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Smad1 and Smad5 Act Downstream of Intracellular Signalings of BMP-2 That Inhibits Myogenic Differentiation and Induces Osteoblast Differentiation in C2C12 Myoblasts

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Bone morphogenetic protein-2 (BMP-2) inhibits terminal differentiation of C2C12 myoblasts and converts them into osteoblast lineage cells (Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., and Suda T. (1994) *J. Cell Biol.* 127, 1755–1766). In the present study, we examined the possible involvement of Smad proteins, vertebrate homologues of *Drosophila* Mothers against decapentaplegic, in the BMP effects on the differentiation of C2C12 myoblasts. C2C12 cells expressed Smad1, Smad2, Smad4, and Smad5 mRNAs, and expression levels were not altered by treatment with BMP-2 or TGF-β1. When Smads were transiently transfected into C2C12 cells, both Smad1 and Smad5 induced alkaline phosphatase (ALP) activity and decreased the activity of myogenin promoter/chloramphenicol acetyltransferase (myogenin-CAT) without BMP-2. When C-terminal-truncated Smad1 and Smad5 were transfected into constitutively active BMP receptor type IB (BMPR-IB)-expressing C2C12 cells, BMP signals were blocked, resulting in an increase in myogenin-CAT activity. On the other hand, Smad1 and Smad5 decreased myogenin-CAT activity but did not induce ALP activity in MyoD-transfected NIH3T3 fibroblasts. These results suggest that both Smad1 and Smad5 are involved in the intracellular BMP signals which inhibit myogenic differentiation and induce osteoblast differentiation in C2C12 cells, and that the conversion of the two differentiation pathways is regulated independently at a transcriptional level. © 1997 Academic Press

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, were first identified as the factors that induce ectopic bone formation in vivo, when implanted into muscular tissues (1,2). BMPs play important roles in organogenesis and embryogenesis in vertebrates, and mutations of their genes cause embryonic death or defects in the development of certain tissues including skeletal patterning (3,4).

The receptors for members of the TGF-β superfamily possess transmembrane serine/threonine kinases and are classified as type I or type II based on structural and functional characteristics (3-6). For the propagation of ligand-inducing signals, it is necessary to form a heteromeric complex of a ligand, and type I and type II receptors (7), and to phosphorylate other cytoplasmic protein(s) by a type I receptor kinase (8). For BMP signaling, two structurally related type I receptors (BMPR-IA and BMPR-IB), and a type II receptor (BMPR-II) were identified (9-14). In the molecular mechanism of the signal transduction of TGF-β and related factors, however, intracellular signalings of those factors have not well been characterized. Sekelsky et al. (15) first identified an intracellular signaling protein for *Drosophila* decapentaplegic (dpp), the Mothers against dpp (Mad). Several mammalian and amphibian homologues of Mad (Smad) were cloned and their specificity and relation to the ligands characterized (16). At least, 6 related Smads have been identified in mammals (17-25). Smad1 is a signaling molecule for BMP-2/4 (17-21), and Smad2 and Smad3 are signaling molecules for activin and TGF-β, respectively (21-24). Smad4 which was first identified as a putative tumor suppressor gene (DPC4) in colorectal cancers (26,27) is now thought to be a signaling partner of the TGF-β response (23,28). It is necessary for the signal transduction of TGF-β and BMP responses that Smad1 and Smad2 are phosphorylated by the respective activated kinases of BMP and TGF-β type I receptors (29,30).

