expression, and that Smad4 is a critical mediator of the signaling pathways activated by these growth factors. Therefore, understanding the mechanisms by which Smad4 regulates gene expression is essential for understanding the biological functions of TGF-β and activin signaling pathways.

EXPERIMENTAL PROCEDURES

Two-hybrid Library Screening—A full-length Hoxc-8 coding sequence was cloned into pGBT9 (CLONTECH) to generate the pGBT9/Hoxc-8 bait plasmid. The human ubiquitous osteoblast-like cell PGCT2 cDNA library (CLONTECH) was screened with the pGBT9-Hoxc-8 bait plasmid according to the manufacturer’s instruction (CLONTECH).

Immunoprecipitation and Western Blot—FLAG-tagged full-length, amino-terminal domain with linker region (Smad6NLI), and carboxy-terminal domain (Smad6C) of Smad6 were subcloned into a mammalian expression vector pcDNA3 (Invitrogen). HA-tagged Hoxc-8 expression vector was constructed previously (8). Constitutively active BMP type IA (ALK3) expression plasmid was provided by Dr. Jeffrey L. Wrana (The Hospital for Sick Children, Toronto, Ontario, Canada). COS-1 cells were transfected with expression constructs as indicated in Fig. 1 using Tfx-50 according to the manufacturer’s description (Promega). Cells were lysed 48 h post-transfection, and lysates were immunoprecipitated with anti-HA antibody (Mouse anti-HA antibody, Babco). Gel Shift Assay—Gel shift assays were performed as described previously (18). Smad1/4 cDNAs were obtained from Dr. R. Derynck (University of California, San Francisco, CA). GST fusion constructs of Smad1, 1, and Hoxc-8 were generated in our previous study (8). Smad6 cDNA, obtained from Dr. Ali Hemmati-Brivanlou (The Rockefeller University), and Smad7 cDNA, obtained from Dr. Peter ten Dijke (Ludwig Institute for Cancer Research, Sweden), were cloned into pGEX-KG vector. The GST constructs described above were transformed into BL21. The expression and purification of the fusion proteins were performed as described (16). OPN5 DNA fragments were used as the probe for the gel shift assays (8).

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‡ The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic proteins; GST, glutathione S-transferase; FAST, forkhead activin signal transducer; PAI-1, plasminogen activator inhibitor-1; TFE3, transcription factor cF3; HA, hemagglutinin; Hox-pGL3, mutant Hox-pGL3; GFp, green fluorescent protein.
Specific interaction of Smad6 with Hoxc-8 in a yeast two-hybrid system

The interaction was assayed in liquid culture of mutant yeast strain Y190, which requires His, Leu, and Trp to grow. pGBT9-Hox c-8 and pACT2-Smad6 plasmids carry Trp and Leu as their selective markers, respectively. The interaction between Smad6 and Hoxc-8 enables the yeast to synthesize His and induces β-gal expression. The arbitrary units of β-gal activities for yeast bearing different plasmids were plotted as shown.

| Group | Bait Prey | β-Gal activity |
|-------|-----------|----------------|
| 1 | pGBT9 | pACT2 | 1 |
| 2 | pGBT9 | pACT2-Smad6 | 3 |
| 3 | pGBT9 | pACT2-Smad6c | 8 |
| 4 | pGBT9-Hoxc-8 | pACT2 | 2 |
| 5 | pGBT9-Smad1 | pACT2-Hoxc-8 | 59 |
| 6 | pGBT9-Hoxc-8 | pACT2-Smad6 | 286 |
| 7 | pGBT9-Hoxc-8 | pACT2-Smad6c | 1957 |

RESULTS AND DISCUSSION

We have previously demonstrated that Smad1 interacts with Hoxc-8 in response to BMP stimulation (8). Hoxc-8 functions as a transcription repressor in BMP signaling. The interaction of Smad1 with Hoxc-8 dislothes Hoxc-8 binding from its element resulting in initiation of gene transcription (8). To characterize the Hoxc-8-mediated transcription mechanism in BMP-induced gene activation, we used a yeast two-hybrid system to identify transcription factors that interact with Hoxc-8. An intact Hoxc-8 cDNA fused with the Gal4 DNA binding domain was used as a bait plasmid to screen a human U2 OS osteoblast-like cell cDNA library constructed in pACT2 plasmid. After two rounds of screening, we obtained 26 positive clones. DNA sequence analysis identified one clone as Smad6 (Table 1). Smad6 and Smad7 are immunolocalized in the nucleus of rat epiphyseal plate (22), Xenopus embryo (23), and Mink lung epithelial (Mv1Lu) cells (24). The interaction of Smad6 with Hoxc-8 suggests that Smad6 may have a novel antagonistic function in the nucleus.

