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Induction of Smad6 mRNA by Bone Morphogenetic Proteins

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Members of the transforming growth factor-β (TGF-β) superfamily transduce signals via Smad proteins. Smad2 and Smad3 mediate TGF-β signaling, whereas Smad1 and Smad5 transduce bone morphogenetic protein (BMP) signals. Smad4 is a common mediator required for both pathways. Smad6 and Smad7 are recently identified members in the Smad family; they inhibit the signaling activity of the other Smad proteins. Here we show that expression of the Smad6 mRNA is dramatically induced by BMP-2 or osteogenic protein-1 (OP-1)/BMP-7 in various cells. BMP-2 induced expression of Smad7 in one cell type, although much less potently than that of Smad6. Smad6 message was induced by TGF-β1 in TGF-β1-responsive Mv1Lu cells, but the induction was transient in contrast to the induction by BMPs. These results indicate that Smad6 may form a feedback loop to regulate the signaling activity of BMPs.

Members of the transforming growth factor-β (TGF-β) superfamily transduce signals through two different types of serine/threonine kinase receptors, type I and type II (1). After ligand binding, type II receptor transphosphorylates the type I receptor mainly at the GS domain located upstream of the serine/threonine kinase region, which results in the activation of type I receptor serine/threonine kinase. Thus, the type I receptors act as downstream components in the signaling pathway, and determine the specificity of intracellular signals.

Smad proteins are 50-70 kDa intracellular proteins, which transduce signals from the serine/threonine kinase receptors for the TGF-β superfamily proteins (2–5). *Drosophila* Mothers against dpp (Mad) was first identified in the Smad family, which transduces intracellular signals of *Drosophila decapentaplegic* (*dpp*) gene product (6). Certain Smads act in a lineage-specific manner in the signal transduction pathway. Smad2 and Smad3 are activated by TGF-β type I receptor (TβR-I) or activin type IB receptor (ActR-IB; also termed activin receptor-like kinase 4 or ALK-4), whereas Smad1 and Smad5 are activated by type I receptors for bone morphogenetic proteins (BMPR-IA and BMPR-IB, also termed ALK-3 and ALK-6, respectively). These pathway-restricted Smads are phosphorylated by serine/threonine kinase receptors, and form heteromeric complexes with Smad4 (also termed DPC-4). The heteromeric complexes translocate into the nucleus and regulate the transcription of various genes, and thus, the TGF-β superfamily proteins regulate growth, differentiation, metabolism and apoptosis of various cell types.

Smad6 and Smad7 have recently been identified in mammals, which are distantly related to other Smads (7–9). Interestingly, Smad6/Smad7 act as inhibitors in the TGF-β superfamily signals. Although pathway-restricted Smads interact with type I receptors only transiently and they are released from the receptors after phosphorylation (10–12), Smad6/Smad7 stably bind to the serine/threonine kinase receptors, and they may interfere with the phosphorylation of lineage-specific Smads by the receptors. *Drosophila* Daughters against dpp (Dad) is structurally similar to Smad6/Smad7, and also acts as an inhibitory Smad in the Mad signal transduction pathway (13).

It is intriguing how these inhibitory Smads are regulated in the signal transduction pathway. Topper *et al.* (14) reported that expression of Smad6 and Smad7 is induced by laminar shear stress in vascular endothelial cells, but not by turbulent shear stress. More importantly, the inhibitory Smads are regulated by ligand

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stimulation; Dad has been shown to be induced by Dpp stimulation (13), and Smad7 is induced by the addition of TGF-β1 (8). Here we have studied the regulation of Smad6 mRNA expession by ligand stimulation in various cell types.

MATERIALS AND METHODS

Reagents. Human recombinant bone morphogenetic protein-2 (BMP-2) and osteogenic protein-1 (OP-1) were purified from Chinese hamster ovary cells transfected with the respective cDNAs (15). TGF-β1 was obtained from Dr. H. Ohashi at Kirin Brewery Co., Ltd. (Maebashi, Japan). Smad7 cDNA is a gift from Drs. A. Nakao and P. ten Dijke (Uppsala, Sweden).