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We reported that BMP-2 not only stimulated maturation of osteoblast progenitor cells, but also induced differentiation of pluripotent fibroblastic C3H10T1/2 cells into osteoblastic cells (31,32). To elucidate the molecular mechanism of ectopic bone formation induced by BMP in muscular tissues, we examined the effects of BMP-2 on myogenic differentiation. BMP-2 inhibited the differentiation of C2C12 myoblasts and myogenic transcription factor-introduced C3H10T1/2 cells and NIH3T3 fibroblasts into mature myotubes by suppressing the transcriptional activity of myogenic factors such as MyoD and myogenin (33,34). Moreover, BMP-2 induced the expression of typical osteoblast phenotypes such as alkaline phosphatase (ALP) activity, parathyroid hormone response, and osteocalcin production in C2C12 and C3H10T1/2 cells, but not in NIH3T3 cells (31,33,34). TGF-β1 also inhibited the terminal differentiation of C2C12 cells, but did not induce the osteoblast phenotype (33). These results suggest that the induction of osteoblast differentiation in C2C12 cells occurs specifically in response to BMPs. The in vitro differentiation of C2C12 cells appears a useful model to elucidate the specific signaling of BMP-2. Recently, we reported that a kinase domain-truncated form of BMPR-IA (ΔBMPR-IA) blocked BMP-2-induced signals in C2C12 cells, and that ΔBMPR-IA-expressed C2C12 cells did not differentiate into osteoblastic cells even in the presence of BMP-2 (35). Although C2C12 cells did not express BMPR-IB (35,36), a constitutively active mutant of BMPR-IB transduced BMP-2 signals in C2C12 cells even in the absence of the ligand (36). These results suggest that both signals from BMPR-IA and BMPR-IB are transduced in C2C12 cells.

In the present study, we examined the effects of BMP signals via Smads on myogenic and osteoblast differentiation in C2C12 cells. We show here that overexpression of either Smad1 or Smad5 induces the osteoblast phenotype and inhibits muscle specific gene expression in the absence of BMP-2. Although Smad1 and Smad5 also decreased the activity of myogenic transcription factors in MyoD-overexpressed NIH3T3 cells, they did not induce ALP activity in those cells. These results indicate that both BMP effects are induced by the intracellular signaling via either Smad1 or Smad5, and the induction of the osteoblast phenotype and the inhibition of myogenic differentiation are regulated independently at a transcriptional level.

MATERIALS AND METHODS

Cell culture. The mouse myoblast cell line, C2C12 (37), was purchased from the American Type Culture Collection (Rockville, MD). IB19a (36) and ΔIA-12 (35) cells were established by stably transfecting C2C12 cells with a constitutively active BMPR-IB and a kinase domain-truncated BMPR-IA, respectively. NIH-D22A cells were established by stably transfecting NIH3T3 fibroblasts with mouse MyoD (34). C3H10T1/2 fibroblasts (38) were obtained from the RIKEN Cell Bank (Tsukuba Science City, Japan). All the cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemicals, St. Louis, MO) containing 15% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) and antibiotics (100 U/ml of Penicillin-G and 100 μg/ml of Streptomycin). For the induction of muscle specific gene expression or the treatment with growth factors, the medium was replaced with DMEM containing 5% FBS with or without recombinant human BMP-2 (300 ng/ml) (2) or TGF-β1 (10 ng/ml) (R&D systems, Minneapolis, MN). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cloning of Smad cDNA. Human Smad1 (17), Smad3 (23) and Smad4 (26) cDNAs were cloned from HT-1080 fibrosarcoma by RT-PCR using restriction site-linked primers GCTCTAGAGTAATTTCTACTCTTCTGGACTTCAAACTAAGAAG and GCTCTAGATGGGGCCATTTAAGATACAGATGAAATAG, GCTCTAGACCCGGCGTCCCGTCGAGCCCAG and GCTCTAGAGCCTGCCCTCCCCTACCATACTTGAT, and GCTCTAGAGCTGTTGTTTTTCACTGTTTCCAAAGG and GCTCTAGATAGTCCACCATCCTGATAAGGTTAAGGG, respectively. Mouse Smad2 (22) and Smad5 (39) were cloned from C2C12 myoblasts by using primers GCGAATTCGGGCTTTTTCTGAGTGTGGATTGTTACC and GCGAATTCTCTTTGATGGGTTTACGACATGCTTGAG, and GCTCTAGATAAATGTCACTCCCCGCCTCCACTTG and GCTCTAGATTCAACAGGGGCAAAATACTCTACATCGTT, respectively. C-terminal-truncated Smads were generated by introducing a stop codon into the conserved alanine residue at 422 for Smad1, 424 for Smad2, 381 for Smad3, and 422 for Smad5, to delete 43 amino acids. Obtained cDNAs were verified by sequencing and subcloned into the mammalian expression vector, pMIKHygB, that carries SRα promoter (provided by Dr. K. Maruyama, University of Tokyo, Japan).

