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BMP signaling is responsible for serum-induced Id2 expression

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ABSTRACT

Ids function as negative regulators of basic helix–loop–helix transcription factors and their expression is rapidly induced by serum stimulation in various cell types. In this study, we investigated the molecular basis of serum-induced expression of the mouse *Id2* gene in NIH3T3 cells. A small-molecule inhibitor of bone morphogenetic protein (BMP) type I receptor kinases blocked the serum induction of *Id2* mRNA. The chemical compound and several inhibitory proteins specific for BMP signaling suppressed the serum-induced activation of the luciferase construct with the mouse *Id2* 4.6-kb promoter region. Importantly, serum stimulation evoked rapid phosphorylation of Smad1/5/8 and significant activation of the reporter plasmid containing the recently identified BMP-responsive element (BRE) of the mouse *Id2*. Mutation analysis demonstrated that the binding sites for Smad proteins in the *Id2* BRE were critical for serum response of the 4.6-kb whole construct. Gel shift and chromatin immunoprecipitation (ChIP) assays confirmed the serum-inducible binding of Smad1/5/8 and Smad4 to the *Id2* BRE *in vitro* and *in vivo*. Finally, a knockdown experiment revealed the functional importance of Smad1 in the serum induction of *Id2* expression. Thus, we concluded that BMP signaling is primarily responsible for the serum-induced *Id2* expression. Our results also suggest that some of the cellular effects caused by serum are mediated through BMP signaling.

1. Introduction

Id proteins are negative regulators of basic helix–loop–helix transcription factors that control cell fate determination. Four members of the Id family, Id1 to Id4, have been identified in mammals. Extensive analyses of knock-out mice have demonstrated that Ids play essential roles in various processes including neurogenesis, angiogenesis, and immune cell development [1].

The expression of the *Id* genes is up-regulated in response to diverse stimuli [1]. Above all, it is rapidly induced by stimulation with serum [2–5]. While serum stimulates cell proliferation, it maintains stem or progenitor cells in undifferentiated states. Deprivation of serum from culture medium induces differentiation of embryonic stem cells into neural precursors [5] and of myoblasts into myotubes [2]. These effects are tightly associated with down-regulation of the *Id* genes, suggesting that Ids function as key molecules to prevent differentiation in these cells. Although serum exerts the cellular effects through multiple signaling pathways, it remains unclear which pathway is responsible for the serum-induced *Id* gene expression.

Another representative stimulus that can induce *Id* gene expression is bone morphogenetic protein (BMP), which displays pleiotropic activities in cell differentiation, pluripotency, and tissue morphogenesis [6]. BMP signaling is initiated by binding of the ligands to their transmembrane type I and type II receptors [6]. Both types of receptors encode serine/threonine kinases and the activated type I receptor phosphorylates the receptor-regulated (R) Smads, Smad1, Smad5, and Smad8, in the cytoplasm. The phosphorylated R-Smads form complexes with the common-partner (Co) Smad, Smad4, and then move into the nucleus, where the complexes bind to the regulatory regions of the downstream target genes [6]. Recently, we have identified the BMP-responsive element (BRE) in the promoter region of the mouse *Id2* gene [7]. Similar BREs are present in those of *Id1* [8] and *Id3* [9].

Here we provide evidence that BMP signaling is a primary pathway responsible for serum-induced expression of the *Id2* gene. We show that inhibition of BMP signaling leads to a significant decrease in the serum induction of *Id2* expression and demonstrate

Abbreviations: BMP, bone morphogenetic protein; BRE, BMP-responsive element; ChIP, chromatin immunoprecipitation; R-Smad, receptor-regulated Smad; Co-Smad, common-partner Smad; FBS, fetal bovine serum; TGF-β, transforming growth factor-β; dn, dominant negative.

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that Smad proteins play a pivotal role. Our results suggest that some of the cellular effects caused by serum are mediated through BMP signaling.

2. Materials and methods

2.1. Cell culture, drugs, and antibodies

Mouse fibroblast NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT). For serum stimulation, cells were starved by culture in DMEM containing 0.2% FBS for 24 h and the medium was then replaced with DMEM containing 10% FBS. PD98059 and SB203580 were purchased from Calbiochem (San Diego, CA). SP600125, LY294002, dorsomorphin, SB431542, puromycin, and normal rabbit IgG were from Sigma–Aldrich (St. Louis, MO). Antibodies against phosphorylated Smad1/5/8 (#9511; Cell Signaling, Beverly, MA), Smad1/5/8 (N-18), Smad4 (B-8), NF-YA (H-209) (Santa Cruz Biotechnology, Santa Cruz, CA), NF-YB (Diagnode, Liège, Belgium), and Smad1 (Zymed, South San Francisco, CA) were used.

2.2. Western blot analysis

Western blot analysis was basically carried out as described previously [10]. Forty micrograms of total protein was resolved by 10% SDS–PAGE, transferred onto a nitrocellulose filter, and immunoblotted with various antibodies.

