cDNA Cloning and Chromosomal Mapping of Rat Smad2 and Smad4 and Their Expression in Cultured Rat Articular Chondrocytes

MAKOTO OSAKI, TOMOO TSUKAZAKI*, NORIO ONO**, AKIHIKO YONEKURA, YASUHiro HIROTA, YOICHI MIYAZAKI, HIROYuki SHINDO, SHIN-ICHI SONTA** AND SHUNICHI YAMASHITA*

Department of Orthopaedic Surgery, Nagasaki University School of Medicine, Japan
*Department of Nature Medicine, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, Japan
**Department of Genetics, Institute for Developmental Research, Aichi Human Service Center

Abstract. Smad proteins are known to transduce signalling of TGF-β receptor superfamily. We report here the entire sequences of rat Smad2 and Smad4 which have not been identified yet. Entire sequences were identified by degenerated polymerase chain reaction and following phage library screening and 5′ RACE. The predicted amino acid sequences of rat Smad2 and Smad4 are highly conserved among rat, human and mouse. We also mapped these Smads to chromosome 18q12.3. Unlike endothelial cells, TGF-β1 stimulates articular chondrocyte proliferation as well as extracellular matrix production, and acts as a repairing agent against cartilage destruction. Since both Smad2 and Smad4 are essential factors for TGF-β signalling, we examined their expression and regulation in cultured articular chondrocytes. Northern blot analysis showed that TGF-β1 significantly increased the mRNA level of Smad2 but not of Smad4 in a dose- and time-dependent manner, suggesting that the augmentation of TGF-β1 action is caused by increasing the expression of the downstream signalling molecule.

Key words: Smad2, Smad4, Rat, Mapping, Chondrocyte

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Correspondence to: Tomoo Tsukazaki, M.D., Department of Nature Medicine, Atomic Bomb Disease Institute, Nagasaki University School of Medicine 1-12-1 Sakamoto, Nagasaki 852-8523, Japan

TYPE I transforming growth factor beta (TGF-β1), one of the members of TGF-β superfamily, is involved in a range of biological activities including cell growth, morphogenesis, development and immune responses [1, 2]. TGF-β mediates its signalling through the heteromeric complex of serine/threonine kinase type I and type II receptors. Upon ligand binding, constitutively active type II receptor phosphorylates the GS domain of type I receptor, resulting in the activation of type I receptor [3]. Recently Smad proteins have been identified as a direct substrate of the TGF-β receptor superfamily, in which Smad1 and Smad5 transduce signals from bone morphogenic proteins and Smad2 and Smad3 for TGF-β and activin, whereas Smad4 acts as a common signalling component [1, 4]. For example, phosphorylated Smad2 by the TGF-β type I receptor forms a heteromer with Smad4, and then translocates into the nucleus whereby this complex activates target genes [5].

The majority of reports concerning biological function of TGF-β1 have been focused on defining the mechanism of growth-inhibitory effect, but in some kinds of cells, TGF-β1 stimulates cell growth [6–11]. We have reported that TGF-β1 is a potent stimulator for chondrocyte cell growth as well as differentiation [12, 13]. However, the stimulatory mechanisms in chondrocytes have not been studied yet. Further, although the importance of Smad family proteins as signal transducer of TGF-β has been proved, their expression and function in cartilage are
not clear. To assess the mechanism of stimulatory effect of TGF-β1 signal transduction, we tried to isolate novel Smad proteins that are specific for chondrocytes.

We reported here the isolation of homologues of human Smad2 and Smad4 from rat articular chondrocyte cDNA library, although we could not isolate a novel Smad. We also studied the expression and regulation of Smad2 and Smad4 mRNAs in cultured rat articular chondrocytes.

Materials and Methods

Articular chondrocyte culture and RNA preparation

Articular chondrocytes were prepared from 5-week-old male Sprague-Dawley rats as described previously [12]. Isolated articular chondrocytes were cultured in monolayer and passaged once three days later. Total RNA was extracted from the subconfluent cells by RNA extraction kit Isogen (Wako, Japan)

Degenerated PCR and cloning of PCR products

A cDNA library in pGBT9 prepared from rat articular chondrocytes (made by Yonekura, unpublished data) was subjected to polymerase chain reaction (PCR) with degenerated primers [upstream primer; 5’-GT(A/C/G/T)GA(C/T)GG(A/C/G/T)T(A/T)(C/T)(A/G)(A/C/G/T)GA(C/T)CC-3’, downstream primer; 5’-CC(C/T)TT(A/C/G/T)AC(A/G)AA(A/C/G/T)(C/G)(A/T)CAT(A/C/G/T)C-3’] designed on conserved sequence of C-terminal domain of Smads. The PCR products were cloned into TA cloning vector and sequenced.

cDNA library and Phagemid library

A rZAP cDNA library from rat thyroid was screened with specific probes for Smad2 or Smad4 as described elsewhere [14].

5’ RACE

Using TAKARA 5’ RACE kit, cDNA was synthesized from rat articular chondrocyte according to the manufacturer’s instruction. Hybrid RNA was degenerated by RNase H and then single strand DNA was ligated by T4 RNA ligase. Nested PCR was performed with primers as shown in Figure 1 and 2. The PCR products were subcloned into TA cloning vector and sequenced.

Northern blot analysis

Twenty-five micrograms of total cellular RNA were electrophoresed in a 1.0% agarose gel under denaturing condition and transferred to Hybond-N nylon membrane. Specific probes for Smad2 and Smad4 were prepared by amplifying the linker regions by PCR. cDNA labeling and Northern blot were performed as described previously [12]. The membranes were washed under the high stringency condition. The intensity of each mRNA was quantitated by densitometric scanning of the autoradiographs using NIH Image.

Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) was carried out according to the procedures published previously [15, 16] with slight modifications [17]. Briefly, chromosome preparations for FISH were prepared from fibroblasts of male Wistar/Slc rat using the thymidine synchronization and BrdU release technique for R- and Q-bands. Denatured chromosomal DNA was hybridized with biotin-labeled almost full length cDNA, and hybridization signals were detected with avidin-FITC and biotinylated anti-avidin D antibody. FITC signals and R-bands on propidium iodide-stained chromosomes were visualized through a U-MWIB fluorescent cube (Olympus, Tokyo). For delineation of Q-band patterns by excitation with ultraviolet light, the same metaphase preparations were sequentially observed using a U-MWU fluorescent cube (Olympus, Tokyo). A numbering system of rat chromosomes at 400-band stage proposed by Satoh et al. was adopted [18].

Results

The analysis of the degenerative PCR products revealed four different types of Smad cDNAs despite
no evidence of a novel type of Smad in the rat articular chondrocyte cDNA library. As a result of sequencing among 70 subcloned PCR products, the number of Smad cDNAs was as follows: Smad1, 29; Smad2, 26; Smad4, 14; and Smad5, 1. As rat Smad1 and Smad5 have already been sequenced and our results of partial sequence were completely identical with them, we performed a clarification of entire sequences of Smad2 and Smad4 because of no report on rat Smad2 and Smad4. The rat Smad2 cDNA encodes 2113 bp and the predicted amino acid is 467 (Fig. 1A). Compared to the known human and mouse Smad2 protein, rat Smad2 shows complete match with that of human and mouse except for only 4 amino acids located in MH1 domain (Fig. 1B). The full length rat Smad4 cDNA is 3641 bp and predicted amino acid is 552. Rat Smad4 protein also shows a high homology with human and mouse (Fig. 2A and B).

FISH analysis using Smad2 and Smad4 probes resulted in a specific labeling on the long arm of rat chromosome 18 (Fig. 3). Of 97 metaphases examined, 74 (76.3%) showed 85 double-dot hybridization signals. These signals were specifically assigned.
to chromosome 18 at band q12.3. Sites with more than two signals were not observed other than the band 18q 12.3. These results of FISH indicate that both rat smad2 and 4 genes are located at band 18q12.3.

To confirm the presence of Smad2 and Smad4 mRNAs in articular chondrocytes, we extracted total RNA from the cultured rat articular chondrocytes and performed Northern blot analysis with specific Smad2 or Smad4 probe prepared from linker region. Rat articular chondrocyte had approximately 2.0 kb and 3.0 kb single transcript for Smad2 and Smad4, respectively (data not shown). We next studied the regulation of each Smad in response to TGF-β1 stimulation. Time course study with 0.1 ng/ml TGF-β1 showed that expression of Smad2 was potentiated after 1 hr of stimulation and this increase was steady until 12 hrs, whereas the increase of Smad4 was small compared with Smad2 (Fig. 4A). We also observed that the up-regulation of Smad2
and Smad4 expression was started from 0.1 ng/ml and was steady even at the higher concentrations of TGF-β1 (Fig. 4B).

**Discussion**

Recent advances in cloning of Smad families make it possible to realize the molecular mechanisms by which TGF-β can potentiate growth inhibition [1, 2]. Several candidate signallings have been suggested to be involved or interacted by cross-talking mechanism. However, the growth stimulatory mechanism of TGF-β action on articular chondrocytes is entirely unknown. We therefore tried at first to find a novel type of Smad gene families by degenerative homologous RT-PCR method, but all of our PCR products were known Smad cDNAs. However, we clarified the entire sequences of rat Smad2 and 4 cDNAs and examined the chromosomal mapping which has not yet been analyzed. From the predicted amino acid sequences we concluded these proteins to correspond with Smad2 and Smad4, respectively.

It is known that human Smad2 and Smad4 genes are closely linked to each other on chromosomal band 18q21.1 [4]. The present results of FISH indicate that these genes are also closely linked to each other in rat genome as found in human. These facts suggest that a region of rat chromosome 18 including the band q12.3 is synthetic to a region of the long arm of human chromosome 18.

TGF-β signalling requires the phosphorylation of Smad2 and following heterometric complex with Smad4. This pathway is specific for TGF-β and activin signalling but not for BMP signalling. Exogenous introduction of Smad2 and Smad4 results in synergistic activation of TGF-β signalling, whereas transfection of dominant negative form of these Smads diminishes the signalling [19, 20]. We have reported that TGF-β is a potent stimulator for
chondrocyte proliferation and differentiation [12]. Furthermore, TGF-β1 stimulates the production of extracellular matrix in vitro and in vivo [12, 21, 22]. These results indicate that Smad2 and Smad4 may be key regulators for chondrocyte proliferation. Therefore, we examined the expression and regulation of these Smads in cultured rat articular chondrocytes. The present study revealed that mRNA expression of Smad2 was significantly up-regulated by TGF-β1, while up-regulation of Smad4 was weak. These findings are inconsistent with the previous report observed in Mv1Lu and SW1736 cells [23], however, and may suggest that TGF-β1 augments its own action by stimulating Smad2 and Smad4 gene expression in cultured rat articular chondrocytes. Further experiments including intracellular cross-talking would be necessary to clarify the proliferative mechanism of TGF-β in articular chondrocytes.

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References


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