Transfection and reporter assays. C2C12, ΔIA-12, and NIH-D22A cells were transfected with the expression vectors by cationic liposomes (DOTAP; Boehringer Mannheim, Germany) to examine transient expression. After transfection, cells were cultured for 3 days in growth medium and stained for alkaline phosphatase (ALP) activity as described below.

For the chloramphenicol acetyltransferase (CAT) reporter assay, myogenin-CAT, which contained a 4 kbp sequence of the promoter region of mouse myogenin gene (40), was used. Cells were co-transfected with each Smad expression vector, a myogenin-CAT reporter vector and a β-galactosidase expression vector by the calcium phosphate-DNA co-precipitation method. These cells were incubated for 12 h after transfection with DMEM containing 15% FBS then cultured for 2 days with DMEM containing 5% FBS (low serum medium). The cells were washed with phosphate-buffered saline (PBS), and lysed using Reporter Lysis Buffer (Promega, Madison, WI). Aliquots of the cell lysates were subjected to CAT assay and β-galactosidase assay (Promega) for normalization of the transfection efficiency.

Histochemical analyses. To examine ALP activity histochemically, cells were fixed for 10 min with 3.7% formaldehyde at room temperature. After washing with PBS, the cells were incubated for 30 min with a mixture of 0.1 mg/ml of naphthol AS-MX phosphate (Sigma), 0.5% N, N-dimethylformamide, 2 mM $MgCl_2$, and 0.6 mg/ml of fast blue BB salt (Sigma) in 0.1 M Tris-Cl, pH 8.5, at room temperature.

RNA preparation and RT-PCR. Total cellular RNAs were prepared from cultured cells using TRIZOL (GIBCO BRL, Grand Island, NY). Ten μg of RNAs was reverse-transcribed using SuperscriptII (GIBCO), and aliquots of the obtained cDNA pools were subjected to PCR. For confirmation of Smad mRNA expression, the following specific primers for mouse Smads were used: GTATTTCTACCTTTCAAACCGCAGTTCCAAGAAG and CACAGGTCTTTTAAGACACGGATGAAATAG for Smad1, and GCCATTGGTTTTCACTGCCTTCAAAAG and TAGTCCACCATCCTGGAAATGGTTAGGG for Smad4. Primers for mouse Smad2 and Smad5 have been described above. For the detection of PCR products, [α-^{33}P]dATP (3000 Ci/mmol; DuPont/NEN, Boston, MA) was added to the reaction mixtures. As an inter-

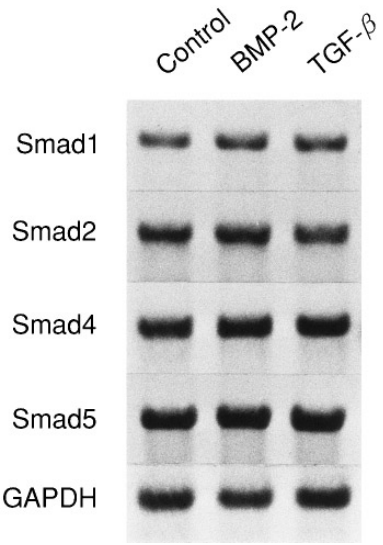

FIG. 1. C2C12 myoblasts express Smad1, Smad2, Smad4 and Smad5 mRNAs. C2C12 cells were cultured in DMEM containing 5% FBS with or without BMP-2 or TGF-β1. After culture for 3 days, total cellular RNAs were prepared and subjected for RT-PCR. Aliquots of PCR products were resolved by electrophoresis, transferred to a nylon membrane, and autoradiographed. As an internal control, mouse GAPDH cDNA was co-amplified with each Smad cDNA.

