Smad proteins exist as monomers in vivo and undergo homo- and hetero-oligomerization upon activation by serine/threonine kinase receptors

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Smad proteins are signal transducers for the members of the transforming growth factor-β (TGF-β) superfamily. Here we show that, in the absence TGF-β stimulation, Smads exist as monomers in vivo. Smad2 and Smad3 form homo-oligomers upon phosphorylation by the constitutively active TGF-β type I receptor, and this oligomerization does not require Smad4. Major portions of Smad4, Smad6 and Smad7 are also present as monomers in vivo. Analysis using a cross-linking reagent suggested that the Smad2 oligomer induced by receptor activation is a trimer. Studies by gel chromatography demonstrated that the Smad2–Smad4 heteromer is not larger than the Smad2 homomer. Moreover, overexpression of Smad4 prevented Smad2 from forming a homo-oligomer. These findings suggest that Smad2 may form a homotrimer, or heterotrimers with Smad4, which are probably composed of two and one, or one and two molecules of Smad2 and Smad4, respectively, depending on the amount of each protein. Gel-mobility shift assay revealed that the Smad3 homo- and Smad3–Smad4 heteromer constitute DNA-binding complexes. Transition of the Smad proteins from monomers to oligomers is thus a critical event in the signal transduction of the TGF-β superfamily members.

Keywords: oligomer/serine/threonine kinase receptor/signal transduction/Smad/TGF-β

Introduction

Transforming growth factor-βs (TGF-βs), activins and bone morphogenetic proteins (BMPs) are structurally related proteins, and are collectively termed the TGF-β superfamily. Members of the TGF-β superfamily play pivotal roles in a wide variety of biological processes, including morphogenesis during development, tissue repair and tumorigenesis (reviewed in Derynck and Feng, 1997). Signals of the TGF-β superfamily proteins are transduced by two different types of cell surface receptors, termed type I and type II, both of which have intracellular serine/threonine kinase domains.

Activation of serine/threonine kinase receptors upon ligand binding has been well characterized in the TGF-β receptor system (Wrana et al., 1994). TGF-β receptor type II (TβR-II) and type I (TβR-I) exist as homodimers on the cell surface, and form a heterotetramer upon TGF-β binding (Chen and Derynck, 1994; Henis et al., 1994; Yamashita et al., 1994; Weis-Garcia and Massagué, 1996; Gilboa et al., 1998). In the receptor complex, TβR-II transphosphorylates TβR-I at its juxtamembrane domain, mainly in the conserved glycine- and serine-rich domain (GS domain), which results in activation of the TβR-I kinase. Replacement of Thr204 in the GS domain by aspartic acid results in constitutive activation of TβR-I. Expression of this TβR-I mutant induces TGF-β-specific responses such as growth inhibition and extracellular matrix deposition in the absence of ligand or TβR-II (Wieser et al., 1995). Changes in the corresponding amino acid residues to aspartic acid or glutamic acid in other type I receptors also leads to constitutive activation of the receptors. These findings demonstrate that type I receptors are effector molecules in the receptor complex, and thus determine the specificity of the intracellular signals (Cárdenas et al., 1994; Feng and Derynck, 1997).

Intracellular events following serine/threonine kinase receptor activation have recently been shown to be mediated by Smad proteins (reviewed in Heldin et al., 1997; Massagué et al., 1997). Mothers against dpp (Mad) identified in Drosophila melanogaster and sma gene products in Caenorhabditis elegans share conserved amino acid sequences, and genetic studies have strongly suggested that proteins in this family mediate signals by the TGF-β superfamily members (Sekelsky et al., 1995; Savage et al., 1996). Biochemical and biological studies using mammalian and Xenopus systems have revealed the molecular basis of the actions of the Smad proteins (Baker and Harland, 1996; Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996; Meersseman et al., 1997).

