Structural basis for the functional difference between Smad2 and Smad3 in FAST-2 (forkhead activin signal transducer-2)-mediated transcription

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Smad2 and Smad3 are signalling proteins that are involved in mediating the transcriptional regulation of target genes downstream of transforming growth factor- β and activin receptors. Although they are structurally very similar, Smad2 and Smad3 have some functional differences in transducing signals for these receptors. In FAST-2 (forkhead activin signal transducer-2)-mediated transcriptional regulation using the activin-responsive element derived from *Xenopus* Mix.2 promoter as a reporter, Smad3 but not Smad2 alone was able to stimulate the transcription. In addition, Smad3 was able to inhibit the transactivation of the promoter induced by co-expression of Smad2, Smad4 and an active activin type-I receptor. We used a series of chimaeras between Smad1 and Smad3 and found that the Mad homology 1 (MH1) domain of Smad3 was indispensable for the dual regulatory function of Smad3. However, this Smad3-specific

function could not be manifested in Smad2 mutants that were devoid of the two amino acid insertions (at the MH1 domain) that comprise the major structural difference between Smad2 and Smad3, indicating that other structural motifs are involved in determining the regulatory activity of Smad3. By using chimaeras between Smad2 and Smad3, we found that the most N-terminal portion of Smad3 was crucial for its function. Taken together, these results suggest that, as compared with Smad2, the unique function of Smad3 in modulating the FAST-2-mediated transcription is contributed to by a subtle difference in the structural features at the MH1 domain.

Key words: activin, serine kinase, signal transduction, TGF- β , transcription factor.

INTRODUCTION

Smad proteins are intracellular signalling molecules that serve as a link between receptors and regulation of target genes in signal transduction by members of the transforming growth factor- β (TGF- β) superfamily of cytokines including TGF- β s, activin and bone morphogenetic proteins (BMPs) [1,2]. The receptors of these factors, after activation by ligand binding, interact with pathway-specific Smad proteins and activate them by phosphorylation via the intrinsic serine kinase activity of the receptors [3–5]. The phosphorylated Smads form hetero-oligomeric complexes with a common Smad, Smad4 [6], and the complexes migrate to the nucleus [7], in which the Smad complexes regulate the transcription of the target genes by two major mechanisms, either directly by DNA binding or indirectly by associating with other specific transcription factors [8]. The different functions of Smad proteins are fulfilled by their distinct structural motifs. Most Smad proteins are composed of three domains, the N-terminal Mad homology 1 domain (MH1 domain), the C-terminal MH2 domain and a linker domain in between [1,2]. The MH1 domain is mainly responsible for DNA binding. The MH2 contains transactivating activity and is also involved in homo- and hetero-oligomerization with other Smad proteins.

One of the major biological functions of TGF- β family members involves regulation of cell growth and proliferation [1]. The principal function of TGF- β s is to negatively regulate cell-cycle progression by induction of inhibitors of cyclin-dependent

kinases that are critical for G₁-to-S transition. This cell growth regulatory function of TGF- β is evidenced by the finding that many tumour cells lose their responsiveness to TGF- β 's negative effect on cell proliferation [1]. Disruption of TGF- β signalling in tumour cells can be achieved by different mutations at either the receptor or downstream signalling molecules. For example, mutations of TGF- β type-II receptor have been found in hereditary non-polyposis colorectal cancer (HNPCC) as a result of microsatellite instability caused by a defect of the mismatch-repair proteins [9]. Furthermore, recent studies have indicated that the Smad proteins downstream of TGF- β receptors also play an active role in cancer formation. Mutations or deletions of Smad4 have been reported in about half of human pancreatic cancers [6]. Smad4 mutations have also been observed in cancers of the breast, ovary and many other tissues [10]. Smad2 mutations are associated with human colorectal cancer [11]. Smad3-knock-out mice die after 4–6 months after developing colorectal carcinomas [12].

So far, eight distinct mammalian Smad proteins have been identified [1,2]. A large majority of functional and biochemical studies have indicated that both Smad2 and Smad3 are the cognate Smad proteins downstream of TGF- β as well as activin receptors. However, more recent studies have indicated that Smad2 and Smad3, although structurally similar, are functionally different. For example, Smad2 and Smad3 behave differently in regulating the transcription of a homeobox gene, *goosecoid*, through different interaction with Smad4 and a transcription factor FAST-2 (forkhead activin signal transducer-2) [13]. The

Abbreviations used: ALK, activin-like kinase; ARE, activin-responsive element; BMP, bone morphogenetic protein; FAST-2, forkhead activin signal transducer-2; PAI-1, plasminogen activator inhibitor-1; TGF- β , transforming growth factor- β ; MH1 domain, Mad homology 1 domain; AP-1, activator protein-1; HEK293, human embryonic kidney 293; CA, constitutively active.

