Formation of a Stable Heterodimer between Smad2 and Smad4*  

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Smad proteins mediate transforming growth factor β signaling from the cell membrane to the nucleus. Upon phosphorylation by the activated receptor kinases, the receptor-regulated Smad, such as Smad2, forms a heterocomplex with the co-mediator Smad, Smad4. This heterocomplex is then translocated into the nucleus, where it associates with other transcription factors and regulates expression of ligand-responsive genes. The stoichiometry between receptor-regulated Smad and co-mediator Smad is important for understanding the molecular mechanisms of the signaling process. Using purified recombinant proteins, we demonstrate that Smad2 and Smad4 form a stable heterodimer and that the Smad4 activation domain is important for the formation of this complex. Many tumor-derived missense mutations disrupt the formation of this heterocomplex in in vitro interaction assays. Mapping these mutations onto the structures of Smad4 and Smad2 identifies a symmetric interface between these two Smad proteins. Importantly, two previous models on the formation of a heterocomplex are incompatible with our observations and other reported evidence.

TGF-β1 signaling regulates a broad range of cellular responses, including growth, differentiation, and cell fate specification, in all animals (1, 2). TGF-β signaling from the cell membrane to the nucleus is mediated by the Smad family of proteins, which contains at least nine distinct members in vertebrates and two of which, Smad2 and Smad4, have been identified as tumor suppressors in humans (3–6).

The Smad proteins are functionally divided into three distinct classes: (i) co-mediator Smads (Co-Smads), namely, Smad4 in mammals and Smad10 (also known as Smad4β) in Xenopus, which participate in signaling by diverse TGF-β family members, (ii) receptor-regulated Smads (R-Smads), including Smad1, Smad2, Smad3, Smad5, and Smad8, each of which is involved in a specific signaling pathway, and (iii) inhibitory Smads, which include Smad6 and Smad7 and negatively regulate these pathways (3–5, 7).

A TGF-β response is initiated by the binding of a specific TGF-β ligand to a pair of specific transmembrane receptors, the type I and II receptors, leading to the activation of the Ser/Thr kinase in the cytoplasmic domain of the type I receptor (8). The signal is then propagated by the type I receptor-mediated phosphorylation of specific R-Smads. For example, Smad1, Smad5, and Smad8 are phosphorylated by the bone morphogenetic protein receptors, whereas Smad2 and Smad3 are phosphorylated by the activin and TGF-β receptors. The phosphorylated R-Smad hetero-oligomerizes with Co-Smad Smad4, translocates into the nucleus, and associates with sequence-specific DNA-binding protein(s), resulting in the positive or negative regulation of ligand-responsive genes.

The Smad proteins are conserved across species, particularly in the N-terminal MH1 domain and the C-terminal MH2 domain. The MH2 domain, to which most of the tumor-derived mutations map, is responsible for receptor recognition, transactivation, interaction with transcription factors, and homo- and hetero-oligomerization among Smads. The MH1 domain, on the other hand, exhibits sequence-specific DNA binding activity and negatively regulates the functions of the MH2 domain.

Formation of a heterocomplex between Co-Smad and R-Smad is indispensable for the signaling process (2). The only known Co-Smad in mammals, Smad4, forms a homotrimer in a concentration-dependent manner both in vivo and in vitro (9–11). The R-Smads, however, exhibit several distinct oligomeric states at the basal state (11, 12). The complex between Co-Smad and R-Smad has been suggested to be a heterohexamer (9), a heterotrimer (10, 12), or, more recently, a heterodimer (11). Understanding this stoichiometry has important implications for understanding the molecular mechanisms of transcriptional regulation by Smad proteins. To address this controversy, we have undertaken a biochemical and biophysical approach, using purified homogeneous Smad proteins. Results from both gel filtration and ultracentrifugation analyses demonstrate that Smad2 and Smad4 form a stable heterodimer. In addition, 15 tumor-derived missense mutations were introduced into these two Smad proteins to assess several prevailing models of heterocomplex formation. Our results suggest a novel arrangement between Smad2 and Smad4.

MATERIALS AND METHODS

Site-directed Mutagenesis and Protein Preparation—Point mutations were generated using a standard polymerase chain reaction-based cloning strategy, and the identities of individual clones were verified through double-strand plasmid sequencing. All Smad4 proteins were overexpressed in Escherichia coli strain BL21(DE3) at room temperature as a GST-fusion protein using a pGEX-2T vector (Amersham Pharmacia Biotech). The soluble fraction of the GST-Smad4 fusion in the E. coli lysate was purified over a glutathione-Sepharose column, cleaved by thrombin, and further purified by anion-exchange chromatography (Source-15Q; Amersham Pharmacia Biotech) and gel filtration chromatography (Superdex-75, Amersham Pharmacia Biotech). All recombinant Smad2 proteins were overexpressed in E. coli strain...
and 2 mM dithiothreitol. All fractions were collected at 0.5 ml each.