nal control, primers for mouse GAPDH cDNA (Clontech, Palo Alto, CA) were added for co-amplification with Smad cDNAs. After the cycles within the linear-phase amplification, aliquots were resolved by electrophoresis in a 1.0% agarose gel, transferred to a membrane (Hybond-N+; Amersham, Buckinghamshire, UK), and autoradiographed.

RESULTS

C2C12 Myoblasts Express Smad1, Smad2, Smad4, and Smad5 mRNAs

We first examined the expression of each Smad mRNA by RT-PCR in C2C12 cells (Fig. 1). C2C12 cells expressed Smad1, Smad2, Smad4 and Smad5 mRNAs. The expression levels were not affected appreciably by the treatment with BMP-2 or TGF-β1, when compared with the control culture (Fig. 1).

Overexpression of Smad1 and Smad5 Induces ALP Activity in C2C12 Myoblasts and C3H10T1/2 Fibroblasts but Not in NIH-D22A Cells

To ascertain which Smads transduce BMP signals, we transiently transfected C2C12 cells with each Smad expression vector, and cultured them for 3 days. ALP-positive cells appeared in the cultures transfected with Smad1 or Smad5 even in the absence of BMP-2 (Fig. 2, a and c). However, ALP-positive cells did not appear in the cultures transfected with Smad2 (Fig. 2, b), Smad3 or Smad4 (data not shown). We also transfected each Smad into ΔIA-12 cells, subclonal C2C12 cells that expressed a kinase domain-truncated BMPR-IA (ΔBMPR-IA). They did not respond to BMP-2 (35). As in the parental C2C12 cells, ALP-positive cells appeared in the cultures of ΔIA-12 cells transfected with Smad1 or Smad5 (Fig. 2, d and f). Although similar results were obtained in C3H10T1/2 cells transfected with each Smad (Fig. 2, g to i), no ALP positive cells appeared in NIH-D22A cells transfected with any Smad (Fig. 2, j to l), which constitutively expressed exogenous MyoD and induced muscle specific gene expression when cultured with the low serum medium (34).

Overexpression of Smads Inhibits Muscle-Specific Gene Expression

We examined the role of the Smads in the inhibition of myogenic differentiation in C2C12 cells. C2C12 cells were co-transfected with each Smad expression vector and a myogenin-CAT reporter vector, then cultured for 2 days in low serum medium. When either Smad1, Smad2, Smad3 or Smad5 was overexpressed in C2C12 cells, myogenin-CAT activity was similarly decreased (Fig. 3). Co-expression of Smad4 with Smad1 or Smad5 did not alter the effect of Smad1 or Smad5 on myogenin-CAT activity. Similar results were obtained in ΔIA-12 and NIH-D22A cells (Fig. 3).

C-Terminal-Truncated Smad1 and Smad5 Block BMP Signals

To examine further the involvement of Smads in the signal transduction of BMP-2, dominant negative effects of C-terminal-truncated Smads were investigated. For this purpose, IB19a cells, subclonal C2C12 cells that stably expressed constitutively active BMPR-IB, were used. These cells expressed osteoblast-specific genes even in the absence of BMP-2 (36). We generated C-terminal-truncated Smads (ΔSmads; see Materials and Methods), that failed to transduce ligand-induced signals in a dominant negative manner (23,28,41). When IB19a cells were co-transfected with a ΔSmad1 or ΔSmad5 expression vector and a myogenin-CAT reporter gene and cultured in low serum medium, myogenin-CAT activities were increased when compared with the cells co-transfected with an empty vector (Fig. 4). No increase in the myogenin-CAT activity was observed on transfection with a ΔSmad2 or ΔSmad3 expression vector (Fig. 4). Co-transfection with ΔSmad1 and ΔSmad5 did not increase further the myogenin-CAT activity induced by each ΔSmad transfection (Fig. 4).