2.3. RNA isolation, Northern blot analysis, and RT-PCR

Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA). For Northern blot analysis, 5 μg of RNA was separated by electrophoresis on a 1.2% agarose-formaldehyde gel and transferred onto a nylon membrane using standard protocols. The membrane was hybridized with [α-^{32}P]dCTP-labeled DNA probes for mouse *Id2* and *Gapdh* [7]. For semiquantitative RT-PCR analysis, reverse transcription and PCR were executed as described previously [11], with slight modification. Cycle conditions were as follows: 30 or 35 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min. The primers used for PCR are listed in Supplementary Table 1.

2.4. Plasmids

All the firefly luciferase plasmids were constructed using pGL4.12 (Promega, Madison, WI). pGL4.12-Id2/4.6-kb was as previously reported [12] and pGL4.12-Id2/BRE was derived from pGL3-Id2/BRE [7]. Mutations of the BRE were introduced into pGL4.12-Id2/4.6-kb by site-directed mutagenesis. As an internal control, phRL-TK (Promega) was used. The coding regions of mouse ALK1, ALK2, ALK3, and ALK5 were amplified by RT-PCR. Their dominant-negative forms, ALK1(K228R), ALK2(K235R), ALK3(K261R), and ALK5(K232R), were generated by substituting arginine for the critical lysine in the kinase domains and then subcloned into pFLAG-CMV-14 (Sigma). pcDNA3-FLAG-mouse Smad6 was kindly provided by Dr. K. Tsuchida (Fujita Health University). To silence the mouse Smad1 expression, the target sequence was chosen as follows: 5′-GGATGGACAAGTCAGACAG-3′ [13]. The annealed oligonucleotides were inserted into pSUPER-puro (Oligoengine, Madison, WI) to create pSUPER-mSmad1.

2.5. Luciferase assay

NIH3T3 cells were transfected with plasmids using FuGENE 6 reagent (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. After 24 h, the cells were serum-starved by culture in DMEM containing 0.2% FBS for a further 24 h. The medium was then replaced with fresh DMEM containing 10% FBS and the cells were incubated for an additional 8 h. Luciferase assays were performed using the Dual-Glo Luciferase Assay System (Promega), as previously described [10]. The firefly luciferase activity was measured and normalized to the renilla luciferase activity conferred by phRL-TK.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as described previously [7]. Ten micrograms of nuclear extract incubated with [γ-^{32}P]dATP-labeled oligonucleotides was electrophoresed on a 5% polyacrylamide gel. For the supershift experiment, 2 μg of anti-Smad1/5/8 or 0.5 μg of anti-Smad4 antibody was added to the reaction.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted as described previously [7,12]. In brief, cells were cross-linked with 1% formaldehyde in medium for 10 min at room temperature, followed by quenching with 125 mM glycine for 5 min. Two and half micrograms of each antibody was used for immunoprecipitation. The samples were resuspended in 100 μl of TE for input DNA and 20 μl of TE for ChIP DNA, and analyzed by PCR using the following primer pairs: BRE, 5′-CTTGCAGGCATTGATCAGCTG-3′ (−2903) and 5′-TTTTGTTCTCAC AGCTGTGTCCATT-3′ (−2630); proximal promoter, 5′-ACACTGTACT CAATTTGCCACCC-3′ (−226) and 5′-GCTGCTCGTAGGAGGAGAG ACC-3′ (+76).

2.8. RNA interference

NIH3T3 cells were transfected with pSUPER-mSmad1 or the control vector using FuGENE HD reagent (Roche). Two days after transfection, the cells were subjected to puromycin (2 μg/ml) selection for 10 days. Drug-resistant clones were then isolated and the positive clones were selected by immunoblotting with anti-Smad1 antibody.

3. Results

3.1. Dorsomorphin suppresses serum-induced expression of the mouse Id2 gene

It has been reported that expression of the human and mouse *Id2* genes is rapidly induced by serum stimulation in various cell types [2,4,12]. However, it remains to be determined which intracellular signaling pathway is responsible for serum-induced *Id2* gene expression. To address this issue, we treated serum-stimulated NIH3T3 cells with several kinase inhibitors: PD98059 for MEK, SB203580 for p38 MAPK, SP600125 for JNK, LY294002 for PI3K, dorsomorphin for the BMP type I receptors, and SB431542 for the transforming growth factor-β (TGF-β) type I receptors. Northern blot analysis showed that dorsomorphin completely blocked the induction of *Id2* mRNA, whereas the other inhibitors did not reduce it (Fig. 1A). We also examined the effect of dorsomorphin on the serum-induced *Id2* expression using the luciferase construct with the mouse *Id2* 4.6-kb promoter region (see Fig. 3A). The reporter plasmid exhibited a marked response to serum (about 16-fold increase) and the response was suppressed by dorsomorphin in a dose-dependent manner (Fig. 1B). These observations suggest that BMP signaling is involved in the serum induction of *Id2* expression.