The EMBO Journal Vol.17 No.4 pp.996–1005, 1998

The L3 loop: a structural motif determining specific interactions between SMAD proteins and TGF-β receptors

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Signal transduction specificity in the transforming growth factor-β (TGF-β) system is determined by ligand activation of a receptor complex which then recruits and phosphorylates a subset of SMAD proteins including Smads 1 and 2. These then associate with Smad4 and move into the nucleus where they regulate transcription. We have identified a discrete surface structure in Smads 1 and 2 that mediates and specifies their receptor interactions. This structure is the L3 loop, a 17 amino acid region that protrudes from the core of the conserved SMAD C-terminal domain. The L3 loop sequence is invariant among TGF-β- and bone morphogenetic protein (BMP)-activated SMADS, but differs at two positions between these two groups. Swapping these two amino acids in Smads 1 and 2 induces a gain or loss, respectively, in their ability to associate with the TGF-β receptor complex and causes a switch in the phosphorylation of Smads 1 and 2 by the BMP and TGF-β receptors, respectively. A full switch in phosphorylation and activation of Smads 1 and 2 is obtained by swapping both these two amino acids and four amino acids near the C-terminal receptor phosphorylation sites. These studies identify the L3 loop as a determinant of specific SMAD–receptor interactions, and indicate that the L3 loop, together with the C-terminal tail, specifies SMAD activation.

Keywords: L3 loop/receptor interactions/SMAD proteins/transforming growth factor-β

Introduction

Transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), activins and related factors signal their many responses via pairs of transmembrane serine/threonine kinase receptors. In the ligand-induced complex, one of the two kinases, called the type II receptor, phosphorylates and activates the other kinase, the type I receptor, which then phosphorylates substrates propagating the signal (Massagué, 1996). The type I receptor substrates include a recently identified protein family, the SMAD proteins, that plays a central role in the relay of TGF-β signals from the receptors to target genes in the nucleus (Derynck and Zhang, 1996; Wrana and Attisano, 1996; Massagué *et al.*, 1997).

A subclass of SMADs known as 'receptor-regulated' SMADs are phosphorylated by specific receptors in a ligand-dependent manner (Hoodless *et al.*, 1996; Kretzschmar *et al.*, 1997b). The receptor-regulated SMADs physically associate with the ligand-activated receptor complex (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996) and undergo phosphorylation at the C-terminus, release from the receptor, association with the related protein Smad4, which acts as a shared partner. This complex translocates into the nucleus and participates in transcriptional complexes (Eppert *et al.*, 1996; Hoodless *et al.*, 1996; Lagna *et al.*, 1996; Liu *et al.*, 1996, 1997; Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996, 1997; Kretzschmar *et al.*, 1997b). Although much progress has been made in understanding the TGF-β/SMAD pathway, nothing is known about the protein structures that determine the specificity of this pathway at the level of receptor–SMAD interaction.

Specificity in this system is provided by the ability of the receptors to discriminate among SMADs. In vertebrates, Smad1 (Graff *et al.*, 1996; Hoodless *et al.*, 1996; Liu *et al.*, 1996), and presumably its close homologs Smad5 (Yingling *et al.*, 1996) and Smad8 (Chen *et al.*, 1997; Watanabe *et al.*, 1997), are phosphorylated by BMP receptors (Hoodless *et al.*, 1996; Kretzschmar *et al.*, 1997b) and mediate BMP responses (Graff *et al.*, 1996; Liu *et al.*, 1996; Thomsen, 1996). Smad2 and its close homolog Smad3 are phosphorylated by TGF-β receptors (Eppert *et al.*, 1996; Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996; Kretzschmar *et al.*, 1997b; Nakao *et al.*, 1997a) and mediate TGF-β and activin responses (Baker and Harland, 1996; Eppert *et al.*, 1996; Graff *et al.*, 1996; Lagna *et al.*, 1996; Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). In *Drosophila*, Mad (a close homolog of Smad1 and the founding member of this family) mediates the effects of the BMP-like factor, Dpp (Sekelsky *et al.*, 1995; Wiersdorff *et al.*, 1996; Newfeld *et al.*, 1997). In *Caenorhabditis elegans*, Sma-2 and Sma-3 function downstream of Daf-4, which is a receptor for a BMP-related factor (Savage *et al.*, 1996).

SMAD proteins consist of three regions: a conserved N-terminal domain (referred to as the N or MH1 domain), a conserved C-terminal domain (the C or MH2 domain) and a more divergent linker region (Massagué *et al.*, 1997). The MH2 domain has effector function as determined in transcriptional assays (Liu *et al.*, 1996) and in *Xenopus* mesoderm formation assays (Baker and Harland, 1996). The MH1 domain inhibits this effector function (Baker and Harland, 1996; Liu *et al.*, 1996; Hata *et al.*, 1997). This inhibitory effect is relieved by receptor-mediated phosphorylation on the C-terminal sequence SS(V/M)S present in receptor-regulated SMADs (Macias-Silva *et al.*, 1996; Kretzschmar *et al.*, 1997b). Recently, the MH1 domain of Mad has been shown to bind to DNA (Kim

et al., 1997). Therefore, the MH1 domain may also subserve the gene activation role of the SMADs, and the MH1 and MH2 domains may have a mutually inhibitory effect in the basal state. The linker region contains serine residues whose phosphorylation by MAP kinases in response to tyrosine kinase receptor activation inhibits Smad1 translocation to the nucleus (Kretzschmar *et al.*, 1997a). Smad6, Smad7 and *Drosophila* Dad, which act as antagonists of TGF-β signaling, have a more divergent MH1 domain (Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997b; Topper *et al.*, 1997; Tsuneizumi *et al.*, 1997; Hata *et al.*, 1998).

Smad2 and *Smad4* are tumor suppressor genes in chromosome 18q21, and mutations in these genes have been found in various types of cancers, principally carcinomas of the pancreas and colon (Eppert *et al.*, 1996; Hahn *et al.*, 1996). Many of the tumor-derived missense mutations, as well as missense mutations in defective alleles of *Mad*, *Sma-2* and *Sma-3*, map to the MH2 domain (summarized in Shi *et al.*, 1997). The recent resolution of the crystal structure of the Smad4 MH2 domain has provided insights into how the naturally occurring mutations interfere with SMAD function (Shi *et al.*, 1997). Smad4 MH2 domain monomers assemble into a trimer, with each monomer resembling an open-sided cradle containing a core β-sandwich structure. Combining structural and functional insights, naturally occurring MH2 domain mutations can be grouped into those that disrupt the core structure of the MH2 domain and destabilize the protein, those that disrupt the trimer interface, and those that fall in a protruding structure referred to as the L3 loop. The latter mutations inhibit Smad4 from associating with receptor-activated Smad2, thus preventing the formation of a functional Smad2–Smad4 complex (Shi *et al.*, 1997). Mutations in the MH1 domain augment its affinity for the MH2 domain, thus increasing the autoinhibitory function (Hata *et al.*, 1997).