DISCUSSION

We previously reported that BMP-2 inhibited terminal differentiation of C2C12 cells by suppressing the transcriptional activity of MyoD family proteins and

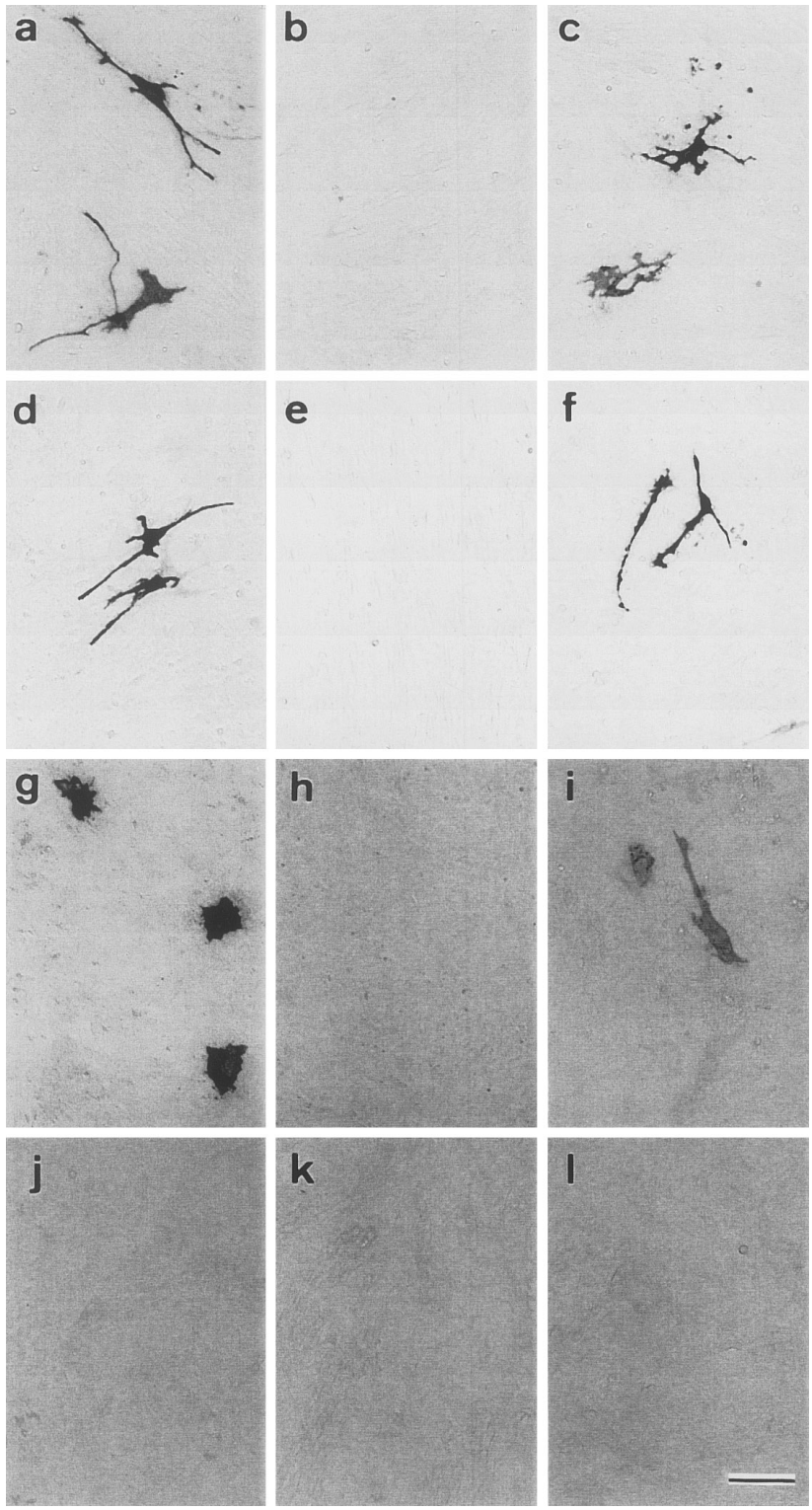

FIG. 2. Overexpression of either Smad1 or Smad5 induces ALP activity in C2C12 myoblasts and C3H10T1/2 fibroblasts but not in MyoD-expressing NIH3T3 fibroblasts. C2C12 cells (a to c), ΔIA-12 cells (d to f), C3H10T1/2 cells (g to i) or NIH-D22A cells (j to l) were transfected with Smad1 (a, d, g and j), Smad2 (b, e, h and k) or Smad5 (c, f, i and l) expression vector by lipofection, and cultured in DMEM containing 15% FBS. After 3 days, the cells were fixed and stained for ALP. Bar, 100 μm.

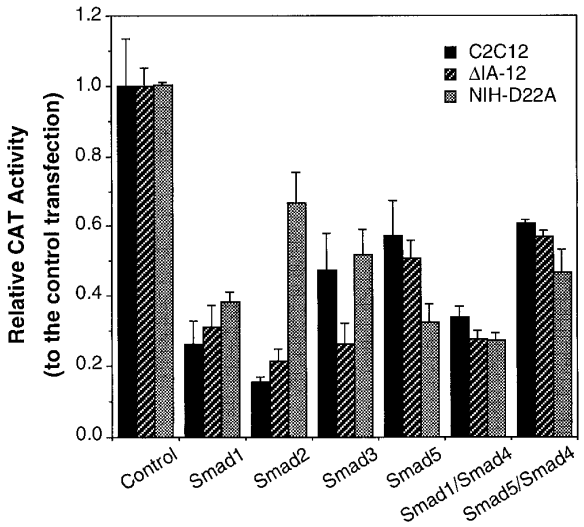

FIG. 3. Overexpression of Smads suppresses myogenin promoter activity in C2C12 myoblasts and MyoD-expressing NIH3T3 fibroblasts. C2C12, ΔIA-12 or NIH-D22A cells were co-transfected with each Smad expression vector, a myogenin-CAT reporter vector and a β-galactosidase expression vector. After culture for 12 h with DMEM containing 15% serum, cultures were transferred to fresh DMEM containing 5% FBS (low serum medium). Two days later, CAT and β-galactosidase activities were measured. Relative CAT activities to each control transfection (empty vector) were calculated. Data are the means ± SE of at least three experiments.

induced characteristics typical of an osteoblast phenotype such as ALP activity (33). In the present study, we showed that overexpression of either Smad1 or Smad5 induced ALP activity and inhibited promoter activity of myogenin in C2C12 cells in the absence of BMP-2. These results suggest that both Smad1 and Smad5 act as mediators of BMP-2 signals in C2C12 cells.