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major structural difference between Smad2 and Smad3 is at the MH1 domain, in which Smad2 has two short insertions (or loops) in the most N-terminal part corresponding to the amino acids 21–30 and 79–108, as compared with Smad3 and other Smads. Analysis of the crystal structure of the Smad3 MH1 domain bound to DNA has indicated that the second insertion at the MH1 domain of Smad2 may interfere with DNA binding [14]. This may explain the finding that only the MH1 domain of Smad3 but not Smad2 is able to directly bind DNA [14–16]. Interestingly, deletion of the second loop of Smad2 is naturally present in mammalian cells as a product of alternative splicing with a deletion of exon 3 of the *Smad2* gene [17]. It was also found that the absence of this insertion in the Smad2 variant is correlated with a gain of function in binding the activator protein-1 (AP-1) sites of the p3TP-lux promoter, which contains three repeats of AP-1 elements and the plasminogen activator inhibitor-1 (PAI-1) promoter [17].

Although it appears relatively clear that the extra amino acid insertions may disrupt the DNA-binding ability of Smad2, the functional importance of this insertion with respect to other biological functions of Smad proteins remains uncharacterized. To further delineate the functional difference between Smad2 and Smad3, we investigated the importance of the MH1 domains of Smad2 and Smad3 as well as the two extra amino acid loops of Smad2 in the regulation of FAST-2-mediated transcriptional regulation. By analysing the functions of a series of chimaeras between Smad1 and Smad3, as well as chimaeras between Smad2 and Smad3, we have localized the structural determinants of these Smad proteins in modulating the FAST-2-mediated transcriptional regulation.

MATERIALS AND METHODS

Cell culture and cell transfection

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and supplemented with penicillin and streptomycin. Transient cell transfection was performed by the calcium phosphate method as described in [18].

Promoter assay

For the pAR3-lux promoter assay, about 5×10^4 cells/well were seeded in 6-well plates and transfected with different combinations of plasmid DNA. The pCMV- β -galactosidase gene was used as an internal control for the transfection efficiency. The pcDNA3 plasmid (Invitrogen) was used to make up a total of 5 μ g of DNA for the transfection in each well. The cells were harvested at 48 h after transfection by lysis with 400 μ l of Nonidet P40 lysis buffer [19]. The lysate was used in the β -galactosidase assay (20 μ l) as reported in [20] and in the luciferase assay (10 μ l) with a Promega luciferase assay kit and counted for 10 s in a luminometer (ZyLux).

Plasmid construction

Cloning of the mouse FAST-2, rat Smad1 and rat Smad3 genes have been reported before [18,20]. The constitutively active (CA) TGF- β family receptors including ALK (activin-like kinase)-2, ALK-3, ALK-4 and ALK-5 have been described before ([20–22], see also [21a]). All the plasmids used in this study were subcloned into pcDNA3 for expression in mammalian cells. In addition, DNA sequencing was performed for all the mutants to confirm that no sequence error was introduced. The details of the Smad chimaera and deletion constructs as well as the restriction sites

Figure 1 Generation of Smad mutants

The rat Smad1, mouse Smad2 and rat Smad3 were used to generate different chimaeras or deletion mutants used in this study. The amino acid positions at each junction for the chimaeras are indicated underneath. The restriction sites introduced by PCR or already present in the nucleotide sequence are indicated by arrows.

used are illustrated in Figure 1. To generate chimaeras between Smad1 and Smad3, a *SgrA*I restriction site was engineered into Smad3 at the same position as for Smad1 by PCR. In detail, the codons for amino acid residues 136–138 of Smad3 were changed from 5'-CCTCCAGTG-3' to 5'-CCACCGGTG-3' (containing a *SgrA*I site) to generate a same-sense mutation. A natural *SgrA*I site was present in Smad1, spanning the amino acid residues 136–138. In addition, a *Hpa*I site (5'-GTTAAC-3') was present in the corresponding positions of both Smad1 (spanning amino acid residues 320–321) and Smad3 (residues 277–278). Different combinations of Smad1/Smad3 chimaeras were then generated by swapping between the two genes by using the unique *SgrA*I and *Hpa*I sites.