Protein–protein interactions are critical determinants of specificity and fidelity in signal transduction pathways (Hill and Treisman, 1995). Various protein modules have been identified that mediate specific protein–protein interactions in signal transduction pathways in a wide range of cellular processes (Pawson and Scott, 1997). In order to identify protein structures that may mediate and specify protein–protein interactions in the receptor serine/threonine kinase signaling system, we have investigated TGF-β and BMP signaling pathways whose surface receptors are prototypic of this system. We report here that the L3 loop in the MH2 domain of receptor-regulated SMADs is crucial for their specific interaction with the receptors.

Results

The C-tail is dispensable for Smad2 association with the TGF-β receptor

Receptor-regulated SMADs are phosphorylated by activated receptors at conserved C-terminal serine residues (Macias-Silva *et al.*, 1996; Kretzschmar *et al.*, 1997b). According to the crystal structure of the Smad4 C-domain, which is thought to be conserved in the receptor-regulated SMADs (Shi *et al.*, 1997), these residues are located at the end of an 11 amino acid region (here referred to as the 'C-tail') following α-helix 5 (Figure 1A). As a substrate for the TGF-β type I receptor kinase, the C-tail might mediate the previously observed docking of Smad2 to the receptor complex (Macias-Silva *et al.*, 1996). We examined this possibility by testing the receptor-binding activity of a Smad2 construct lacking the C-tail [Smad2(1–456)]. Receptor-binding activity was assayed by co-transfection of TβR-I, TβR-II and Flag epitope-tagged Smad2 constructs into cells, then affinity-labeling the receptors by cross-linking to bound [^{125}I]TGF-β1, and finally co-immunoprecipitating the labeled receptors with Smad2 via the Flag epitope (Figure 2A) (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). Surprisingly, the receptor interaction was stronger with Smad2(1–456) than with wild-type Smad2 (Figure 2A), indicating that removal of the C-tail increased the Smad2–receptor interaction. This suggests that the physical contact between the C-tail of Smad2 and the catalytic cleft of the TβR-I kinase during the phosphotransfer reaction does not contribute significantly to Smad–receptor association. Smad2 docking to the receptor must therefore be mediated by a region of Smad2 other than the C-tail.

It has been shown that the interaction between the TGF-β receptor complex and Smad2 is increased when TβR-I is made catalytically inactive by a mutation in the kinase domain or when the C-terminal phosphorylation sites in Smad2 are eliminated by mutation to alanine (Macias-Silva *et al.*, 1996) [see Figure 2A, Smad2(3A) construct]. In light of our observation that removal of the C-tail increases the receptor interaction, these results suggest that docking is inhibited when the C-tail is phosphorylated.

The Smad2 MH2 domain can associate with the receptor complex

In order to localize the region of Smad2 required for association with the receptor, we tested various Smad2 deletion mutants for receptor-binding activity (Figure 3). To facilitate the analysis without altering the C-terminus of Smad2, we used the kinase-defective TβR-I(K232R) receptor construct, taking advantage of its enhanced Smad2 binding phenotype (Macias-Silva *et al.*, 1996). Deleting half of the MH1 domain [Smad2(100–467) construct] or the entire MH1 domain [Smad2(186–467)] had no appreciable effect on Smad2–receptor association. Consistent with this, the MH1 domain (1–185) alone had no detectable affinity for the receptor complex (data not shown). Furthermore, the MH2 domain alone [Smad2(248–467)] was still capable of associating with the receptor complex, albeit more weakly. This could be due to the fact that the MH2 domain forms homo-oligomers less stably than the full-length protein (Hata *et al.*, 1997), and that this homomeric complex might associate cooperatively with the receptor complex. As with the full-length Smad2, the MH2 domain interacted with the wild-type TβR-I more stably when the C-terminal phosphorylation sites of Smad2 were mutated [Smad2(248–467/3A) construct] (Figure 3).

L3 loop involvement in Smad2 docking

Given these results, our search for a critical determinant of receptor docking focused on the MH2 domain of Smad2 excluding the C-tail. Two missense mutations in this region have been shown to inhibit receptor-mediated phosphorylation. A colorectal tumor-derived mutant form