It has been reported that Smad1 is a mediator of the intracellular signaling of BMP-2/4 for inducing ventral mesoderm in Xenopus (17-21). Smad5, also termed dwarfin-C, is thought to act downstream of BMP signaling pathways, because of the high homology with Smad1, termed dwarfin-A (95% homologous) (39). Smad5 induces ventral mesoderm in Xenopus embryo, suggesting that Smad5 acts as a signal mediator of BMP-2/4 as well (42). In the present study, we showed that overexpression of not only Smad1 but also Smad5 induced ALP activity in C2C12 cells even in the absence of BMP-2. Moreover, these effects were reproduced by transfection into kinase domain-truncated BMPR-IA-expressing C2C12 cells, that differentiated into multinucleated myotubes but did not express osteoblast specific genes even in the presence of BMP-2 (35). These results indicate that overexpression of exogenous Smad1 and Smad5 induce ALP activity without involving endogenous BMP-2 or other factors via BMPR-IA at least in C2C12 cells. Recent reports suggest that ligand-induced phosphorylation of C-terminal serine residues of Smads are necessary for nuclear accumulation of those proteins (29, 30). Therefore, overexpression of Smad protein in transfected cells may lead to the apparent increase of the concentration of that protein in the nucleus. These effects were specific to Smad1 and Smad5, because overexpression of Smad2, Smad3 and Smad4 did not induce ALP. Of great interest is that dwarfin-A and dwarfin-C are phosphorylated by TGF-β treatment but only dwarfin-A is phosphorylated by BMP-2 treatment in L6 myoblasts (39). In C2C12 myoblasts, however, BMP-2 induced ALP activity but TGF-β did not (33). This may be due to the difference of experimental conditions and cell lines used, because both BMP-2 and TGF-β1 inhibited myogenic differentiation but did not increase ALP activity at low basal levels in L6 myoblasts (43).