1 complex of Smad2-Smad4 (250 mM NaCl, 2 mM dithiothreitol, and 5% glycerol. After prerunning for at least 45 min to allow equilibrium to be reached. The flow rate was 0.5 ml/min, and the buffer contained 25 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM dithiothreitol. Relevant fractions were visualized on SDS-polyacrylamide gel electrophoresis. The chromatographs and the calibration of the Superdex-200 column are shown on the right. The arrows indicate the starting points for all four chromatographic runs. B, electrophoretic mobility shift assays under non-denaturing conditions. C, mutual exclusion of a Smad2-Smad4 complex and a Smad2-SARA complex. GST-SARA was first bound to glutathione resin, and a stoichiometric amount of a 1:1 Smad2-Smad4 complex was allowed to flow through the resin. The resin was washed four times with assay buffer, and aliquots of the last wash were visualized on SDS-polyacrylamide gel electrophoresis.

**RESULTS AND DISCUSSION**

Smad2 Forms a Stable Heterodimer with Smad4—The full-length Smad2 is unable to form a complex with Smad4 in the absence of phosphorylation in its C-terminal SS*MS* sequence. This is likely due to an autoinhibitory interaction between the MH1 and MH2 domains that can be relieved by phosphorylation (14). Indeed, removal of the MH1 domain in Smads results in constitutively active transcriptional activity (15) and allows the formation of a stable heterocomplex in the absence of phosphorylation (9, 14, 16). Hence, we chose to focus on the MH1-deleted proteins of Smad2 (residues 241–467) and Smad4 (residues 251–552). These recombinant proteins were overexpressed in bacteria and purified to homogeneity.

To examine the stoichiometry between Smad2 and Smad4, we devised an in vitro interaction assay employing size exclu-
We also used equilibrium sedimentation analytical ultracentrifugation to characterize the molecular mass of the hetero-oligomer. Because Smad proteins exhibit concentration-dependent homo-oligomerization, we selected the concentration ranges in which both proteins behave as monomers on gel filtration analysis (Fig. 1A). The elution volume for Smad2 corresponds to a molecular mass of \( \approx 22 \text{ kDa} \) (Fig. 1A, panel 1), consistent with its calculated molecular mass of 25 kDa. The elution volume for Smad4, which contains a 30-residue flexible loop (residues 460–490) between helices H3 and H4 (9), corresponds to a molecular mass of about 39 kDa (Fig. 1A, panel 2), in reasonable agreement with its calculated molecular mass of 33 kDa. When equimolar amounts of Smad2 and Smad4 were used, the vast majority of Smad2 was shifted to earlier fractions, indicating a 1:1 stoichiometry (Fig. 1A, panel 3). In addition, the elution volume for the complex corresponds to an apparent molecular mass of \( \approx 54 \text{ kDa} \), consistent with the expected mass of a complex composed of one Smad2 and one Smad4 (Fig. 1A, panel 3). To further demonstrate the formation of a heterodimer, 1.5 molar equivalents of Smad2 were mixed with one molar equivalent of Smad4. In this case, the excess amount of Smad2 was eluted from the size exclusion column as a monomer (Fig. 1A, panel 4). During the course of size exclusion chromatography, there is little dissociation between Smad2 and Smad4, suggesting stable complex formation. Nevertheless, excess Smad2 (Fig. 1A, panel 4) or higher concentrations of both proteins (Fig. 1A, panel 5) led to more complete formation of a heterodimer.

To estimate the binding affinity between Smad2 and Smad4 and to further confirm the formation of a heterodimer, electrophoretic mobility shift assays under nondenaturing conditions were employed (Fig. 1B). Under these conditions, Smad2 does not enter the gel (Fig. 1B, lane 1) whereas Smad4 migrates as a single band (Fig. 1B, lane 2). With increasing concentrations of Smad2, a distinct heterocomplex is formed (Fig. 1B, lanes 3–8). Neither excess Smad4 (lanes 3 and 4) nor excess Smad2 (lanes 6–8) resulted in more than one heterocomplex. Quantitation of the binding experiment revealed a dissociation constant of \( \approx 1 \mu\text{M} \).