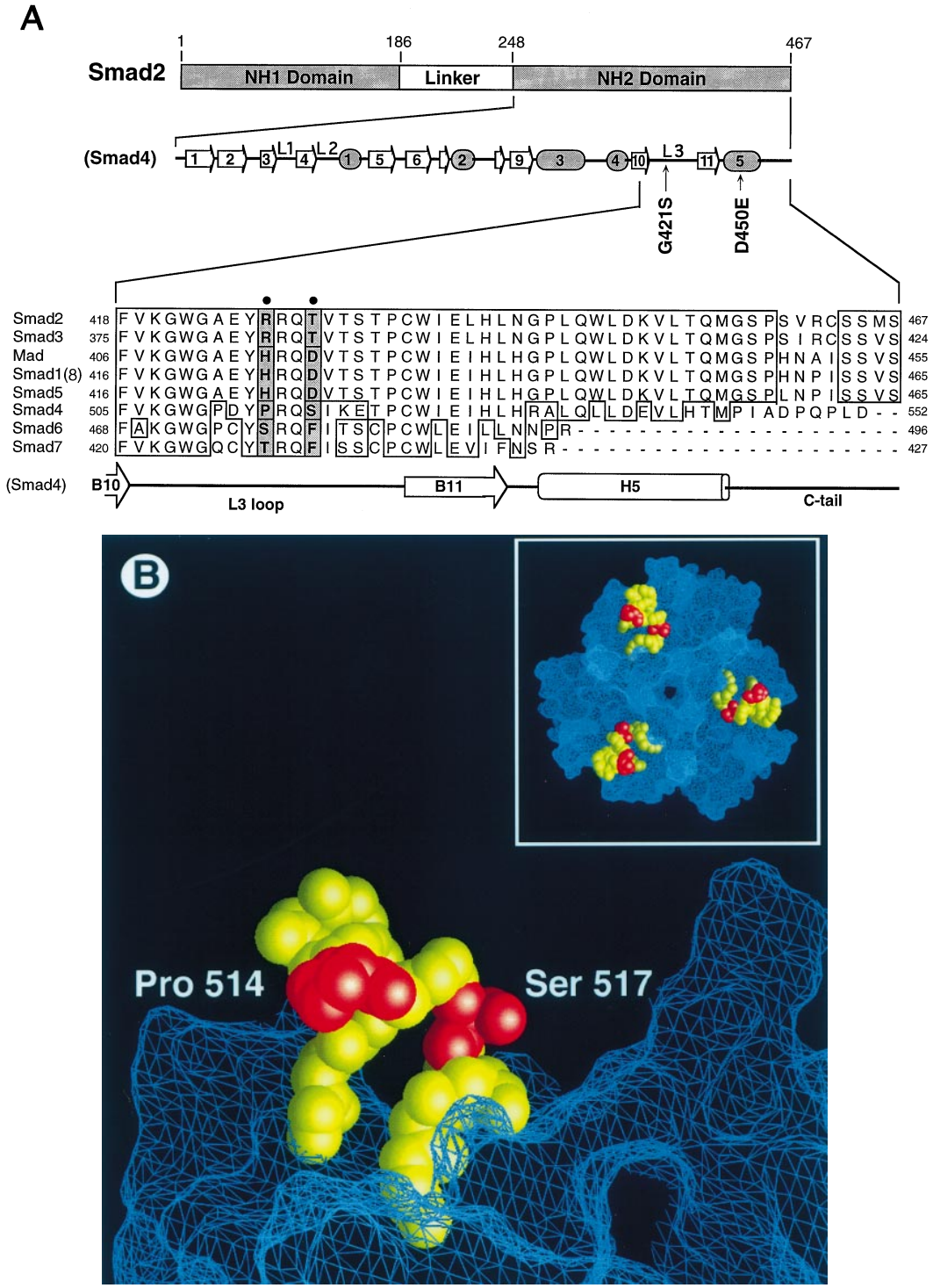

Fig. 1. (**A**) Diagrammatic representation of Smad2, its MH2 domain structure based on Smad4 (Shi *et al.*, 1997), and amino acid sequence alignment of the SMADs starting from the L3 loop to the end. In the MH2 domain structure, arrowheads (1–11) represent β-sheets; L1–L3 represent loops; filled circles represent α-helices. In the amino acid sequence alignment, the conserved amino acids are boxed. The two residues in the L3 loop which are distinct among different SMAD groups are highlighted. (**B**) Inset: the structure of the Smad4 MH2 domain trimer highlighting the L3 loop in each monomer. The close-up shows the L3 loop (yellow) protruding from the core structure (adapted from Shi *et al.*, 1997). The two group-specific amino acids are indicated in red.

of Smad2 with an aspartic acid to glutamic acid mutation (D450E) is defective in receptor-dependent phosphorylation (Eppert *et al.*, 1996; Figure 2B). However, this mutant was able to bind to the receptor as effectively as did the Smad2(3A) mutant (Figure 2A), suggesting that the D450E mutation interferes with Smad2 phosphorylation and, as a result, enhances Smad2 binding to the receptor.

A different result was obtained with another mutant, Smad2(G421S). This is a highly conserved glycine residue whose mutation to serine in *Drosophila* Mad (Sekelsky

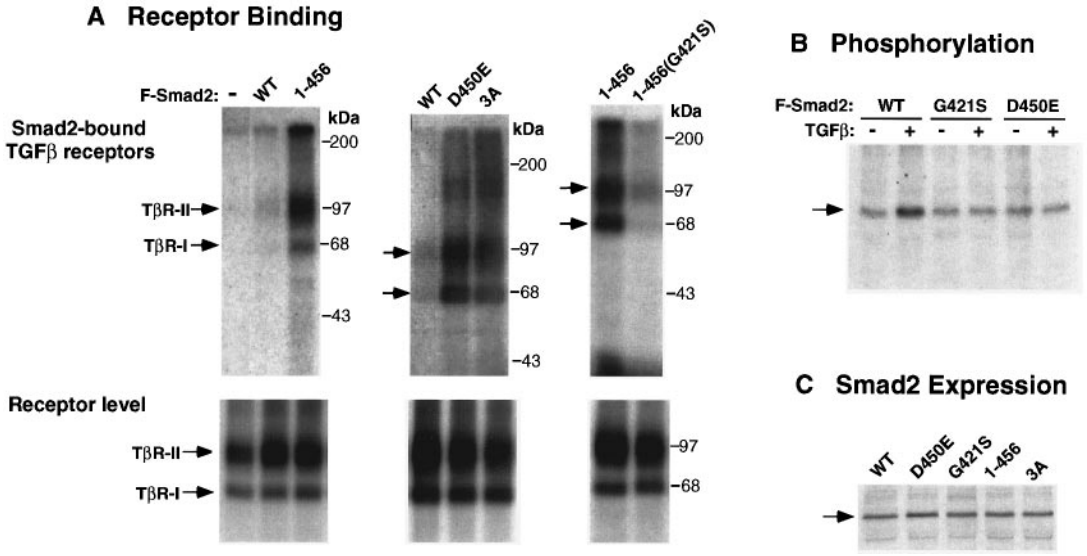

Fig. 2. Smad2 association with the TGF-β receptor does not require its C-tail and is affected by Smad2 phosphorylation. (**A**) Smad2–TGF-β receptor interaction was determined by co-transfecting Flag-tagged wild-type or mutant Smad2 with wild-type TβR-I and TβR-II receptors into COS-1 cells, affinity-labeling by cross-linking to [^{125}I]TGF-β1, then co-immunoprecipitating the Smad2–receptor complex using anti-Flag antibody. The immunoprecipitates and aliquots of whole cell lysates were subjected to SDS–PAGE and autoradiography to visualize the Smad2-bound receptors (upper panel) and the total receptor levels (lower panel), respectively. (**B**) Smad2 phosphorylation was determined by transfecting Flag-tagged wild-type or mutant Smad2 alone (–) or together (+) with TβR-I into R1B/L17 cells. After 48 h, cells were labeled with [^{32}P]orthophosphate for 2 h and stimulated with (+) or without (–) TGF-β1 for 30 min. Cell lysates were immunoprecipitated with anti-Flag antibody and the immunoprecipitates analyzed by SDS–PAGE and autoradiography. (**C**) Expression of Smad2 constructs was checked by transfecting Flag-tagged Smad2 into COS-1 cells. At 48 h post-transfection, cell lysates were resolved by SDS–PAGE and transferred onto a membrane support. Western blotting was carried out using anti-Flag antibody. The expression level of Smad2(1–456/G421S) was similar to that of Smad2(1–456) (data not shown).

et al., 1995) or to aspartic acid in *C.elegans* Sma-2 (Savage *et al.*, 1996) causes null or severe developmental phenotypes. The corresponding mutation in Smad1 inhibits BMP-induced phosphorylation of Smad1 (Hoodless *et al.*, 1996). In Smad2, the (G421S) mutation inhibited TGF-β-dependent phosphorylation (Figure 2B). Unlike the D450E mutation, however, the G421S mutation inhibited Smad2 binding to the receptor (Figure 2A). This suggested that Gly421 is involved, directly or indirectly, in Smad2 association with the receptor, and mutation of this residue may inhibit phosphorylation by preventing this association.