It is not clear why overexpression of Smad1 and Smad5 induced ALP activity in C2C12 cells in a ligand-independent manner. We showed that the expression levels of endogenous Smad mRNAs were not altered by the treatment with BMP-2 or TGF-β1 in C2C12 cells. The ligand-independent stimulation of TGF-β, activin, or BMP responsive gene expression has previously been reported in some cell lines transfected with Smad expression vectors (23,43) and also in Xenopus animal cap assays injected with Smad mRNAs (20-21,42). Moreover, wild-type Smads have been reported to be distributed throughout the cells in the absence of the ligands and they are accumulated into the nucleus

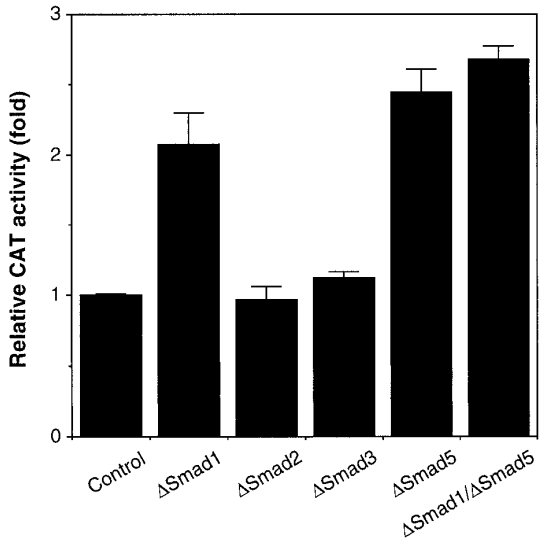

FIG. 4. C-terminal-truncated Smad1 and Smad5 specifically inhibit intracellular BMP signaling. IB19a cells (see *Materials and Methods*) were co-transfected with each truncated Smad expression vector, a myogenin-CAT reporter vector and a β-galactosidase expression vector. After culture for 12 h with DMEM containing 15% serum, cultures were transferred to fresh DMEM containing 5% FBS (low serum medium). Two days later, CAT and β-galactosidase activities were measured. Relative CAT activities to each control transfection (empty vector) were calculated. Data are the means ± SE of at least three experiments.

upon the ligand addition (17,18,43,44). In accordance with those observations, overexpression of Smad proteins in transfected cells may lead to an apparent increase in the concentration of the proteins in the nucleus.

C2C12 myoblasts constitutively express MyoD in the proliferation phase (33). When these cells are cultured in low serum medium, several muscle specific genes including myogenin and myosin heavy chain (MHC) are induced and the cells differentiate into mature myotubes. BMP-2 inhibited muscle specific gene expression by suppressing the transcriptional activity of myogenic transcription factors in C2C12 cells, which in turn did not differentiate into MHC-positive myotubes (33,34). The effects were reproduced in myogenic factor-overexpressed C3H10T1/2 and NIH3T3 fibroblasts (34). In the present study, overexpression of Smad1, Smad2, Smad3 or Smad5 decreased the promoter activity of myogenin in C2C12 cells and MyoD-overexpressing NIH3T3 cells. These results suggest that the inhibitory effect on myogenic differentiation is mediated via Smads, which are commonly involved in the signal transduction of proteins belonging to the TGF-β superfamily including BMPs (45).

