Biosci. Biotechnol. Biochem., **67** (9), 2042–2044, 2003

Note

Activin A Induces Phosphorylation of Smad2 but Not Complex Formation of Smad2 with Smad4 in Human Colon Cancer Cell Line HT-29

Pimara PHOLNUKULKIT, Kei SONOYAMA,† and Jun KAWABATA

Laboratory of Food Biochemistry, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

Received April 23, 2003; Accepted June 10, 2003

Western blotting coupled with immunoprecipitation showed that activin A treatment induced phosphorylation of Smad2 but not complex formation of Smad2/4 in human colon cancer-derived HT-29 cells. Because HT-29 cells expressed neither Smad4 mRNA nor Smad4 protein, it is suggested that deletion of Smad4 leads to a defect of formation of Smad2/4 complex upon activin A stimulation in HT-29 cells.

Key words: activin A; Smad2; Smad4; colon cancer; HT-29

Activins, which are members of the transforming growth factor-β (TGF-β) superfamily, have been shown to regulate a number of different cell functions.[1,2] Our previous study suggested that activin A plays a role in the regulation of the cell renewal process in the intestinal epithelium *via* an autocrine/paracrine mechanism.[3] Theoretically, it would be possible that the activin A pathway is related to colorectal carcinogenesis, since disregulation of the renewal process in the epithelium is pathologic of colorectal cancer.

TGF-β/activin A signal transduction is mediated by a heterotetrameric receptor complex consisting of two ligand-binding type II receptors and two signal transducing type I receptors.[4] Once the ligand binds to the type II receptor, it phosphorylates the type I receptor on multiple serine and threonine residues. To transmit the signal intracellularly, type I receptors then phosphorylate Smad2 and Smad3 proteins. Phosphorylation of Smad2/3 results in the release of Smad2/3 from the receptor complex and then the association with Smad4. The Smad2/3/4 complex translocates to the nucleus, whereupon transcriptional activation of a variety of genes occurs. Therefore some disregulations in this signaling pathway of TGF-β/activin A would be possibly associated with carcinogenesis. Actually, mutations of Smad4[5] and Smad2[6] have been reported in colorectal tumors, and Smad3 mutant mice uniformly developed metastatic colorectal cancer.[7]

It has been generally known that cancer cells are resistant to the growth-suppressing effect of TGF-β/activin A. Indeed, we observed that cell cycle of the human colon cancer cell line HT-29 was unaffected by treatment with activin A,[8] while DNA synthesis of the nontransformed rat intestinal cell line IEC-6 was significantly suppressed by activin A.[3] In this study, therefore, comparisons were made of activin A signaling in HT-29 and IEC-6 cells.

HT-29 and IEC-6 cells were maintained under standard conditions as previously described.[8] Preconfluent cells were cultured in the serum-free medium for 3 h, followed by stimulation with 100 ng/ml of recombinant human activin A (Genzyme, MA, U.S.A.) in fresh serum-free medium for 0, 5, 15, 30, 60, and 120 min. Cells were then lysed for 1 h on ice in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 150 mM NaCl, 0.25% sodium deoxycholate, 1% NP40, and 1 tablet/10 ml of a protease inhibitor cocktail tablet (Complete Mini, Roche, Mannheim, Germany). Cell lysates were cleared of debris by centrifugation and then immunoprecipitated with anti-Smad2/3 antibody (Santa Cruz Biotechnology, CA, U.S.A.). The immunoprecipitates were put through 7.5% SDS-PAGE followed by electrotransfer to nitrocellulose membranes (Hybond C extra, Amersham Biosciences, Tokyo, Japan). Blots were probed with either anti-Smad2 antibody (Santa Cruz Biotechnology), anti-phosphorylated Smad2 antibody (Upstate Biotechnology, NY, U.S.A.), or anti-Smad4 antibody (Santa Cruz Biotechnology), and immune complexes were detected with horseradish peroxidase conjugated with specific secondary antiserum (Santa Cruz Biotechnology). Cell lysates without immunoprecipitation in untreated HT-29 and IEC-6 cells were also put through Western blotting using anti-Smad2 and anti-Smad4 antibodies. In a separate experiment, total RNA was isolated from preconfluent HT-29 and IEC-6 cells in addition to the human normal fibroblast cell line CCD-18 and put through RT-PCR for detection of