Formation of a functional Smad2-Smad4 complex may be antagonized by the formation of a complex between SARA and Smad2 (17, 18). To further demonstrate the functional relevance of the observed heterodimer, the Smad2-Smad4 complex was applied to glutathione resin preimmobilized with GST-SARA (Fig. 1C). As expected, the flow-through fraction contained less Smad2 than the input (Fig. 1C, lane 5), suggesting disruption of a Smad2-Smad4 complex by SARA. Indeed, the eluted fraction contained a SARA-Smad2 complex (Fig. 1C, lane 7), demonstrating that SARA does compete with Smad4 for binding to Smad2.

To complement the gel filtration analysis, the molecular mass of the hetero-oligomer was analyzed by analytical ultracentrifugation. The complex was prepared in 1:1 ratio, based on the conclusion from the gel filtration results that this complex forms a heterodimer. The complex was analyzed at four loading concentrations and four rotor speeds. At 5 and 10 \( \mu\text{M} \), the protein complex is fully consistent with that of a heterodimer, with molecular masses of 58,900 and 59,800 daltons, respectively (Table I). If Smad2 and Smad4 form heterotrimers instead, then the apparent molecular mass would be significantly reduced because one of the two proteins would be in significant excess and would have contributed to a significant reduction in the reported molecular mass. In Fig. 2, we show the fit of the 10 \( \mu\text{M} \) data to a heterodimer model (Fig. 2). At 2.5 \( \mu\text{M} \), we see evidence for a dynamic equilibrium between monomers and...
Table I

Analytical ultracentrifugation analysis of a Smad2-Smad4 complex

The Smad2-Smad4 complex exhibits concentration-dependent molecular masses, indicating higher order oligomerization. Two-state models were analyzed for these complexes to assess which model would best fit the data. Models tested included heterodimer(-heterotrimer (where both 2:1 or 1:2 ratios of Smad2:Smad4 were considered) and both heterodimer and heterotrimer self-association (1:1=) where 1 equals either the heterodimer or heterotrimer unit and = N 2, 3, 4, 5, and 6 units of each heteromer). Least squares analysis disfavors all models involving heterotrimer formation. The best two-state model is a self-associating model for the heterodimer with N = 4.

| Concentration of complex | Molecular mass |
|--------------------------|----------------|
| 2.5 µM                   | 37,800 ± 3,900 |
| 5.0 µM                   | 58,900 ± 4,200 |
| 10 µM                    | 59,800 ± 7,900 |
| 20 µM                    | 72,200 ± 5,800 |

heterodimers, whereas at 20 µM, there is some evidence for minor aggregation (Table I). Indeed, Smad2 by itself starts to homo-oligomerize and/or aggregate at the 20 µM concentration. The best-fit two-state model to account for the aggregation is a heterodimer-hetero-octamer model. In summary, ultracentrifugation analysis demonstrates that Smad2 and Smad4 form a heterodimer.

The Smad4 Activation Domain Is Required for the Formation of a Stable Heterocomplex—To investigate the sequence requirement for the formation of a stable Smad2-Smad4 complex, we created deletion mutants in Smad2 and Smad4 and examined their interaction with each other. The results indicate that neither MH2 domain is sufficient for heterocomplex formation (Fig. 3A). In the case of Smad2, a 28-residue extension N-terminal to the MH2 domain (residues 269–467) is required for stable interaction with Smad4 (Fig. 4A), presumably due to the structural requirement that this fragment contribute an additional β-strand that packs against a hydrophobic surface (19). On the other hand, neither the MH2 domain of Smad4 (residues 319–552) (Fig. 3B, upper panel) nor a longer fragment with 19 additional amino acids (300–552) was able to form a stable heterocomplex with Smad2, as judged by their progressive propensity toward dissociation upon size exclusion chromatography. Inclusion of the full Smad4 activation domain (SAD) (20) restored its ability to form a stable heterodimer with Smad2 (Fig. 3B, bottom panel). The coupling of efficient formation of a heterocomplex with the requirement for SAD may have functional implications for TGF-β signaling.

Tumorigenic Mutations Disrupt the Formation of the Smad2-Smad4 Heterodimer—Most of the tumor-derived missense mutations map to the MH2 domain in Smad4 proteins (6, 9, 21). Because the formation of a functional Smad2-Smad4 complex is important for signaling, some of these missense mutations may act by disrupting this complex. Although previous work shows that this is indeed the case in vivo (14), only four mutations were examined by immunoprecipitation, which precluded a conclusion on whether or not these mutations directly prevented formation of a heterocomplex.