Eight mammalian members of the Smad family have been reported so far (Heldin et al., 1997; Massagué et al., 1997). Based on their structures and functions, Smad proteins can be sorted into three classes: pathway-restricted Smads, common mediator Smads and inhibitory Smads. Smad2 and Smad3 transduce TGF-β and activin signals. Smad1, Smad5 and possibly Smad8/MADH6 (Chen, Y. et al., 1997; Watanabe et al., 1997) mediate BMP signals. These pathway-restricted Smads are direct substrates of the type I receptor kinases (Macias-Silva et al., 1996; Zhang et al., 1996; Liu,X. et al., 1997; Nakao et al., 1997b). Smad4/DPC4 was originally identified as a candidate tumor suppressor gene product in pancreatic cancers (Hahn et al., 1996). Pathway-restricted Smads form complexes with Smad4 upon phosphorylation by the type I receptors. Smad4 thus functions as a partner of different pathway-restricted Smads, and is therefore a common mediator of distinct signals (Lagna et al., 1996; Candia et al., 1997; Wu et al., 1997). Heteromeric Smad com-
plexes then translocate into the nucleus, where they activate expression of target genes. In Xenopus embryos, activin induces transcription of the Mix.2 gene (Chen et al., 1996). FAST-1 was identified as a transcription factor with a winged-helix domain which binds to the Mix.2 promoter. The Smad2–Smad4 heteromer was shown to interact with FAST-1, forming a transactivation complex on the Mix.2 promoter (Chen, X. et al., 1997; Liu, F. et al., 1997). Smad proteins have also been shown to bind directly, to specific DNA sequences (Kim et al., 1997; Liu, F. et al., 1997; Yingling et al., 1997; Zawel et al., 1998). Smad6 and Smad7 play an inhibitory role in signalling by the TGF-β superfamily members (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997a; Hata et al., 1998). Both of these Smads stably bind to type I receptors, and thereby inhibit phosphorylation of the pathway-restricted Smads. Smad6 can also form a complex with Smad1, which may lead to the inhibition of BMP signals at this step (Hata et al., 1998).

Smad proteins share conserved domains, denoted Mad homology domain-1 (MH1) and -2 (MH2). MH1 domain resides in the N-terminal portion of the Smad proteins, and MH2 domain is located in the C-terminal region. MH1 and MH2 domains are separated by a linker region with variable amino acid sequence and length. MH2 domain is the effector domain with intrinsic transactivation activity, whereas MH1 domain is the regulatory domain that binds MH2 domain and suppresses its function (Hata et al., 1997). Binding to specific DNA sequences occurs via MH1 domains (Kim et al., 1997; Zawel et al., 1998). Inhibitory Smads either lack MH1 domain or contain a diverged MH1 domain. Pathway-restricted Smads have, at their C-terminals, the serine–serine–X–serine (SSXS) motif (Macías-Silva et al., 1996), in which the last two serine residues are phosphorylated by the type I receptors (Abdollah et al., 1997; Souchelnytskyi et al., 1997).

Determination of the three-dimensional structure of the MH2 domain of Smad4 indicated that Smad4 forms a homotrimer in vitro (Shi et al., 1997). Analogously, pathway-restricted Smads have been proposed to exist as homotrimers. Phosphorylation of pathway-restricted Smads may thus induce the formation of heterohexamer complexes between the pathway-restricted Smad homotrimer and Smad4 homotrimer (Shi et al., 1997). However, it was shown that Smad2 and Smad3, which are 91% identical in amino acid sequence, can also form a heteromer. Moreover, the complex containing Smad2, Smad3 and Smad4 appeared to be most efficient in transcriptional activation of the TGF-β-responsive promoter reporter construct, p3TP-lux (Nakao et al., 1997b). However, the structure of the heteromeric complex containing Smad2, Smad3 and Smad4 remains unknown.

Here we present evidence that Smad proteins exist as monomers in vivo in the absence of ligand stimulation, and form homo- and hetero-oligomeric complexes upon activation by the receptors. The Smad2 homomer appears to be a trimer, and the heteromeric complexes composed of Smad2 and Smad4 may be heterotrimers, containing two and one, or one and two molecules each of Smad2 and Smad4, respectively. We also show that not only the Smad3–Smad4 heteromer, but also Smad3 homomer form DNA-binding complexes.

**Results**

**Monomeric Smad2 and Smad3 form homo-oligomers upon TGF-β receptor activation**

Based on the three-dimensional structure of the MH2 domain of Smad4, which exists as a homotrimer when expressed in Escherichia coli, other Smad proteins have also been suggested to exist as homotrimers in vivo (Shi et al., 1997). In order to demonstrate the formation of homo-oligomers of pathway-restricted Smads, we transfected N-terminally FLAG- and myc-tagged Smad2 into COS7 cells, and homo-oligomer formation was detected using immunoprecipitation by anti-FLAG M2 antibody, followed by immunoblotting with anti-myc 9E10 antibody. However, we detected no Smad2 homo-oligomer in the transfected COS7 cells in the absence of the constitutively active form of TβRI, TβRII(TD). The homo-oligomer was detected only in the presence of TβRII(TD) (Figure 1A). The intensity of the Smad2 homo-oligomer band was similar to that obtained by immunoblotting using the anti-myc antibody, in which an aliquot (~6%) of the cell lysate was subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) without immunoprecipitation. Smad3 was also observed as a monomer in the absence of TβRI(TD), and formed a homo-oligomer in the presence of the active form of the receptor (Figure 1B). These findings show that pathway-restricted Smads exist as monomers in vivo, and form homo-oligomers after phosphorylation by the serine/threonine kinase receptors.