Cell cultures. C2C12 cells, C3H10T1/2 cells (clone 8), and mink lung epithelial cells (Mv1Lu) were from American Type Culture Collection (Bethesda, MD, USA). F9 cells and ST2 cells were obtained from Riken Cell Bank (Tsukuba, Japan). Mv1Lu cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 units of penicillin and 50 μg of streptomycin per ml. C2C12 cells and F9 cells were cultured in DMEM with 15% FBS and the antibiotics. C3H10T1/2 cells and ST2 cells were cultured in Basal Medium Eagle with Earle's salts and RPMI 1640, respectively, containing 10% FBS and the antibiotics. When C2C12 cells were treated with BMP-2, OP-1 or TGF-β1, medium was replaced by DMEM containing 5% FBS and antibiotics. The cells were kept in 5% CO_2 humid atmosphere at 37°C.

Poly(A)$^+$ RNA isolation and Northern blotting. Total RNA was isolated from the cells by using Isogen (Wako), and poly(A)$^+$ RNA was obtained using Oligotex dT-30 Super latex beads (Takara Biochemicals) according to the manufacturer's method. Poly(A)$^+$ RNA (2 μg) from cells treated with BMP-2 (300 ng/ml), OP-1 (300 ng/ml), or TGF-β1 (25 ng/ml) for various time periods were electrophoresed in 1.2% agarose gels in the presence of 2.2 M formaldehyde, and blotted to Hybond N membranes (Amersham). The complete coding regions of mouse Smad6 and human Smad7 cDNAs were labelled by [α-^{32}P]dCTP using Random Primer Labelling Kit (Takara Biochemicals). Hybridization was performed in a solution containing 5 × SSC, 1% SDS, 5 × Denhardt's solution and 10 μg/ml salmon sperm DNA at 65°C overnight, and the filter was washed at 65°C with 2 × SSC, 1% SDS for 20 min twice, 0.5 × SSC, 1% SDS for 30 min, and 0.2 × SSC, 1% SDS for 10 min. The filters were stripped by boiled distilled water containing 0.1% SDS and rehybridized. The amounts of mRNAs were verified by rehybridizing the filters with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe (not shown). The signals were detected either by autoradiography or by Fuji BAS 2000 Bio-Imaging Analyzer (Fuji Photo Film).

RESULTS

Smad6 mRNA Is Induced by BMP-2 in Various BMP-Responsive Cells

Various BMP-responsive cells (16-19) were treated with BMP-2 (300 ng/ml) for 6 h, and expression of Smad6 mRNA was analyzed by Northern blotting. In all the cells investigated in the present study, mRNA for Smad6 of 3.0 kb was dramatically induced by the treatment with BMP-2 (Fig. 1A, left panel). The Smad6 message in F9 cells was relatively low and a result by a phosphoimager is also shown (Fig. 1A, right panel). In mouse muscle myoblast C2C12 cells, Smad6 mRNA was only very weakly detected in the untreated cells, but we could see strong induction of the Smad6 mRNA after 6 h with BMP-2. In contrast to the increase of the Smad6 mRNA, increase of the Smad7 mRNA was much less remarkable in these cells; in the C2C12 cells, Smad7 mRNA was slightly induced compared to the untreated cells, but not in the other cell types tested in the present study (Fig. 1B).

Induction of Smad6 mRNA was studied at various time intervals in C2C12 cells. Induction of Smad6 mRNA was observed at 3-24 h after the addition of BMP-2 or OP-1 (Fig. 2). In contrast, the expression of Smad7 mRNA was not significantly induced compared to Smad6 (data not shown). These data indicate that Smad6 expression is positively regulated by the members in BMP family, whereas Smad7 may not be directly regulated by BMPs.

Expression of Smad6 mRNA in Mv1Lu Cells Treated with TGF-β1

TGF-β1 was shown to induce expression of Smad7 in a TGF-β-responsive cell line, Mv1Lu (8). Regulation of the Smad6 mRNA expression by TGF-β1 was tested in the same cell line. Smad6 mRNA was moderately induced by TGF-β1 at 1 h. However, after 4-8 h with TGF-β1 stimulation, the expression level of Smad6 became less than that in the untreated cells (Fig. 3). Similar data regarding the transcriptional regulation of Smad6 by TGF-β1 could be observed in the C2C12 cells (data not shown), indicating that Smad6 is transiently induced after the TGF-β stimulation, but it is repressed afterwards.