To address this issue systematically, we generated a total of 18 missense mutations into either Smad2 (residues 241–467) or Smad4 (residues 251–552), purified the mutant proteins to homogeneity, and examined their interactions with their wild-type counterparts. To rule out the possibility of misfolding or aggregation, each mutant protein was carefully compared with the wild-type Smad2 or Smad4 by gel filtration, dynamic light scattering, and thermodenaturation analyses. With the exception of three insoluble mutants (L440R and P445H in Smad2 and I527R in Smad4), these analyses demonstrated that each of the mutant proteins was well folded and exhibited very similar solution properties. Each of eight tumor-derived mutations was introduced in both Smad2 and Smad4. For example, D450H in Smad2 has been reported in colon cancers (22). Both D450H in Smad2 and the corresponding D537H in Smad4 were generated. Each of the three tumor-derived mutations in Smad4 (D351H, R361H, and V370D), as well as their corresponding mutations in Smad2 (D300H, R310H, and V319D), disrupted the formation of a heterocomplex (Table II; Fig. 4A). On the other hand, D450H in Smad2 and D537H in Smad4 failed to disrupt the formation of a heterodimer (Table II; Fig. 4B). Two additional tumorigenic mutations (P346W in Smad2 and R420H in Smad4) and their corresponding mutations (W398V in Smad4 and W319D) exhibited no effects on heterocomplex formation (Table II; Fig. 4). Interestingly, each of the tumorigenic mutations in the original Smad and the corresponding mutation in the other Smad has identical effects on heterocomplex formation (Fig. 4). This result suggests that Smad2 and Smad4 may form a pseudosymmetric heterodimer, in which each of the two Smad proteins uses similar surface motifs to interact with the other. Because three of the four deleterious mutations affect residues that are located in the L1 and L2 loop region (9) (Fig. 4B), this loop-helix region must be directly involved in mediating the formation of a heterodimer between Smad2 and Smad4. In support of this observation, this loop-helix region contains the overwhelming majority of the most highly conserved and solvent-exposed residues in Smad proteins (9).
Comparison of Several Distinct Models for Heterocomplex Formation—Assessment of the effects of these tumorigenic mutations and other reported evidence allows us to evaluate several existing models on the formation of a heterocomplex between Smad2 and Smad4.

### Table II
Mutational analysis of a Smad2-Smad4 complex

| Mutation in Smad4 | Interaction with Smad2 | Mutation in Smad2 | Interaction with Smad4 |
|-------------------|------------------------|-------------------|-----------------------|
| WT                | Yes (1 μM)             | SE*ME*            | Yes (0.4 μM)          |
| D351H             | No                     | D300H             | No                    |
| R361H             | No                     | R310H             | No                    |
| V370D             | No                     | V319D             | No                    |
| R420H             | Yes                    | W368H             | Yes                   |
| R441P             | Yes                    | N367P             | n/a                   |
| D493H             | No                     | Y406H             | n/a                   |
| W398V             | Yes                    | F346V             | Yes                   |
| I527R             | n/a (insoluble)        | L440R             | n/a (insoluble)       |
| A532H             | No                     | P445H             | n/a (insoluble)       |
| D537H             | Yes                    | D450H             | Yes                   |

**Fig. 4.** Tumorigenic mutations inactivate the heterodimer between Smad2 and Smad4. Ten tumor-derived missense mutations were introduced in Smad4 (251–552) and Smad2 (241–467). These mutant proteins were purified to homogeneity and assayed for their ability to interact with their wild-type counterparts by gel filtration. A, representative results showing that three Smad4 mutants (indicated by a purple line) are unable to form a heterocomplex with Smad2, whereas one Smad4 mutant and one Smad2 mutant (highlighted by an orange line) retain their ability to form a heterodimer. B, schematic representation of the residues affected by the missense mutations. The MH2 domain of Smad4 is shown in blue. Mutation of the purple residues results in disruption of heterodimer formation. Mutation of the orange residues does not affect the formation of a heterodimer. Mutations in parentheses are introduced in Smad2. The two corresponding mutations in Smad2 and Smad4 exhibit an identical effect on the formation of a heterocomplex.