**Major portions of Smad4, Smad6 and Smad7 are monomers in vivo**

We then examined whether Smad4 is present as an oligomer in vivo. N-terminally FLAG- and myc-tagged Smad4 constructs were transfected into COS7 cells, and homo-oligomer formation was detected by immunoprecipitation using the anti-FLAG antibody, followed by immunoblotting using the anti-myc antibody. Only a very weak Smad4 homo-oligomer band was detected (Figure 2A); the intensity of the Smad4 homo-oligomer band was 1:100 of that obtained by immunoblotting using the anti-myc antibody, in which an aliquot (~6%) of the cell lysate was directly subjected to immunoblotting. This indicates that a major portion of Smad4 exists as a monomer intracellularly, which was confirmed by gel exclusion chromatography (see below). It has been shown that TGF-β does not induce phosphorylation of Smad4 (Lagna et al., 1996), and oligomer formation of Smad4 was not induced by TβRII(TD) (data not shown).

Homo-oligomer formation by Smad6 or Smad7 was also examined by transfection into COS7 cells. Weak homo-oligomer bands were detected for Smad6 and Smad7 in vivo (Figure 2B and C); the intensities of the Smad6 and Smad7 homo-oligomer bands were only 1:15 and 1:30, respectively, of those in which ~6% aliquots were directly subjected to immunoblotting. Phosphorylation of Smad6 is not induced by ligand stimulation (Imamura et al., 1997), and homo-oligomer formation by Smad6 was not induced by TβRII(TD) (data not shown).

An N-terminally truncated form of Smad6 has been reported in humans (Riggins et al., 1996; Topper et al., 1997), although its physiological significance is unknown. The N-terminally truncated Smad6 has been shown to
Pathway-restricted Smads exist as monomers in vivo.

(A) COS7 cells were transfected with N-terminally FLAG- and myc-tagged Smad2 with or without C-terminally HA-tagged TβR-I(TD). Cell lysates were immunoprecipitated (IP) with the anti-FLAG antibody followed by immunoblotting (Blot) using the anti-myc antibody. The ProBlott membrane was stripped and reblotted with the anti-FLAG antibody to detect the expression of FLAG–Smad2. Aliquots of the cell lysates (~6%) were subjected directly to immunoblotting using the anti-myc or anti-HA antibody. (B) COS7 cells were transfected with N-terminally FLAG- and myc-tagged Smad3 constructs with or without TβR-I(TD)–HA, and complex formation was determined by immunoprecipitation followed by immunoblotting.

Fig. 1. Pathway-restricted Smads exist as monomers in vivo.

(A) COS7 cells were transfected with N-terminally FLAG- and myc-tagged Smad2 with or without C-terminally HA-tagged TβR-I(TD). Cell lysates were immunoprecipitated (IP) with the anti-FLAG antibody followed by immunoblotting (Blot) using the anti-myc antibody. The ProBlott membrane was stripped and reblotted with the anti-FLAG antibody to detect the expression of FLAG–Smad2. Aliquots of the cell lysates (~6%) were subjected directly to immunoblotting using the anti-myc or anti-HA antibody. (B) COS7 cells were transfected with N-terminally FLAG- and myc-tagged Smad3 constructs with or without TβR-I(TD)–HA, and complex formation was determined by immunoprecipitation followed by immunoblotting.

form a complex with Smad7 or Smad4 (Topper et al., 1997); however, we found that full-length Smad6 forms a complex only very weakly with Smad7 or Smad4 (Figure 2D). The intensities of the Smad6–Smad4 and Smad6–Smad7 hetero-oligomer bands were 1:80 and 1:30, respectively, of those obtained by immunoblotting using the anti-myc antibody, in which aliquots (~6%) of the cell lysate were directly subjected to immunoblotting. Smad7 did not efficiently form a complex with Smad4 either (data not shown). Our findings thus showed that major portions of Smad4, Smad6 and Smad7 are present as monomers intracellularly, and that only small portions of these Smads spontaneously form homo- and hetero-oligomers.