To generate the first-loop deletion of Smad2, two nested primers (5'-GTCCATTCTGCTCTCCTTTTTCATCCAG-AAG-3' and 5'-CTTCTGGGATGGAAAAAGGAGAGCA-GAATGGAC-3') and two outside primers (covering 5' upstream and 3' downstream of the first loop of the *Smad2* gene) were used in PCR. To generate the second-loop deletion of Smad2, a similar strategy as for the first-loop deletion was used except that a *Sac*II site (5'-CCGCGG-3') was engineered into the position covering the amino acid residues 78–109 (after the second loop was deleted). The *Sac*II site at this position did not change the encoded amino acids.

To generate the chimaeras between Smad2 and Smad3, a *SgrA*I site was engineered into Smad2 using the same strategy as for Smad3 and this site covered the amino acid residues 176–178 of Smad2. A *Sac*II site was also engineered into Smad3 at amino

acid positions 68–69. The Smad2/Smad3 chimaeras were then generated by swapping between these two Smad genes using the unique *Sac*II and *Sgr*A1 sites.

RESULTS

Specificity of receptor serine kinases and Smad proteins in mediating FAST-2 stimulation of the Mix.2 activin-responsive element (ARE)

We and others recently cloned a mouse transcription factor, FAST-2 [13,20,23,24], that is closely related to the *Xenopus* FAST-1 ([25], see also [25a]). To further determine the specificity of mouse FAST-2 in transducing the signalling of receptor serine kinases of the TGF- β superfamily, we investigated the ability of FAST-2 to mediate induction of pAR3-lux in HEK293 cells by different Smad proteins and TGF- β family receptors. The pAR3-lux contains three tandem repeats of the ARE upstream of the luciferase gene. As shown in Figure 2, expression of a CA-ALK-3, a BMP2/4 type-I receptor, or its cognate Smad, Smad1 [26,27], was not able to stimulate pAR3-lux in the presence of mouse FAST-2. Addition of Smad4 with Smad1 and the CA-ALK-3 could not induce pAR3-lux stimulation either. Activation of the ALK-2 pathway by expression of CA-ALK-2 or its cognate Smad, Smad8 ([21], see also [21a]), was not able to stimulate the Mix.2 ARE in the presence of FAST-2. However,

Figure 2 Specificity of FAST-2 in mediating transcriptional regulation by different Smad proteins and receptor serine kinases

HEK293 cells were transiently transfected with vector (pcDNA3), pAR3-lux (0.2 μ g), pCMV- β -galactosidase (0.2 μ g), FAST-2 (0.6 μ g), different Smad-expressing plasmids (0.5 μ g) and different CA type-I receptors (1.0 μ g). The fold change of luciferase activity shown as means $\pm$ S.D. was derived by comparing with the value of the vector-transfected cells (set to 1) and normalized by β -galactosidase activity. The same experiment was performed three times with similar outcomes and the data from one representative experiment are shown here.

Figure 3 Smad3 inhibits CA-ALK-4- and Smad2-mediated Mix.2 ARE stimulation

HEK293 cells were transiently transfected with vector pcDNA3, pAR3-lux (0.2 μ g), pCMV- β -galactosidase (0.2 μ g), FAST-2 (0.6 μ g), CA-ALK-4 (0.02 μ g), Smad2 (0.2 μ g), Smad4 (0.2 μ g) and different amounts of Smad3 (0.05, 0.4, 1 and 4 μ g). The fold change of luciferase activity shown as means $\pm$ S.D. was derived by comparing with the value of the vector-transfected cells (set to 1) and normalized by β -galactosidase activity. The same experiment was performed more than three times with similar results.

expression of the CA activin type-I receptor (CA-ALK-4) or CA TGF- β type-I receptors (CA-ALK-5) were able to strongly stimulate ARE through FAST-2. Expression of Smad2 alone could slightly stimulate the promoter, whereas expression of Smad3 alone was able to moderately stimulate pAR3-lux in the presence of FAST-2, indicating the self-stimulatory function of Smad3. Smad4 could also synergize with Smad2 or Smad3 in the promoter stimulation. In addition, we also observed that the mouse FAST-2 is required for the stimulation of pAR3-lux by Smad3 or the activated TGF- β and activin type-I receptors because Smad3 and these receptors could not induce any transactivation of the promoter in the absence of FAST-2 (results not shown). Taken together, these data indicated that the mouse FAST-2 specifically mediates signalling downstream of TGF- β and activin receptors, but not BMP2/4 receptor and ALK-2.