Recently, it was reported that Smad4 associates with Smad1 in response to BMP-4 and with Smad2 in response to activin or TGF-β (28), and co-expression of Smad4 with Smad3 co-operatively induces promoter activity of plasminogen activator inhibitor-1 in Mv1Lu cells (23). However, whether Smad1 and Smad4 interact to induce osteoblast differentiation of C2C12 cells is not clear, because of the lack of specific reporter assay systems for BMP signaling. In our preliminary study, when Smad5 was co-transfected with C-terminal-truncated Smad4 into C2C12 cells, the inhibitory effect of Smad5 on the promoter activity of myogenin was reduced (data not included). However, we could not confirm the co-operative effect of the co-transfection of Smad5 with Smad4 on the inhibition of myogenin promoter activity. Further studies are needed to elucidate the interaction of Smad1/Smad5 with other proteins including Smad4 in BMP signalings in C2C12 cells.

Smads have two highly conserved domains in N- and C-terminals. It is known that C-terminal serine residues are phosphorylated in response to the ligands by type I receptor kinases (29), and that the ligand-induced Smad phosphorylation is necessary for association with Smad4 or other proteins, nuclear accumulation and transcriptional activation at physiological condition (30). Thus, it is likely that Smads are inactivated by truncation of several amino acids present in the conserved C-terminal domain (23,41) or substitution of C-terminal serine residues into alanine (29,30). These mutated Smads inhibit ligand-induced intracellular signalings in a dominant negative manner (23,41). To confirm the effect of C-terminal-truncated Smads (ΔSmads) on BMP signalings in C2C12 cells, we used a subclonal cell line of C2C12 cells that expressed constitutively active BMPR-IB, named IB19a (36). This mutant BMPR-IB constitutively transduces BMP signals even in the absence of BMP-2, and the mutant cells expressing that mutant receptor do not differentiate into mature myotubes (36). When ΔSmads were co-transfected with myogenin-CAT into IB19a cells, ΔSmad1 and ΔSmad5 increased the promoter activity of myogenin in those cells, but ΔSmad2 and ΔSmad3 did not. These results suggest that C-terminal-truncated Smad1 and Smad5 specifically inhibit BMP signals in constitutively active BMPR-IB-expressing C2C12 cells. Taken together with the ALP-inducing activity of full length Smad1 and Smad5, the present study clearly indicates that the two BMP effects, induction of osteoblast differentiation and inhibition of myogenic differentiation, are mediated by either Smad1 or Smad5 in C2C12 cells. Further studies are necessary to elucidate the functional differences between Smad1 and Smad5, since C2C12 cells express both Smad mRNAs.

It is not clear how BMP-2 signals regulate the conversion of the pathway of differentiation of C2C12 myoblasts into mature myotubes and osteoblastic cells. Our results suggest that the regulation neither pathway occurs in the cytoplasm. We recently reported that BMP-2 inhibited muscle specific gene expression in MyoD-overexpressed NIH3T3 fibroblasts and C3H10T1/2 fibroblasts (34). Moreover, BMP-2 induced ALP activity in MyoD-overexpressed C3H10T1/2 cells but not in MyoD-overexpressed NIH3T3 cells (34). Overexpression of Smads inhibited the promoter activity of myogenin (Fig. 3) but did not induce ALP-positive cells in MyoD-overexpressed NIH3T3 cells (Fig. 2). Taken together, it is concluded that the two signals of BMP-2 are regulated independently in the nucleus. Failure of osteoblast-specific gene expression may be due to the lack of unknown nuclear factor(s) necessary for the induction of osteoblast differentiation by BMPs in the NIH3T3 cells. A search for such a factor is in progress in our laboratory.

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