Smad4 is not required for oligomer formation by Smad2 and Smad3

Smad2 and Smad3 form hetero-oligomers with Smad4, and Smad4 plays an important role in the efficient signalling activity by the pathway-restricted Smads (Zhang et al., 1996; Nakao et al., 1997b). Since Smad4 is expressed in various cell types, the occurrence of oligomer formation by pathway-restricted Smads observed in COS7 cells may be supported by the presence of endogenously expressed Smad4. In order to determine whether Smad4 is required for the homo-oligomerization of Smad2, we used the SW480.7 colon carcinoma cell line, which lacks endogenous Smad4 (Zhang et al., 1996). Similar to the results obtained with COS7 cells, homo-oligomerization of Smad2 (Figure 3A) and Smad3 (data not shown) was induced in the presence of TβR-I(TD) in the SW480.7 cells.

We previously reported that Smad2 and Smad3 associate with each other upon stimulation by TβR-I(TD) in COS7 cells (Nakao et al., 1997b). Similar to the Smad2 homomer, heteromers between Smad2 and Smad3 were also formed in the absence of Smad4 (Figure 3B), indicating that oligomers of pathway-restricted Smads can be induced in the absence of Smad4.

Cross-linking analysis of the Smad2 oligomer

In order to determine the structure of Smad2 homomer, we prepared cell lysates from COS7 cells transfected with FLAG-tagged Smad2, cross-linked with various concentrations of a chemical cross-linking reagent, disuccinimidyl suberate (DSS), and performed SDS–PAGE after immunoprecipitation with the anti-FLAG antibody. In the absence of TβR-I(TD), most of the Smad2 protein was observed as a 65 kDa monomer. Stimulation by TβR-I(TD) induced oligomer formation; in the presence of high concentrations of DSS, we detected a 140 kDa complex as well as a 220 kDa complex (Figure 4), which may represent homodimeric and homotrimeric forms of Smad2, respectively. These findings suggest that Smad2 forms a homotrimer; however, due to the low cross-linking efficiency of DSS, only a small fraction of Smad2 was observed as the 220 kDa complex. Higher concentrations of DSS induced aggregation of the cell lysates, and we therefore were unable to specifically immunoprecipitate the FLAG-tagged Smad2.

Notably, we detected a 90 kDa cross-linked complex in the presence of DSS. We also detected complexes which migrated slightly slower than the 140 and 220 kDa complexes. This suggests that a protein of 25 kDa may associate with Smad2 after ligand stimulation. After longer exposure of the X-ray film, we detected weak bands of 90 kDa and 140 kDa complexes in the absence of TβR-I(TD). This suggests that if Smad2 is overexpressed in the COS7 cells, it spontaneously forms oligomers and associates with the 25 kDa component.
Oligomer formation of Smad monomers

Fig. 2. Common mediator Smad and inhibitory Smads are present as monomers. (A) N-terminally FLAG- and myc-tagged Smad4 constructs were transfected into COS7 cells and oligomer formation was detected by immunoprecipitation (IP) with the anti-FLAG antibody followed by immunoblotting (Blot) using the anti-myc antibody. Aliquots of the cell lysates (~6%) were directly subjected to immunoblotting using the anti-myc or anti-FLAG antibody. Smad6 (B) and Smad7 (C) constructs were transfected into COS7 cells and subjected to immunoprecipitation followed by immunoblotting. Aliquots of the cell lysates (~6%) were directly subjected to immunoblotting using the anti-myc or anti-FLAG antibody. (D) Smad6 was transfected into COS7 cells with Smad7 or Smad4 and subjected to immunoprecipitation followed by immunoblotting. Aliquots of the cell lysates (~6%) were directly subjected to immunoblotting using the anti-myc antibody.

Analysis by Superose 6 gel chromatography

The molecular structures of the Smad complexes were studied by gel exclusion chromatography. N-terminally FLAG-tagged Smad2 or Smad4 was transfected into COS7 cells and cell lysates were fractionated with a Superose 6 column. In agreement with the results obtained by immunoprecipitation followed by immunoblotting of the cell lysates (Figure 1), Smad2 eluted in fractions corresponding to its monomer size (Figure 5A; upper panel). When cells were transfected with Smad2 together with TβR-I(TD), ~65% of Smad2 eluted in fractions corresponding to a higher molecular weight (Figure 5A; middle panel). Although the Smad2 monomer was also observed in the presence of TβR-I(TD), the phosphorylated form of Smad2 detected by the anti-phosphoserine antibodies was mainly observed in the higher molecular weight fractions. Immunoblotting with the anti-hemagglutinin (HA) antibody to detect HA-tagged TβR-I(TD) failed to reveal the receptor in the Smad complexes (data not shown).