Interestingly, we observed that co-expression of Smad2 and Smad4 could greatly enhance the ability of the activated ALK-4 and ALK-5 to stimulate the Mix.2 ARE. However, expression of the activated ALK-4 and ALK-5 had no additive effect on Smad3/Smad4-mediated pAR3-lux stimulation. Therefore, Smad3 and Smad2 behaved differently in the FAST-2-mediated Mix.2 ARE induction. Our data indicated that Smad3 alone is able to mediate FAST-2 induction of the Mix.2 ARE independent of receptor activation but Smad2 requires activation by TGF- β or activin receptors to mediate FAST-2 induction of the promoter.

Smad3 inhibits CA-ALK-4/Smad2/Smad4 stimulation of the ARE

To further investigate the role of Smad3 in mediating the transcriptional regulation of the Mix.2 promoter by FAST-2, we expressed different amounts of Smad3 in HEK293 cells and examined its ability to regulate CA-ALK-4- and Smad2-mediated Mix.2 ARE regulation. As shown in Figure 3, Smad3 expression

Figure 4 The MH1 domain of Smad3 is indispensable for its dual regulatory functions

Different wild-type or chimaeric Smads (2 µg/well) were transfected into HEK293 cells together with pAR3-lux, FAST-2 and other plasmids described in Figure 3 and the luciferase assay and data analysis were performed as for Figure 3. The relative positions of the MH1, linker and MH2 domains of Smad proteins are indicated at the top of the Smad1 diagram.

alone led to a receptor-independent activation of pAR3-lux in the presence of FAST-2. Expression of CA-ALK-4 with Smad2 and Smad4 could stimulate the promoter to about 80-fold. However, Smad3 was able to inhibit the stimulation of the Mix.2 ARE mediated by CA-ALK-4 and Smad2/Smad4 in a dose-dependent manner. We also found that Smad3 was able to inhibit the stimulation of the Mix.2 ARE mediated by CA-ALK-4 alone (results not shown). This phenomenon is very similar to what has been observed of the inhibitory activity of Smad3 to the goosecoid promoter [13]. Taken together, these data suggested that Smad3 has dual activity in regulating FAST-2-mediated transcription, i.e. the self-stimulatory and inhibitory activities.

The MH1 domain of Smad3 is necessary for both its self-stimulatory and inhibitory activities

To determine which domain(s) of Smad3 is responsible for its stimulatory and inhibitory functions, we used a series of chimaeras between Smad1 and Smad3. Smad1 is the signalling mediator of the BMP pathway and is not able to stimulate pAR3-lux with or without co-expression of a constitutively activated ALK-3 (Figure 2). These chimaeras were generated by swapping portions corresponding to the MH1, MH2 and linker regions of Smad1 and Smad3. Using a luciferase assay with co-transfection of pAR3-lux and FAST-2, we examined the ability of these chimaeras to (i) stimulate the ARE transcription and (ii) inhibit the CA-ALK-4/Smad2/Smad4-mediated transcriptional stimulation. As shown in Figure 4, three chimaeras containing the MH1 domain of Smad1 were not able to stimulate pAR3-lux to a level as high as that of wild-type Smad3. In addition, these

three chimaeras could not inhibit the stimulation of the reporter by co-expressed CA-ALK-4/Smad2/Smad4. In contrast, we found that all three chimaeras containing the MH2 domain of Smad3 retained the phenotype of wild-type Smad3 in both the self-stimulatory and inhibitory effects. Taken together, these data indicated that the MH1 domain of Smad3 is the principal structural determinant that confers the dual activity of Smad3 in regulating FAST-2-mediated transcription. Furthermore, these data also suggested that the transactivation function of the MH2 domain is interchangeable between these two Smads, because chimaeras containing the MH2 domain of either Smad1 or Smad3 were able to stimulate the transcription in the absence of the activated receptor, as long as the Smad3 MH1 domain was preserved. This finding was consistent with the previous report in which the MH2 domain from different Smads showed similar transactivating activity when fused with a heterologous DNA-binding domain [27].