The initial Smad6 cDNA clone (Smad6C in Table 1) encodes amino acids 281 to 496 of a 496-amino acid protein. The interaction between Hoxc-8 and Smad6 was further confirmed with a β-gal filter lift assay (data not shown) and quantified by a liquid β-gal assay (Table 1). When the full length of Smad6 fused with the Gal4 transcriptional activation domain was tested in the two-hybrid system, it showed a weaker interaction with Hoxc-8 in comparison with the carboxy-terminal domain (Smad6C). Deletion of the Smad6 amino-terminal domain may change the protein conformation in a way that the carboxy-terminal region becomes easier to interact with Hoxc-8. The assays of both empty bait vector (pGBT9) with Smad6C or Smad6 full-length cDNAs in prey plasmids and empty prey vector (pACT2) with full-length Hoxc-8 in bait vector showed very little activity. Compared with the interaction between Smad1 and Hoxc-8, the interaction of Smad6 with Hoxc-8 is about 5 times stronger (Table 1).

To investigate the interaction of Smad6 with Hoxc-8 in mammalian cells and the effect of BMP stimulation on this interaction, COS-1 cells were transiently co-transfected with expression plasmids for FLAG-Smad6, HA-Hoxc-8, and/or constitutively active BMP type I receptor ALK3 (Q233D). The cell lysates were immunoprecipitated with anti-FLAG M2 antibody, and the resulted complexes were analyzed by Western blotting with anti-HA antibody. The expression levels of Smad6 were shown by Western blot with anti-FLAG M2 antibody (middle panel), and Hoxc-8 expression levels were shown by Western blot with anti-HA antibody (bottom panel).

FIG. 1. Interaction of Smad6 with Hoxc-8 in vivo. FLAG-tagged Smad6, FLAG-tagged Smad6 carboxy-terminal domain (Smad6C), FLAG-tagged Smad6 amino-terminal domain with linker region (Smad6NL), and HA-tagged Hoxc-8 were co-transfected in COS-1 cells with or without ALK3 (Q233D). Cell lysates were immunoprecipitated with anti-FLAG M2 antibody, and the resulted complexes were analyzed by Western blotting with anti-HA antibody. The expression levels of Smad6 were shown by Western blot with anti-FLAG M2 antibody (middle panel), and Hoxc-8 expression levels were shown by Western blot with anti-HA antibody (bottom panel).

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Hoxc-8 is also specifically involved in the BMP signaling pathway. Smad7 was tested for its interaction with Hox proteins (Fig. 2b). Like Smad2 and -3, Smad7 did not interact with either Hoxc-8 or Hoxa-9. Like Smad2 and -3, Smad7 did not interact with Smad6. Smad7 was tested for its interaction with Hoxc-8 and Hoxa-9 (Fig. 2c). Instead of preferentially involved in BMP signaling, Smad4, the common partner for all receptor-regulated Smads and interaction with Hoxc-8, was examined for the same purpose (Fig. 2c). Indeed, Smad4 did not block the interaction of Smad6 with Hoxc-8 completely (Fig. 2c, lanes 7 and 9). This suggests that Smad4 can only be passively translocated into the nucleus by forming hetero-oligomers with any of the receptor-regulated Smads. In our experiments, these results support that Smad6 is an important antagonist for Hox proteins in the nucleus.

To investigate whether the Smad6-Hoxc-8 complex inhibits the interaction of Smad1 with Hoxc-8 in activating gene transcription, we utilized the model described in our earlier studies (8). Overexpression of the Smad1-Hoxc-8 interaction domain linked to a nuclear localization signal (Smad1B) stimulates BMP downstream gene expression and induces osteoblast differentiation from osteogenic cells (8, 30). When the BMP-inducible construct (Hox-pGL3) was co-transfected in Mv1Lu cells with the Smad1B expression plasmid, the luciferase activity was stimulated in a dose-dependent manner (Fig. 3a). This model provides an ideal assay to directly examine the Smad6 antagonistic function in the nucleus. Because Smad1B mimics BMP-induced gene transcription without BMP receptor phosphorylation involving and interaction with Smad6 of Smad1 (13, 14, 30), this assay avoids Smad6 inhibitory function in the cytoplasm. Hox-pGL3 construct was co-transfected in Mv1Lu cells with Hoxc-8 and/or Smad6 expression plasmid. As shown in Fig. 3b, overexpression of Hoxc-8 or Smad6 alone moderately inhibited Smad1B-induced transcription activity. Most importantly, Smad6 completely abolished the Smad1B-induced luciferase activity. To validate this observation, we transfected Mv1Lu cells with a mutated construct, Hox-pGL3, in which the core nucleotides of the Hoxc-8 binding site were mutated from TAAT to GCCG. Transfection of the mutant construct dramatically reduced Smad1B-induced reporter activity. As expected, the inhibition mediated by co-transfection of Smad6 and Hoxc-8 was also reduced (Fig. 3c).

Finally, protein localization analysis demonstrated that both Smad6 and Hoxc-8 are highly expressed in the nucleus (Fig. 3d). Differing from Smad7, Smad6 nuclear exportation was not induced with BMP-4. Taken together with immunoecoprecipitation of Smad6 with Hoxc-8 and formation of the Smad6-Hoxc-8 complex in gel shift assays, these data suggest co-localization of Smad6 with Hoxc-8 in the nucleus. This is the first to demonstrate that Smad6 has an antagonistic function toward BMP signaling in the nucleus in addition to its interaction with BMP type I receptor and Smad1 in the cytoplasm.

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