On Superose 6 column chromatography, Smad4 eluted slightly faster than Smad2 (Figure 5B, upper panel). Since a Smad4 mutant (D537E), which is defective in oligomer formation (Shi et al., 1997), also eluted in similar fractions (data not shown), we concluded that Smad4 is also present as a monomer.

We next investigated the structure of the Smad2–Smad4 heteromer by gel chromatography. COS7 cells were transfected with Smad2 and Smad4 together with TβR-I(TD), and proteins were separated with the Superose 6 column. Interestingly, the Smad2–Smad4 heteromer eluted faster than the Smad2 or Smad4 monomer, but slower than the Smad2 homo-oligomer (Figure 5A and B, lower panels). Phosphorylated Smad2 was observed in the fractions containing the Smad2–Smad4 heteromer. Similar results were obtained with another gel chromatography column, Superdex 200. These observations are inconsistent with the previous model in which homo-trimers of Smad2 and Smad4 form a hetero-hexamer after activation by the receptors (Shi et al., 1997).
Smad4 competes with Smad2 in oligomer formation

In order to further examine the structure of the Smad2–Smad4 heteromer, we tested whether Smad2 and Smad4 compete with each other in heteromer formation. FLAG- and myc-tagged Smad2 were transfected together with increasing amounts of myc-tagged Smad4. In the absence of Smad4, Smad2 formed a homo-oligomer in the presence of TβR-I(TD). When myc-tagged Smad4 and myc-tagged Smad2 were expressed in similar amounts, both were co-immunoprecipitated with FLAG–Smad2 (Figure 6). However, with an excess amount of Smad4, only myc–Smad4 co-immunoprecipitated with FLAG–Smad2. Taken together, these findings indicate that Smad4 competes with Smad2 in the process of hetero-oligomer formation.

Smad3 and Smad4 participate in DNA-binding complexes

To investigate whether Smad proteins can form DNA-binding complexes, we tested the binding of Smad2, Smad3 and Smad4 to a DNA probe containing activating protein (AP)-1 sites (Yingling et al., 1997), which was prepared from the p3TP-lux promoter reporter construct (Cárcamo et al., 1995). COS7 cells were transfected with various combinations of Smads and TβR-I(TD), and binding to the DNA probe was tested by a gel-mobility shift assay (Figure 7). Smad2 did not form a DNA-binding complex in the presence of Smad4 and TβR-I(TD) (data not shown). Since a recent study revealed the binding of Smad3 and Smad4, but not of Smad2, to specific DNA sequences (Yingling et al., 1997; Zawel et al., 1998), we then tested DNA binding using Smad3. Smad3 and Smad4 bound to the probe only in the presence of TβR-I(TD). When FLAG–Smad3 and myc–Smad4 were cotransfected, two DNA-binding complexes (complexes 1 and 2, Figure 7A) were observed. Incubation of the gel-shift mixture with the anti-FLAG antibody resulted in supershifts of both complexes, while incubation with the anti-myc antibody shifted only the slower-migrating complex (complex 2). Moreover, in the presence of the anti-FLAG and anti-myc antibodies, a supershift with progressively slower mobility was observed. This suggests that complex 1 contains only Smad3, whereas complex 2 contains both Smad3 and Smad4. This was confirmed by an experiment using a Smad4 mutant, Smad4(D537E), which cannot form homo- or hetero-oligomers (Shi et al., 1997). Smad4(D537E) was efficiently expressed in COS7 cells (Figure 7B). In the presence of Smad4(D537E), only complex 1, but not complex 2, was observed, and supershifted by the anti-FLAG but not by the anti-myc antibody. These findings
Fig. 5. Superose 6 gel chromatography. (A) COS7 cells were transfected with expression constructs for FLAG-tagged Smad2 alone, or in combination with myc–Smad4 and TβR-I(TD)–HA. Cell lysates were applied to a Superose 6 HR 10/30 gel filtration column. The column was run at 0.5 ml/min and fractions of 0.5 ml were collected. Eluates were subjected to immunoprecipitation using the anti-FLAG antibody followed by immunoblotting using the same antibody. Filters were stripped and rebotted with the anti-phosphoserine antibodies. (B) COS7 cells were transfected with FLAG-tagged Smad4 alone, or in combination with myc–Smad2 and TβR-I(TD)–HA. Cell lysates were subjected to Superose 6 gel chromatography and eluted fractions were analyzed by immunoprecipitation with the anti-FLAG antibody, followed by immunoblotting using the same antibody. The column was calibrated by standard mol. wt markers (Pharmacia-Biotech, Inc.). Void volume, 7.8 ml.
Smad2 and Smad4 compete in the oligomer formation. COS7 cells were transfected with various amounts of plasmid of myc-tagged Smad4 with or without FLAG- and myc-tagged Smad2 constructs and TβR-I(TD)-HA. Cell lysates were immunoprecipitated with the anti-FLAG antibody followed by immunoblotting using the anti-myc antibody. Filters were stripped and reblotted with the anti-FLAG antibody. Aliquots of the cell lysates were directly subjected to immunoblotting using the anti-myc or anti-HA antibody.