Deletion of either or both N-terminal loops in Smad2 does not change its phenotype to that of Smad3

The main structural difference between Smad2 and Smad3 is the presence of the two extra insertions or loops in the MH1 domain of Smad2 [14]. The first loop consists of 10 amino acid residues in positions 21–30. The second loop covers a 30 amino acid stretch in positions 79–108. Since our experiment had indicated that the MH1 domain of Smad3 was sufficient to implement the dual activity of Smad3 in the regulation of FAST-2-mediated transcription and that Smad2 did not possess these two activities, we next examined whether or not the difference

Figure 5 Deletion of the extra N-terminal loops of Smad2 is not sufficient to change its phenotype to that of Smad3

(A) Graphic illustration of the Smad2 loop mutants. Smad2 (loop 1), Smad2 without loop 1; Smad2 (loop 2), Smad2 without loop 2; Smad2 (loop 1 + 2), Smad2 without loops 1 or 2. (B) The same transcriptional assay as in Figure 3 was used in HEK293 cells using the loop mutants of Smad2 (2 μ g/well).

between Smad2 and Smad3 was due to the presence or absence of these two loops. To address this issue, we generated different Smad2 mutants that contained deletion of either one or both of these loops. These mutants were then expressed in HEK293 cells together with FAST-2 and pAR3-lux and both the stimulatory and the inhibitory activities of these mutants were compared with wild-type Smad2 and Smad3. As shown in Figure 5, all three loop mutants of Smad2 behaved similarly to the wild-type Smad2, but not to the wild-type Smad3. None of them were able to stimulate the transcription by themselves nor to inhibit the ARE stimulation mediated by CA-ALK-4/Smad2/Smad4. Therefore, these data suggested that the functional difference between Smad2 and Smad3 in FAST-2-mediated transcription was not solely contributed to by the two extra insertions present in Smad2.

Characterization of N-terminal chimaeras between Smad2 and Smad3

In order to further characterize the structural motifs inside the MH1 domain that confers the self-stimulatory and inhibitory activities of Smad3, we used two chimaeric mutants between Smad2 and Smad3, this time swapping only two short regions within the MH1 domain. As shown in Figure 1, one of the chimaeras contained the first 68 amino acids of Smad3; all of the C-terminal portion of this construct was derived from Smad2 that was devoid of the second loop. The other chimaera contained the 78 amino acids of Smad2 (still having the first loop), followed by a short stretch of Smad3 (positions 69–137 of Smad3) and the remaining C-terminal portion of Smad2. When these chimaeras were transfected into HEK293 cells in the presence of FAST-2, the chimaera containing the very N-terminal end of Smad3 behaved similarly to the wild-type Smad3 in both stimulatory and inhibitory functions (Figure 6). In contrast, the chimaera containing a short Smad3 sequence in the second half of the MH1 domain was not able to stimulate the transcription or to inhibit the Smad2/Smad4/receptor-mediated transactivation of the promoter (Figure 6). Therefore, these data indicated that the unique activities of Smad3 in modulating FAST-2-mediated

Figure 6 The unique function of Smad3 is determined by the most N-terminal end

The same transcriptional assay as described in Figure 3 was used in HEK293 cells using chimaeras between Smad2 and Smad3 (2 μ g/well).

Figure 7 The most N-terminal end of Smad3 is critical for the transactivation of p3TP-lux promoter

HEK293 cells were transiently transfected with vector pcDNA3, p3TP-lux (0.5 μ g), pCMV- β -galactosidase (0.5 μ g), wild-type Smad2 and Smad3 (2 μ g each), Smad2 with deletion of the two N-terminal loops [Smad2 (loop1 + 2); 2 μ g] and the chimaeras between Smad2 and Smad3 (2 μ g each). The fold change of luciferase activity (means $\pm$ S.D.) was derived by comparing with the value of the vector-transfected cells (set to 1) and normalized by β -galactosidase activity. The same experiment was performed more than three times with similar results.

transcription are conferred by the most N-terminal portion of the Smad3 protein.

To further address whether the most N-terminal part of Smad3 is required for its transactivating activity, we examined the ability of the Smad2/Smad3 chimaeras to stimulate p3TP-lux, which contains PAI-1 promoter and PMA-responsive elements. This promoter has been used extensively to measure the transcriptional regulation by TGF- β and activin. As shown in Figure 7, Smad3 but not Smad2 was able to stimulate p3TP-lux. The Smad2 mutant with deletions of the two N-terminal loops had minimal ability to stimulate this promoter. However, the chimaera between the most N-terminal part of Smad3 and the rest of the Smad2 sequence was also able to fully stimulate p3TP-lux. This observation indicated further that the transcription-regulatory activity of Smad3 is mainly contributed to by its most N-terminal sequence.

