Smad7 Is an Activin-inducible Inhibitor of Activin-induced Growth Arrest and Apoptosis in Mouse B Cells*

(Received for publication, April 10, 1998, and in revised form, June 30, 1998)

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Members of the transforming growth factor-β (TGF-β) family, which includes the activins, relay signals from serine/threonine kinase receptors in membrane to nucleus via intracellular Smad- and Mad-related (Smad) proteins. Inhibitory Smad proteins were found to prevent the interaction between the serine/threonine kinase receptors and pathway-restricted Smad proteins. Smad7 was identified as a TGF-β-inducible antagonist of TGF-β signaling, and it may participate in a negative feedback loop to control TGF-β signaling. Here we demonstrate that the mRNA expression of Smad7 is induced by activin A in mouse B cell hybridoma HS-72 cells, which undergo growth arrest and apoptosis upon exposure to activin A. The ectopic expression of mouse Smad7 in HS-72 cells suppressed the activin A-induced cell cycle arrest in the G1 phase by abolishing the activin A-induced expression of p21cip1/14-3-3, and hypophosphorylation of retinoblastoma protein. Furthermore, Smad7 expression suppressed activin A-induced apoptosis in HS-72 cells. Thus, our data indicate that Smad7 is an activin A-inducible antagonist of activin A-induced growth arrest and apoptosis of B lineage cells.

Activins belong to the transforming growth factor-β (TGF-β) family (1) and were originally isolated as factors stimulating secretion of a follicle-stimulating hormone from anterior pituitary cells (2, 3). In addition to regulation of the reproductively endocrine system, activins are also implicated in regulation of erythroid differentiation (4), mesoderm induction of an embryo (5–8), and negative cell growth of various cell types, including gonadal cells and adrenal cells (9–12). Recently, activin A has been isolated from the cultured media of activated mouse macrophages as a factor that inhibits the growth of plasmacytic cells, including mouse B cell hybridomas and mouse and human myeloma cells (13–15). We have reported that activin A induces the cell cycle arrest in the G1 phase and apoptosis in mouse B cell hybridomas HS-72 cells (16) and that signals for both growth arrest and apoptosis induced by activin A are mediated through a type IB activin receptor (ActR-IB) in HS-72 cells (17). However, regulation of intracellular signal transduction for activin remains to be clarified.

TGF-β family members elicit their multifunctional effects through heteromeric complexes of type I and type II serine/threonine kinase receptors (18–20). Two type I receptors, i.e. ActR-I and in particular ActR-IB (21–24) and two type II receptors, i.e. ActR-II and ActR-IB (25–27), have been implicated in transducing activin signals. Upon activin binding to type II receptor with constitutively active kinase, the type I receptor is recruited and phosphorylated and activated by type II kinase receptor (28, 29).

Members of the Smad family are known to play pivotal roles in intracellular TGF-β signaling (30). Smad1, Smad2, Smad3, and Smad5 become phosphorylated by specific activated type I serine/threonine kinase receptors and thus act in a pathway-restricted fashion. Smad4 forms hetero-oligomeric complexes with pathway-restricted Smad proteins, which translocate into the nucleus and activate transcriptional responses. Smad6 and Smad7 function as inhibitors of TGF-β family signaling (31–33). Smad7 is most closely related to Smad6, with 36 and 56% sequence identities in the amino-terminal domain and the carboxyl-terminal Mad homology 2 domain, respectively. They bind to type I receptors and interfere with the phosphorylation of the pathway-restricted Smad proteins (30–33) or interfere with complex formation between pathway-restricted and common-mediator Smad proteins (34).

In this study, the levels of Smad7 mRNA were examined in HS-72 cells stimulated with activin A. In addition, HS-72 cells were transfected with mouse Smad7 expression plasmid to examine the effect of Smad7 on activin A-induced responses. Here we report the functional role of Smad7 in the regulation of growth arrest and apoptosis induced by activin A.

EXPERIMENTAL PROCEDURES

Cell Culture—Establishment and characterization of mouse hybridoma HS-72 cells were described previously (13). HS-72 cells were cultured in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc.) containing 10% heat-inactivated fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Plasmid and Transfection—Construction of the Smad7 expression plasmid pcDNA3-FLAG-Smad7 was reported previously (31). HS-72 cells were transfected with plasmid by electroporation using an Electroprober II (InVitrogen, San Diego, CA) at 200 V, 1000 microfarads, and then selected by cultivation with G418 (1 mg/ml). Single-cell clones were obtained by limiting dilution.

MTT Assay—The cells (2 × 10³ cells/well in 96-well plates) were incubated with Iscove’s modified Dulbecco’s medium containing 5%
Smad7 Inhibits Activin-induced G1 Arrest and Apoptosis

RESULTS AND DISCUSSION

Northern blot analysis of Smad7 revealed that Smad7 mRNA was induced in HS-72 cells stimulated with activin A (Fig. 1). The expression of Smad7 mRNA was most vigorously induced after 3 h of activin A stimulation, and decreased time-dependently, indicating that Smad7 is an activin A-responsive gene. TGF-β1 and activin A were found to induce Smad7 mRNA expression in the mink epithelial cell line Mv1Lu (31).