Gly421 is located in a highly conserved segment of the Smad2 MH2 domain (Figure 1A). The crystal structure of the Smad4 MH2 domain reveals that this segment forms a solvent-exposed loop, the L3 loop, protruding from the β-sandwich core structure of the MH2 domain (Figure 1B) (Shi *et al.*, 1997). The L3 loop is predicted to participate in SMAD interaction with other proteins (Shi *et al.*, 1997). To test whether the integrity of the L3 loop is required for Smad2–receptor association, we substituted various residues that are absolutely conserved in this loop (G423, Y426 and RQ428,429; see Figure 1A) with alanine. Gly423 of Smad2 corresponds to Gly348 in Sma-3, which is converted to arginine in a developmental mutant allele (Savage *et al.*, 1996). As inferred from the Smad4 crystal structure, these mutations should not destabilize the overall folding of Smad2. Indeed, these mutants were indistinguishable from the wild-type Smad2 in their expression levels and their ability to form homo-oligomers (Table I). However, these mutations greatly diminished (G423A) or abolished (Y426A and RQ428, 429AA) Smad2 binding to the TGF-β receptor complex (Table I). Defective binding to the receptor was accompanied by defective TGF-β-induced phosphorylation and defective association with Smad4 (Table I), the latter as measured by co-immunoprecipitation with a co-transfected epitope-tagged Smad4 construct (Lagna *et al.*, 1996; and see below).

The effect of these mutations strongly suggested that the L3 loop plays a crucial role in mediating Smad2–receptor interactions. Several other mutations in the L3 loop also inhibited Smad2 association with the receptor. These include R427P, R427A, T432K, T432A and S433A (Table I). We also mutated various highly conserved residues in other regions of the Smad2 MH2 domain that are surface-exposed as predicted from the tertiary structure of the related Smad4 MH2 domain (Shi *et al.*, 1997). Mutations in α-helix 2 (P360R; QRY364–366YHH; W368F), in α-helix 3 (A392Q) and in α-helix 4 (A404T; Q407E) did not diminish the binding of Smad2 to the receptor complex (data not shown), suggesting that the integrity of these other regions is not essential for SMAD–receptor association.

The L3 loop specifies SMAD–receptor interactions

A sequence comparison of the TGF-β-activated SMADs (Smads 2 and 3) and the BMP/Dpp-activated SMADs (Smads 1, 5, 8 and Mad) reveals that the L3 loop is invariant within each group, but differs at two positions (corresponding to residues 427 and 430 in Smad2) between these two groups (Figure 1A and B). To determine whether the L3 loop can define the specificity of SMAD–receptor interaction, we first compared the ability of Smad1 and Smad2 to associate with the TGF-β receptor complex (Figure 4A). The relative binding of Smad1 versus Smad2 to the TGF-β receptor complex was assessed in three different co-transfection schemes that optimize the TGF-β receptor–SMAD interaction: wild-type SMAD with

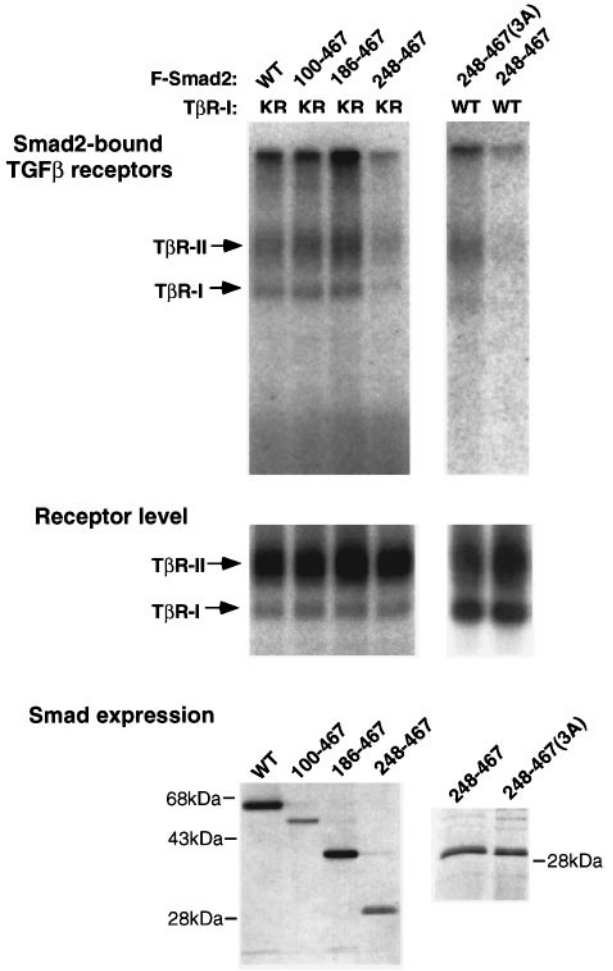

Fig. 3. The Smad 2 MH2 domain retains the receptor docking ability. COS-1 cells were co-transfected with Flag-tagged wild-type or mutant Smad2, wild-type (WT) or kinase-defective (KR) TβR-I, and wild-type TβR-II, and were affinity-labeled with [^{125}I]TGF-β1. The Smad2-bound and total receptors were resolved by SDS–PAGE and autoradiography as described in Figure 2A. Smad2 expression was determined in parallel by Western blotting.

kinase-defective receptor; wild-type receptor with SMAD C-tail deletion constructs; and wild-type receptor with SMAD C-tail serine to alanine mutations. All three schemes yielded consistent results, showing that Smad2 associated with the TGF-β receptor complex 5- to 15-fold more effectively than Smad1 (Figure 4A).

We then tested whether the L3 loops of Smad1 and Smad2 could account for this differential affinity. To this end, we created a Smad2 construct containing the Smad1 L3 loop (by introducing the mutations R427H and T430D), and the reciprocal Smad1 construct. This Smad2 construct, hereafter referred to as Smad2(L1), had poor TGF-β receptor-binding ability compared with Smad2, whereas the reciprocal construct Smad1(L2) was able to bind the TGF-β receptor complex as effectively as did Smad2 (Figure 4B). Switching the C-tails of Smads 1 and 2 in addition to the L3 loop [Smad1(LC2) and Smad2(LC1) constructs] had no additional effect on receptor binding (Figure 4B), consistent with our observation that the Smad2 C-tail does not contribute to docking to the receptor (Figure 2A). As expected, C-tail chimeras [Smad1(C2) and Smad2(C1) constructs] behaved like their wild-type counterparts with regard to binding to the receptor (data not shown). Thus, we conclude that the SMAD L3 loop critically determines the specificity of the SMAD–TGF-β receptor interactions.