DISCUSSION

Transcriptional regulation of the Mix.2 ARE results from interaction between Smad proteins and a specific transcription factor, FAST-1 [25]. Lately, a mammalian homologue of *Xenopus* FAST-1 was cloned from human and mouse, named as human FAST-1 and mouse FAST-2 [13,20,23,24,28]. We report here that the FAST-2-mediated ARE regulation is specific for TGF- β and activin signalling as only the constitutively activated type-I receptors for TGF- β and activin but not for BMPs were able to stimulate the FAST-2-mediated ARE transactivation. Although they are structurally very similar to each other, Smad2 and Smad3 exhibit functional differences in FAST-2-mediated transcriptional regulation. Smad3 was able to stimulate the ARE in the absence of receptor activation. This phenomenon may be related to the ability of Smad3 to migrate to the nucleus in the absence of receptor activation [18]. In contrast, Smad2 by itself was not able to stimulate the ARE transcription. When a constitutively activated TGF- β type-I receptor was co-expressed with Smad2, a high level of transcriptional stimulation could be achieved. However, this Smad2- and receptor-induced transcription was inhibited by co-expressed Smad3. The functional difference between Smad2 and Smad3 as we observed here is very similar to what was found for the FAST-2-mediated regulation of the gooseoid promoter, in which Smad3 may compete with

Smad4 for association with a Smad-binding element of the promoter [13]. It is further supported by our recent observation that Smad3 may also compete with Smad4 for binding with FAST-2 in regulating the transcription of Mix.2 promoter [20]. Yeo et al. [29] have found that the MH1 domain of Smad3 is able to directly bind Mix.2 ARE, and this binding is largely enhanced by the binding of *Xenopus* FAST-1 to the ARE. It remains to be determined if such a direct binding between Smad3 and the ARE plays any role in the negative regulation imparted by Smad3.

In our attempts to characterize the structural features responsible for the unique dual activities of Smad3 in the regulation of FAST-2-mediated transcription, we found that the MH1 domain of Smad3 was essential for both the stimulatory and inhibitory functions. Our analysis with the Smad1/Smad3 chimaeras has indicated that the MH1 domain is critical for the dual activities of Smad3 and that the transactivating activity of the MH2 is interchangeable between Smad1 and Smad3. In addition, we found that the two extra loops present in Smad2 are not sufficient for determining the functional difference between Smad2 and Smad3 in FAST-2-mediated ARE regulation, as deletion of either or both of the extra loops of Smad2 appears not able to change the phenotype of Smad2 to that of Smad3. Recently, it has been shown that deletion of the second loop of Smad2 could lead to binding of the protein to the AP-1 site of the p3TP-lux promoter [17]. In addition, Dennler et al. [30] also found that the second loop of Smad2 is responsible for its loss of interaction with the CAGA motif in the PAI-1 promoter. These observations are consistent with the crystal-structure analysis of the MH1 domain of Smad3, in which it has been proposed that the second loop of Smad2 may hinder the interaction of Smad with DNA, as this 30-residue insertion would be located immediately prior to the DNA-binding motif and could interfere with DNA recognition [14]. Our observation that the deletion of the second loop of Smad2 did not convert it into the phenotype of Smad3 would indicate that the unique activity of Smad3 in the regulation of FAST-2-mediated transcription could not be accounted for by the absence of the extra loop(s) present in Smad2, but rather determined by other structural motif(s).

The hypothesis that a structural motif(s) other than the absence of the extra loops in Smad2 is involved in the dual activities of Smad3 is further supported by our observation that the most N-terminal end of Smad3 is indispensable for conferring the dual

regulatory activities of Smad3. The Smad2/Smad3 chimaera that possessed the phenotype of wild-type Smad3 had only 78 amino acid residues belonging to Smad3, with the rest of the sequence encoded by the *Smad2* gene. In comparison with the wild-type mouse Smad2, this particular chimaera protein had amino acid changes of Val to Leu at position 11, Gln to Glu at position 32 (only mouse Smad2 has this Gln, other species have Glu at this position), Arg to Gln at position 47 and Cys to Val at position 60, as well as the absence of both N-terminal loops present in Smad2. However, when two of the amino acid residues of Smad2 at positions 47 and 60 were changed to the corresponding residues present in Smad3, these mutations could not switch the phenotype to that of Smad3 (results not shown). Based on these findings, we hypothesize that the unique functionality of Smad3 in FAST-2-mediated transcriptional regulation might be determined by a combination of those single amino acid changes with the absence of the loop(s) present only in Smad2. Future studies along this line would definitely aid in elucidating the exact role that Smad2 and Smad3 play in mediating and modulating the transcriptional control of the target genes downstream of TGF- β family receptors.

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