Discussion

Smads exist as monomers in vivo

Analysis of the three-dimensional structure of the MH2 domain of Smad4 indicated that Smad4 expressed in E.coli exists as a homotrimer (Shi et al., 1997). Since Smad4 was shown to form a hetero-oligomer with Smad2, formation of a heterohexamer composed of a homotrimer of Smad2 and that of Smad4 has been proposed to occur. However, our results indicate that Smad2 and Smad3 exist as monomers intracellularly, and form homomers only after activation by TβR-I(TD). A possibility may still remain that Smads exist as oligomers without stimulation in vivo, but are unstable under the experimental conditions used in the present study; receptor activation may induce the formation of more stable Smad oligomers. Drosophila Mad gene product was also present as a monomer when expressed in COS7 cells, and formed a homo-oligomer upon activation by the constitutively active form of Dpp type I receptor, Thick veins (Tkv) (Inoue et al., 1998).

Smad4, Smad6 and Smad7 also exist as monomers, although small portions of them spontaneously formed homo-oligomers in vivo. Although about two-thirds of Smad2 were eluted as an oligomer on the Superose 6 gel chromatography (Figure 5A), only ~6% of Smad2 was detected as an oligomer after immunoprecipitation by the anti-FLAG antibody (Figures 1A and 3A). This suggests that the anti-FLAG antibody brought down ~10% of the FLAG-tagged Smad2. Provided that the efficiency of immunoprecipitation by the anti-FLAG antibody is 10% for the other Smads, we can estimate the incidence of Smad oligomers by the intensities of the bands (Figure 2A–D); although ~60% of Smad2 was observed as an oligomer after TβR-I(TD) stimulation, incidences of Smad4, Smad6 and Smad7 homo-oligomers spontaneously formed in vivo are 0.6, 4 and 2%, respectively, and those of Smad6–Smad4 and Smad6–Smad7 hetero-oligomers were 0.8 and 2%, respectively.

In the absence of pathway-restricted Smads, a major portion of Smad4 exists as a monomer in the presence
and absence of TβR-I(TD) stimulation. In contrast, Smad4 expressed in vitro has a tendency to form an oligomer (Shi et al., 1997). These observations suggest that certain other component(s) may be required for Smad oligomer formation in vivo, or that there is a mechanism in the cell that prevents spontaneous oligomer formation of Smads. In this context, it is interesting to note that Smad2 was cross-linked with a 25 kDa component upon TβR-I(TD) stimulation. Identification and characterization of the 25 kDa component remains to be performed.

Complex formation between pathway-restricted Smads

Upon activation by the serine/threonine kinase receptors, pathway-restricted Smads form homo- and hetero-oligomers. Since complex formation was also observed in the SW480.7 colon carcinoma cell line, oligomer formation by Smad2 and Smad3 does not require Smad4. At present, the function of oligomers composed only of pathway-restricted Smads is unknown. We have shown here that the Smad3 homomer participates in DNA-binding complex (Figure 7); however, Yingling et al. (1997) reported that DNA binding is dispensable from the transcriptional activation of p3TP-lux by Smads. Whether the homo-oligomers composed only of pathway-restricted Smads have certain biological functions in vivo remains to be determined.