Switching SMAD activation

As suggested by the results in Table I, optimal TGF-β receptor binding for Smad2 appeared to be necessary for the optimal phosphorylation of the C-tail. Consistent with this notion, TGF-β stimulation failed to phosphorylate Smad2(L1) and Smad2(LC1), which are defective in binding to the TGF-β receptor, but still phosphorylated Smad2(C1) almost as efficiently as wild-type Smad2 (Figure 5A). Furthermore, Smad1(LC2) was phosphorylated as efficiently as Smad2 in response to TGF-β stimulation (Figure 5A). However, Smad1(L2) was phosphorylated in response to TGF-β less extensively than were Smad2 or Smad1(LC2), even though all three constructs could bind to the TGF-β receptor similarly well (see Figure 4B).

We also investigated the requirements for SMAD phosphorylation by activated BMP receptors. Smad1 phosphorylation in response to BMP stimulation was decreased, but not lost, when its L3 loop was swapped with that of Smad2 [Smad1(L2) construct] (Figure 5B). Upon BMP stimulation, Smad1 containing the C-tail of Smad2 [Smad1(C2) construct] was phosphorylated to a similar extent as wild-type Smad1 (Figure 5B). However, when both the L3 loop and the C-tail were swapped [Smad1(LC2) construct], this chimeric Smad1 lost BMP-stimulated phosphorylation (Figure 5B). On the other hand, Smad2 containing the Smad1 L3 loop [Smad2(L1) construct] gained BMP-induced phosphorylation (Figure 5B). When the Smad1 C-tail, in addition to the Smad1 L3 loop, was also introduced into Smad2 [Smad2(LC1) construct], the gain of BMP-induced phosphorylation was increased further (Figure 5B).

To corroborate that the switch in receptor docking and phosphorylation specificity by exchanging both the L3 loop and the C-tail between Smad1 and Smad2 resulted in the activation of Smad1(LC2) by TGF-β or Smad2(LC1) by BMP, we determined the ability of these constructs to associate with Smad4. Smad1(LC2), as with wild-type Smad2, was able to associate with Smad4 in response to TGF-β stimulation, whereas Smad2(LC1) was not. Similarly, Smad2(LC1), but not Smad1(LC2), was able to associate with Smad4 in response to BMP stimulation (Figure 6A). Note that swapping both the L3 loop and the C-tail between Smads 1 and 2 had no detectable effect on their ability to form homo-oligomers with their wild-type counterparts (Figure 6B). The ability of the TGF-β receptors and BMP receptors to induce SMAD nuclear translocation was also switched in the Smad1(LC2) and Smad2(LC1) mutants (Figure 7). Like Smad2, Smad1(LC2) was translocated to the nucleus in response to TGF-β but not to BMP. On the other hand, like Smad1, Smad2 (LC1) was translocated to the nucleus in response to BMP but not to TGF-β. Thus, the receptor input necessary to induce association of Smad1 or Smad2 with Smad4 and their movement to the nucleus is provided through a receptor interaction that is dependent on the L3 loop, and specified by the L3 loop together with the C-tail.

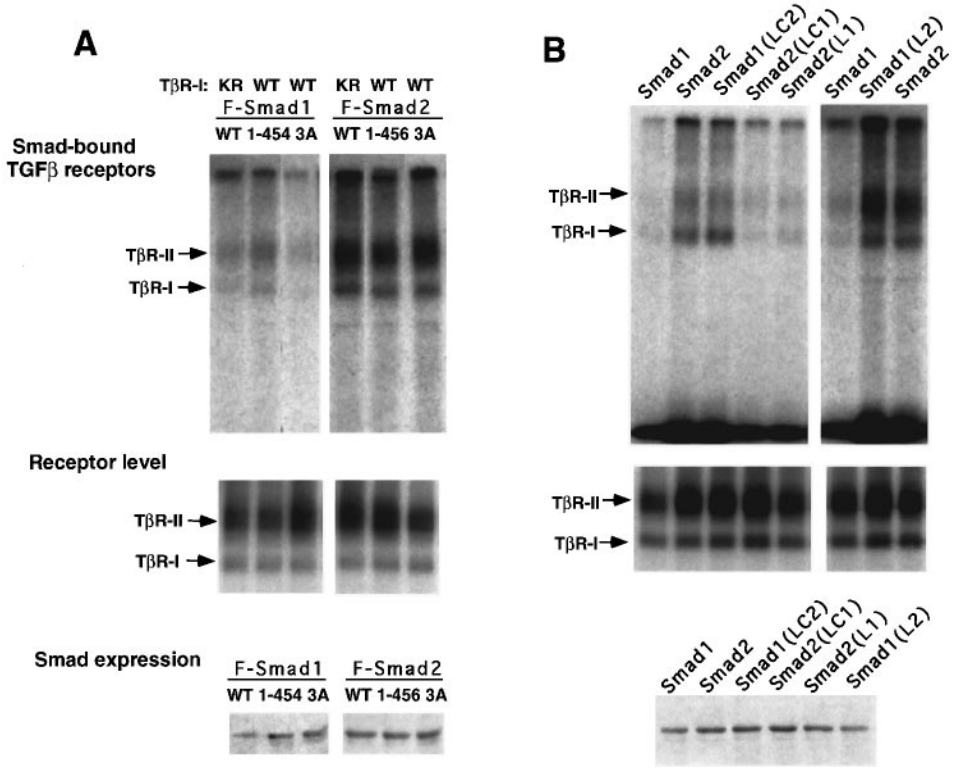

Fig. 4. The L3 loop specifies SMAD–receptor interaction. (**A**) Differential binding affinity of Smad1 and Smad2 to the TGF-β receptor complex. COS-1 cells were transiently co-transfected with wild-type or kinase-defective TβR-I, wild-type TβR-II and Flag-tagged wild-type and mutant Smad1 or Smad2 as indicated. The interaction between the indicated SMAD constructs and the TGF-β receptor complex was assessed as described in Figure 2A. (**B**) The L3 loop determines the specificity of SMAD–receptor interaction. COS-1 cells were transiently co-transfected with the kinase-defective TβR-I, wild-type TβR-II and Flag-tagged wild-type and mutant Smad1 or Smad2. The interaction between the indicated SMAD constructs and the TGF-β receptor complex was assessed as described in Figure 2A. SMAD construct expression levels as determined by anti-Flag immunoblotting are shown in the bottom panel.

Discussion

Specificity is an essential property of signal transduction pathways. In the TGF-β signaling system, specificity is determined by ligand activation of a particular receptor combination which, in turn, recruits and phosphorylates a particular subset of SMAD proteins. In the present study, we have investigated the SMAD–receptor interaction and the molecular basis for its specificity. Our results identify the L3 loop as a discrete surface structure in SMAD proteins that is necessary for the SMAD–receptor interaction and sufficient to dictate its specificity.