† To whom correspondence should be addressed. Fax: +81-11-706-2496; E-mail: ksnym@chem.agr.hokudai.ac.jp

Smad2 and Smad4 transcripts as previously described.[8] The products were analyzed by agarose gel electrophoresis. Smad2 cDNA (1440 bp) was amplified using the primers 5′-GGTAAGAACATGTCGTCCATC-3′ (forward) and 5′-TTTCCATGGGACTTGATTGG-3′ (reverse). Smad4 cDNA (1445 bp) was amplified using the primers 5′-ATACACCGACAAGCAATGACG -3′ (forward) and 5′-GCCAGCAGCAGCAGACAGACT -3′ (reverse).

Figure 1A shows the representatives of Western blots of immunoprecipitates with anti-Smad2/3 antibody. In both cell lines, Smad2 was detected throughout the incubation period, indicating successful immunoprecipitation. In nontransformed IEC-6 cells, the signals of Smad4 and phosphorylated Smad2 were first detected at 5 min and reached a maximum by 15–30 min. The results indicated the phosphorylation of Smad2 and formation of Smad2/4 complex upon activin A stimulation. In contrast, activin A treatment did not cause Smad4 to coprecipitate with Smad2 whereas Smad2 was phosphorylated in colon cancer-derived HT-29 cells (Fig. 1A). Thus it seems that activin A treatment in HT-29 cells induced the phosphorylation of Smad2 but not complex formation of Smad2 with Smad4. Western blotting of cell lysates (Fig. 1B) and RT-PCR analysis (Fig. 1C) showed that HT-29 cells expressed undetectable levels of Smad4 mRNA and protein. The results suggest that deletion of Smad4 leads to a defect of formation of Smad2/4 complex upon activin A stimulation in HT-29 cells. Therefore it would be possible that the unresponsiveness of HT-29 cells to the growth-suppressing effect of activin A is due to deletion of Smad4. The Smad4 mutation is not associated with only resistance to the growth-suppressing effect of TGF-β/activin A. Takaku *et al.* reported that introducing the Smad4 mutation into the Apc gene knockout mice, a model for human familial adenomatous polyposis, made intestinal polyps into malignant tumors showing an extensive stromal cell proliferation, submucosal invasion, cell type heterogeneity, and *in vivo* transplantability.[9] It was thus indicated that the Smad4 mutation plays a significant role in the malignant progression of colorectal tumors.

Very recently, Fink *et al.* demonstrated by immunofluorescence staining the translocation of Smad2/3 to the nucleus upon TGF-β stimulation in a colon cancer cell line, VACO-9M, which is Smad4 null.[10] The findings suggested that Smad4 was not required for nuclear translocation of Smad2/3. Because the phosphorylation of Smad2 upon activin A stimulation was demonstrated in HT-29 cells, which express undetectable levels of Smad4 in the present study, the phosphorylation may be a crucial step for the nuclear translocation of Smad2 (and probably also Smad3). However, the nuclear translocation of Smad2/3 without Smad4 was reportedly not sufficient for triggerring TGF-β-induced transcriptional responses,[10] even though it still remains debatable whether the nuclear translocation of Smad2/3 is a totally silent event. Understanding the relationship between the biological function of TGF-β/activin A and the intracellular signal transduction requires further investigation.