Smad2 and Smad3, which exhibit 91% amino acid sequence identity, can also form heteromers with each other (Nakao et al., 1997b). Notably, despite their high sequence similarity, Smad2 and Smad3 have different functions, e.g. difference in binding to specific DNA sequences (Yingling et al., 1997; Zawel et al., 1998). Thus, heteromers between Smad2 and Smad3 may have functions different from those of homo-oligomers composed only of Smad2 or Smad3. In agreement with this theory, we have shown that Smad2 and Smad3 together have higher transcriptional activity than either Smad alone in the p3TP-lux assay (Nakao et al., 1997b). Smad2 did not participate in the DNA-binding complex to p3TP-lux (Yingling et al., 1997). The mechanism by which Smad2 induces transcriptional activation of the p3TP-lux construct is currently unknown.

Structure of the Smad homo-oligomers

Analysis using a cross-linking reagent revealed that Smad2 forms a homotrimer after activation by the receptor (Figure 5), although the possibility of higher-order oligomer formation has not been completely excluded because of the low efficiency of cross-linking. In contrast, we did not detect oligomers of Smad4 even in the presence of Smad2 and TβR-I(TD) (data not shown). This suggests that certain lysine residues, which are located on the molecular surface of Smad2 and used for cross-linking, are not present in Smad4. On Superose 6 gel chromatography, Smad2 homo-oligomer was observed in fractions which corresponded to a larger molecular weight than expected, the reason for this is currently unknown. Similar results were obtained using a Superdex 200 gel chromatography column; it is thus possible that the Smad2 homomer has a loose structure.

Hetero-oligomeric Smad complexes

Previous studies revealed that hetero-oligomer formation between pathway-restricted Smads and the common mediator Smad results in higher transcriptional response (Zhang et al., 1996; Nakao et al., 1997a). It has been suggested that the heteromeric complex is a hexamer; however, our results using gel chromatography showed that the Smad2–Smad4 heteromer is not larger than the Smad2 homomer. Smad4 may stabilize the oligomer structure containing pathway-restricted Smads, and the heteromer may thus have a more compact structure than the homomer. The fact that Smad4 competed with Smad2 in complex formation (Figure 6) indicates that the heteromer is not a hexamer, but suggests that it may be a heterotrimer composed of two and one, or one and two molecules of Smad2 and Smad4, respectively. However, it is possible that the hetero-oligomer may occur not as a trimer, but as other forms, e.g. a heterodimer. Drosophila Smads also form similar complexes. We found that MAD formed a homo-oligomer only in the presence of the constitutively active form of Tkv (Inoue et al., 1998). MEDEA formed a heteromer with MAD, but in the presence of an excess amount of MEDEA, the MAD homomer was not observed (our unpublished data).

Proposed model for mode of action of Smad oligomers

The present study revealed that Smads exist as monomers in vivo. Activation by the serine/threonine kinase receptors
(TβRI and TβRII) resulted in the phosphorylation of the C-terminal SSXS motif of pathway-restricted Smads (Smad2 and Smad3), leading to oligomer formation (Figure 8). In the absence of Smad4, pathway-restricted Smads form homomers. Smad2 and Smad3, which are structurally highly similar, can also form heteromers with each other. The oligomer composed only of Smad3 can form a DNA-binding complex, although its in vivo functions remain to be determined. In the presence of common mediator Smad (Smad4), pathway-restricted Smads form heteromers. Smad4 may stabilize the structure of the oligomer complexes; the complexes can then bind to various DNA-binding proteins, e.g. FAST-1, or directly bind to DNA. In the absence of Smad4, the Smad oligomer is composed of three molecules of pathway-restricted Smads. In the presence of Smad4, the complexes are composed of two and one, or one and two molecules of pathway-restricted Smads and Smad4. Whether differences in the number of Smad4 in the heteromer affect its biological functions remains to be determined.

Materials and methods

cDNA constructs
pcDNA3-HA was prepared by inserting an annealed oligonucleotide between the XhoI and XbaI sites of pcDNA3 (Invitrogen) (Oeda et al. 1998). FLAG-pcDNA3 and myc-pcDNA3 were previously described (Imamura et al., 1997). 6myc-pcDNA3 containing six tandem copies of the myc-epitope tag was provided by J.-i.Hanai. The original constructions of TβR-1(TD), Smad2, Smad3, Smad4, Smad6 and Smad7 were as reported (Imamura et al., 1997; Nakao et al., 1997a). A Smad4 mutant with a glutamic acid instead of Asp537 was constructed by site-directed mutagenesis using the Chameleon mutagenesis kit (Stratagene). In order to obtain efficient expression of proteins, some inserts were subcloned into another expression vector, pcDEF3 (Goldman et al., 1996). For efficient detection by the anti-myc antibody, some myc-constructs were subcloned into 6myc-pcDNA3. All of the polymerase chain reaction (PCR) products were sequenced.