The differential ability of Smads 1 and 2 to associate with the TGF-β receptor complex is consistent with their known responsiveness to these receptors: Smad2, which mediates TGF-β signaling, associates with the TGF-β receptor complex ~10-fold better than Smad1, which is primarily a mediator of BMP signaling (Derynck and Zhang, 1996; Wrana and Attisano, 1996; Massagué *et al.*, 1997). This receptor interaction is required for Smad2 phosphorylation, since docking-defective mutants of Smad2 are not phosphorylated in response to TGF-β. However, the Smad2 phosphorylation sites themselves, along with the adjacent sequence in the 11 amino acid C-tail region, are dispensable for the receptor interaction. This conclusion is based on our observation that the TGF-β receptor can associate with a Smad2 deletion mutant lacking the C-tail.

We have identified a structural motif, the L3 loop, as an important determinant of SMAD recognition by the receptor. The L3 loop is a highly conserved region within the MH2 domain that, by analogy to the crystal structure of the Smad4 MH2 domain, is predicted to form a highly solvent-exposed structure that is poised for protein–protein interactions (Shi *et al.*, 1997). Introduction of various mutations into the L3 loop, including developmental mutations previously observed in *Drosophila* Mad (Sekelsky *et al.*, 1995) and *C.elegans* Sma-2 and -3 (Savage *et al.*, 1996), diminishes the ability of Smad2 to associate with the TGF-β receptor complex. None of these mutations has appreciable effects on the Smad2 expression level or its ability to homo-oligomerize, as predicted from the fact that the L3 loop is not part of the SMAD MH2 domain core structure (Shi *et al.*, 1997).

The sequence of the L3 loop, which is invariant among TGF-β-activated SMADs (Smads 2 and 3) and among SMADs thought to be activated by BMPs (Smads 1, 5 and 8) or Dpp (Mad), differs at two positions between these two groups. These two amino acids also differ in Smad4 as well as in Smads 6 and 7 (Figure 1A). In Smad4, these two positions are highly exposed (Figure 1B), and the same is likely to occur in other SMADs given their overall structural similarity to Smad4 (Shi *et al.*, 1997). As further testament to the importance of the L3 loop, switching these two amino acids in Smads 1 and 2 induces a gain or a loss, respectively, in their ability to bind to the TGF-β receptor complex. This gain or loss of TGF-β receptor binding is reiterated in TGF-β receptor-

Table I. Properties of L3 loop mutants of Smad2

L3 loop mutation	Expression level	Homo-oligomer	Receptor binding	TGF-β-induced phosphorylation	Smad4 binding
Wild-type	+	+	+++	+	+++
G421S	+	+	+/–	–	+/–
G423A	+	+	+/–	nd	+/–
A424P	+	+	+++	+	+++
Y426A	+	+	–	–	–
R427P	+	+	–	–	–
R427A	+	+	–	nd	–
RQ428,429AA	+	+	–	–	–
T432K	+	+	–	–	–
T432A	+	+	–	nd	+/–
S433A	+	+	+/–	nd	+/–

The expression level of Flag-tagged Smad2 constructs was determined by anti-Flag immunoblotting. Homo-oligomeric Smad2 interactions were assessed by co-transfection of Flag-tagged Smad2 mutants and HA-tagged wild-type Smad2. Smad4 binding to Smad2 was determined by co-transfection of Flag-tagged Smad2 constructs and HA-tagged Smad4. In both cases, cell lysates were immunoprecipitated with anti-Flag antibody and the precipitates immunoblotted using anti-HA antibody. Receptor binding was determined by the level of [^{125}I]TGF-β1-labeled receptors that were co-immunoprecipitated with Flag-tagged Smad2 following two co-transfection schemes: kinase-defective TβR-I with full-length Smad2 constructs or wild-type TβR-I with C-tail deletion versions of each Smad2 construct. The two transfection schemes yielded similar results with each Smad2 mutant. TGF-β1-stimulated phosphorylation of Flag-tagged Smad2 constructs was determined as described in Materials and methods. In the binding assays, +++ indicates a wild-type level of binding, +/– indicates a binding level 5-fold less than wild-type, and – indicates no detectable binding. nd, not determined.

mediated phosphorylation of these SMADs, indicating that the L3 loop-dependent receptor interaction is necessary and sufficient for TGF-β receptor phosphorylation. Thus, it appears that the L3 loop-mediated SMAD–TGF-β receptor interaction is crucial or rate-limiting for TGF-β receptor-mediated phosphorylation of its SMAD substrates. It should be noted, moreover, that the homologous C-tail containing the phosphorylation sites and the adjacent sequence may ensure an optimal TGF-β receptor-mediated phosphorylation.

Testing whether the BMP receptor shares the same structural requirements for SMAD phosphorylation as the TGF-β receptor revealed an intriguing difference. Whereas Smad2 containing the Smad1 L3 loop lost TGF-β-induced phosphorylation completely, Smad1 containing the Smad2 L3 loop was still phosphorylated in response to BMP, albeit to a lesser extent than wild-type Smad1. This difference suggests that, in selecting among different SMAD substrates, the BMP receptor may have a more permissive requirement for the optimal L3 loop sequence, with the SMAD C-tail region playing a complementary role in substrate recognition. Consistent with this notion, physical interaction between the BMP receptor complex and the SMADs appears to be much more transient than that between the TGF-β receptor complex and the SMADs, as both affinity-labeled wild-type and kinase-defective BMPR-IA or -IB, in conjunction with wild-type BMPR-II, could not be co-immunoprecipitated with Smad1 (our unpublished observations). In any case, swapping both the L3 loop and the C-tail allows Smad1 and Smad2 to be phosphorylated respectively by the TGF-β and BMP receptors, and a switch in agonist-induced association with Smad4 and nuclear translocation accompanies this switch in phosphorylation.