Since the kinase domains of ActR-IB and type I TGF-β receptor (TβRI) are nearly identical (24), activin A and TGF-β may share the same or similar signals for induction of Smad7 mRNA expression.

To investigate whether Smad7 controls the growth arrest and apoptosis induced by activin A, HS-72 cells were stably transfected with mouse Smad7 expression plasmid (pcDNA3-FLAG-Smad7) or control plasmid (pcDNA3). Four clones (HS-72SM1, HS-72SM2, HS-72SM3, HS-72SM4) were

Fig. 1. Activin A induces Smad7 mRNA in HS-72 cells. HS-72 cells were exposed to activin A (50 ng/ml) for various times. The total RNA (10 μg) were separated and analyzed for levels of Smad7 mRNA by Northern blotting. The same blot was serially hybridized with GAPDH cDNA probe.

fetal calf serum and antibiotics in the presence of various concentrations of activin A or human bone morphogenetic protein (hBMP)-2 for 48 h and then examined for cell viability by a calorimetric assay with MTT (Sigma) (13). Absorbance was determined at a wavelength of 570 nm with background subtraction at 620 nm.

Detection of Apoptotic Cells—To detect apoptotic nuclei, the cells (2 × 10⁶) were suspended in a hypotonic solution (3.4 mM sodium citrate, 0.1% Triton X-100, 0.1 mM EDTA, 1 mM Tris-HCl (pH 8.0)), stained with 5 μg/ml propidium iodide and analyzed by a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) (35). In the DNA fragmentation assay, the DNA was extracted from HS-72 cells (5 × 10⁶) according to the method of Moore et al. (36). The DNA was electrophoresed in a 2% agarose gel and stained with ethidium bromide.

Immunoblot Analysis—Anti-FLAG monoclonal antibody (anti-FLAG M2) was purchased from Estman Kodak Co. Anti-retinoblastoma protein (Rb) monoclonal antibody (G3-245) was purchased from PharMingen (San Diego, CA). Anti-p21CIP1/WAF1 monoclonal antibody (Ab-4) was purchased from Calbiochem-Novabiochem Corp. The cells were lysed in 50 mM Tris-HCl (pH 6.8), 2% SDS, boiled for 5 min, and then centrifuged at 12,000 × g. The extracted proteins (60 μg) in supernatants were separated in 7, 10, or 12.5% polyacrylamide gels containing 0.1% SDS and then electroblotted on polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). Immunodetection was performed using an ECL Western blotting detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). Blots were stained with Coomassie Brilliant Blue, and each lane was confirmed to contain a similar amount of protein.

Northern Blot Analysis—Total RNA was extracted from the cells using a phenol RNA extraction kit (Nippon Gene, Tokyo, Japan). Five or ten micrograms of total RNA were electrophoresed in a formaldehyde-agarose gel and blotted on a nylon membrane (Hybond-N or ten micrograms of total RNA were electrophoresed in a formaldehyde-agarose gel and blotted on a nylon membrane (Hybond-N) using an Isogen RNA extraction kit (Nippon Gene, Tokyo, Japan). Five micrograms of total RNA were electrophoresed using a formaldehyde-agarose gel and blotted on a nylon membrane (Hybond-N). Antibodies were detected using ECL Western blotting detection system (Amersham Pharmacia Biotech). Mouse Smad7, mouse p21 CIP1/WAF1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA fragments were isolated from pcDNA3-FLAG-Smad7 (31), pCMW35T3, and pK5321 (37), respectively, and labeled with [α-32P]dCTP using a Multiprime DNA labeling system (Amersham Pharmacia Biotech). Hybridization and washing were performed as described previously (38).

RESULTS AND DISCUSSION

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Fig. 2. Ectopic expression of Smad7 blocks activin A-induced G1 arrest in HS-72 cells. A, HS-72 cells were transfected with plasmid pcDNA3-FLAG-Smad7. FLAG-Smad7 expression was detected using anti-FLAG monoclonal antibody by immunoblotting. B, the cells were cultured with various concentrations of activin A for 48 h, and the cell viabilities were monitored by a MTT assay. Percent viability was calculated by the following formula: percent viability = 100 × (A₅₇₀–₆₂₀ nm without activin A)/ A₅₇₀–₆₂₀ nm with activin A) × 100. C, the DNA content was analyzed by flow cytometry.
found to express high levels of mouse FLAG-Smad7 (48 kDa) (Fig. 2A). Control clones (HS-72C1, HS-72C2, HS-72C3, HS-72C4) showed growth arrest in response to activin A, however, Smad7 overexpressing clones showed no growth arrest in response to activin A in a MTT assay (Fig. 2B). Furthermore, cultivation with activin A for 24 h increased the population of the cells in the G1 phase from 33 to 81% with a reduction of those in the S phase from 61 to 11% in HS-72C4 cells. In contrast, activin A showed no effect on the cell cycle distributions of Smad7 overexpressing clones (Fig. 2C). These results indicate that an overexpression of Smad7 in HS-72 cells abolishes the cell cycle arrest in the G1 phase caused by the activin A treatment.