DISCUSSION

Smad6, Smad7 and *Drosophila* Dad comprise a novel class in the Smad family. They share the MH2 (Mad homology 2) region conserved among the Smad proteins, but lack the MH1 region. Dad inhibits Dpp signaling (13) and Smad6 and Smad7 inhibit TGF-β and/or BMP signals (7–9). Dad was initially cloned as a molecule whose transcription was induced by Dpp, and was subsequently demonstrated to block Dpp activity. It was thus proposed that Dad plays a role of negative feedback in Dpp signaling. Likewise, the expression of Smad7 was shown to be induced by TGF-β and thus Smad7 may participate in an autoregulatory feedback circuit in TGF-β signaling. Here we have shown that the expression of Smad6 is induced by BMPs.

Smad6 mRNA was remarkably induced by BMPs in all the cells tested in this study, whereas that of Smad7 remained uninduced in most of the cells. The induction of Smad6 by BMPs increased at least until 24 hours after ligand stimulation. Thus, BMPs seem to consistently induce Smad6 but not Smad7. TGF-β1 greatly upregulated the expression of Smad7 (8). The expression of Smad6 was also induced by TGF-β1 in Mv1Lu

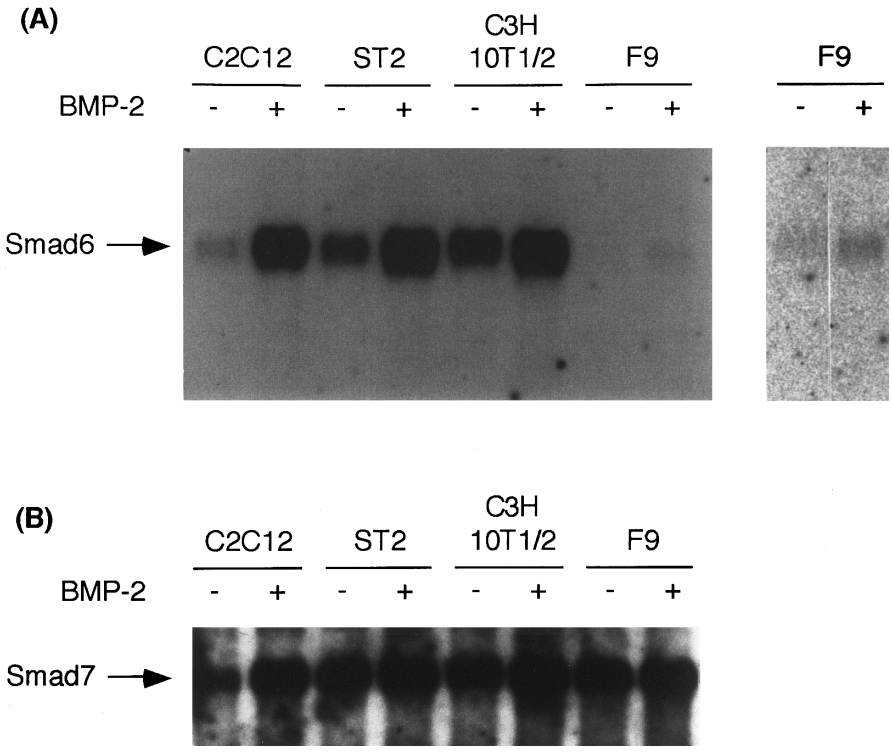

FIG. 1. Induction of Smad6 and Smad7 mRNA in various cell lines. Mouse muscle myoblast C2C12 cells, ST2 mouse bone marrow stromal cells, mouse embryo fibroblast C3H10T1/2 cells, and F9 mouse embryonal carcinoma cells were treated for 6 h with 300 ng/ml of BMP-2. mRNAs were isolated, electrophoresed in an agarose-formaldehyde gel, hybridized with Smad6 (A) or Smad7 (B) probes, and subjected to autoradiography. The right panel of (A) is the detection of Smad6 message in F9 cells by a phosphoimager.

cells, but the induction was transient and less than that by BMPs in various BMP-responsive cells. Taken together, Smad6 may form a feedback circuit that regulate BMP signals, whereas Smad7 seems to play a similar role in TGF-β signaling.