Unlike the receptor-regulated SMADs, Smad4 lacks a C-terminal SS(V/M)S phosphorylation motif and does not appear to associate with the receptors on its own (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). What then is the function of the L3 loop in Smad4? Based on structural considerations and the observation that a mutation (G508S) in the Smad4 L3 loop abolishes the ability of Smad4 to associate with Smad2, we have proposed that the Smad4 L3 loop mediates the association with receptor-activated SMADs (Shi *et al.*, 1997). We have corroborated the importance of the Smad4 L3 loop for Smad2–Smad4

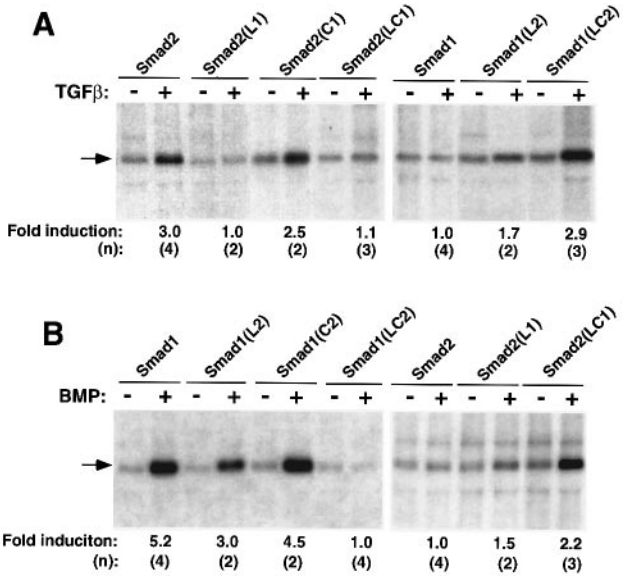

Fig. 5. Role of the L3 loop and C-tail in the phosphorylation of SMADs by the type I receptors. (**A**) The L3 loop of Smad2 is necessary for Smad2 phosphorylation in response to TGF-β and allows Smad1 to be phosphorylated in response to TGF-β. The Smad2 C-tail supports optimal phosphorylation. (**B**) Both the L3 loop and C-tail of Smad1 determine the ability of the BMP receptor to phosphorylate SMAD substrates. To determine the inducibility of SMAD phosphorylation by TGF-β1 or BMP4, R1B/L17 cells were transfected with the indicated Flag-tagged SMAD constructs alone (–) or together (+) with either TβR-I or BMPR-IB and BMPR-II. Cell were labeled with [^{32}P]orthophosphate for 2 h and then incubated with (+) or without (–) TGF-β or BMP for 30 min. SMAD construct expression levels were similar as determined by anti-Flag immunoblotting (data not shown). The arrow indicates SMAD proteins. Fold induction values represent averages of 2–4 independent experiments, as indicated (*n*).

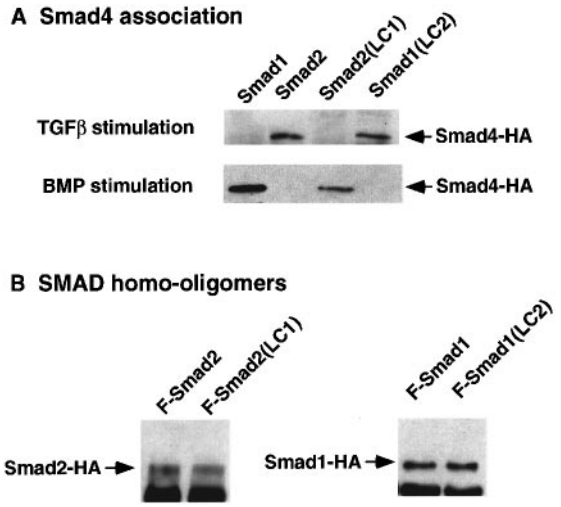

Fig. 6. (**A**) Association of the receptor-regulated SMADs with Smad4. COS-1 cells transfected with the indicated Flag-tagged Smad1 or 2 constructs, HA-tagged Smad4 and activated TβR-I(T204D) or activated BMPR-IB(Q203D) and wild-type BMPR-II were treated with either TGF-β1 or BMP4 for 1 h. After SMAD complexes were immunoprecipitated using anti-Flag antibody, Smad4 was visualized by Western blotting with anti-HA antibody. (**B**) COS-1 cells were transfected with wild-type Smad2 C-terminally tagged with HA epitope (Smad2-HA) and wild-type and mutant Smad2 N-terminally tagged with Flag epitope (F-Smad2) (left panel), or transfected with wild-type Smad1 C-terminally tagged with HA epitope (Smad1-HA) and wild-type and mutant Smad1 N-terminally tagged with Flag epitope (F-Smad1) (right panel). After 48 h, cells were lysed and immunoprecipitation was carried out with anti-Flag antibody, and SMAD homomeric complexes were visualized by anti-HA immunoblotting.

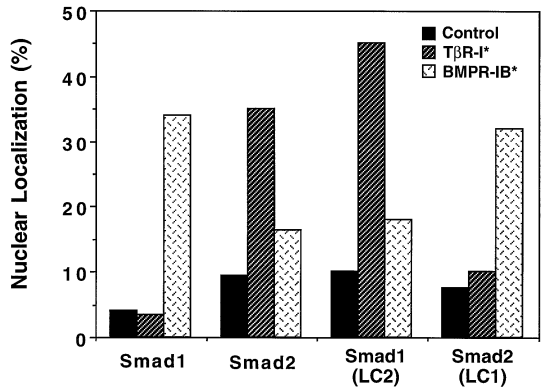

Fig. 7. Nuclear translocation of Smad1, Smad2 and their derivatives in response to TGF-β or BMP. Vectors encoding the indicated Flag-tagged SMAD constructs alone (Control) or together with either TβR-I(T204D) (TβR-I*) or BMPR-IB(Q203D) (BMPR-IB*) were transfected into HepG2 cells. At 48 h post-transfection, cells were incubated with TGF-β1 or BMP2, and immnuofluorescence was visualized with primary mouse anti-Flag antibody and secondary FITC-conjugated goat anti-mouse antibody. Nuclear localization was confirmed with DAPI DNA staining. The percentage of SMADs localized in the nucleus was determined by counting 200–300 immunofluorescence-positive cells for each sample.

interaction by showing that mutations of other residues in the Smad4 L3 loop (Y513A; and RQ515,516AA) also lead to the loss of TGF-β-inducible Smad2–Smad4 association in transfected COS-1 cells (our unpublished observations). Smad4 is required for various responses to TGF-β, activin and BMP by acting as a partner for the corresponding receptor-activated SMADs (Lagna *et al.*, 1996; X.Chen *et al.*, 1997; Liu *et al.*, 1997; Zhang *et al.*, 1997). In addition, Smad4 can associate with these SMADs in yeast, suggesting that the interaction may be direct (Hata *et al.*, 1997; Wu *et al.*, 1997). SMAD L3 loops, therefore, are implicated in two distinct types of interactions. Among the receptor-regulated SMADs, the L3 loop may mediate SMAD–receptor interactions, whereas the more divergent Smad4 L3 loop (see Figure 1A) may mediate Smad4 interaction with receptor-activated SMADs. It will be interesting to determine whether the L3 loop of receptor-regulated SMADs has a dual function as a receptor-interacting region and, upon phosphorylation of the C-tail, as a Smad4-interacting region.