p21CIP1/WAF1 is known to suppress the activity of cyclin-dependent kinase (CDK)-4 (39). We have demonstrated that activin A induces the expression of p21CIP1/WAF1 and blocks the Rb kinase activity of CDK-4, resulting in the accumulation of hypophosphorylated Rb (pRb) (16). pRb binds to, and negatively regulates, the activities of transcriptional factors of the E2F family, whose function is important for the G1 to S transition (40, 41).

To obtain more insight into mechanism by which Smad7 suppresses the activin A-induced cell cycle arrest in the G1 phase, we examined the effect of Smad7 on the activin A-induced expression of p21CIP1/WAF1 by immunoblot and Northern blot analyses. Immunoblot analysis showed that the untreated control clone (HS-72C4) contained undetectable levels of p21CIP1/WAF1 (21 kDa) (Fig. 3A). Upon exposure to activin A (50 ng/ml), expression of p21CIP1/WAF1 (21 kDa) was detected as early as 6 h, and its level increased time-dependently in the control clone. However, Smad7 overexpressing clones (HS-72SM1 and HS-72SM2) contained undetectable levels of p21CIP1/WAF1, even when cultured with activin A (50 ng/ml). Northern blot analysis showed that p21CIP1/WAF1 mRNA was rapidly induced in the control clone (HS-72C4) after 3 h of activin A stimulation (Fig. 3B). However, p21CIP1/WAF1 mRNA was not detected in the Smad7 overexpressing clone (HS-72SM1 and HS-72SM2), even when cultured with activin A (50 ng/ml). These results suggest that Smad7 abolishes p21CIP1/WAF1 expression by attenuating the activin signal, which induces the p21CIP1/WAF1 mRNA expression in HS-72 cells. We examined the effect of Smad7 on activin A-induced hypophosphorylation of Rb by immunoblot analysis. As shown in Fig. 3C, activin A (50 ng/ml) decreased the levels of hyperphosphorylated Rb (ppRb) in the control clone (HS-72C4) at 12 and 24 h, resulting in an increase in the levels of pRb time-dependently. On the contrary, activin A showed no effect on the phosphorylation status of Rb in Smad7 expressing clones (HS-72SM1 and HS-72SM2) during the 24-h culture. Taken together, these findings suggest that the decreased level of p21CIP1/WAF1 by overexpression of Smad7 in HS-72 cells results in continuous phosphorylation of Rb by activated CDK-4.

In addition, we examined the effect of Smad7 on activin A-induced apoptosis, which is usually seen after the cell cycle arrest in the G1 phase. Gel electrophoresis of cellular DNA showed that fragmented DNA was detected much less in ac-
Smad7 Inhibits Activin-induced G1 Arrest and Apoptosis

We examined the effect of TGF-β family, such as TGF-β, BMP-2, and inhibit, on the responses of HS-72 cells. Among these factors, hBMP-2 induced the growth arrest in HS-72 cells, and the overexpression of Smad7 in HS-72 cells inhibited the hBMP-induced growth arrest (data not shown). The precise mechanism by which Smad7 suppresses the hBMP-2-induced growth arrest is under the investigation in our laboratory. We have reported that concanamycin A induces apoptotic cell death of HS-72 cells (42). Concanamycin A is known as a strong inhibitor of cellular ATPases. We have reported that concanamycin A induces apoptotic cell death of HS-72 cells (42). In this study, we found that concanamycin A sensitivities of Smad7 overexpressing cells (HS-72SM1, HS-72SM2) were essentially the same as that of the control clone (HS-72C4) concerning the induction of apoptosis (data not shown). These results suggest that the ability of activin A and concanamycin A to promote apoptosis could be differently regulated in HS-72 cells.

In summary, we demonstrated that Smad7 mRNA in HS-72 cells was induced in response to activin A stimulation. Over-expression of mouse Smad7 was found to suppress activin A-induced cell cycle arrest in the G1 phase by abolishing the activin A-induced expression of p21CIP1/WAF1 and hypophosphorylation of Rb. Furthermore, Smad7 suppressed activin A-induced growth arrest in HS-72 cells. These results indicate that Smad7 may play an important role in regulating the growth arrest and apoptosis induced by activin A stimulation.

Acknowledgment—We thank Y. Eto (Ajinomoto Co., Ltd.) for kindly providing us with activin A. We thank Yamamouchi Pharmaceutical Co., Ltd. for kindly providing us with hBMP-2.

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