Cell culture and cDNA transfection
COS7 cells were obtained from American Type Culture Collection. The SW480.7 colon carcinoma cell line was provided by E.J.Stanbridge (Goyette et al., 1992). The cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 U/ml penicillin, and 10 μg/ml of gentamycin. For transient transfection, cells in 6-well plates or in 10 cm cell culture dishes were transfected using DMRIE-C (Gibco-BRL) or FuGENE6 (Boehringer Mannheim) transfection reagents following manufacturers’ protocols.

Immunoprecipitation and immunoblotting
COS7 cells were transfected with expression constructs for Smads alone, or in combination with TβR-I(TD). Forty-eight hours after transfection, cells were washed, scraped and solubilized in a buffer containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1% aprotinin and 1 mM phenethylsulfonyl fluoride (PMSF). After 20 min on ice, the cell lysates were centrifuged and the supernatants were incubated with anti-FLAG M2 (Eastman Kodak Co.) or anti-myc 9E10 antibodies (PharMingen) for 2 h, followed by incubation with protein G–Sepharose beads for 20 min at 4°C. The beads were washed four times with the buffer used for cell solubilization. The immune complexes were then eluted by boiling for 3 min in the SDS sample buffer (100 mM Tris–HCl pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS) containing 10 mM dithiothreitol and subjected to SDS–PAGE. Aliquots of the cell lysates were directly subjected to SDS–PAGE without immunoprecipitation. Proteins were electrotransferred to a ProBlott membranes (Applied Biosystems) and immunoblotted with the anti-FLAG M2, anti-myc 9E10, anti-HA 3F10 (Boehringer Mannheim), or anti-phosphoserine antibodies (Zymed Laboratories, Inc.) and detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). For re-blotting, the membranes were stripped following the manufacturer’s protocol.

Cross-linking of Smads in the cell lysates
COS7 cells were transfected with expression constructs for FLAG- or myc-tagged Smads with TβRI(TD). Cell lysates were prepared as described above, except for using 20 mM HEPES instead of 20 mM Tris–HCl, and incubated with various concentrations of DSS (Pierce Chemical Co.) for 15 min on ice. Samples were then subjected to immunoprecipitation using the anti-FLAG antibody, followed by immunoblotting using the same antibody.

Gel chromatography
COS7 cells in 6-well plates were transfected with expression constructs for Smads alone, or in combination with TβR-1(TD). Forty-eight hours after transfection, cell lysates were prepared as described above, and applied to a Superose 6 HR 10/30 gel filtration column (Pharmacia Biotech, Inc.) pre-equilibrated with 50 mM Tris buffer (pH 8.0), 200 mM NaCl and 5 mM dithiothreitol. After Application of the cell lysates (0.5 ml), the column was run at 0.5 ml/min and fractions of 0.5 ml were collected. Eluates were subjected to immunoprecipitation using the anti-FLAG antibody, followed by immunoblotting using the same antibody. Filters were then rebotted using the anti-phosphoserine antibodies.

Gel-mobility shift assay
Gel-mobility shift assay was performed as previously described (Yingling et al., 1997). Whole-cell extracts were prepared from COS7 cells transfected with Smad and TβR-1(TD) expression constructs. Cells were lysed in a lysis buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 0.5 mM PMPSF, 1 mM dithiothreitol, 1 μg/ml aprotinin, 2 μg/ml leupeptin and 1 μg/ml pepstatin. The probe containing AP-1 sites (77 bp) was created by digestion of p3TP-lux with Ndel and SphI, purified with the Qiagen PCR purification kit (Qiagen), and subjected to [α-32P]dCTP Klenow labeling. Whole-cell lysates (3 μl, containing 3 μg of protein) were added to a premix solution (13.4 μl) containing 1 μg of poly(dI–dC) and 1 μl of the probe labeled to an activity of 5.5×104 c.p.m./μl (Datto et al., 1995). The final concentration of NaCl in the samples was adjusted to 110 mM using hypotonic and hypertonic lysis buffers. For supershift analysis, the anti-FLAG and/or anti-myc antibodies (1 μl each) were added to the whole-cell lysates, incubated for 15 min at room temperature and then mixed with the 32P-labeled probe. Complexes were resolved on a 4% polyacrylamide gel, and analyzed by autoradiography.

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