**Fig. 5.** Comparison of proposed models of heterocomplex between Co-Smad and R-Smad. In A and C, Co-Smad and R-Smad are colored green and blue, respectively. The critical interface residues from Co-Smad and R-Smad are shown in red. A, proposed heterotrimer model. This model is inconsistent with several experimental observations (see “Results and Discussion”). This model shows a 2:1 complex between Co-Smad and R-Smad. The discussion in the text also applies to the other scenario, in which Co-Smad and R-Smad form a 1:2 complex. B, formation of a stable heterodimer between Smad4 (251–552) and a mutant Smad2 (241–467) in which the two C-terminal Ser residues (455 and 467) are replaced by Glu (SEME). Equimolar amounts of these two proteins were incubated together for 45 min before assay by size exclusion chromatography. C, two possible models of a heterodimer. Neither is consistent with our mutational analysis.
data contradict this model. Asp-450 in Smad2 and the corresponding Asp-537 in Smad4 play a central role in this model, each making three intersubunit hydrogen bonds (9). Mutation of this residue is expected to completely disrupt a network of hydrogen bonds, leading to the disruption of the heterotrimeric packing (Fig. 5A). However, neither D450H in Smad2 nor D537H in Smad4 disrupted the formation of a heterocomplex (Table II; Fig. 5). Second, according to the heterotrimer model, SARA binding to Smad2 should not interfere with Smad2-Smad4 interactions because the SARA-binding surface of Smad2 is far away from the proposed heterotrimeric interface (19); however, SARA-Smad2 and Smad2-Smad4 complexes appear to antagonize each other (17, 18) (Fig. 1). Third, the heterotrimer model is inconsistent with the observation that the SAD domain is important for the formation of a heterocomplex (Fig. 3) because SAD is located in the periphery of the proposed heterotrimer (Fig. 5A). Fourth, a Smad2-Smad4 heterotrimer would have a larger molecular mass than that of a Smad2 homotrimer, which is inconsistent with the observation that the apparent molecular mass of a functional complex is less than 300 kDa (20).

Smad4 is a constitutively active Smad (21). In our studies, we used the unphosphorylated Smad2 and Smad4 proteins. Could this affect our final conclusion? We think that the answer is likely to be no. The major role of phosphorylation is to relieve the inhibitory effect of the MH1 domain and to release R-Smads from SARA and other proteins (14, 17). With the removal of the MH1 domain, the resulting Smad proteins are fully able to form heterocomplex between R-Smad and Co-Smad and are constitutively active in transcriptional assays (14–16). In fact, the MH2 domain of Smad2 is far away from the proposed heterotrimeric interface (19); however, SARA-Smad2 and Smad2-Smad4 complexes appear to antagonize each other (17, 18) (Fig. 1). Third, the heterotrimer model is inconsistent with the observation that the SAD domain is important for the formation of a heterocomplex (Fig. 3) because SAD is located in the periphery of the proposed heterotrimer (Fig. 5A). Fourth, a Smad2-Smad4 heterotrimer would have a larger molecular mass than that of a Smad2 homotrimer, which is inconsistent with the observation that the apparent molecular mass of a functional complex is less than 300 kDa (20).

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On the basis of our biochemical and biophysical analysis, Smad2 clearly forms a heterodimer with Smad4. There are three scenarios for a heterodimer. Two scenarios involve packing interactions similar to that of the homotrimer, except that the relative positions of Smad2 and Smad4 could be switched (Fig. 5C); a third scenario involves a novel interaction interface, possibly pseudosymmetric. Our current data support the third scenario because the results with mutations D450H in Smad2 (D537H in Smad4) and D351H (D300H in Smad2) and R361H (R310H in Smad2) in Smad4 are not compatible with either of the first two scenarios.

**Smad2 versus Smad3**—Given the strong sequence similarity among R-Smads, it appears likely that Smad4 forms a heterodimer with other R-Smads. For example, Smad2 shares 92% sequence identity with Smad3, and both proteins are involved in signaling by TGF-β and activin. Nevertheless, the basal states of Smad2 and Smad3 differ considerably (11). Thus, the states of their heterocomplexes with Smad4 may also be different, as is the case for their biological functions. For example, Smad3 exhibits the highest sequence-specific DNA binding affinity; but Smad2 does not bind DNA because of an obstructing insertion immediately before the DNA-binding β-hairpin (25). Ectopic expression of the ubiquitin E3 ligase, Smurf2, selectively reduces the steady-state levels of Smad2 but not Smad3 (26). More importantly, Smad3-null mice are viable, but Smad2-null mice are not. Thus, it remains to be seen how Smad3 or Smad1 forms a heterocomplex with Smad4.

In summary, we conclude that the R-Smad Smad2 forms a stable heterodimer with Co-Smad Smad4. The formation of this complex requires the SAD domain in Smad4 and can be disrupted by a number of tumor-derived missense mutations in both Smad2 and Smad4. This finding should have broad implications in the interpretations of a range of biological experiments.

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