Autoregulatory signals have been demonstrated in other signaling systems. Recently, a family of SH2 proteins, SOCS/JAB/SSI, have been shown to act as antagonists of the JAK-STAT signaling pathways (20–22). Moreover, they are induced by cytokine signals, indicating that similar to inhibitory Smads, SOCS/JAB/SSI proteins act as autoregulatory switch-off signals.

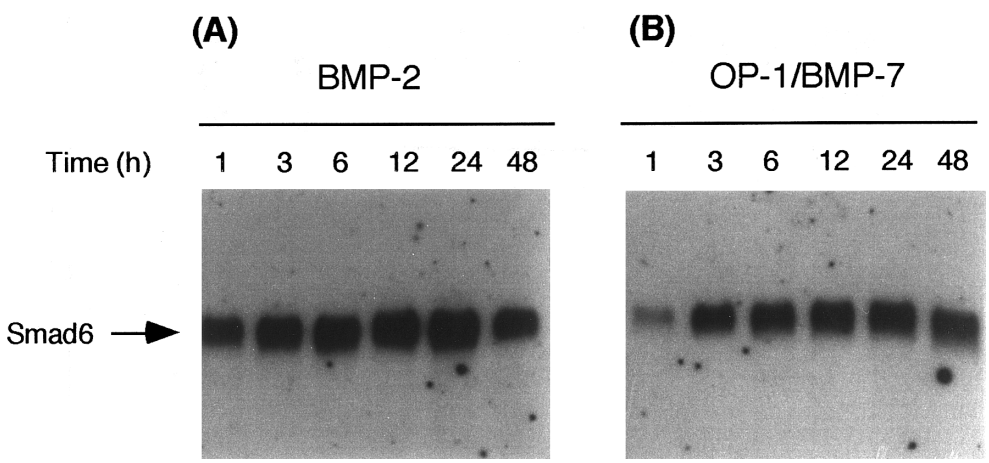

FIG. 2. Induction of the Smad6 mRNA by BMP-2 or BMP-7/OP-1 in C2C12 cells. C2C12 cells were treated with BMP-2 (300 ng/ml) (A) or OP-1/BMP-7 (300 ng/ml) (B) for various time periods. mRNAs were separated in agarose-formaldehyde gels and hybridized with Smad6 probe.

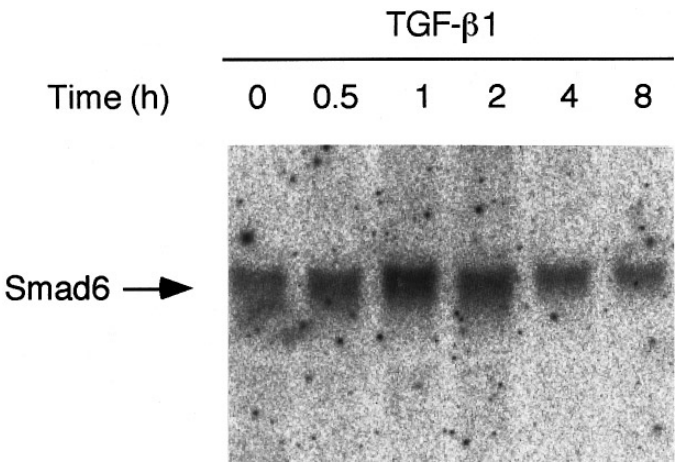

FIG. 3. Expression of Smad6 mRNA after treatment with TGF-β1 in Mv1Lu cells. Mv1Lu cells were treated with 25 ng/ml of TGF-β1 for various time periods. mRNAs were separated in an agarose-formaldehyde gel and hybridized with Smad6 probe.

Smad6 and Smad7 were shown to be induced by steady laminar shear stress in vascular endothelial cells (14). Our future question would be how these inhibitory Smads are regulated in physiological and pathological conditions *in vivo*.

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