Since the C-tail of receptor-regulated SMADs serves as a substrate for the type I receptor kinase, it must physically contact the receptor. However, this interaction apparently does not contribute significantly to the stability of the interaction that precedes phosphorylation, at least as determined with Smad2 and the TGF-β receptor. In fact, the TGF-β receptor–Smad2 interaction is weakened upon phosphorylation by the receptor, as either phosphorylation-defective Smad2 mutants or a kinase-defective TGF-β type I receptor mutant enhance SMAD–receptor association (Macias-Silva *et al.*, 1996; this work). It is not clear how SMAD phosphorylation may promote its dissociation from the receptor. A gain of affinity for Smad4 might contribute to Smad2 dissociation from the receptor upon phosphorylation. However, the Smad2(3A) mutant still showed an elevated receptor-binding activity as compared with the wild-type Smad2 in the Smad4-deficient colorectal carcinoma cell line SW480.7 (our unpublished observation). Thus, an increased affinity for Smad4 may not be the only event driving dissociation of the phosphorylated Smad2 from the receptor complex.

Although two residues in the L3 loop are sufficient to dictate the specificity of the SMAD–receptor interaction, the entire L3 loop may not be sufficient to support this interaction fully. Attempts to demonstrate direct binding between receptors and SMADs or their L3 loops have not yet provided concrete evidence. It could be that a direct SMAD–receptor interaction is weak and requires oligomeric forms of both the receptors and the SMADs for cooperative binding. Alternatively, the SMAD–receptor interaction might be indirect, requiring a hitherto unidentified adaptor protein. Regardless of the mechanism, the evidence at hand identifies the L3 loop as a critical determinant of specific SMAD–receptor interactions.

Materials and methods

Expression vectors

Human Smad1, Smad2 and Smad4 mutations were made by a PCR-based strategy as described previously (Hata *et al.*, 1997). All PCR-generated fragments were subcloned into wild-type SMADs in cytomegalovirus (CMV) promoter-based mammalian expression vectors pCMV5 or pCS2 and verified by sequencing.

Transfection, metabolic labeling and immunoprecipitation

For *in vivo* labeling with [^{35}S]methionine or [^{32}P]orthophosphate and for co-immunoprecipitation studies, cells were transiently transfected by the DEAE–dextran method as previously described (Y.G.Chen *et al.*, 1997). To examine the phosphorylation of Flag-tagged Smad1 and Smad2 constructs, R-1B/L17 cells (Boyd and Massagué, 1989) were co-transfected with either TβR-I or BMPR-IB and BMPR-II. At 40–48 h post-transfection, cells were washed and pre-incubated with phosphate-

free media for 1 h. The cells were then incubated with the same phosphate-free media containing 1 mCi/ml [^{32}P]phosphate for 2 h at 37°C and then stimulated with either TGF-β1 (1 nM) or BMP4 (10 nM) for 30 min. Subsequently, labeled and ligand-stimulated cells were lysed in TNE buffer (10 mM Tris, pH 7.8; 150 mM NaCl; 1 mM EDTA; 1% NP-40) containing protease and phosphatase inhibitors, and the lysates were subjected to immunoprecipitation with anti-Flag M2 monoclonal antibody (IBI; Eastman Kodak). Protein expression of SMADs was determined by either metabolic labeling or Western blotting. COS-1 cells that have been transiently transfected for 40–48 h were washed and pre-incubated in methionine-free media and then labeled with trans-[^{35}S]methionine for 3 h. Lysis and immunoprecipitation were performed as for [^{35}P]phosphate-labeled cells. Immunoprecipitates were visualized by SDS–PAGE followed by autoradiography. For Western blotting, a fraction of the total cell lysate was separated by SDS–PAGE and assayed by immunoblotting as indicated.

For Smad4 association studies, Flag-tagged Smad1 or Smad2 constructs were transiently co-transfected with HA-tagged Smad4 into COS-1 cells (Hata *et al.*, 1997). At 40–48 h post-transfection, cells were washed in Dulbecco's modified Eagle's medium (DMEM) containing 0.2% fetal calf serum and treated with the indicated ligand (200 pM TGF-β1 or 5 nM BMP4). Following ligand stimulation, cells were lysed in TNE buffer containing protease inhibitors. Cells lysates were then subjected to immunoprecipitation with anti-Flag M2 monoclonal antibody. Immunoprecipitates were washed, separated by SDS–PAGE and transferred to PVDF membranes (Immobilon-P; Millipore). HA-tagged Smad4 was detected using anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim), followed by donkey anti-mouse antibody conjugated with horseradish peroxidase (Sigma) and chemiluminescence (ECL, Amersham).

Receptor affinity labeling

COS-1 cells transiently transfected for 40–48 h by the DEAE–dextran method were affinity-labeled with [^{125}I]TGF-β as previously described (Massagué, 1987). Briefly, cells were pre-incubated at 37°C in Krebs–Ringer–HEPES (KRH) buffer containing 0.5% bovine serum albumin (BSA), washed with cold KRH/0.5% BSA, and affinity-labeled using 200 pM [^{125}I]TGF-β in KRH/0.5% BSA for 3.5 h at 4°C. Then, the cells were washed four times in ice-cold KRH containing 0.5% BSA and once more with KRH alone. Subsequently, cell surface-bound [^{125}I]TGF-β was cross-linked to the receptor complex by incubation for 15 min at 4°C with 60 μg/ml disuccinimidyl suberate in KRH; cross-linking was terminated by washing the cells twice with ice-cold STE (0.25 M sucrose, 10 mM Tris–HCl, pH 7.4 and 1 mM EDTA). Cells were then lysed in TNT [20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 (v/v)] (Y.G.Chen *et al.*, 1997) containing protease and phosphatase inhibitors and the cell lysate subjected to anti-Flag immunoprecipitation. Labeled receptor complexes in the immunoprecipitates and in the total cell lysates were then visualized by separation on SDS–PAGE and autoradiography.

Immunofluorescence

HepG2 cells were transfected overnight using the standard calcium phosphate–DNA precipitation method. Twenty four hours after transfection, cells were transferred onto chamber slides (Nunc, Inc.). At 40–48 h post-transfection, cells were stimulated with 5 nM BMP4 or 1 nM TGF-β for 30 min and processed for immunofluorescence as described (Kretzschmar *et al.*, 1997a). Immunostaining was performed using anti-Flag M2 monoclonal antibody and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Pierce).

Acknowledgements

We would like to thank A.Hata, F.Liu, D.Wotton and C.Pouponnot for reagents and helpful insights, C.Zhang for excellent technical assistance and the Genetics Institute for generously providing BMP. This work was supported by NIH grants to J.M. (CA34610) and MSKCC. Y.G.C. and J.M. are a Research Associate and an Investigator, respectively, of the Howard Hughes Medical Institute.

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Received September 23, 1997; revised December 8, 1997; accepted December 12, 1997