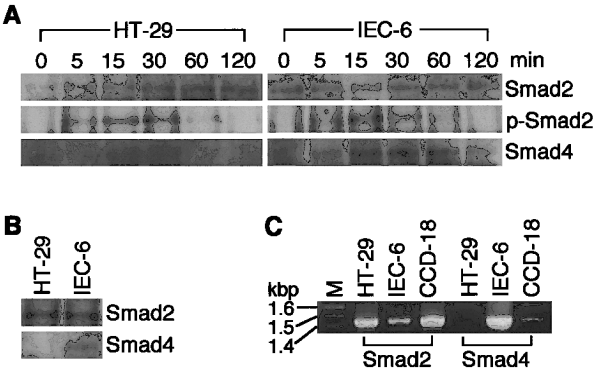

Fig. 1. Phosphorylation and Complex Formation (A), Protein (B) and mRNA (C) Expression of Smads in Human Colon Cancer-derived HT-29 Cells and Rat Nontransformed Intestinal IEC-6 Cells.

A: HT-29 and IEC-6 cells were incubated with 100 ng/ml of recombinant human activin A for the indicated times, and the cell lysates were immunoprecipitated with anti-Smad2/3 antibody and analyzed by Western blotting. The representative blots probed with Smad2, phosphorylated Smad2 (p-Smad2), and Smad4 are shown. B: Cell lysates from HT-29 and IEC-6 cells were analyzed by Western blotting. The representative blots probed with Smad2 and Smad4 are shown. C: Total RNA were isolated from the human normal fibroblast cell line CCD-18 as well as HT-29 and IEC-6 cells, and analyzed by RT-PCR to detect Smad2 (1440 bp) and Smad4 (1445 bp) transcripts. The representatives of ethidium bromide staining of RT-PCR products separated on agarose gel are shown. M represents the size marker (100 bp ladder).

Acknowledgments

This work was partly supported by a grant-aid from The Ministry of Education, Science, Sports, and Culture of Japan.

References

1) Kingsley, D. M., The TGF-β superfamily: New members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.*, **8**, 133–146 (1994).
2) Massagué, J., The transforming growth factor-β family. *Annu. Rev. Cell. Biol.*, **6**, 597–641 (1990).
3) Sonoyama, K., Rutatip, S., and Kasai, T., Gene expression of activin, activin receptors, and follistatin in intestinal epithelial cells. *Am. J. Physiol.*, **278**, G89–G97 (2000).
4) Massagué, J., TGF-beta signal transduction. *Annu. Rev. Biochem.*, **67**, 753–791 (1998).
5) Takagi, Y., Kohmura, H., Futamura, M., Kida, H.,

Tanemura, H., Shimokawa, K., and Saji, S., Somatic alterations of the DPC4 gene in human colorectal cancers *in vivo*. *Gastroenterology*, **111**, 1369–1372 (1996).
6) Eppert, K., Scherer, S. W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L. C., Bapat, B., Gallinger, S., Andrulis, I. L., Thomsen, G. H., Wrana, J. L., and Attisano, L., MADR2 maps to 18q21 and encodes a TGF-beta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell*, **86**, 543–552 (1996).
7) Zhu, Y., Richardson, J. A., Parada, L. F., and Graff, J. M., Smad3 mutant mice develop metastatic colorectal cancer. *Cell*, **94**, 703–714 (1998).
8) Sonoyama, K., Pholnukulkit, P., Toyoda, M., Rutatip, S., and Kasai, T., Upregulation of activin A gene by butyrate in human colon cancer cell lines. *Am. J. Physiol.*, **284**, G989–G995 (2003).
9) Takaku, K., Oshima, M., Miyoshi, H., Matsui, M., Seldin, M. F., and Taketo, M. M., Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell*, **92**, 645–656 (1998).
10) Fink, S. P., Mikkola, D., Willson, J. K. V., and Markowitz, S., TGF-β-induced nuclear localization of Smad2 and Smad3 in Smad4 null cancer cell lines. *Oncogene*, **22**, 1317–1323 (2003).